The Structure of the yFACT Pob3-M Domain, Its Interaction with the DNA Replication Factor RPA, and a Potential Role in Nucleosome Deposition

Andrew P. VanDemark,1 Mary Blanksma,1 Elliott Ferris,1 Annie Heroux,2 Christopher P. Hill,1,* and Tim Formosa1,2*
1Department of Biochemistry
University of Utah School of Medicine
Salt Lake City, Utah 84132
2Biology Department
Brookhaven National Laboratory
Upton, New York 11973

Summary

We report the crystal structure of the middle domain of the Pob3 subunit (Pob3-M) of S. cerevisiae FACT (yFACT, facilitates chromatin transcription), which unexpectedly adopts an unusual double pleckstrin homology (PH) architecture. A mutation within a conserved surface cluster in this domain causes a defect in DNA replication that is suppressed by mutation of replication protein A (RPA). The nucleosome reorganizer yFACT therefore interacts in a physiologically important way with the central single-strand DNA (ssDNA) binding factor RPA to promote a step in DNA replication. Purified yFACT and RPA display a weak direct physical interaction, although the genetic suppression is not explained by simple changes in affinity between the purified proteins. Further genetic analysis suggests that coordinated function by yFACT and RPA is important during nucleosome deposition. These results support the model that the FACT family has an essential role in constructing nucleosomes during DNA replication, and suggest that RPA contributes to this process.

Introduction

FACT (facilitates chromatin transcription) is an essential chromatin reorganizing factor (Belotserkovskaya and Reinberg, 2004; Formosa, 2002; O’Donnell et al., 2004). The complex from the yeast S. cerevisiae, yFACT, is composed of three proteins: Spt16/Cdc68 (120 kDa), Pob3 (63 kDa), and Nhp6 (11 kDa). FACT subunits are highly conserved among eukaryotes, although in metazoans the Pob3 and Nhp6 orthologs are fused to form SSRP1. Of these components, only the structure of the nonspecific DNA binding protein Nhp6 has been reported (Allain et al., 1999; Masse et al., 2002).

Purified yFACT increases the accessibility of some nucleosomal DNA sites to nucleases (Formosa et al., 2001; Rhoades et al., 2004; Ruone et al., 2003). In contrast to ATP-dependent chromatin remodeling factors (Langst and Becker, 2004; Vignali et al., 2000), yFACT enhances digestion of nucleosomal DNA without translocating histone octamers to expose the affected sites and without hydrolyzing ATP (Orphanides et al., 1998; Rhoades et al., 2004; Ruone et al., 2003). Although the physical state of the nucleosomes altered by yFACT remains speculative, we have called the activity of yFACT “reorganization” to distinguish it from remodeling.

yFACT has at least two important roles in transcription. First, yFACT promotes normal initiation. Mutation of SPT16 or POB3 or overexpression of SPT16 causes the Spt− phenotype, which results from abnormal transcription initiation site selection (Formosa et al., 2002; Malone et al., 1991; Rowley et al., 1991; Schlesinger and Formosa, 2000). Consistent with a direct role in initiation, yFACT enhances the otherwise inefficient interaction between TATA binding protein (TBP) and TFIIA with a nucleosomal TATA site both in vitro and in vivo (Biswas et al., 2005). Second, FACT promotes normal elongation of transcription by RNA polymerase II on chromatin templates in vitro; this activity was used to purify human FACT (Orphanides et al., 1998, 1999). FACT also associates with RNA Pol II complexes throughout transcribed regions in yeast and plants (Kim et al., 2004; Mason and Struhl, 2003; Duroux et al., 2004) and colocalizes with RNA Pol II in flies (Saunders et al., 2003). Also consistent with a role in elongation, some spt16 mutations cause sensitivity to 6-azauracil, which perturbs rNTP pool balance and inhibits elongation (Formosa et al., 2002; John et al., 2000; Orphanides et al., 1999). yFACT subunits also display a range of physical and genetic interactions with other transcription initiation and elongation factors (Costa and Arndt, 2000; Formosa et al., 2002; Krogan et al., 2002; Shimijima et al., 2003; Squazzo et al., 2002), suggesting that FACT performs several different functions by interacting with multiple complexes.

In addition to these roles in transcription, FACT is also required for DNA replication. yFACT binds directly to DNA polymerase (Pol) α/primase, and yFACT subunits interact genetically with several replication factors (Budd et al., 2005; Formosa et al., 2002; Schlesinger and Formosa, 2000; Wittmeyer and Formosa, 1997; Wittmeyer et al., 1999; Zhou and Wang, 2004). A subset of SPT16 and POB3 mutations causes sensitivity to hydroxyurea (HU; Formosa et al., 2002; Schlesinger and Formosa, 2000; O’Donnell et al., 2004), which inhibits ribonucleotide reductase activity, decreasing dNTP production and thus interfering with DNA synthesis. POB3 mutations that caused HU sensitivity also delayed S phase progression and made cells more dependent on the S phase checkpoint mediated by Mec1 (Schlesinger and Formosa, 2000). Further, FACT is needed for normal levels of DNA replication in frog oocyte extracts (Okuhara et al., 1999), and it is associated with DNA replication foci in mouse cells (Hertel et al., 1999).

The broad range of FACT functions can be explained by the need to overcome the inhibitory effects of nucleosomes at many steps during chromatin-based processes. In this view, reorganization of nucleosomes by FACT provides access to blocked DNA sites without requiring nucleosomal translocation. Alternatively, FACT could be responsible for promoting the formation of stable nucleosomes, and mutated FACT could cause deposition of defective nucleosomes during replication or...
during transcriptional repression, leading to abnormal behavior of the resulting chromatin. A role for FACT in nucleosome deposition is consistent with several observations. First, purified human FACT has been shown to promote the assembly of nucleosomes in vitro (Belotserkovskaya et al., 2003). Second, inappropriate transcriptional initiation from cryptic promoters in some yFACT mutants has been interpreted as a failure to re-form normal chromatin after passage of RNA polymerase II (Kaplan et al., 2003). Third, some yFACT mutations are lethal when combined with mutations in the Hir/Hpc complex, indicating greater reliance on a pathway that has been implicated in nucleosome deposition (Formosa et al., 2002). These two views of FACT function, relieving nucleosomal inhibition and promoting nucleosome assembly, are not incompatible, as both could arise from an ability to chaperone nucleosomal components or to interconvert intermediates.

