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Structural and Functional Analysis of the Spt16p N-terminal Domain Reveals Overlapping Roles of yFACT Subunits*[S]

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yFACT (heterodimers of *Saccharomyces cerevisiae* Spt16-Pob3 combined with Nhp6) binds to and alters the properties of nucleosomes. The essential function of yFACT is not disrupted by deletion of the N-terminal domain (NTD) of Spt16 or by mutation of the middle domain of Pob3, but either alteration makes yeast cells sensitive to DNA replication stress. We have determined the structure of the Spt16 NTD and find evidence for a conserved potential peptide-binding site. Pob3-M also contains a putative binding site, and we show that these two sites perform an overlapping essential function. We find that yFACT can bind the N-terminal tails of some histones and that this interaction is important for yFACT-nucleosome binding. However, neither the Spt16 NTD nor a key residue in the putative Pob3-M-binding site was required for interactions with histone N termini or for yFACT-mediated nucleosome reorganization *in vitro*. Instead, both potential binding sites interact functionally with the C-terminal docking domain of the histone H2A. yFACT therefore appears to make multiple contacts with different sites within nucleosomes, and these interactions are partially redundant with one another. The docking domain of H2A is identified as an important participant in maintaining stability during yFACT-mediated nucleosome reorganization, suggesting new models for the mechanism of this activity.

yFACT (yeast facilitator of chromatin transcription or transactions) is a heterodimer of the *Saccharomyces cerevisiae* Spt16 and Pob3 proteins that is assisted *in vivo* and *in vitro* by the high mobility group type B domain DNA-binding protein Nhp6 (1, 2). *In vitro*, yFACT binds to histones (3, 4) and can alter the accessibility of DNA within nucleosomes without hydrolyzing ATP and without repositioning the histone octamer core relative to the DNA (5–7). This activity is different from ATP-dependent chromatin remodeling and has been called nucleosome reorganization (6). yFACT and related FACT complexes from other eukaryotes are needed for both normal regulation of transcription (5, 8–11) and for DNA replication (12–20). Reorganization activity therefore appears to be important in a range of chromatin-based processes, including initiation and elongation of transcription, establishment and maintenance of normal chromatin, and survival during DNA replication stress. Consistent with this broad functional importance, FACT family members have been found in all eukaryotes examined, and at least one of the subunits is essential for viability in all cases reported (9, 21, 19, 22).

FACT complexes contain several distinct structural domains (16, 23), but little is known about how these domains contribute to FACT function. The middle domain of Pob3 (Pob3-M) forms two pleckstrin homology (PH)[4] folds that are closely juxtaposed (23), with highly conserved surface residues forming a patch in a region often associated with binding sites in PH domain proteins (23). Altering this patch caused increased sensitivity to hydroxyurea (HU) (23), a toxin that blocks dNTP synthesis and therefore causes replication stress. This suggests that the Pob3-M domain contributes to a binding interaction that is of increased importance when yeast cells encounter replication stress. Consistent with a role as a protein-binding module, Pob3-M was shown to interact physically and genetically with Rfa1 (23), a subunit of the eukaryotic single-stranded DNA binding factor RPA. yFACT and RPA appear to have overlapping functions in a process that affects nucleosome deposition during DNA replication (23). However, mutations in the conserved putative interaction surface on Pob3-M did not disrupt the yFACT-RPA interaction *in vitro* (23). Pob3-M may therefore have multiple binding partners, with each interaction contributing to different functions of yFACT in different contexts.

The N-terminal domain (NTD) of Spt16 forms an independent structural unit (16, 23). Surprisingly, although this domain is conserved among all known Spt16 homologs, it is not essential for viability in yeast cells, although it is required for normal growth in the presence of high levels of HU (16). The Spt16 NTD shares limited sequence similarity with a class of aminopeptidases, but it does not have peptidase active site residues (see Ref. 24 and this study). These observations suggest that the

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The atomic coordinates and structure factors (code 3BIP, 3BIQ, 3BIT) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Methods, Figs. S1–S5, and Table S1.
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[4] The abbreviations used are: PH, pleckstrin homology; WT, wild type; HU, hydroxyurea; NTD, N-terminal domain; TEV, tobacco etch virus; PDB, Protein Data Bank; 5-FOA, 5-fluoroorotic acid.

Spt16 NTD may have peptide binding activity but that this role is dispensable for the core activity of yFACT.

Here we report the structure of the Spt16 NTD and the results of studies examining the role of this domain in yFACT function. The Spt16 NTD is structurally similar to aminopeptidases, and the most highly conserved surface residues line a cleft equivalent to the aminopeptidase substrate-binding site. The presence of potential peptide-binding sites in each subunit of yFACT led us to examine candidate substrates, initially focusing on the attractive possibility that these sites bind to the N-terminal tails of histones that extend beyond the structured core of the nucleosome and are known to influence yFACT functions *in* vivo (6, 25). The N-terminal tails of histones were found to have an important role in yFACT function and to be bound by yFACT with high affinity. However, this activity and other measurable functions of yFACT *in vitro* remained intact after mutating Pob3-M or deleting the Spt16 NTD. Instead, the Spt16 NTD and Pob3-M domains were found to have overlapping roles in a process that involves the C-terminal extension of H2A. This stirrup-like "docking domain" of histone H2A acts to stabilize the binding of H2A-H2B dimers to $(H3\text{-}H4)_2$ tetramers within histone octamers (26–28). These results suggest that yFACT makes multiple additive contacts with nucleosomes during reorganization, and that some of these contacts are important after yFACT has induced the reorganization of the nucleosome. The docking domain of H2A is identified as an important contributor to this process, perhaps tethering nucleosomal components together or controlling the insertion of H2A-H2B dimers during nucleosome formation.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—DNA fragments encoding Spt16 residues 1–451 or 1–465 were amplified by PCR and inserted into a modified pET bacterial expression vector that fused eight histidines and a TEV protease site to the N termini. TEV cleavage leaves the sequence "GHM . . . " at the N terminus in place of the native methionine. Each protein was expressed in Codon+(RIL) cells (Stratagene) and purified by nickel chelation chromatography (Qiagen). After TEV protease digestion and nickel chelation chromatography to remove tagged N termini, proteins were further purified by gel filtration on Superdex-200 (GE Healthcare) in 10 mM HEPES, pH 7.5, 150 mM NaCl, 2% glycerol, 1 mM 2-mercaptoethanol. The Spt16 NTD fragments eluted as apparent monomers and were then concentrated in gel filtration buffer.

Spt16-Pob3 complexes with 12 histidines and a TEV site fused to the N terminus of Pob3 were purified from yeast cells overexpressing each protein from the *Gal1* promoter after growth in galactose medium, as described (19). Complexes were purified from extracts using nickel chelation and gel filtration as above, except using Sephacryl S300 (GE Healthcare) and omitting the TEV cleavage.

Nucleosomes were prepared by dialysis from high ionic strength solutions using derivatives of a sea urchin rDNA nucleosome positioning sequence and chicken histones or bacterially expressed yeast histones, as described previously (7; see the supplement material for details).

Crystallography—Single plate crystals of Spt16-(1–451) were grown at 4 °C over 2–3 weeks by sitting drop vapor diffusion against a reservoir solution of 200 mM NaCl, 100 mM sodium acetate, pH 4.5, and 35% polyethylene glycol 300. The drop consisted of 1 μl of protein concentrated to 10–15 mg/ml, 1 μl of reservoir solution, and 0.4 μl of 100 mM cysteine. Selenomethionine-substituted Spt16-(1–451) was expressed as described (29) and then purified and crystallized using conditions similar to those described above. Crystals were cryoprotected by emersion in reservoir solution made up with 25% glycerol and then flash-frozen in liquid nitrogen. SAD data were collected at National Synchrotron Light Source beamline X26-C and processed with HKL2000 and SCALEPACK (30). Nine of the 10 possible selenium positions in the two molecules in the asymmetric unit were located by SOLVE (31), and an initial model was built into the experimental electron density maps using RESOLVE (32).

Two visually indistinguishable but distinct crystal forms (space groups P1 and $P2_1$) of native Spt16-(1–465) were grown overnight at 22 °C from a single drop with 1.2 μl of 14 mg/ml protein solution, 1.2 μl of reservoir solution, and 0.4 μl pentanediol by vapor diffusion against a reservoir solution of 25% pentaerythritol ethoxylate (15/4 EO/OH) and 100 mM sodium acetate, pH 4.5. Data were collected and processed as above. The refined model of Spt16-(1–451) was used as a starting model for molecular replacement (PHASER; 33) into both native datasets. Model building for all structures reported here was performed using COOT (34). Refinement used REFMAC implemented within CCP4i (35), and the TLSMD server to generate TLS parameters (36). The structures have been deposited into the Protein Data Bank under the codes 3BIP, 3BIQ, and 3BIT.

Genetic Methods—Strains used are described in supplemental Table S1. Standard yeast methods and media were as described (37).

RESULTS

Structure of the Spt16 NTD—The boundaries of the Spt16 NTD were determined as described previously (16, 23), and soluble fragments, including residues 1–451 and 1–465, were expressed and crystallized. Experimental phases were determined by the SAD method using selenomethionine-substituted protein, and the model was refined to R_{factor}/R_{free} values of 18.0/22.3% against data to 1.9 Å resolution (crystallographic statistics are given in Table 1). This model was used in molecular replacement calculations to determine two different crystal forms of native Spt16 NTD that were refined to R_{factor}/R_{free} values of 17.6/24.4% and 15.1/21.0% against data to 1.94 and 1.75 Å resolution, respectively. The five independent Spt16 NTD molecules in these three crystal forms displayed only minor differences, with a maximum root mean square deviation of 0.7 Å for overlap on 440 pairs of C-α atoms in pairwise comparisons.

Comparison with Other Structures—The Spt16 NTD includes a smaller N-terminal lobe (residues 1–175) and a larger C-terminal lobe (residues 176–447; Fig. 1). The extensive packing between the lobes and the similarity of crystallographically independent molecules suggest that the two lobes maintain a fixed relative orientation in solution. The N-termi-

TABLE 1

Data collection and refinement statistics

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

	Native form I	Native form II	Selenomethionine form III
Data collection			
Space group	P1	$P2_1$	$P2_1$
Cell dimensions (Å)	a = 51.1, b = 60.1, c = 85.9	a = 60.3, b = 50.8, c = 79.2	a = 40.7, b = 144.6, c = 88.5
Unit cell angles (°)	α = 72.6, β = 77.1, γ = 89.9	β = 107.1	β = 101.4
Resolution (Å)	50-1.94	50-1.75	40-1.90
Detector edge shell (Å)[a]	2.44-2.30	2.38-2.20	
Resolution outer shell (Å)	2.01-1.95	1.81-1.75	1.97-1.90
No. of observations	361,089	522,903	3,070,941
No. of unique reflections	51,635	33,504	73,990
R_{sym} (%)[b]	7.2 (40.7)	10.2 (32.6)	9.6 (45.2)
$I/\sigma(I)$	22.5 (3.1)	15.7 (2.4)	20.3 (2.4)
Completeness to detector edge (%)	93.2	98.4	
Completeness to outer shell (%)	73.1 (24.9)	71.8 (12.4)	94.0 (69.6)
Refinement			
R_{work}/R_{free} (%)[c,d]	17.6/24.4	15.1/21.0	18.0/22.3
No. of atoms			
Protein	7,321	3,643	7,396
Solvent	732	422	1,045
Average isotropic B-factor(Å)^2	26.3	29.4	40.7
Ramachandran plot (non-Gly)			
Most favorable region (%)	91.6	91.3	91.6
Allowed region (%)	8.3	8.7	8.4
Generous allowed region (%)	0.1	0.0	0.0
Disallowed region (%)	0.0	0.0	0.0
Root mean square deviations			
Bond lengths (Å)	0.011	0.012	0.016
Bond angles (°)	1.346	1.314	1.465

[a] Detector edge outer shell represents the bin of data that extended to the edge of the detector. Upon reexamination of the data, it was found that quality data could be obtained in the corner of the diffraction images. The added data were of high quality but limited in completeness; therefore, we report completeness statistics both to the detector edge and including the higher resolution data. The higher resolution data were included throughout structure refinement, and refinement statistics (including R values) were calculated using all the data.

[b] $R_{sym} = (|(\Sigma I - \langle I \rangle|)/(\Sigma I)$, where $\langle I \rangle$ is the average intensity of multiple measurements.