Here we report the crystal structure of the middle domain of Pob3 (Pob3-M). Unexpectedly, Pob3-M has an unusual “double PH” domain architecture. Conserved residues cluster on one face of the structure, indicating a surface that is functionally important. Genetic analysis indicates that this surface functions in DNA replication in a process that also involves the ssDNA binding factor replication protein A (RPA). The responses of the yFACT and RPA mutants to manipulations of histone genes suggest that yFACT and RPA cooperate to promote nucleosome deposition during DNA replication.

Results and Discussion

StructuralDomains of Spt16-Pob3

We characterized the domain structure of yFACT by subjecting purified Spt16-Pob3 to limited proteolysis (Figure 1 and see Figures S1A and S1B in the Supplemental Data available with this article online). These results guided expression of various domains either individually or in combination to determine which were soluble and which were involved in the Spt16-Pob3 dimer interface (Figure S1C). This analysis indicated that Spt16 is composed of four domains (Figure 1): N terminal (N, similar in sequence with a family of aminopeptidases; Ponting, 2002), dimerization (D), middle (M), and C terminal (C). Spt16 is composed of three domains: N terminal and dimerization (N/D), middle (M), and C terminal (C). Similar results based on partial proteolysis have been reported previously (O’Donnell et al., 2004). The involvement of the N/D domain of Pob3 in dimerization with Spt16 is consistent with studies of human FACT (Keller and Lu, 2002). The C-terminal domains of each protein have high fractions of acidic residues and are predicted to be largely unstructured. Genetic analysis suggested that Pob3-M has a specific role in DNA replication (see below), so we initially focused on the structure of this domain.

Pob3-MAdopts a Double PH Domain Structure

Pob3-M was purified and crystallized. Phases were estimated by the SAD method from a selenomethionine-substituted variant protein in which leucines 297, 298, and 300 were replaced with methionines. The structure was refined to an R factor/Rfree of 20.8%/26.9% against 2.2 Å data (Table 1). Residues 237–424 and 434–474 are ordered in the structure, and the two independent molecules in the asymmetric unit superimpose on each other with an rmsd of 0.7 Å for 237 pairs of ordered Cα atoms.

Pob3-M comprises two pleckstrin homology (PH) domain motifs (residues 248–367 and 383–474; Figure 2). This finding was unanticipated because, although the Pob3 sequence is conserved throughout eukaryotes (the SSRP1 family or pfam SSRrecog; Marchler-Bauer et al., 2005; Marchler-Bauer and Bryant, 2004), sequence similarity with other proteins was not apparent. PH domain structures comprise a 7-stranded antiparallel β barrel that is capped at one end by a helix. The two Pob3-M PH domain structures are similar to each other (rmsd of 2.3 Å on 78 pairs of Cα atoms), and they each correspond closely to the standard PH domain motif. For example, Pob3 383–474 overlaps with one of the PH domains of pleckstrin (1PLS, Yoon et al., 1994) with an rmsd of 2.5 Å on 87 pairs of Cα atoms using the program DALI (Holm and Sander, 1998). In addition to the elements of a standard PH domain, Pob3-M 248–367 also contains two strands and a helix (S8, S9, and H2) that are inserted between the last strand of the PH domain (S7) and the β barrel-capping helix (H3), and two strands (S6’ and S6”) that contribute to the relatively long Q308 loop that is inserted between strands S6 and S7 (Figure 2).

Notably, the two PH domains are intimately associated with each other. They are related primarily by a translation such that their β barrels point in the same direction and the sides of the barrels pack against each other. A total of ~500 Å2 of solvent-accessible surface area would be exposed if the two domains were separated from each other without other conformational changes. Further, numerous side chains that have been conserved as hydrophobic are buried in this interface (e.g., L288, F315, V317, Y396, L398, F403, and L405; see Figure S2). Thus, consistent with the limited proteolysis data, the Pob3-M domain should be viewed as one double PH domain rather than as two adjacent but relatively independent PH domains.

The many PH domain-like structures that have been observed belong to six distinct subgroups that each adopt the same overall fold but lack significant sequence similarity with one another (Marchler-Bauer et al., 2005; Marchler-Bauer and Bryant, 2004). The Pob3-M domain constitutes a seventh such subgroup. PH domains are best known as inositol lipid binding modules that function in regulated membrane binding, although this

![Figure 1. yFACT Domain Structure](Image)

Partial proteolysis and the solubility of singly or coexpressed fragments (Figure S1) were used to delineate structural domains of Spt16-Pob3, named as described in the text. Sites digested by trypsin (T), chymotrypsin (C), and proteinase K (K) are indicated for Pob3; other boundaries were defined as noted in Figure S1.
Table 1. Data Collection and Refinement Statistics for the Pob-M and Pob-M(Q308K) Structures

<table>
<thead>
<tr>
<th></th>
<th>Pob-M</th>
<th>Pob-M(Q308K)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data Collection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P21;2;1</td>
<td>P21</td>
</tr>
<tr>
<td>Unit cell dimensions (Å)</td>
<td>a = 57.11, b = 57.77, c = 157.53</td>
<td>a = 57.89, b = 57.89, c = 157.53</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50.2–2.21</td>
<td>40.3–2.55</td>
</tr>
<tr>
<td>Outer shell (Å)</td>
<td>2.29–2.21</td>
<td>2.64–2.55</td>
</tr>
<tr>
<td>Number of reflections</td>
<td>36,598</td>
<td>28,670</td>
</tr>
<tr>
<td>Unique</td>
<td>334,160</td>
<td>286,760</td>
</tr>
<tr>
<td>Mean I/Fo,0 (%)</td>
<td>60.4 (30.1)</td>
<td>15.1 (2.01)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.3 (96.3)</td>
<td>91.7 (92.8)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R factor/Rfree (%)</td>
<td>20.8/26.9</td>
<td>22.0/30.3</td>
</tr>
<tr>
<td>Nonhydrogen atoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4062</td>
<td>7652</td>
</tr>
<tr>
<td>Solvent</td>
<td>254</td>
<td>78</td>
</tr>
<tr>
<td>Rsmd from ideal geometry</td>
<td>0.013</td>
<td>0.013</td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>1.436</td>
<td>1.497</td>
</tr>
<tr>
<td>Bond angles (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average isotropic B values (Å²)</td>
<td>41.4</td>
<td>40.7</td>
</tr>
<tr>
<td>Ramachandran plot, nonglycine residue in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most favorable region (%)</td>
<td>90.2</td>
<td>86.4</td>
</tr>
<tr>
<td>Additional allowed region (%)</td>
<td>9.1</td>
<td>12.7</td>
</tr>
<tr>
<td>Generous allowed region (%)</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Disallowed region (%)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Values in parentheses correspond to those in the outer resolution shell.

*a R_{free} = \left(\frac{\langle I \rangle - <I>}{\langle I \rangle}\right)\langle I \rangle$, where <I> is the average intensity of multiple measurements.

*b R factor = \frac{\sum_{hkl}||F_{obs}(hkl)|| - F_{calc}(hkl)||}{\sum_{hkl}F_{obs}(hkl)}$.

*c $R_{free}$ = the crossvalidation R factor for 5% of reflections against which the model was not refined.

activity is limited to just one of the superfamily’s subgroups (Jacobs et al., 1999; Lemmon, 2004). Other PH-like families are also associated with binding to specific ligands, but their ligands are diverse, including lipids, peptides containing phosphosyrosine, proline, and other peptides or proteins (Jacobs et al., 1999; Lemmon, 2004). The presence of a PH domain therefore suggests a binding function but does not indicate the nature of the ligand (Yu et al., 2004). Furthermore, several different regions of the PH-fold surface are used for ligand binding by the various subgroups, so it is not possible to propose a specific binding surface of Pob3-M solely on the basis of the PH domain architecture.

Previous sequence analysis suggested weak similarity between residues 4–104 and 231–475 of Pob3 (Ponting, 2002). Pob3 (374–475) forms a standard PH fold (Figure 2), so the N terminus of Pob3 might also adopt this architecture and could form another binding site. This organization is consistent with formation of multiple contacts between Pob3 and nucleosomes or members of other complexes.

### A Conserved Pob3-M Surface Functions in DNA Replication

We aligned a diverse subset of the approximately 60 sequences of members of the Pob3/SSRP1 family (Altschul et al., 1997) and displayed the invariant residues on the Pob3-M structure (Figure 2 and Figures S2 and S3). Many of the invariant surface residues cluster in one patch (red in Figure 2). The equivalent region is the binding site for inositol phosphates in pleckstrin (Ferguson et al., 2000), and this region of the PH domain of moesin is the binding site for a peptide (Pearson et al., 2000). Therefore, at least some PH-fold domains use this surface as a ligand binding site, and the high degree of conservation suggests that this is a functionally important region of Pob3.

A genetic screen revealed a role for the conserved surface of Pob3-M in DNA replication. HU decreases the rate of dNTP synthesis, slowing DNA replication and increasing the risk of replication-fork stalling. Therefore, mutations in many replication factors that are required for elongation or for the checkpoint response to DNA damage cause HU sensitivity (Parsons et al., 2004). Mutations that physically destabilize Pob3 increase sensitivity to HU (Schlesinger and Formosa, 2000), but this relatively mild effect is probably due to a decrease in the total yFACT activity available in the cell. To identify regions of Pob3 specifically involved in DNA replication pathways, we randomly mutagenized the entire Pob3 gene and then sought mutants that were highly sensitive to HU but were able to grow at 37°C, indicating that the mutated Pob3 protein remained stable but was unable to perform some replication function. Twenty-three independent mutants of this type were obtained, and the Pob3 locus from each was sequenced. Surprisingly, while some isolates contained multiple mutations, all 23 mutants contained either a Q308R (14 isolates) or a Q308K (9 isolates) mutation in Pob3.

Q308 is within Pob3-M, surrounded by the highly conserved patch of residues noted above (Figures 2 and 3). It is located at the end of S6, which together with S6’ forms a β ladder within a loop between strands S6 and S7 that is unusually long when compared with other PH domain folds (Figure 3). The main chain NH and CO groups of Q308 and T311 form hydrogen bonds, and the Q308 side chain is largely buried in a hydrophobic pocket. Although its orientation is not defined, one side chain N/O atom forms a hydrogen bond with the phenolic oxygen of the invariant residue Y257, and the other N/O atom is within 4.0 Å of the invariant P253 side chain and is exposed to solvent. This environment suggests that Q308 might affect the local conformation of the conserved surface patch in Pob3-M.

The pob3-Q308K mutation also causes the Spτ− phenotype (indicated by growth on medium lacking lysine; see Figure 4), indicating diminished control of transcription. Importantly, the Q308K substitution does not cause Pob3 instability, as Western blot analysis indicates that protein levels are unchanged in mutant strains relative to the wild-type (Figure S4). Further, Pob3-Q308K forms a stable heterodimer with Spt16 (see below), and we have been able to purify and determine the structure of Pob3-M(Q308K) at a resolution of 2.5 Å (Table 1). The mutant protein is essentially indistinguishable from normal Pob3-M (rmsd of 0.8 Å over 220 Cα atoms), indicating that its effects in vivo are not caused by gross structural changes. Therefore, while Q308 is at the center of a highly conserved region of Pob3-M, the Q308K mutation does not destabilize Pob3 or Spt16, but it does...
It disturb the ability of yFACT to promote both normal transcription and normal DNA replication.