[c] $R_{work} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$

[d] R_{free} = the cross-validation R factor for 5% of reflections against which the model was not refined.

nal lobe structure is similar to the RuvC/RNase H family, with a root mean square deviation of 3.3 Å for overlap with RuvC (38; PDB 1HJR) over 89 pairs of C-α atoms (supplemental Fig. S1). Structures in this family include a 3-layer $\alpha/\beta/\alpha$ sandwich in which the central β-sheet is composed of five strands (order 32145) that, with the exception of strand 2, are parallel to each other. The RuvC active site is formed by a cluster of four acidic residues that lie in a deep cleft formed by both the β-sheet and the helical regions on one side of the sheet. The Spt16 NTD lacks these catalytic residues and contains an additional helix (residues 36–62) that fills this cleft. Furthermore, the charged nucleic acid binding surface of RuvC and RNase H is not conserved in the Spt16 NTD. The limited structural similarity therefore does not indicate a shared biochemical activity of Spt16 with RuvC/RNase H, consistent with the earlier finding that yFACT does not bind DNA in affinity purification or electrophoretic mobility shift experiments (6, 19).

The Spt16 NTD C-terminal lobe adopts a "pita bread" fold, in which two $\alpha\alpha\beta\beta\beta$ motifs associate with approximate 2-fold symmetry to form a half-barrel structure (Fig. 1). This architecture is also found in the aminopeptidases that share limited sequence similarity with the Spt16 NTD, including methionine aminopeptidase, prolidase, and aminopeptidase P. Other structurally similar proteins include creatinase (39, 40) and the ErbB-3 receptor-binding protein (Ebp1; 41, 42). The C-terminal lobe shows closest structural similarity to the prolidase enzyme from *Pyrococcus furiosus* (PDB 1PV9; 43), which overlaps with a root mean square deviation of 2.0 Å over 215 pairs of C-α atoms that share 23% sequence identity (Fig. 2).

The cleft formed at the open side of the half β-barrel in enzymes with a pita bread fold is often found to house the active site. The Spt16 NTD displays a cleft at this position that measures ~22 × 20 Å and is ~10 Å deep. A portion of this cleft is loosely covered by a loop (residues 267–276), forming a short tunnel or covered region within the cleft (*orange* in Figs. 1 and 2). Surface residues that are evolutionarily conserved among Spt16 homologs cluster within the tunnel region and the adjacent section of the cleft (see Fig. 5 below), suggesting that this region is functionally important in Spt16.

Prolidase, methionine aminopeptidase, and aminopeptidase P are peptidases that cleave Xaa-Pro dipeptides, Met-Xaa, and Xaa-Pro peptides, respectively. Consistent with the related chemistry, their active sites are similar structures that feature a dinuclear metal ion cluster. Creatinases are also hydrolases that cleave a C–N bond, although in this case creatine is cleaved to sarcosine and urea. Notably, although ligand complex structures demonstrate that the creatinase and peptidase active sites overlap spatially (Fig. 2), creatinase uses a histidine side chain rather than coordinated metal ions to drive catalysis. Spt16 does not contain either the metal ion coordinating residues of the peptidases or the histidine of creatinase. Additionally, yFACT purified from yeast cells was not found to contain metal atoms,[5] and neither yFACT nor the Spt16 NTD displayed peptidase activity with a variety of substrates (see supplemental material).[6] We therefore disfavor the model that Spt16 NTD is

[5] D. Winge, University of Utah, personal communication.

[6] M. Rechsteiner, University of Utah, personal communication.

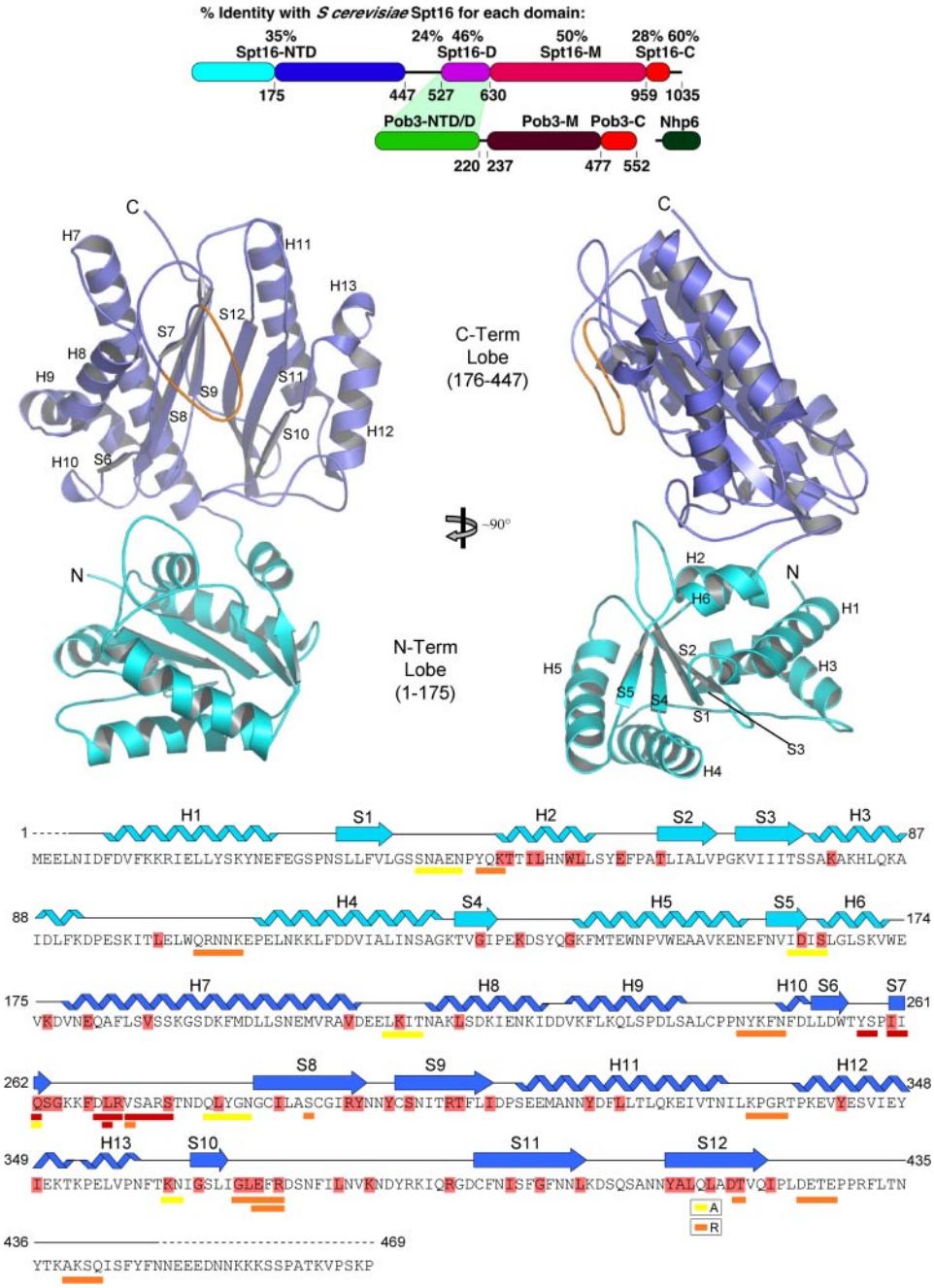

FIGURE 1. **Structure of the Spt16 NTD.** *Top,* the residues in each structural domain of Spt16-Pob3 are indicated (16, 23). 31 Spt16 homologs chosen to include the full spectrum of eukaryotes were aligned and found to be about 40% identical to the yeast sequence overall. The percent identity to the *S. cerevisiae* sequence varied for each domain as indicated. All homologs included the NTD. The C-terminal domain is broken into two regions for this calculation, a less conserved but 50% acidic region (960–1008) and a more highly conserved and neutral region (1009–1029). *D* indicates the dimerization interface. *Middle,* ribbon diagrams of the Spt16 NTD structure. The N-terminal lobe is shown in *turquoise*; the C-terminal lobe containing the putative binding cleft in *blue*, and the loop that encloses the cleft in *orange*. Labels indicate the secondary structure features. *Bottom,* secondary structures within the Spt16 NTD are aligned with the sequence. Residues that are conserved among over 70% of the 31 Spt16 homologs compared are highlighted in *red*. *Bars* below the text indicate regions targeted for site-directed mutagenesis, coded according to the severity of the synthetic defect with *pob3-Q308K* (Table 2).

an enzyme, although the remarkable divergence of the peptidase and creatinase active sites cautions that this possibility cannot be completely discounted. Our preferred hypothesis is that the Spt16 NTD groove is a binding site, perhaps for a peptide ligand from a nucleosome or other associated protein. Like the Spt16 NTD, the pita bread fold domain of Ebp1 appears to lack enzymatic activity but retains a distinctive cleft that might function as a binding site (41, 42).

The structural similarity with prolidase and aminopeptidase P is not limited to the Spt16 NTD C-terminal lobe but also extends over the N-terminal lobe. In these peptidases, the N-terminal lobe mediates oligomerization (forming tetramers of aminopeptidase P and dimers of prolidase; see 43). Despite the structural similarity, it seems unlikely that the Spt16 NTD promotes analogous self-association because relevant interactions are not seen in the crystal structures; analytical ultracentrifugation shows that yFACT is a heterodimer of 1:1 stoichiometry (19); the Spt16 NTD is not required for the interaction between Spt16 and Pob3 (16, 23 and see below); and both analytical ultracentrifugation and gel filtration indicate that the isolated Spt16 NTD fragment is a monomer even at very high protein concentrations (not shown). Notably, although monomeric aminopeptidases exist that are missing the N-terminal lobe (methionine aminopeptidase, for example; PDB 1MAT, see Ref. 44), no Spt16 homologs lacking the N-terminal lobe have been identified. The N-terminal lobe of Spt16 may therefore act as a protein-protein interaction domain but presumably with partners outside of the yFACT complex.

Functional Overlap between the Spt16 NTD and Pob3-M—Although the NTD represents 43% of the *SPT16* gene, and deletion of the entire gene is lethal (9), yeast cells survive deletion of residues 2–484 (16). We confirmed this result in different genetic backgrounds by constructing a genomic deletion allele that removes residues 2–468 but causes no other alteration of the genome. Strains with this allele of *SPT16* (*spt16-ΔNTD*) grew normally under a variety of conditions, although they displayed a moderate retardation of growth when exposed to high concentrations of HU, a toxin that causes DNA replication stress by inhibiting deoxynucleotide synthesis (supplemental Fig. S2). Screens for additional phenotypes caused by deletion of the NTD revealed only weak effects (supplemental material). Nota-

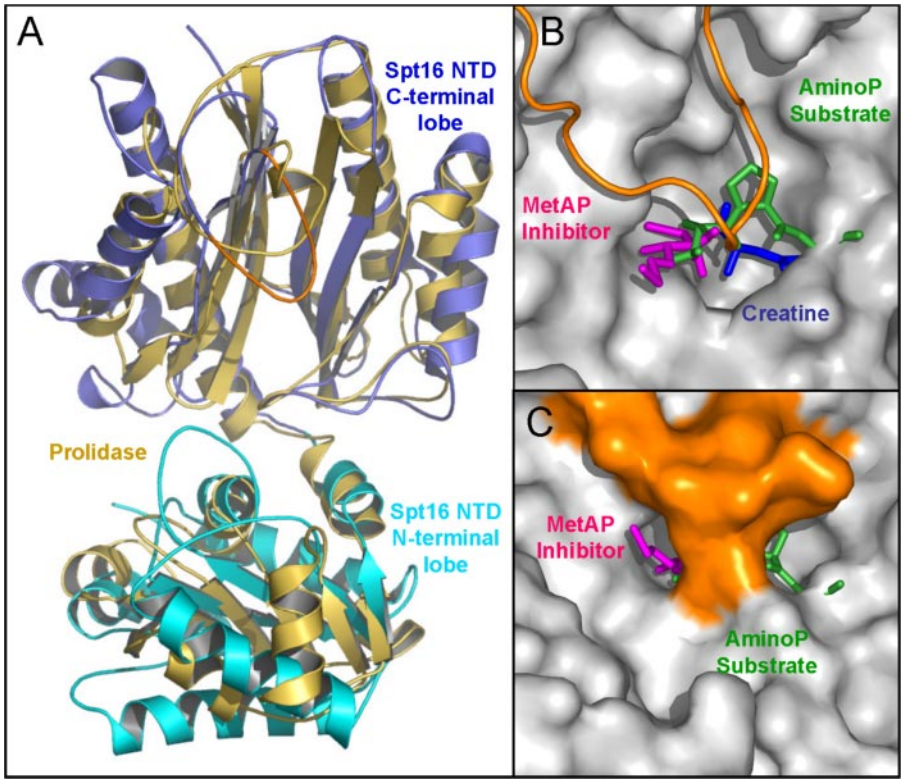

FIGURE 2. **The Spt16 NTD aligns with other pita bread fold proteins.** *A,* Spt16 NTD (*turquoise* and *blue*) is superimposed with prolidase (*yellow*). The loop of Spt16 that encloses the putative binding cleft is colored *orange. B* and *C,* a peptide substrate in aminopeptidase P (*AminoP*) (*green*), an inhibitor of methionine aminopeptidase (*MetAP*) (*pink*), and the substrate creatine in creatinase (*blue*) are superimposed on the Spt16 NTD structure. The surface of the loop is omitted in *B* and included in *C* to view different aspects of the tunnel and cleft.

bly, the strains were able to grow normally at elevated temperatures and displayed only a very weak Spt$^-$ phenotype (Fig. 3; the Spt$^-$ phenotype is assayed here as growth on media lacking lysine because of use of aberrant transcription initiation sites within the *lys2-128∂* allele; see Ref. 45). This shows that deletion of the NTD does not destabilize the protein significantly and leaves yFACT able to perform its role in regulating transcription initiation site selection normally. The stability of the Spt16-ΔNTD protein was also confirmed by Western blotting (supplemental Fig. S4). The Spt16 NTD is therefore dispensable under normal laboratory growth conditions but provides a significant selective advantage in some contexts.