To further investigate the importance of Pob3-M, we mutated several highly conserved residues. This included changing residues T252, R254, R256, and D258 simultaneously to alanines; residues K271, T272, and Y273 to EAA; deleting Q308 and Q310 simultaneously; and mutating Q308 to alanine or aspartate. These changes caused very mild or no effects and in particular did not cause sensitivity to HU (Figure S5). The high degree of conservation observed in this region of the Pob3-M surface suggests that substitutions are detrimental on an evolutionary time scale, but our results show they are tolerated briefly under laboratory conditions. Yeast cells are therefore able to perform DNA replication, and to a lesser extent transcription, fairly well when the conserved surface patch in Pob3-M is modified, but not if a basic residue is substituted at position 308. If the conserved patch is a binding site with an important function in DNA replication, this pattern of responses to mutations suggests that the binding interaction includes multiple, partially redundant sites of contact.

pob3-Q308K Is Suppressed by an Intragenic Mutation

To analyze the physiological function disrupted by the Q308K mutation, we sought suppressors of the HU sensitivity. One HU-resistant strain was found to contain both the original Q308K mutation and a new T311A change within Pob3. T311 is highly conserved (Figure 2 and Figure S2) and forms two main chain hydrogen bonds with Q308. The T311A change within Pob3 is modified, but not if a basic residue is substituted at position 308. If the conserved patch is a binding site with an important function in DNA replication, this pattern of responses to mutations suggests that the binding interaction includes multiple, partially redundant sites of contact.

Pob3-M Interacts Functionally with RPA

Analysis of one extragenic suppressor of the HU sensitivity caused by pob3-Q308K produced the surprising result of HU resistance and Spt2 (Figure S6). The defect in DNA replication caused by pob3-Q308K can therefore be separated from the defect in transcription, indicating that both pathways use the conserved patch in Pob3-M but that their requirements differ.

Figure 2. Structure of Pob3-M

(Left) Ribbon representation of Pob3-M. N-terminal (green) and C-terminal (blue) PH domains are shown. Strands S6, S9, and helix H2 (gray) are not found in a minimal PH fold. The loop between strands S6 and S7 (pink) is unusually long, includes two additional strands (S6' and S6''), and contains residue Q308 (yellow). Disordered residues are modeled as dashed lines. (Right) A surface representation of Pob3-M in a similar orientation. Surface residues that are invariant in 12 Pob3/SSRP1 homologs (Figure S2) are colored red. Only two invariant surface residues are not visible in this view (Figure S3). Q308 (yellow) is highly conserved but not invariant (Figure S2). (Bottom) The Pob3-M sequence is shown with secondary structural elements. Invariant residues are shown on a red background. Q308 and T311 are indicated with yellow dots.

Figure 3. Close-up View of Q308 and Surrounding Residues

Residues within 5 Å of Q308 are shown in stick representation. Orientation is similar to Figure 2, and colors are the same as Figure 2. The H bond between the Q308 side chain and Y257 is shown as a dashed line.
result that the suppressing mutation itself caused HU sensitivity. Thus, strains that have either the pob3-Q308K mutation or the suppressor mutation alone are HU sensitive, but a strain with both mutations is resistant. This pattern of mutual suppression can indicate that the two affected proteins cooperate to promote a similar function. Three lines of evidence show that the suppressing mutation is in RFA1, the gene that encodes the large subunit of the ssDNA binding factor RPA. First, a plasmid with only RFA1 complemented the HU sensitivity caused by the suppressor mutation. Second, the suppressor mutation was mapped and found to be about 8 cM from ADE1, consistent with the physical distance of about 12 kbp between RFA1 and ADE1. Third, the RFA1 locus from wild-type and suppressor strains was found to differ at a single site, a G262C mutation leading to an A88P change. The rfa1-A88P mutation therefore causes HU sensitivity and also almost completely suppresses the HU sensitivity caused by pob3-Q308K (Figure 4).

RPA is an essential factor in multiple facets of DNA metabolism, including replication, recombination, and repair (Bell and Dutta, 2002; Binz et al., 2004; Brill and Stillman, 1991; Iftode et al., 1999). It accomplishes these various roles partly by binding preferentially to ssDNA and partly by binding to other replication factors, including DNA Pol α/primase (Bae et al., 2003; Braun et al., 1997; Dornreiter et al., 1992; Kim and Brill, 2001). RPA has four main DNA binding domains, but none of these are affected by the A88P mutation (Figure 5A). Instead, this region forms a discrete structural domain that has been implicated in DNA damage checkpoint signaling and in protein-protein interactions (Jacobs et al., 1999). For example, the N-terminal domain of human RFA1 binds to a region of p53 (Bochkareva et al., 2005), consistent with the possibility that the A88P mutation disturbs a binding interaction.

Suppression of HU sensitivity by rfa1-A88P is allele specific. pob3-F133S and pob3-2 each cause temperature sensitivity, HU sensitivity, and the Spt^ phenotype, but none of these phenotypes were suppressed by rfa1-A88P (Figure 4). This specificity shows that rfa1-A88P does not suppress defects in Pob3 function by simply bypassing the need for yFACT function, as this would be expected to affect all pob3 mutants. Instead, rfa1-A88P specifically ameliorates a defect caused by the pob3-Q308K mutation, suggesting restored functional cooperation between Pob3 and Rfa1 in a common process that is disturbed by each single mutation.

The rfa1-A88P mutation suppresses the HU sensitivity caused by pob3-Q308K to essentially wild-type levels (Figure 4) but has only a small effect on the Spt^ phenotype (Figure 4; the double mutant remains significantly Lys^ relative to the wild-type). We interpret this to mean that the Q308K mutation causes defects in both replication (HU sensitivity) and transcription (Spt^), but only the replication defect is reversed by the rfa1-A88P mutation.

Pob3-M and RPA Interact Directly
A simple model to explain the phenotypes caused by pob3-Q308K and rfa1-A88P mutations is the following: the Pob3-M domain mediates yFACT-RPA binding, either mutation disrupts the binding, and the mutated proteins regain the ability to bind to one another. To test this model, we first determined whether purified yFACT and RPA could be coimmunoprecipitated in vitro. Antiserum generated against Pob3 protein precipitated RPA only if Spt16-Pob3 was present, showing that RPA interacts with Spt16-Pob3 (Figure 5B). In the reciprocal experiment, intact Spt16-Pob3 interacted nonspecifically with antibodies generated against Rfa1. This required
the acidic C-terminal domain of Pob3, as a spontaneous proteolytic fragment lacking this domain (Pob3*) was recovered with Rfa1 antiserum only if RPA was present, again showing direct interaction between Spt16-Pob3 and RPA, but they suggest that this interaction is weak or transient. The data do not support the model that the Pob3-M domain and the N-terminal domain of Rfa1 act as simple autonomous binding modules whose affinity is altered by the pob3-Q308K and rfa1-A88P mutations. The effects of these mutations therefore appear to be more complicated than just loss and recovery of affinity between single binding surfaces on each protein. Therefore, while a direct interaction may be important for cooperation between yFACT and RPA, neither the HU sensitivity caused by the single mutants nor the robust mutual genetic suppression appears to be a consequence of simple changes in the affinity of this interaction. Instead, the suppression may involve interactions between yFACT and RPA and other replication factors, posttranslational modifications missing from the purified proteins, or changes in protein conformation or dynamics that only occur in specific contexts, such as during S phase.

Effect of Histone Manipulations on pob3-Q308K
We next sought to determine whether the common replication function promoted by yFACT and RPA is related to the known activity of yFACT in altering the properties of nucleosomes. We previously reported that the defects caused by some pob3 alleles can be partially suppressed by increasing the ratio of H2A-H2B to H3-H4, but these same cells could not tolerate mutations that blocked acetylation of the H4 tail at positions 8 and 16 (Formosa et al., 2002), sites often associated with transcriptional regulation (Zhang et al., 1998). A similar analysis of pob3-Q308K reveals properties more consistent with defective DNA replication. The pob3-Q308K strain is able to tolerate the H4-K8R, K16R mutations, but its growth is severely impaired by H4-K8R, K16R mutations, but its growth is severely impaired by H4-K8R, K16R mutations, but its growth is severely impaired by H4-K8R, K16R mutations, but its growth is severely impaired by H4-K8R, K16R mutations, but its growth is severely impaired by H4-K8R, K16R (Figure 6A; compare rows 6–8 with row 5 on 75 mM HU, a condition that is normally permissive for a pob3-Q308K strain). Notably, the synthetic defect was especially severe with overexpression of H2A-H2B copy 2 (Figure 6B, row 8). This is the only set of histone genes in yeast that is not transcriptionally repressed by the binding responses between wild-type and mutant pairs of Pob3 and RPA (data not shown). Only a small fraction of the tagged Pob3 molecules were capable of binding RPA in these assays, and the isolated N-terminal domain of Rfa1 did not bind Pob3 (data not shown). These results are inconsistent with the simple model outlined above.

Taken together, the in vitro binding data support a direct interaction between purified Spt16-Pob3 and RPA, but they suggest that this interaction is weak or transient. The data do not support the model that the Pob3-M domain and the N-terminal domain of Rfa1 act as simple autonomous binding modules whose affinity is altered by the pob3-Q308K and rfa1-A88P mutations. The effects of these mutations therefore appear to be more complicated than just loss and recovery of affinity between single binding surfaces on each protein. Therefore, while a direct interaction may be important for cooperation between yFACT and RPA, neither the HU sensitivity caused by the single mutants nor the robust mutual genetic suppression appears to be a consequence of simple changes in the affinity of this interaction. Instead, the suppression may involve interactions between yFACT and RPA and other replication factors, posttranslational modifications missing from the purified proteins, or changes in protein conformation or dynamics that only occur in specific contexts, such as during S phase.

Figure 5. Physical Interaction between yFACT and RPA
(A) Schematic map of the three subunits of RPA with the location of the A88P mutation. The four highest affinity DNA binding domains are indicated (DBD-A, -B, -C, -D as in Bastin-Shanower and Brill [2001]).

(B) Purified RPA and Spt16-Pob3 were mixed as indicated, then immunoprecipitated with anti-Pob3 (left) or anti-Rfa1 antisera (right). Proteins from the IP were separated by SDS-PAGE, blotted, then probed with antisera generated against Rfa1 (left) or Pob3 (right). Pob3* is a spontaneous proteolytic fragment of Pob3 lacking the acidic C-terminal domain of Pob3, as a spontaneous proteolytic fragment lacking this domain (Pob3*) was recovered with Rfa1 antiserum only if RPA was present, again showing direct interaction between Spt16-Pob3 and RPA, but they suggest that this interaction is weak or transient. The data do not support the model that the Pob3-M domain and the N-terminal domain of Rfa1 act as simple autonomous binding modules whose affinity is altered by the pob3-Q308K and rfa1-A88P mutations. The effects of these mutations therefore appear to be more complicated than just loss and recovery of affinity between single binding surfaces on each protein. Therefore, while a direct interaction may be important for cooperation between yFACT and RPA, neither the HU sensitivity caused by the single mutants nor the robust mutual genetic suppression appears to be a consequence of simple changes in the affinity of this interaction. Instead, the suppression may involve interactions between yFACT and RPA and other replication factors, posttranslational modifications missing from the purified proteins, or changes in protein conformation or dynamics that only occur in specific contexts, such as during S phase.