Pob3-M forms a double-PH fold with invariant residues clustered at a potential binding surface (23). A Q308K substitution within this surface leads to a stably folded Pob3 protein, but causes sensitivity to high levels of HU and a strong Spt$^-$ phenotype (23) (Fig. 3). These phenotypes show that the normal features of this putative binding pocket on the surface of the Pob3-M domain are needed during replication stress and during transcription. Because *spt16-ΔNTD* and *pob3-Q308K* mutations each affected potential peptide-binding sites, we examined double mutants to see if they affected redundant functions. Double mutants in the W303 genetic background required a plasmid with the WT version of *SPT16* for survival (Fig. 3), indicating that the combination of mutations is lethal. The Spt16 NTD and Pob3-M domains are therefore at least partially redundant for providing an essential function.

Functional overlap between the Spt16 NTD and Pob3-M was also observed in strains from the A364a genetic background, although in this case the double mutant was weakly viable, allowing the defect to be examined more carefully (Fig. 3*B*). Double mutants in A364a displayed a growth defect at all temperatures, inviability at elevated temperatures, and the inability to tolerate even very low levels of HU (Fig. 3*B*). The Spt$^-$ phenotype caused by *pob3-Q308K* was not altered by the loss of the Spt16 NTD, consistent with previous data showing that transcriptional regulation is not significantly affected by deletion of the Spt16 NTD (16).

Although we favor the interpretation that the synthetic defect caused by combining *spt16-ΔNTD* and *pob3-Q308K* mutations is because of loss of redundant functions, an alternative explanation is that the Spt16 NTD and Pob3-M domains interact with one another in a way that stabilizes the proteins. The isolated Spt16 NTD and Pob3-M did not copurify when expressed together (16, 23), and no interaction was detected between the fragments by equilibrium sedimentation (not shown). Furthermore, we found that the Spt16-ΔNTD fragment forms a stable heterodimer with Pob3-Q308K protein even in yeast cells shifted to 37 °C for several hours (see below and supplemental Fig. S4). We therefore conclude that the double mutant yFACT complex Spt16-ΔNTD-Pob3-Q308K lacks an important activity but is structurally intact and stable.

Point Mutations Reveal Functional Overlap of the Spt16 NTD Cleft with Pob3-M—We used site-directed mutagenesis to examine the importance of specific residues in the function of the Spt16 NTD, focusing on surface residues to avoid destabilization of the structure (Table 2). A strain with the entire *SPT16* locus deleted from the genome was constructed and kept alive with a plasmid carrying *SPT16* and *URA3* genes. Mutations in *SPT16* were introduced into a low copy plasmid marked with *LEU2*; transformants with both WT and mutated plasmids were obtained, and strains with only the mutated *SPT16* gene were derived by selection on medium containing 5-FOA, which is toxic to cells with the *URA3* gene (46). Most of the mutations had no effect on growth under the conditions tested, although some caused a mild Spt$^-$ phenotype (Table 2 and supplemental Fig. S3). In particular, the substitutions of surface residues did not cause temperature sensitivity and did not significantly destabilize the Spt16 protein as assayed by Western blotting (supplemental Fig. S4; consistent with previous reports, mutations that perturb the hydrophobic core of the

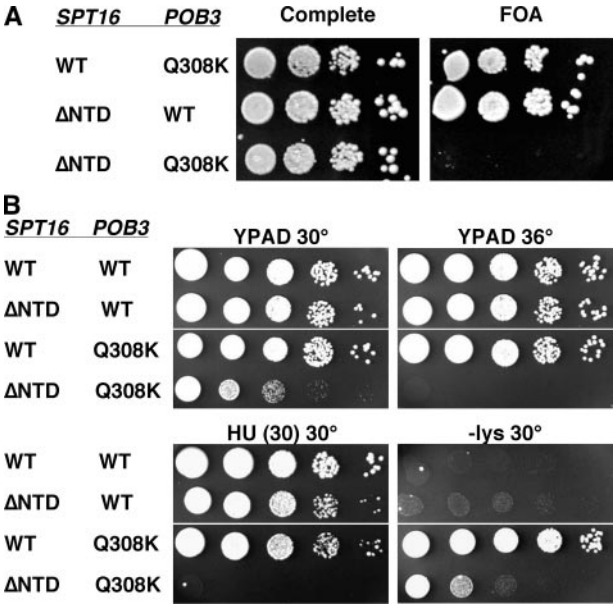

FIGURE 3. **Simultaneous deletion of the Spt16 NTD and mutation of Pob3-M causes a severe synthetic defect.** *A,* strains DY10890, DY11923, and DY12431 (W303 background) with the genotypes indicated and carrying a low copy plasmid with the *POB3* and *URA3* genes were grown to saturation in rich medium. Aliquots of 10-fold dilutions were spotted to complete synthetic medium or medium containing 5-FOA and incubated at 25 °C. Only strains that lose the *URA3* plasmid during nonselective growth are able to grow on plates containing 5-FOA, so lack of growth in the *3rd row* indicates that double mutants are inviable. *B,* strains 8277-26-1, 8137-10-3, 8151-1-2, and 8289-2-4 (A364a background) with the genotypes noted were diluted and tested as indicated. YPAD is rich medium, HU (30) is YPAD with 30 mM hydroxyurea, −lys is synthetic medium lacking lysine (reporting the Spt$^-$ phenotype). Slow growth of the double mutant in the *bottom row* on −lys reflects the slow growth of this strain even on rich medium, not an altered Spt$^-$ phenotype (compare with the same strain on YPAD at 30 °C).

Spt16 NTD did cause both temperature sensitivity and destabilization of Spt16 protein). Furthermore, none of the point mutations that alter surface residues resulted in sensitivity to HU.

We next examined the point mutants to determine which features of the NTD are important for the functional overlap with the Pob3-M domain. A strain with a deletion of *SPT16* and also carrying the *pob3-Q308K* allele was constructed and transformed with the same series of *SPT16* plasmids described above. In this case, a subset of the mutated plasmids was unable to support robust growth on medium containing 5-FOA (Fig. 4*A*), indicating that mutations such as Spt16-IIQ260DIR significantly blocked the function of the Spt16 NTD that overlaps with Pob3-M (alleles with multiple changes are given as the WT sequence, the number of the first affected residue, and the mutated sequence with changes underlined). Most of the *SPT16* alleles tested supported normal growth on rich medium when combined with *pob3-Q308K*, but some of the resulting strains displayed extreme sensitivity to even mildly elevated temperatures or low levels of HU. For example, a *pob3-Q308K* strain with the Spt16-YS257DD plasmid failed to grow at 36 °C or in the presence of 30 mM HU (Fig. 4*B*, *4th row*), conditions that did not affect either single mutant. Tyr-257 and Ser-258 protrude into the Spt16 NTD cleft near the tunnel (Fig. 5). Other mutations such as the triple change QLYGN 279 DLDGR had little or no additive effect with *pob3-Q308K* (Fig. 4*B*). These residues map to the upper portion of the C-terminal lobe of the Spt16 NTD away from the cleft (Fig. 5). Key results obtained from this plasmid-based screen were confirmed by integrating mutations into the genome, and some complex mutations were

TABLE 2

Phenotypes caused by site-directed mutations in the Spt16 NTD

The sequences indicated in the 1st column were mutated to those in the 3rd column by site-directed mutagenesis within a full-length low copy *SPT16* plasmid with the native promoter. The derivatives were shuffled into 7784-1-1 pTF125 (*spt16-*Δ) with the *SPT16* locus deleted from the genome, and then the resulting strains were tested for the ability to grow when challenged with 200 mM HU, incubation at 37 °C, or on media lacking histidine or lysine (the Spt$^-$ phenotype). Some isolates had a moderate Spt$^-$ phenotype, but none displayed sensitivity to HU or elevated temperatures. The screen was repeated in 8319-2-4 (*spt16-*Δ *pob3-Q308K*) using 6, 15, 30, and 60 mM HU, 30, 33, 36, and 37 °C, and media lacking lysine. Dilutions were tested as shown in Fig. 4 and then rated from 0 (no additive defect with *pob3-Q308K*) to 6 (severe additive defect or lethal). No changes were noted for the Spt$^-$ phenotype, which is already severe for *pob3-Q308K* mutants. The combined synthetic defect (SD Severity) was calculated by summing the scores for the Ts$^-$ and HU phenotypes. These scores were used to assign each mutation into mild, moderate, or severe defect classes for use in Figs. 1 and 5.

WT sequence	1st residue	Mutant	Phenotype, Spt$^-$	Phenotype with *pob3-Q308K*				
				HUs	Ts$^-$	Spt$^-$	SD severity	Severity group
			0	0	0	6	0	
SNAEN	39	KNDKK	1	0	1	6	1	Mild
YQK	45	DDD	0	2	3	6	5	Moderate
QRNNK	104	DDDDD	2	3	2	6	5	Moderate
IDIS	163	DRID	0	0	1	6	1	Mild
LKIT	210	DDID	0	1	2	6	3	Mild
NYKFN	245	KDDFD	0	4	3	6	7	Moderate
NYKFN	245	KADFD	0	4	3	6	7	Moderate
YS	257	DD	2	5	5	6	10	Severe
IIQ	260	DIR	3	6	6	6	12	Severe
I	260	D		4	5	6	9	Severe
Q	262	R		1	2	6	3	Mild
DLR	268	KDD	2	5	4	6	9	Severe
L	269	D	1	4	5	6	9	Severe
VSARS	271	DDDDD	2	5	5	6	10	Severe
V	271	D		3	3	6	6	Moderate
QLYGN	279	DLDGR	0	1	1	6	2	Mild
S	289	D		1	3	6	4	Moderate
KPGR	333	DPGD	4	3	3	6	6	Moderate
KN	362	DR	0	0	2	6	2	Mild
EFR	371	AAA	0	1	3	6	4	Moderate
Q	415	R		1	3	6	4	Moderate
Q	415	A		0	1	6	1	Mild
DETE	425	KKKK	0	3	3	6	6	Moderate
AKSQ	439	DDDA	0	4	3	6	7	Moderate

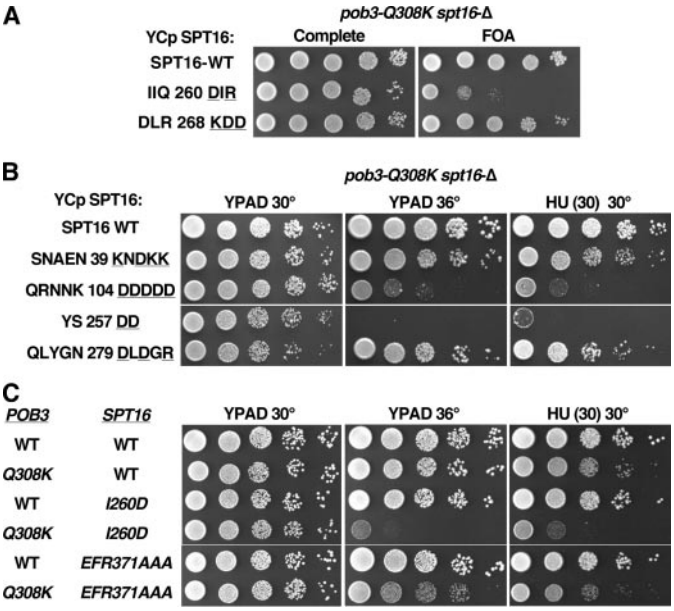

FIGURE 4. **Spt16 NTD point mutations cause synthetic defects with *pob3-Q308K*.** *A*, strain 8319-2-4 (A364a background, *spt16-Δ pob3-Q308K*) carrying a low copy plasmid with the *SPT16* and *URA3* genes was transformed with low copy plasmids carrying the alleles of *SPT16* indicated and the *LEU2* gene. Transformants were grown in medium lacking leucine and then tested on plates containing 5-FOA as in Fig. 3. Poor growth on 5-FOA indicates that the *spt16-IIQ260DIR pob3-Q308K* combination is viable but incapacitated. This allele of *SPT16* supports normal growth in a WT *POB3* strain (Table 2 and supplemental material). *B*, as in *A*, except strains were recovered after treatment with 5-FOA, grown to saturation in rich medium, and then tested on YPAD or YPAD with 30 mM HU at the temperatures indicated. *2nd* and *5th rows* are examples of mild synthetic defects with *pob3-Q308K* (Table 2); *3rd row* is and example of a moderate defect, and the *4th row* is an example of a severe defect. *C*, selected mutations were integrated into the genome (A364a background) and then crossed to a strain with the *pob3-Q308K* mutation. Single and double mutants (8127-5-2, 8324-1-4, 8364-I260D-2d, 8364-I260D-1c, 8364-371-8b, and 8364-371-7c) were diluted and tested as in *B*.

retested to determine the importance of individual residues. For example, IIQ 260 DIR was essentially lethal when combined with *pob3-Q308K*, and the I260D mutation was found to contribute more to this defect than Q262R (Table 2 and Fig. 4*C*). Western blotting was also used to show that these point mutations in Spt16 did not significantly destabilize Spt16 or Pob3 proteins in either *POB3* or *pob3-Q308K* strains (supplemental Fig. S4).

Combining mutations in the Spt16 NTD with *pob3-Q308K* therefore caused a range of effects from no added defect to near lethality. The severity of the synthetic defect is rated in Table 2 and mapped to the structure in Fig. 5. Importantly, the strongest synthetic defects are caused by mutations in residues within the putative peptide binding cleft and tunnel. We conclude that this region is important for performing the role of the Spt16 NTD that functionally overlaps with Pob3-M.

Spt16 NTD Does Not Bind N-terminal Tails of Histones—The four histone proteins all have N-terminal tails that extend beyond the structured core of the nucleosome (47). The similarity of the Spt16 NTD to peptide-binding proteins suggested the obvious possibility that these tails are the substrate for binding by the Spt16 NTD. We tested this idea genetically by looking for interactions between *spt16-ΔNTD* and mutations in the histone tails. Weak interactions consistent with a role of yFACT in nucleosome deposition were observed, but overall the pattern of interactions was not consistent with a direct role for the Spt16 NTD as the binding module for histone tails (supplemental Materials and supplemental Fig. S5). We also asked whether the Spt16 NTD binds to histone tails *in vitro* using several different strategies. In one approach, we found that the N-terminal tails of histones are an important component of the interaction between yFACT and nucleosomes, because nucleosomes treated with trypsin to remove the tails were no longer able to bind to yFACT (Fig. 6*A*). However, yFACT complexes lacking the Spt16 NTD, with a Pob3-Q308K mutation or with both defects, were able to bind to nucleosomes without a decrease in affinity (Fig. 6*B*). These mutated proteins were also able to produce the same increase in accessibility of nucleosomal DNA to nucleases observed with WT proteins (Fig. 6*C*). Finally, yFACT was found to bind to synthetic histone peptides with high affinity using a surface plasmon resonance assay, but the purified Spt16 NTD did not have this activity, and an Spt16-ΔNTD-Pob3 complex lacking the Spt16 NTD retained the ability to bind to peptides (Fig. 6*D*). We were therefore unable to obtain evidence to support the hypothesis that histone N-terminal tails are bound by the Spt16 NTD, although the tails of at least H3 and H4 are bound by some component of yFACT in an interaction that is important for yFACT function.

The H2A C-terminal Extension Is Important for yFACT Function—The specific structural changes that occur during yFACT-mediated nucleosome reorganization are not known, but it has been suggested that H2A-H2B dimers might be partially or fully displaced (8). Nuclease sensitivity results are not consistent with a simple displacement model (1, 7); however, it remains likely that altered contacts among histone proteins within the octamer core contribute to the changes produced by yFACT. Because the N-terminal histone tails do not appear to be the substrate for binding by the Spt16 NTD or the region of Pob3-M disturbed by the Pob3-Q308K mutation, we examined the nucleosome structure for other candidate regions whose structure might be altered if H2A-H2B dimer contacts with $(H3\text{-}H4)_2$ tetramers were broken. Aside from the unstructured N-terminal tails, most of H2B, H3, and H4 exhibit independent tertiary structural organization. In contrast, the C-terminal region of H2A (roughly residues 107–132) extends beyond the globular histone fold region to traverse the surface of the $(H3\text{-}H4)_2$ tetramer (Fig. 7). All but the last 6 residues are ordered in one monomer of the published crystal structure of the yeast nucleosome (27), but because this extended "docking" region of H2A makes extensive contacts with tetramers, it is likely to be unstructured in free H2A-H2B dimers. This stirrup-like domain contributes a large fraction of the buried surface area between H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers (27), and it has been shown to be important in preventing dissociation of dimers and sliding of nucleosomes (26, 28). This region of H2A is therefore a strong candidate for a domain that would be important for factors like yFACT that modulate nucleosome stability.

We therefore tested for genetic interactions between yFACT mutations and mutations in the H2A docking domain. We compared four strains, each with a single *URA3*-marked plasmid as the only source of histone genes. The strains were otherwise either WT or had the single additional genomic muta-

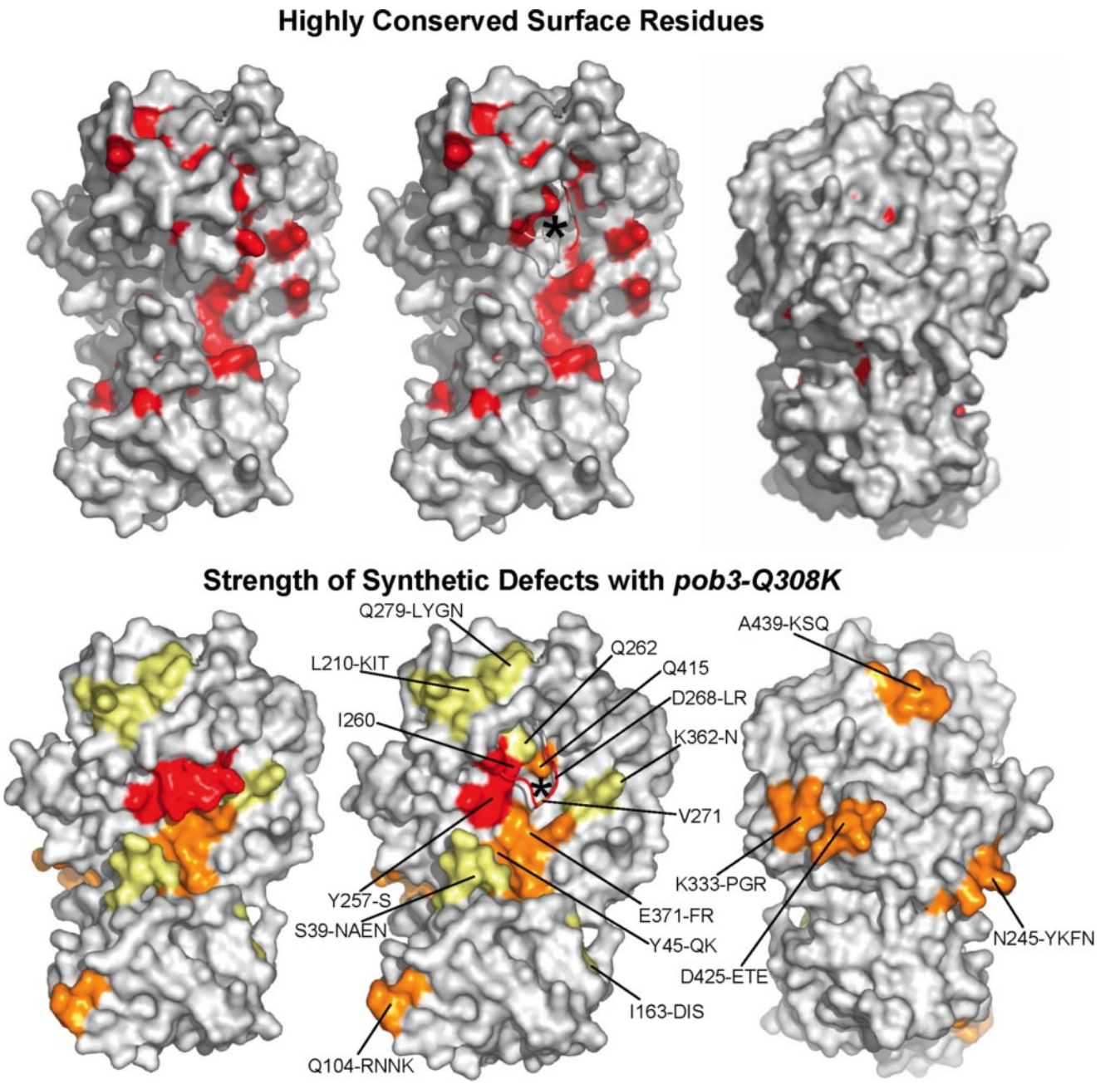

FIGURE 5. **Conservation correlates with the strength of synthetic defects with *pob3-Q308K*.** *Top,* residues that are identical among at least 70% of the 31 Spt16 homologs aligned (see Fig. 1) are indicated in *red*, revealing clustering near the canonical binding/active site cleft for prolidase, methionine aminopeptidase, aminopeptidase P, and creatinase (*asterisk*). The full surface is shown on the *left*, and the loop residues 266–274 are removed in the *middle panel* to reveal the enclosed tunnel region. The *right panel* shows a view rotated 180° about the *vertical axis*. *Bottom,* severity of the synthetic defect when combined with *pob3-Q308K* is indicated (Table 2); severe defects are shown in *red*, moderate defects in *orange*, and mild defects in *yellow*. If residues were tested individually and in multiple mutations, only the score from the single mutation is used here. Otherwise, the score for the complex mutation is assigned to all residues altered. WT sequences are indicated along with the number of the first residue. The orientations are the same as in the *top panels*.

tions *pob3-Q308K*, *spt16-ΔNTD*, or *spt16-11*. The latter allele carries two mutations in the Spt16-M domain (6) and causes strong HU sensitivity, temperature sensitivity, and the Spt$^-$ phenotype, much stronger defects than those caused by *spt16-ΔNTD*. This strain serves as a control to ask whether effects are specific to mutations that alter the putative binding motifs found in Pob3-M and the Spt16 NTD, or are instead general phenotypes associated with yFACT defects. The four strains were transformed with plasmids expressing the normal H2B, H3, and H4 proteins, but expressing a mutant H2A protein. The ability to lose the fully WT histone plasmid was determined by plating on medium containing 5-FOA to select for loss of the *URA3*-marked WT plasmid. Both WT and *spt16-11* strains were able to tolerate G107S, L109S, and H113R mutations in H2A (Fig. 7). In contrast, H2A-G107S could not support viability in either a *pob3-Q308K* strain or an *spt16-ΔNTD* strain (Fig. 7). H2A-H113R also could not support viability in a *pob3-Q308K* strain, but this mutation was not detrimental for the growth of an *spt16-ΔNTD* strain. These tests show that mutations that disturb potential binding domains of yFACT require