Effect of Histone Manipulations on pob3-Q308K
We next sought to determine whether the common replication function promoted by yFACT and RPA is related to the known activity of yFACT in altering the properties of nucleosomes. We previously reported that the defects caused by some pob3 alleles can be partially suppressed by increasing the ratio of H2A-H2B to H3-H4, but these same cells could not tolerate mutations that blocked acetylation of the H4 tail at positions 8 and 16 (Formosa et al., 2002), sites often associated with transcriptional regulation (Zhang et al., 1998). A similar analysis of pob3-Q308K reveals properties more consistent with defective DNA replication. The pob3-Q308K strain is able to tolerate the H4-K8R, K16R mutations, but its growth is severely impaired by H4-K8R, K16R mutations, but its growth is severely impaired by H4-K8R, K16R mutations, but its growth is severely impaired by H4-K8R, K16R mutations, but its growth is severely impaired by H4-K8R, K16R (Figure 6A; compare rows 6–8 with row 5 on 75 mM HU, a condition that is normally permissive for a pob3-Q308K strain). Notably, the synthetic defect was especially severe with overexpression of H2A-H2B copy 2 (Figure 6B, row 8). This is the only set of histone genes in yeast that is not transcriptionally repressed by the binding responses between wild-type and mutant pairs of Pob3 and RPA (data not shown). Only a small fraction of the tagged Pob3 molecules were capable of binding RPA in these assays, and the isolated N-terminal domain of Rfa1 did not bind Pob3 (data not shown). These results are inconsistent with the simple model outlined above.
Hir/Hpc proteins (Recht et al., 1996) and is the condition that showed the most effective suppression of pob3-7 (Formosa et al., 2002). Deletion of one copy of the genes that encode histones H3-H4 was also strongly detrimental in a pob3-Q308K strain (Figure 7), whereas decreased H3-H4 gene copy number had little effect on a pob3-7 strain (Formosa et al., 2002). Aliquots of 10-fold dilutions were placed on medium with or without 5-FOA at 30°C to select for cells lacking DS1700. Deletion of H4 (4–19) is lethal in this background (line 5).

The CAF-1 complex promotes replication-dependent nucleosome deposition, and the Hir/Hpc complex promotes replication-independent deposition (reviewed in Gunjan et al., 2005). However, yeast cells lacking both CAF-1 and the Hir/Hpc complexes are viable, indicating that other deposition pathways must exist (Kaufman et al., 1998; Qian et al., 1998). If yFACT acts in such a pathway, then yFACT mutations that affect this process would be expected to display synthetic defects when paired with CAF-1 or Hir/Hpc mutations. Other pob3 mutants tested were not affected by loss of CAF-1 (Formosa et al., 2002), but a pob3-Q308K mutant displayed moderately enhanced sensitivity to low levels of HU when the Rlf2 subunit of CAF-1 was deleted (Figures 7B and 7C; compare line 4 with line 3), consistent with overlapping functions. Combining pob3-Q308K with loss of the Hir/Hpc complex has more dramatic but less readily interpreted effects. A pob3-Q308K hpc2Δ strain is inviable (data not shown), but this could be due to either of the two known functions of the Hir/Hpc complex: promoting replication-independent nucleosome deposition and regulating histone gene expression. pob3-Q308K could cause a defect in replication-dependent deposition and thereby make the replication-independent process essential. Alternatively, loss of repression by the Hir/Hpc complex causes both increased histone pool production and imbalanced histone pool production, which are each poorly tolerated by pob3-Q308K strains.

Figure 6. Effects of Histone Overexpression or Mutation

(A) Strains 8244-13-2, 8244-18-4, and 8239 (Table S1) carrying plasmid DS1700 (YCp URA3 HHT2-HHF2) were transformed with TRP1-marked plasmids with wild-type versions of both HHT2 (histone H3) and HHF2 (histone H4) or the mutation indicated (Zhang et al., 1998). Aliquots of 10-fold dilutions were placed on selective media at 30°C with or without HU (mM).
If RPA and yFACT cooperate to promote a common step in DNA replication, then RFA1 should share some genetic interaction partners with pob3-Q308K. Like a pob3-Q308K strain but unlike wild-type, an rfa1-A88P strain was unable to tolerate deletion of the N-terminal tail of H3 (Figure 6A, row 2). Further, overexpression of either H3-H4 or H2A-H2B was detrimental to the rfa1-A88P mutant but not to a wild-type strain, resulting in increased HU sensitivity for the mutant (Figure 6B). These overlapping genetic interactions between the replication factor RPA and yFACT with histones are consistent with participation of both RPA and yFACT in a replication function involving histones, presumably nucleosome deposition.

Models of Pob3-M/yFACT Function
The structure of Pob3-M reveals a patch of highly conserved residues on a surface of a PH domain that is used for ligand binding in other proteins with this fold. A mutation within this patch leaves the protein stable but causes sensitivity to the DNA synthesis inhibitor HU and also causes abnormal transcription. These two defects can be separated genetically, and the activity related to HU sensitivity involves the function of the ssDNA binding factor RPA and also requires normal levels of histone proteins and the ability to modify histone H4 at the K5 and K12 positions. yFACT can interact directly with RPA, but it is not yet clear what role this binding plays in the collaboration between these factors. A yFACT mutation and an RPA mutation that each cause HU sensitivity separately result in no HU sensitivity when combined,
and each mutant is more sensitive to HU when histone levels are altered. We interpret these observations as evidence that yFACT and RPA cooperate to perform a step in DNA replication that involves nucleosome deposition, but other possibilities must be considered.

Effects of yFACT mutations on DNA replication might be indirectly caused by altered transcription. We consider this unlikely for the following reasons. First, the HU sensitivity and the transcription phenotype are genetically separable. If the HU sensitivity resulted from a defect in transcription, then suppression of the HU sensitivity would be accompanied by restoration of normal transcriptional regulation, but two of our suppressors do not have this property. Second, pob3-Q308K strains tolerate mutations in histone H4 that prevent normal acetylation at sites associated with transcription, but they do not tolerate mutation of sites associated with nucleosome deposition. If the replication defect were an indirect effect of altered transcription, further disturbance of transcription by mutations in K8 and K16 of H4 would amplify this effect more than mutations in K5 and K12 of H4, but the opposite was observed. Third, the properties of different alleles of pob3 and their suppressors are not consistent with a strict transcription model. We have identified a variety of POB3 and SPT16 mutants by screening for temperature sensitivity (Formosa et al., 2002; Schlesinger and Formosa, 2000). Essentially all of the Ts’ alleles also caused the Spt− phenotype, but only a small subset caused HU sensitivity. Importantly, the strengths of these phenotypes did not correlate with one another. If HU sensitivity resulted only from the most severe defects in transcription, then the alleles that cause HU sensitivity should also be those with the strongest Spt− phenotype, but this is not the case. These observations do not rule out an indirect transcription model but are more consistent with our interpretation that the conserved patch in Pob3-M is important for both replication and transcription for independent reasons.

Another possible indirect explanation for the genetic interaction between yFACT and RPA is that it could involve checkpoint control. Yeast cells respond to HU by triggering a DNA damage checkpoint that uses RPA as a sensor and the protein kinases Mec1 and Rad53 as signal transducers, leading to induced transcription of DNA repair factors and ribonucleotide reductase, the target of HU inhibition (Zou and Elledge, 2003). rfa1-A88P cells could be sensitive to HU because they do not sense the damage, and pob3-Q308K cells could be sensitive to HU because they do not induce the transcriptional response. However, it is not obvious why these defects would suppress one another. One explanation is that the checkpoint response is somehow detrimental in pob3-Q308K cells and that rfa1-A88P rescues the cells by preventing checkpoint activation. Any other mutation in the checkpoint pathway should then also suppress, but we find that neither rad53 nor mec1 mutations have this effect (Figure S6). Instead, rad53 pob3-Q308K double mutants have a severe growth defect and display the same extreme sensitivity to HU as rad53 single mutants (Figure S6). The pob3-Q308K mutation therefore does not suppress and is not suppressed by other checkpoint defects; instead, it causes increased dependence on the DNA damage checkpoint.

Our observations are more consistent with a direct role for yFACT in nucleosome deposition during replication, at a step that also involves the function of RPA. Direct binding observed between purified yFACT and RPA supports such a model, although the behavior of Rfa1-A88P and Pob3-Q308K proteins in binding assays does not conform to the predictions of a simple model in which the robust genetic suppression observed is due to restoration of disrupted binding affinity. This could indicate that the in vitro binding assays do not fully capture the in vivo context of the interaction, or that, instead of disturbing binding affinity, the mutations disrupt some function by altering the geometry or dynamics of binding. An attractive possibility is that yFACT and RPA interact through another factor, perhaps Pol2/primase, which is known to bind directly to both yFACT and RPA (Braun et al., 1997; Domreiter et al., 1992). Other candidates for a coordinating factor are suggested by the recent results linking yFACT to the GINS and MCM complexes, placing yFACT in a context central to the regulation of DNA replication (Gambus et al., 2006). The Pob3-M structure and the various mutant alleles of yFACT and RPA described here will be valuable tools for further dissecting the functional role or roles of these factors in promoting chromatin-dependent processes.

Experimental Procedures

Yeast Methods

Media, strains, and plasmids used are described in the Supplemental Data and in Table S1.

Protein Purification and Structure Determination

Yeast Pob3-M was expressed in E. coli Codon+ (RIL) cells (Stratagene) using a modified PET expression vector encoding an eight-residue, N-terminal histidine tag followed by a TEV protease cleavage site. TEV cleavage results in a protein whose N terminus is GHM, where the M is M220 of the native Pob3 sequence. Pob3-M was purified by nickel affinity chromatography (Qiagen) followed by an overnight digestion with TEV protease and a second round of nickel affinity chromatography to remove His-tagged TEV and any uncleaved Pob3-M. Cleaved Pob3-M was further purified by gel filtration on a Superdex-200 column (Pharmacia), with peak fractions eluting as an apparent monomer. Protein was concentrated to 15 mg/ml in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5% glycerol, 1 mM DTT, using a Vivaspin concentrator (Millipore). The native sequence of Pob3-M contains only two methionine residues, including M220 at the extreme N terminus. To increase the potential anomalous signal, we mutated 397LVL397 to MMVM. Selenomethionine-substituted protein behaved like the native protein in both purification and crystallization.

Single plate-like crystals (300 x 150 x 40 µm) of Pob3-M were grown by the vapor-diffusion method in sitting drops using a reservoir solution containing 21% PEG 3350, 20% glycerol, 200 mM NaCl, 50 mM ammonium sulfate, 100 mM Tris-HCl (pH 7.5) over a period of 3 weeks. Single crystals were flash frozen directly from the mother liquor in liquid nitrogen. Initial diffraction quality was poor (~4 Å resolution with very high mosaicity) but was improved through successive rounds of crystal annealing. Annealing was performed by removing the looped crystal from the cryostream, allowing it to warm at room temperature for 1 min, then returning it to the cryostream. Most crystals only showed a modest improvement in diffraction quality, but occasionally crystals showed a marked improvement.

SAD data were collected at the NSLS beamline X26-C on a crystal of selenomethionine-substituted Pob3-M and were processed with DENZO and SCALEPACK (Otwinski and Minor, 1996). The crystals belong to spacegroup P2,2,2 (a = 57.1, Å, b = 57.8, Å, c = 156.6 Å) and contain two molecules in the asymmetric unit. A total
of eight Se sites were located using SOLVE (Terwilliger and Berz-zen, 1999), and an initial model was built into the experimental elec-
tron-density maps using RESOLVE (Terwilliger, 2003). Subsequent
model building was carried out using COOT (Emsley and Cowtan,
2004), and refinement with TLS parameters was performed using
REFMAC implemented in CCP4 (CCP4, 1994). TLS groups were gen-
erated using the TLSMD server (Panner and Merritt, 2006). Crystallo-
graphic statistics are given in Table 1.