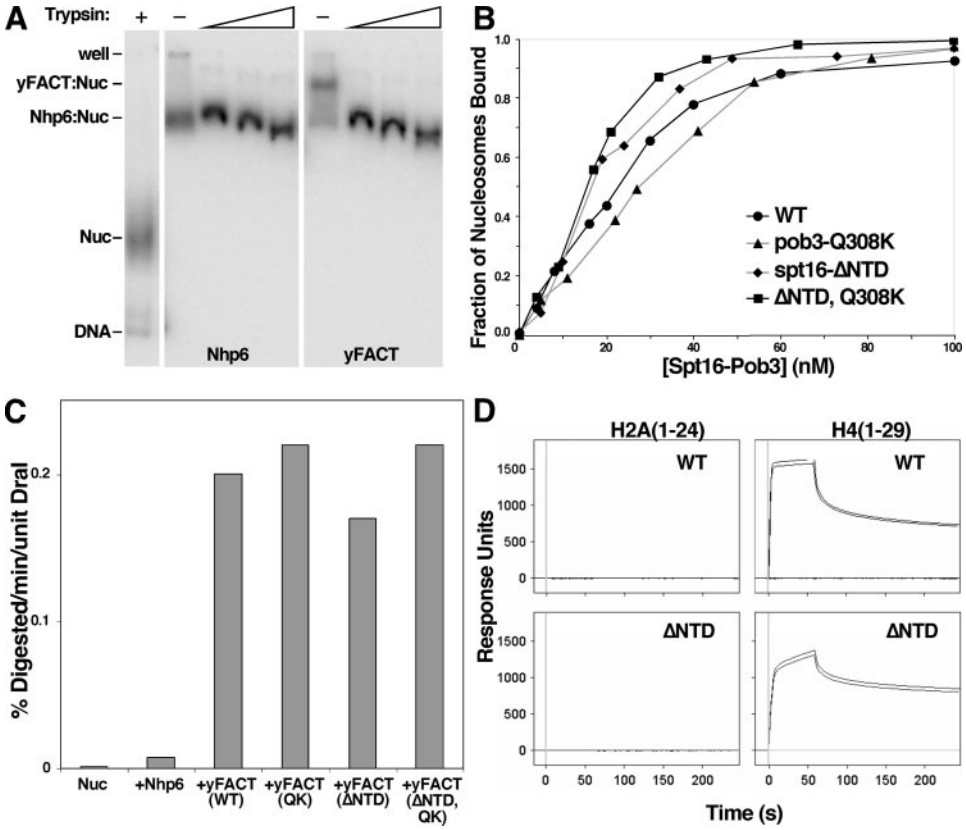

FIGURE 6. **Tests for features important to the direct interaction between yFACT and nucleosomes.** *A,* histone tails contribute to yFACT binding to nucleosomes (*Nuc*). Nucleosomes were reconstituted *in vitro* (supplemental Methods) and then treated with increasing amounts of trypsin, followed by addition of a protease inhibitor. Samples were mixed with Nhp6 or yFACT proteins as indicated then separated by native PAGE. Nhp6 bound to the nucleosomes normally even at the highest level of trypsin digestion, but even the lowest level of trypsin used blocked formation of yFACT-nucleosome complexes. *B,* mutation of putative peptide binding domains does not inhibit yFACT interaction with nucleosomes. Binding of yFACT to nucleosomes was measured using the electrophoretic mobility shift in native polyacrylamide gels, as in *A*. Spt16-Pob3 complexes were titrated using saturating levels of Nhp6, and the percentage of the nucleosomes shifted to the slower migrating form characteristic of complex formation was determined by phosphorimaging. WT (Spt16-Pob3), ΔNTD (Spt16-ΔNTD-Pob3), Q308K (Spt16-Pob3-Q308K), and the double mutant were compared in the same experiment. The concentration of each complex was determined by comparing the amount of intact Pob3 by Coomassie Blue staining after SDS-PAGE and by absorbance at 280 nm; the variability in determining the concentration of intact Spt16-Pob3 was in the range of the small differences in affinity observed here. The affinity was reproducibly slightly higher for complexes lacking the Spt16 NTD. It is therefore clear that deletion of the Spt16 NTD did not decrease the affinity of yFACT for nucleosomes, but we cannot conclude that this does not cause a slight increase in the affinity. *C,* mutation of yFACT does not alter the ability to increase accessibility to restriction endonucleases. The same four protein complexes used in *B* were tested for the ability to enhance digestion by DraI. In each case, the initial rate of digestion was determined by plotting multiple time points and then converted to a rate corrected for the amount of enzyme added as described previously (7). All four complexes displayed equivalent accessibility, indicating that neither the pob3-Q308K mutation nor the deletion of the NTD of Spt16 prevent yFACT from promoting this change in nucleosomes. Similar results were obtained with a different positioning sequence and the enzyme PstI (48; data not shown). *D,* N-terminally biotinylated synthetic peptides representing residues 1–24 of H2A or 1–29 of H4 were immobilized on a streptavidin chip, and binding of WT or Spt16-ΔNTD-Pob3 complexes was detected using surface plasmon resonance in two sequential tests. Binding to H4-(1–29) was complex and could not be fit to simple binding kinetics, but both complexes were able to bind an H4 tail peptide but not an H2A tail peptide. The NTD therefore may influence H4 tail peptide binding by yFACT, but is not required for it. Binding was also detected using an H3 tail peptide (data not shown).

nucleosomes to have normal features in the H2A docking domain. The H2A-G107S mutation was detrimental to an *spt16-11* strain, but this combination was viable, indicating that the requirement for a normal H2A docking domain is at least somewhat specific for yFACT mutations that alter potential binding motifs. Furthermore, Pob3-M and Spt16 NTD mutants require overlapping but distinct features to remain unperturbed within the H2A docking domain, consistent with their overlapping but distinct roles in promoting some essential function, as revealed by their distinct phenotypes when tested alone but synthetic lethality when combined (Fig. 3). These results suggest that the docking domain of H2A plays an important role in yFACT-mediated nucleosome reorganization and that the potential binding sites in the Pob3-M and Spt16 N-terminal domains functionally overlap to perform this role. Several possible functions for these domains are discussed below.

DISCUSSION

FACT homologs alter the properties of nucleosomes in a way that is important for transcription and replication, and this change is likely to include altered contacts between H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers (1, 7, 8, 13). Genetic interactions between yFACT and the Hir-Hpc complex suggest that a normal cycle of reorganization usually includes restoration of normal nucleosome structure after providing increased accessibility to the nucleosomal DNA (13). In this view, yFACT alters a nucleosome, tethers the components together, and then returns them to their initial state. We have shown that the Spt16 NTD and the Pob3-M domain each form potential binding surfaces, and that these sites are functionally redundant for some essential role of yFACT. Our analysis shows that yFACT complexes lacking normal versions of these sites cannot support viability but can still effect nucleosome reorganization *in vitro*. It is therefore likely that the potential binding sites act during the tethering or restoration phases of the reorganization cycle, rather than during initiation of reorganization.

The N-terminal tails of the histones appear to be important during an early stage of reorganization, because nucleosomes lacking these peptides are not bound efficiently by yFACT. Our data do not support a role for the potential binding sites in Pob3-M or the Spt16 NTD in binding these N-terminal tails, so the interaction with the N-terminal histone tails appears to be mediated by other regions of yFACT yet to be defined. However, we find that the stirrup-like C-terminal extension or docking domain of H2A has a role in reorganization that functionally overlaps both Pob3-M and the Spt16 NTD, as any combination of double

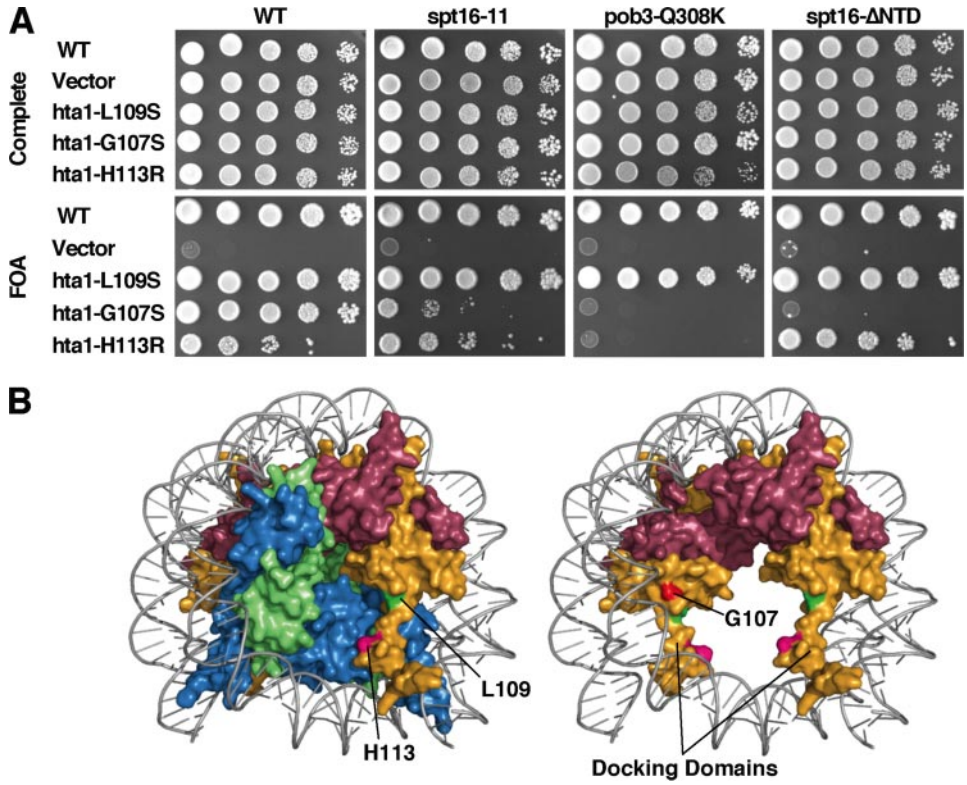

FIGURE 7. **The H2A docking domain is important during yFACT function.** *A,* strains DY9999, DY10003, 8264-17-3, and 8407-10-2 with the genotypes indicated and lacking genomic histone genes but carrying a low copy *URA3* plasmid with all four histone genes were transformed with low copy plasmids with the H2A mutations indicated. Cultures were tested on synthetic complete medium or medium containing 5-FOA as in Fig. 3. *B,* locations of H2A residues mutated in *A* are shown on the yeast nucleosome (PDB 1ID3; 27) Gly-107 is shown in *red*, Leu-109 in *green*, and His-113 in *magenta*. The remainder of H2A is *orange*; H2B is *purple*; H3 is *blue*; and H4 is *green*. The nucleosome is intact in the *left panel* and H3 and H4 are removed in the *right panel* to show Gly-107, which is buried in the docking interface, and to emphasize the stirrup-like extension of the docking domains away from the globular domains of H2A-H2B dimers.

mutants within this set is lethal. Interaction between the H2A docking domain and $(H3\text{-}H4)_2$ tetramers is important for preventing H2A-H2B dimer dissociation and nucleosome sliding (28), making this an interface that is likely to be important for yFACT functions. We therefore consider several models for how the H2A docking domain could be involved in reorganization that are consistent with our results.

First, the H2A docking domain could act during an early step. For example, reorganization by yFACT could be initiated by breaking contacts between the H2A docking domain and the $(H3\text{-}H4)_2$ tetramer surface as this would facilitate rearrangement of the histone core and/or displacement of H2A-H2B dimers. Our results do not address a role in this step because Spt16-ΔNTD-Pob3-Q308K complexes bind to and reorganize nucleosomes normally. However, this model makes specific predictions regarding the mechanism of reorganization that can now be tested.

Second, the H2A docking domain could act to tether nucleosomal components together during reorganization. In one model, contact between Pob3-M and some part of the histone core, between the Spt16 ΔNTD and another part of the histone core, and between the H2A docking domain and $(H3\text{-}H4)_2$ tetramers all contribute independently to maintaining contact among the components of the nucleosome during reorganization. The synthetic lethality observed between any pair of mutations would then be explained if any two points of contact are sufficient to prevent intolerable amounts of nucleosome damage from occurring, but one point of contact is not.

Third, the H2A docking domain could pose a barrier to restoration of nucleosome structure, requiring a chaperone to make insertion of H2A-H2B dimers more efficient. In this model, the H2A docking domain becomes disordered during reorganization because it is no longer in contact with the $(H3\text{-}H4)_2$ tetramer. Restoration of the normal nucleosome therefore requires positioning of this region in a conformation compatible with the docking interaction. The potential binding sites in Pob3-M and the Spt16 NTD could either act as redundant chaperones that can each promote this conformation or each could contribute independently to this outcome. In either case, The H2A-G107S mutation could make it more difficult to achieve the appropriate shape, making loss of either binding site/chaperone lethal.

Many other related models can be imagined. The identification of specific potential roles for domains of yFACT and for features of nucleosomes allows us to make more rigorous, experimentally testable predictions about the mechanism of yFACT-mediated nucleosome reorganization. The insight provided by the structural, genetic, and biochemical results reported here therefore allows us to begin a more detailed analysis of this important component of chromatin-mediated processes.

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Additional methods for analysis of yFACT *in vitro*.

For Fig 6A, nucleosomes were prepared with a duplex DNA derived by PCR from the sea urchin 5S rDNA sequence (1). The product was digested with *Eco*RI and *Sca*I, yielding a 143 bp duplex with a 4 nucleotide single-stranded extension at the left end. The sequence before endonuclease digestion was:
...g·AATTCCAACGAATAACTTCCAGGGATTTATAAGCCGATGACGTCATAACATCCCTGACCC
TTTAAATAGCTTAACTTTCATCAAGCAAGAGCCTACGACCATACCATGCTGAATATACCGGT
TCTCGTCCGATCACCGAAGTCAAGT·act...

Nucleosomes were reconstituted by gradual dialysis from a high salt solution using chicken histone octamers (2). Native polyacrylamide gels for the electrophoretic mobility shift assay (EMSA) were as described previously (3).

For Figs 6B and 6C, the same 5S rDNA sequence was used, but it was amplified with primers that produce the following sequence after *Eco*RI digestion:
AATTCCAACGAATAACTTCCAGGGATTTATAAGCCGATGACGTCATAACATCCCTGACCCTT
TAAATAGCTTAACTTTCATCAAGCAAGAGCCTACGACCATACCATGCTGAATATACCGGTTC
TCGTCCGATCACCGAAGTCAAGCagatatcggctcggttagt

The 162 bp duplex with a 4 nucleotide single-stranded extension was assembled into nucleosomes using recombinant yeast histone octamers. The unique *Dra*I site used to probe accessibility is underlined and is centered about 10 bp from the center of the 147 bp nucleosome positioning sequence (capitalized).

The Spt16 NTD does not appear to be a binding module for N-terminal histone tails.

Genetic analysis reveals weak interactions between the Spt16 NTD and N-terminal histone tails.

We previously showed that the function of yFACT is influenced, both positively and negatively, by modifications of the H3 and H4 tails (5, 3). For example, methylation of H3-K4 by Set1 and acetylation at various sites by different HATs support the role of yFACT, whereas methylation of H3-K36 by Set2 opposes yFACT function (5). These interactions could be mediated through other proteins that recognize modified histones or they could be due to direct binding of the N-terminal tails by yFACT. None of the subunits of yFACT have known sequence motifs associated with recognition of modified residues such as bromodomains or chromodomains, but other domains could have this property.

To test this idea, we initially asked whether the Spt16 NTD contributes to recognition of histone N-terminal tail modifications genetically. If the Spt16 NTD is responsible for recognizing a modified histone tail, and if this interaction is the only role of both the binding domain and the modified tail, then deleting the Spt16 NTD or the histone tail completely should each have the same effect because each will disrupt the interaction completely. Further, a strain with both mutations should have the same phenotype as a strain with either single mutation, because no further disruption of the interaction should be possible. In contrast, mutations that partially inactivate either the ability to bind or the ability to be recognized can have additive effects because either single mutation only partly disrupts the binding, and combining mutations can therefore cause an additive decrease in the interaction.

Deleting the Spt16 NTD causes weak sensitivity to 120 mM HU at 30°, and deleting the N-terminal tail of histone H3 causes somewhat more severe sensitivity (Fig S5A). Combining these deletions causes an additive defect, as the double mutant does not grow under these conditions. This result suggests that Spt16 NTD is not simply a binding module for the H3 N-terminal tail. We were unable to do a similar test with the H4 N-terminal tail because deleting this tail is lethal in the strains we tested (6). We showed previously that some point mutations in yFACT cause strong synthetic defects when combined with point mutations in H4 that block the acetylation pattern that is associated with nucleosome deposition (6). For example, combining *pob3-Q308K* with H4-K5R, K12R caused decreased viability. Each of these mutations only partially disrupts the ability to deposit nucleosomes, as each single mutant is viable and healthy under normal conditions, so in this case additive sensitivity was interpreted

as an indication that yFACT has a function in a pathway that overlaps nucleosome deposition (6). Combining a deletion of the Spt16 NTD with H4-K5R, K12R also caused an additive defect for HU sensitivity (Fig S5A). This pattern of interactions suggests that the Spt16 NTD is not responsible for recognizing the modified H4 N-terminal tail directly. Instead, it appears to have an indirect role in a process that involves nucleosome deposition, and this role becomes more important when cells are subjected to a replication stress. However, it is also possible that the H3 tail, the H4 tail, or the Spt16 NTD contribute to HU resistance in multiple independent and non-overlapping ways.

The Spt16 NTD is not required for nucleosome binding or reorganization by yFACT (additional description of Fig 6)

If the Spt16 NTD is involved in recognizing the N-terminal tails of histones then yFACT complexes lacking this domain should have lower binding affinity for nucleosomes. To test this, we constructed expression plasmids lacking residues 2-468 of the Spt16 NTD, coexpressed this Spt16(469-1035) (Spt16-ΔNTD) in yeast cells along with a His_{12}-tagged Pob3 protein, then purified the Pob3 protein by nickel affinity and size exclusion chromatography. Spt16-ΔNTD copurified as a 1:1 complex with Pob3, consistent with previous results showing that the Spt16 NTD is not required for heterodimer formation (6,7). We titrated this complex and WT complexes with nucleosomes in an EMSA experiment to determine the relative binding affinities. As shown in Fig 6B, the two complexes were able to bind nucleosomes at similar concentrations, indicating that deletion of the Spt16 NTD did not cause decreased affinity for intact nucleosomes. We also asked whether mutation of the potential peptide binding surface on Pob3-M contributes to the interaction between yFACT and nucleosomes using the EMSA assay. Complexes with the Pob3-Q308K mutation and intact Spt16 or Spt16-ΔNTD were readily purified, and both were found to bind nucleosomes with the same affinity as WT complexes in an EMSA test (Fig S4B). This mutation of Pob3 therefore also does not significantly alter the affinity of yFACT for nucleosomes.

yFACT-mediated reorganization of nucleosomes is detected *in vitro* as increased accessibility of nucleosomal DNA to nucleases (4,8). We therefore asked whether the normal binding to nucleosomes observed above with mutant yFACT complexes resulted in normal reorganization. yFACT complexes lacking the Spt16 NTD, containing the Pob3-Q308K mutation, or with both alterations were tested for their ability to alter accessibility of nucleosomal DNA to DNAse I or to the restriction endonuclease *Dra*I. All four versions of yFACT were essentially identical in the DNase I assay (not shown) and in a DraI accessibility assay (Fig 6C). yFACT can therefore bind to and alter the properties of nucleosomes without the Spt16 NTD, with a mutation in Pob3-M, or with both changes. The defects caused by these mutations individually and when combined *in vivo* are therefore not related to a gross inability to effect nucleosome reorganization as detected *in vitro*.

yFACT displayed reduced affinity for nucleosomes whose N-terminal histone tails had been removed with trypsin (Fig 6A), suggesting that yFACT can bind to histone tails. We tested this directly using a surface plasmon resonance assay (SPR). Various biotinylated peptides representing the N-terminal tails of histones were synthesized and immobilized on a streptavidin surface, then different fragments of Spt16-Pob3 were tested for binding to this surface. WT Spt16-Pob3 bound to histone H3 and H4 peptides with high affinity, displaying a Kd of about 2-6 nM. However, this number is a rough approximation because the kinetics of binding and dissociation were complex and did not fit a simple 2-component binding model (Fig 6D and not shown). The interaction appeared to be at least somewhat specific, as no binding was detected with the similarly charged H2A tail peptide assayed in parallel. Spt16-Pob3 therefore is able to bind to isolated histone tail peptides with high affinity but this interaction is complex. The same assay was performed with Spt16-ΔNTD-Pob3 and with the purified Spt16 NTD. Spt16-ΔNTD-Pob3 still bound to histone tail peptides, but the kinetics of binding were somewhat altered compared with WT Spt16-Pob3, suggesting that the Spt16 NTD contributed in some way to the complex interaction with peptides but was not responsible for the interaction itself (Fig S5D). Consistent with this, the isolated Spt16 NTD did not display robust binding to any histone peptides in this assay (not shown;

David Myszka, personal communication). These experiments show that yFACT can interact with the N-terminal tails of histones, but that the Spt16 NTD has no more than a minor role in this binding.

Genetic effects caused by mutation of the Spt16 NTD are not due to instability of yFACT proteins.

The structure of the Spt16 NTD explains the behavior of some previously reported mutant proteins. Partial deletion of the Spt16 NTD and some point mutations in this region cause a Ts- phenotype (failure to grow at elevated temperatures) even though deletion of the entire domain does not (7). This suggested that improper folding of the NTD could destabilize the entire Spt16 protein (7). Consistent with this, mutations such as G132D that cause the Ts- phenotype are found to disturb residues in the hydrophobic core of the structure of the Spt16 NTD, which is expected to make the resulting protein more difficult to fold.

In contrast, substitution of surface residues did not cause the Ts- phenotype or instability of either Spt16 or Pob3 proteins (Fig S4). It was therefore a surprise that combining some of these *spt16* mutations with the *pob3-Q308K* allele caused temperature sensitivity. This result either indicates that the combination of mutations caused yFACT proteins to become unstable or that yeast cells require higher levels of yFACT activity at elevated temperatures. We therefore used quantitative western blots to determine the level of Spt16 and Pob3 proteins in WT, single mutant, and double mutant cells both in cultures grown under permissive conditions (25°) and after shifting cultures to 37° for several hours. Fig S4 shows that Spt16 and Pob3 proteins were stable even in mutant combinations that fail to support growth at elevated temperatures. Levels of yFACT did drop about 2-fold in some cases, but mutations that cause temperature sensitivity on their own, such as *pob3-L78R* and *spt16-T434I*, displayed reductions of 5-10 fold even in cells grown under permissive conditions. We conclude that the 2-fold changes observed with some double mutants may be real but cannot account for the phenotypes observed, because even lower levels of yFACT can be tolerated. Instead, we propose that the Ts- phenotype results from the loss of a function whose role increases in importance as the temperature increases. For example, coordination of events during replication or reassembly of nucleosomes may become more difficult at elevated temperatures. Consistent with the idea that elevated temperatures alone cause replication stress, we note that the toxicity of HU increases at elevated temperatures even with WT strains (Fig S2). Misfolding of the Spt16 NTD therefore can cause defects in yFACT, but the point mutations in surface residues described here appear to disturb a function that overlaps with Pob3-M without causing destabilization of the yFACT complex.

The Spt16 NTD does not appear to have peptidase activity

Due to the low level of sequence similarity between the Spt16 NTD and several types of peptidases, we considered the possibility that yFACT and the Spt16 NTD have protease activity using a standard assay *in vitro*. 100 µl samples containing 110 nM Spt16-Pob3 or 380 nM Spt16 NTD and 100 µM peptide were incubated at RT for 2 hours. Peptidase activity was measured as fluorescence due to liberation of the MCA moiety by hydrolysis of the peptide bond. Consistent with the lack of active site residues in the Spt16 NTD known to participate in proteolysis (the metal-coordinating residues D209, D220, H284, E313 and E327 in the prolidase structure 1PV9 align with S289, N300, S366, S397, and A417 in Spt16, and the conserved active site histidine in creatinase aligns with V271 in Spt16), no significant activity was detected in any reaction, including substrates listed below that should reveal dipeptidase or prolidase activity. Any of the substrates with an unblocked N-terminus should detect peptidase activity. We therefore conclude that yFACT does not have peptidase activity. We thank Greg Pratt and Marty Rechsteiner for performing these assays.