Poib3-M(Q308K) was purified and crystallized using the same
methods as for the wild-type protein. The crystals belonged to a
related space group but, unlike wild-type, were not annealed prior
to data collection. Initial phases were obtained via molecular
replacement using Phaser implemented in CCP4 (CCP4, 1994) utiliz-
ing the wild-type structure as a search model. Refinement of the
model against 2.55 Å data utilizing TLS parameters resulted in an
R factor of 22.0% with an Rfree of 30.3%. Despite the relatively high
Rfree values, simulated annealing on the model with good agreement.

Mutagenesis of Poib3 and Isolation of Suppressors

Primers that anneal about 200 bp outside of the Poib3 insert in plas-
mid pTF139 (Schlesinger and Formosa, 2000) were used to modify
the Poib3 gene using standard PCR conditions. Yeast strain 7787-
4-4 pTF138, with a deletion of POB3 was transformed along with
the URA3 and POB3 genes, which was transformed with a mixture of the
PCR product and the vector YCplac111 (Gietz and Sugino, 1988) di-
gusted with HindIII and EcoRI. Leu + transformants were replica
plated from yeast strain 7787-4-4 pTF138, with a deletion of POB3
growth with 1%–5% of the colonies with the Spt phenotype. These
cells were subjected to forward and reverse mutants with the Spt− phenotype were studied further in this screen. About 500
mutants were then screened for other phenotypes, including Ts−
and ability to grow on medium containing 200 mM HU. Twenty-three
strains that were Ts− at 37°C and tightly HU sensitive were chosen,
the plasmids were isolated by transformation of bacteria, and the
inserts were sequenced.

For integration into the genome, the Poib3 locus with the Q308K
mutation was transferred from the pTF139 plasmid to a similar plas-
mid lacking an origin of replication and containing the URA3 marker.
This was integrated into the yeast genome, and then 5-FOA-resis-
tant colonies that were HU sensitive were tested for popout of the
plasmid, leaving behind the Q308K mutation in an otherwise
unchanged cell. The Poib3 locus was amplified by PCR and sequenced
to ensure accurate excision of the integrated plasmid. To isolate
suppressors, aliquots of these cells were placed on medium con-
taining 200 mM HU, and papillae with suppressing mutations were
isolated for further analysis.

Immunoprecipitation and Nickel Chelation Pull-Downs

RPA was purified as described (Henrickson et al., 1994), and 10 ng
(1 µM final concentration) was incubated for 1 hr at 4°C in binding
buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA,
and 0.3% Triton X-100), either with or without 16 ng (1 µM) of purified
Spt16-Poib3 (Rhoades et al., 2004), in a final volume of 90 µl. One
microliter of antiserum against purified Poib3 (Covance) was added and incubated with mixing for 1 hr at 4°C. Ten microliters
of a slurry of magnetic beads conjugated with protein A (New
England Biolabs) was added and incubated with mixing for 1 hr
at 4°C, and then the beads were collected using a magnet. For nickel
chelation, aliquots of these cells were placed on medium con-
aining 200 mM HU, and papillae with suppressing mutations were
isolated for further analysis.

Supplemental Data

Supplemental Data include six figures and one table and can be
found with this article online at http://www.molecule.org/cgi/
content/full/22/3/363/DC1/.

Acknowledgments

We thank Matthew Weber, Danny Gibbs, Susan Ruone, Amanda
Butler, and Peter Winter for technical assistance; Bob Schackmann
and the University of Utah Biotechnology Core Facility for N-terminal
peptide sequencing; Sharon Dent and David Stillman for providing
plasmids; and Brad Cairns, Jacqui Wittmeyer, and David Stillman
for critical comments on this manuscript. Operations of the National
Syntrophon Light Source (NSLS) are supported by the U.S. Depart-
ment of Energy, Office of Basic Energy Sciences, and by the National
Institutes of Health (NIH). Data collection at the NSLS was funded
by the National Center for Research Resources. This work was sup-
ported by NIH grants GM076242 (C.P.H) and GM064649 (T.F.), and
American Cancer Society grant PF0304001GMC (A.P.V.).

Received: August 24, 2005
Revised: January 10, 2006
Accepted: March 21, 2006
Published: May 4, 2006

References

Allain, F.H., Yen, Y.M., Masse, J.E., Schultz, P., Dieckmann, T.,
Johnson, R.C., and Feigon, J. (1999). Solution structure of the
HMG protein NHLP6A and its interaction with DNA reveals the struc-
tural determinants for non-sequence-specific binding. EMBO J. 18,
2563–2579.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z.,
Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST:
a new generation of protein database search programs. Nucleic
Acids Res. 25, 3389–3402.

Bae, K.H., Kim, H.S., Bae, S.H., Kang, H.Y., Brill, S., and Seo, Y.S.
(2003). Bimodal interaction between replication-protein A and
Dna2 is critical for Dna2 function both in vivo and in vitro. Nucleic
Acids Res. 31, 3006–3015.

four DNA binding domains of replication protein A. The role of
RPA2 in ssDNA binding. J. Biol. Chem. 276, 36446–36453.


Dev. 14, 139–146.

Belotserkovskaya, R., Oh, S., Bondarenko, V.A., Orphanides, G.,

tein A phosphorylation and the cellular response to DNA damage.
DNA Repair (Amst.) 3, 1015–1024.

The yeast FACT complex has a role in transcriptional initiation.

Bochkareva, E., Kaustov, L., Ayed, A., Yi, G.S., Lu, Y., Pineda-
Lucena, A., Liao, J.C., Okorokov, A.L., Milner, J., Arrowsmith, C.H.,
transactivation domain interaction with replication protein A. Proc.
Natl. Acad. Sci. USA 102, 15412–15417.

of murine replication protein A in DNA replication and transcrip-

rum cerevisiae is encoded by three essential genes coordi-
nately expressed at S phase. Genes Dev. 5, 1589–1600.

Budd, M.E., Tong, A.H., Polaczek, P., Peng, X., Boone, C., and
Campbell, J.L. (2005). A network of multi-tasking proteins at the


Accession Numbers

Protein Data Bank entry codes are 2GCL for wild-type Pob3-M and 2GCJ for the Q308K mutant.