Substrates tested:
N-terminus unblocked:
Gly-Pro-MCA
Lys-Ala-MCA
Leu-MCA
Pro-Phe-Arg-MCA

Arg-MCA
Ala-Ala-Phe-MCA
Phe-MCA

N-terminus blocked:
LLE-MCA
LLVY-MCA
LRR-MCA

Supplemental Figures

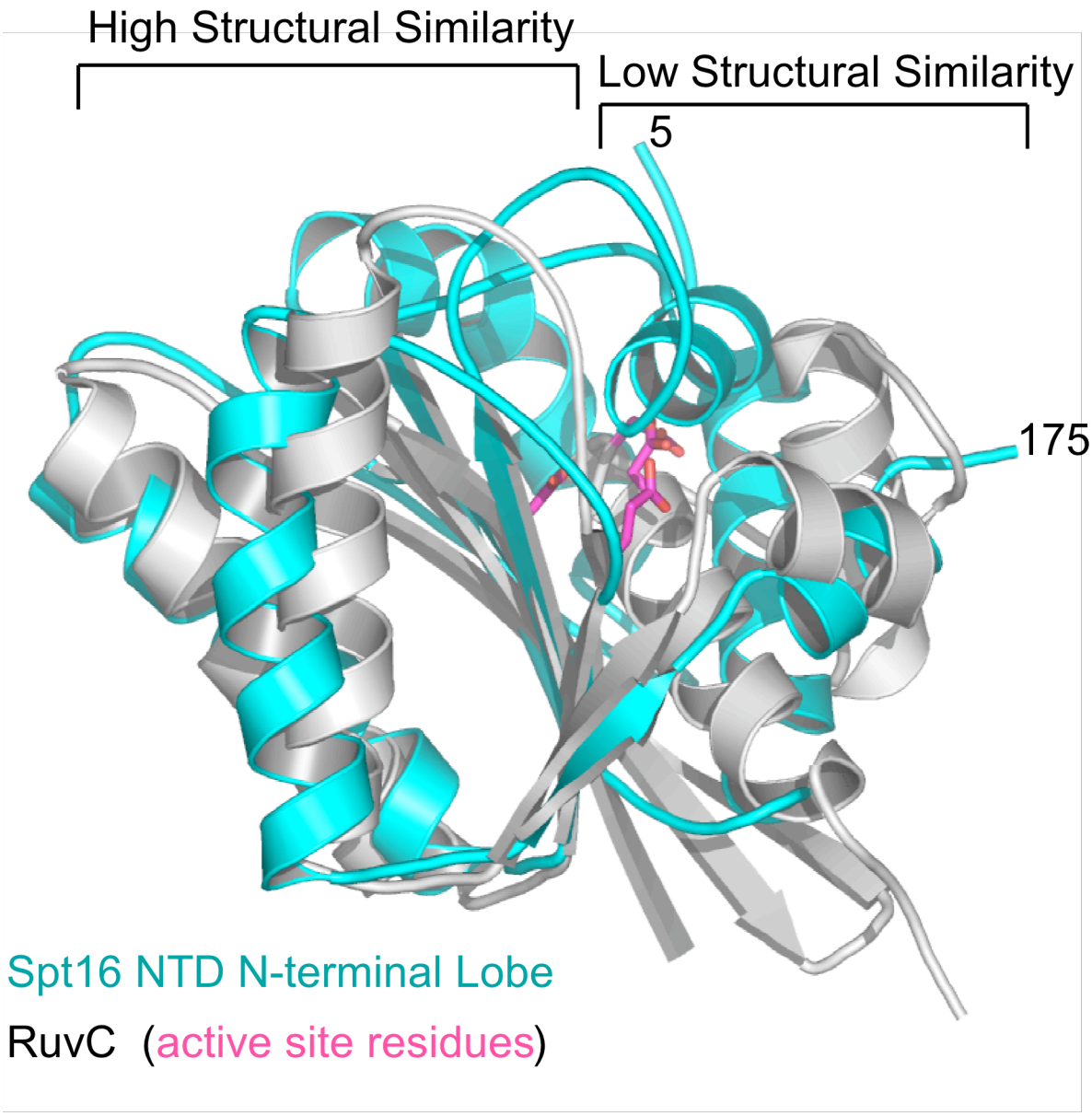

Fig S1 Comparison of RuvC with the Spt16 NTD

The N-terminal lobe of the Spt16 NTD in cyan is aligned with RuvC in grey (9; PDB 1HJR). The structural alignment is stronger in the left portion (Spt16 NTD residues 30-38, 62-77, 105-164), than in the right portion, where the RuvC active site residues (purple) are found.

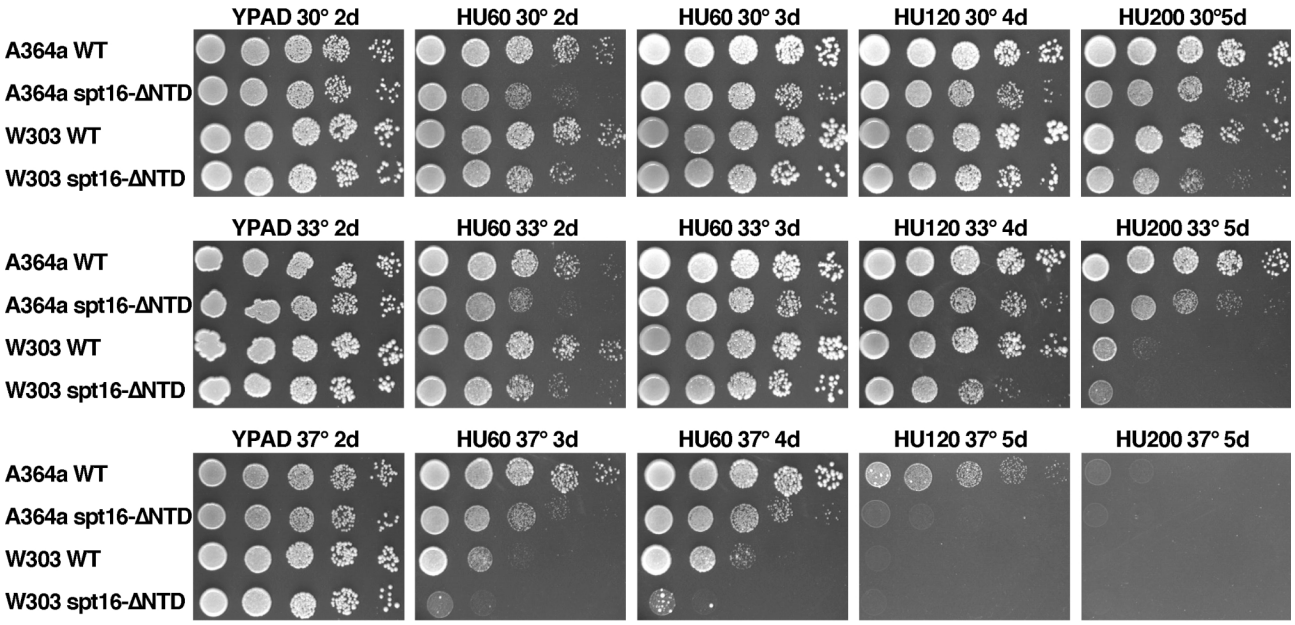

Fig S2 Combined effects of HU and elevated temperature

Strains 8127-7-4 (A364a WT), 8289-12-2 (A364a *spt16*-Δ*NTD*), DY150 (W303 WT), and 8368-3-2 (W303 *spt16*-Δ*NTD*) were grown to saturation in rich medium, then aliquots of 10-fold serial dilutions were placed on plates with no HU or the amount of HU indicated (mM) and incubated at the temperature shown. Deleting the Spt16 NTD consistently caused a growth defect on media containing HU, but the concentration of HU and the temperature at which this defect was maximal varied with time and with genetic background. For example, a clear defect was noted on HU60 in both strain backgrounds after 2 days at 30°, but was no longer evident after 3 days. This shows that the defect is largely a retardation of growth, not a loss of viability. Increasing the temperature or increasing the concentration of HU enhanced the growth defect, but the WT strain was also more severely affected. The W303 strains appear to be inherently less able to withstand these simultaneous stresses, as even the WT failed to grow on HU200 at 33°, a condition tolerated by the A364a strain even with the *spt16*-Δ*NTD* mutation. We conclude that elevated temperatures and the presence of HU are independent, additive stresses even for WT yeast strains and the effect is enhanced by deletion of the Spt16 NTD. This pattern is consistent with intact stress checkpoints but a less effective response to the stress.

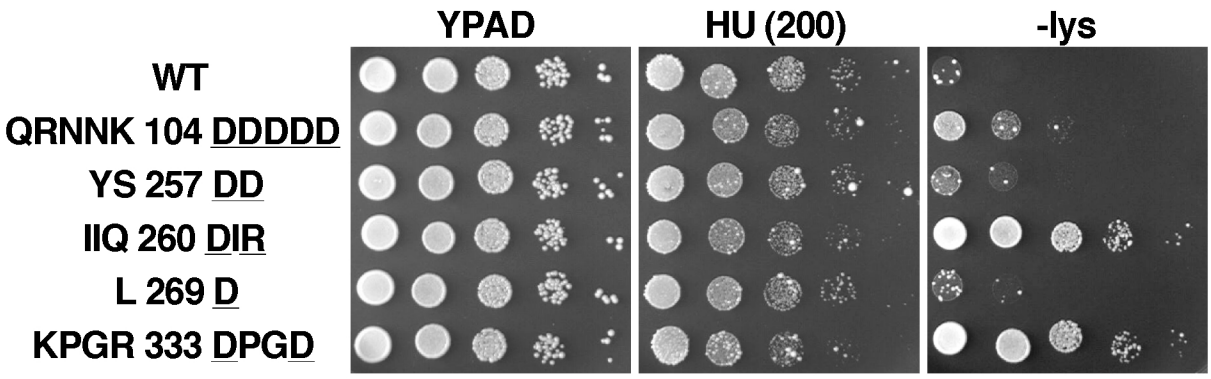

Fig S3 Some point mutations in the Spt16 NTD cause mild phenotypes.

7784-1-1 (*spt16*-Δ, S288c background) carrying *spt16* mutant alleles as indicated on a low copy (YCp) plasmid) was grown to saturation and aliquots of 10-fold serial dilutions were placed on various plates and incubated at 30°. Temperatures including 16° and 38° were also tested but no variation in growth rate was observed (not shown). Other mutants listed in Table 3 were also tested, but no phenotypes were observed (not shown). Other phenotypes tested include the ability to grow on media containing 10 µg/ml camptothecin, 600 mM NaCl, 6 µg/ml phleomycin, 75 µg/ml 6-azauracil, 0.03% MMS, 3% formamide, or 10 mM caffeine. Further, cells were tested for growth on rich medium after exposure to ultraviolet light. No effects of the mutations relative to WT were noted. A strain with the full deletion of the Spt16 NTD was tested for maintenance of repression at telomeres and was found to be normal (no telomere position effect disruption). Kinetics of induction of the *GAL1* promoter were also found to be normal in an *spt16*-Δ*NTD* strain (Debu Biswas, personal communication), consistent with previously published results indicating normal regulation of transcription in *spt16*-Δ*NTD* strains (7).

Fig S4 Stability of Spt16-Pob3 proteins determined by western blotting.

Strains were grown to logarithmic phase at 24° or 25° C as indicated, then shifted to 37° for 2-4 hours. Cells were harvested and lysed by vortexing with glass beads and SDS sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis, then transferred to nitrocellulose. Spt16 and Pob3 were detected with antisera generated against the purified proteins, then infrared-labeled secondary antibodies were used to detect the primary rabbit antibodies. Blots were scanned using a Li-Cor infrared scanner and quantitated using Odyssey software. This method of detection provides a large window of linear detection, enhancing the reliability of quantitation. In each experiment, the level of intact Spt16 or Pob3 detected in each lane was normalized to the total protein in the lane detected by scanning a parallel gel stained with Coomassie Blue dye, then each signal was normalized to the result for the WT strain at 24°, which was defined to be 100%. This allowed experiments performed on different days to be compared, as the absolute intensities of signals from different blots varied. Comparison with purified standards indicated that WT cells contain about 25,000 copies of Pob3 per cell. Spt16 was more difficult to quantitate as it was more prone to proteolysis during extraction, giving roughly 10,000 copies per cell for the intact form. Given the instability of Spt16 and the heterodimeric nature of yFACT, we assume the number for Pob3 is a more reliable estimate of the number of heterodimers in a living cell. These estimates are consistent with the estimates of 10,000-50,000 copies per cell that have been reported previously (10,11, 12).

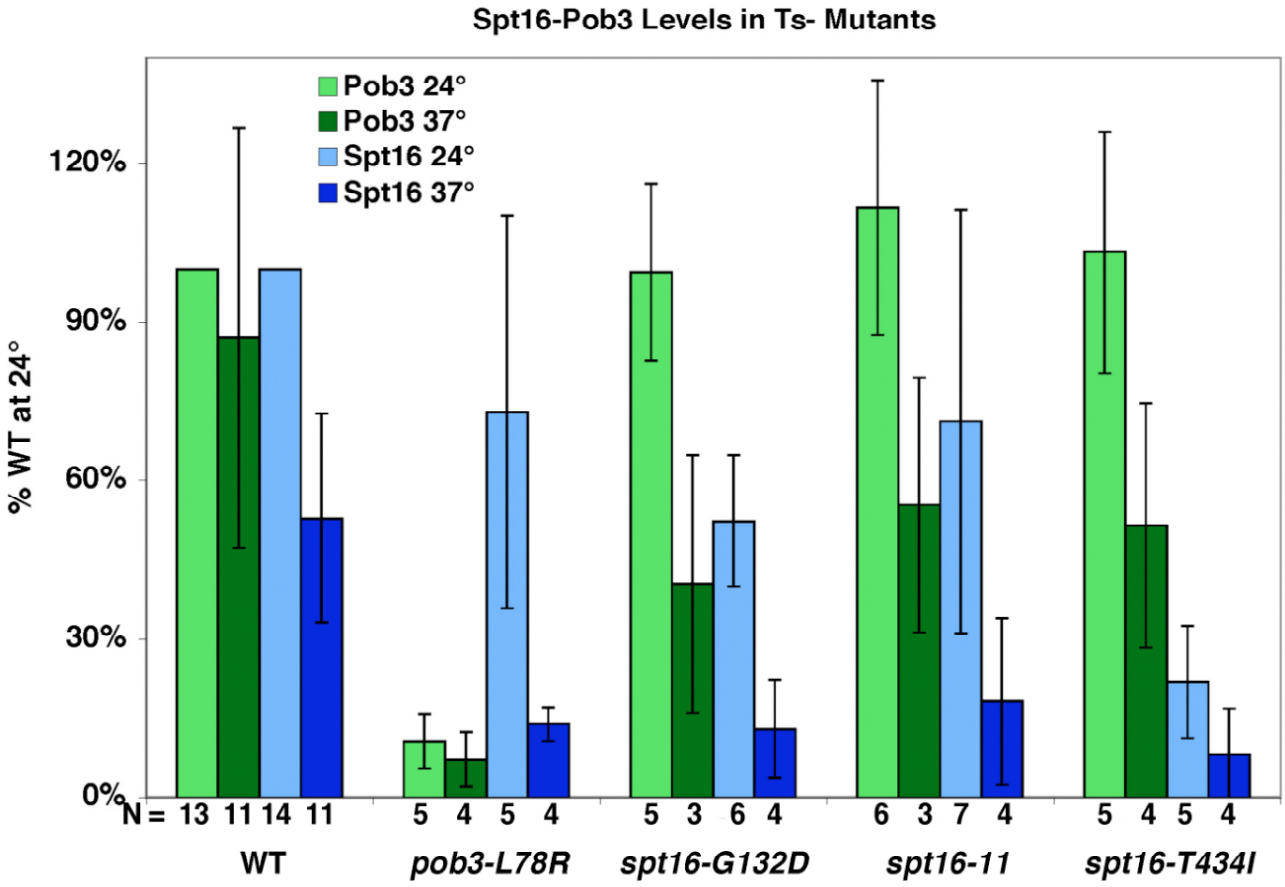

Fig S4A) Several strains with the relevant genotype indicated were tested. Data from A364a, W303, and S288c strain backgrounds were combined; the number of independent experiments for each condition is given (N). Pob3 is stable in a WT strain after a shift to 37°, but the level of Spt16 reproducibly drops about 2-fold. The level of Pob3 detected in a *pob3-L78R* strain growing at the permissive temperature of 24° is decreased about 10-fold relative to a WT strain, and does not decrease dramatically after a shift to 37°. This indicates that cells can grow, although slowly, with about 10% of the normal level of Pob3, and

suggests that the need for Pob3 increases as the temperature increases. Consistent with previous results that showed that Spt16 can be overexpressed independent of Pob3 in yeast cells (11), the level of Spt16 in a *pob3-L78R* strain is close to normal when the cells are growing at 24°, but the excess Spt16 is lost after a shift to 37°. No pools of free Spt16 or Pob3 were detected in normal yeast cells (11), so these results suggest that free Spt16 is unstable at elevated temperatures. Similarly, the level of Pob3 is normal in strains with mutations in *SPT16* that cause temperature sensitivity, and drop after a shift to 37°. Different alleles show somewhat different levels of Spt16 protein under permissive conditions, with Spt16-24 (T434I) consistently displaying the lowest levels at about 20% of the WT. The mutant Spt16 proteins all show some instability at the elevated temperature, but this is difficult to interpret as the magnitude of the decrease is similar to the change observed with WT protein. The instability therefore may contribute to the temperature sensitivity of growth in these strains, but an increased requirement for yFACT may also be responsible.

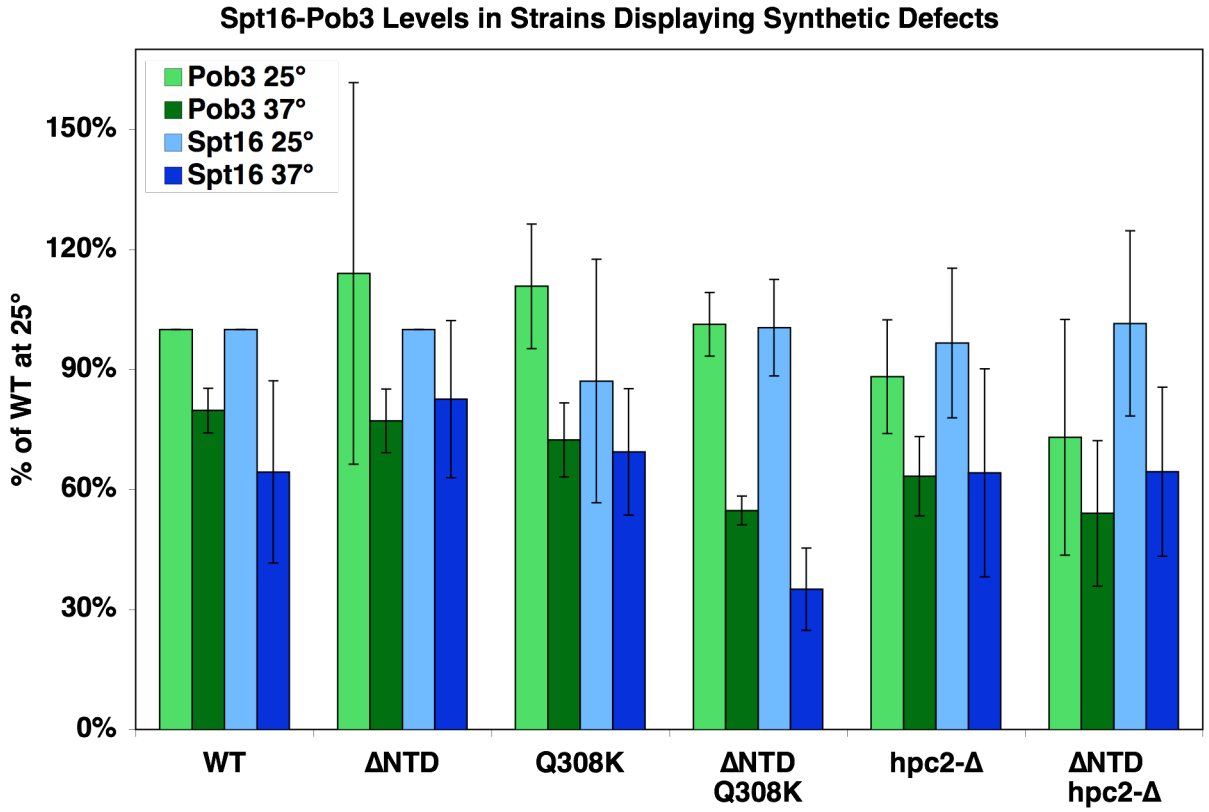

Fig S4B) Pob3 and Spt16 levels were measured in strains lacking the Spt16 NTD, with the *pob3-Q308K* allele, lacking *HPC2*, or with both *spt16-ΔNTD* and the other mutations. The levels of Pob3 and Spt16 decreased 2-3 fold in the *spt16-ΔNTD pob3-Q308K* strain at 37°, but the level of yFACT in these cells remained well above the 10% level shown to be sufficient for growth in panel A. As these cells grew very poorly even at 30° (Fig 3), the 2-3 fold decrease in yFACT level is not likely to be the principle cause of the growth defect. Rather, the cause is likely to be a defect in activity in the doubly mutant complex. Combining a deletion of *HPC2* with *spt16-ΔNTD* also caused a slight drop in yFACT levels at 37°, but comparison with panel A suggests that this is not the cause of the significant growth defect observed with this strain (Fig S5).

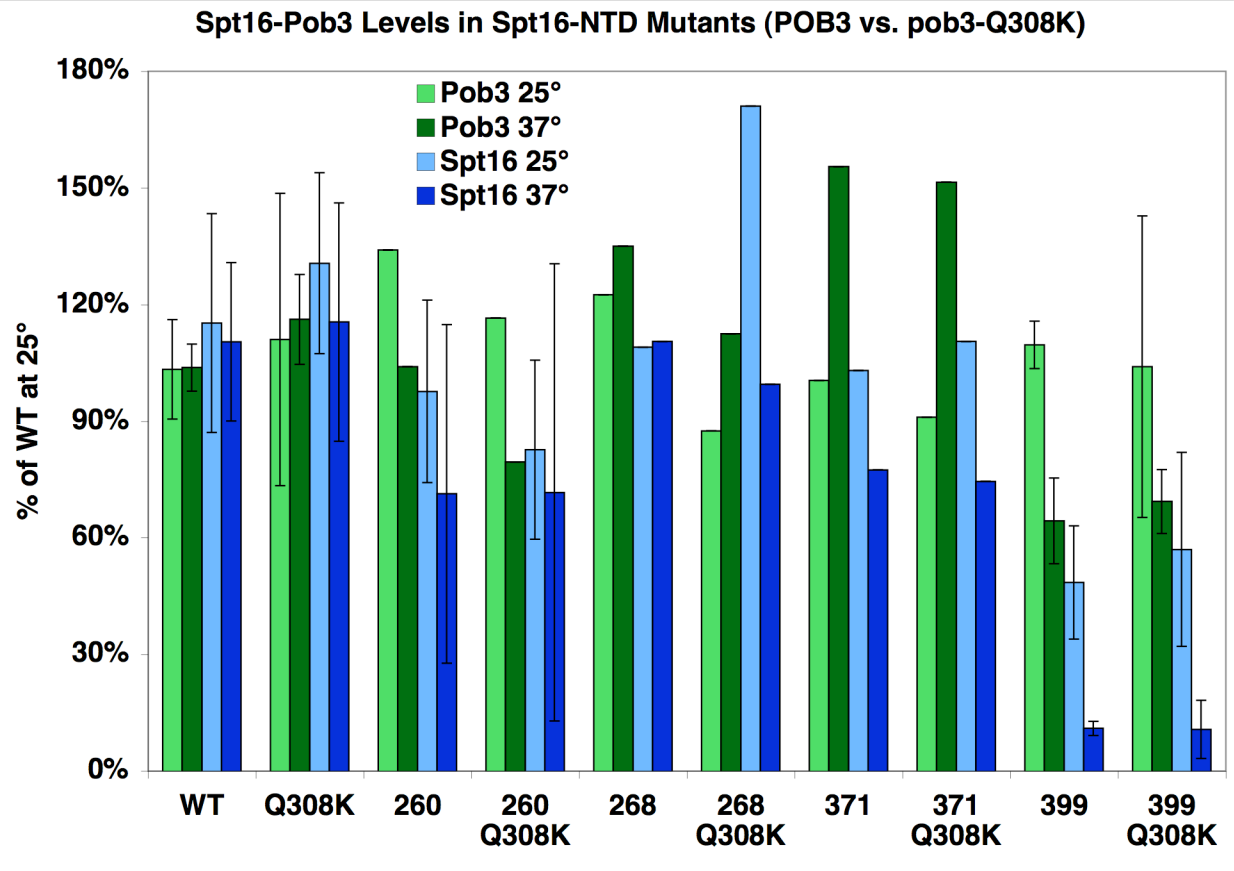

Fig S4C) Pob3 and Spt16 levels in strains with point mutations in the Spt16 NTD integrated into the genome were tested alone or in combination with *pob3-Q308K*. Columns with error bars indicate comparison of 3-5 independent experiments, those without are the average of 2 independent experiments. 260 indicates the IIQ260DIR mutation, 268 is DLR268KDD, and 371 is EFR371AAA. 399 is a G399V mutation isolated in a screen for Ts- mutants in *SPT16* (Huyen Bui, personal communication), and affects a buried residue in the Spt16 NTD. The point mutations in surface residues do not cause the Ts- phenotype and both Spt16 and Pob3 levels are stable, even though these three mutations all cause temperature sensitivity at 37° when combined with *pob3-Q308K* (Table 2). In contrast, the G399V mutation causes Ts- by itself, and leads to gross instability of the Spt16 protein at elevated temperatures in a *pob3-Q308K* strain. This analysis shows that the point mutants in surface residues cause a defect in a function that overlaps with the middle domain of Pob3, rather than causing instability of yFACT.

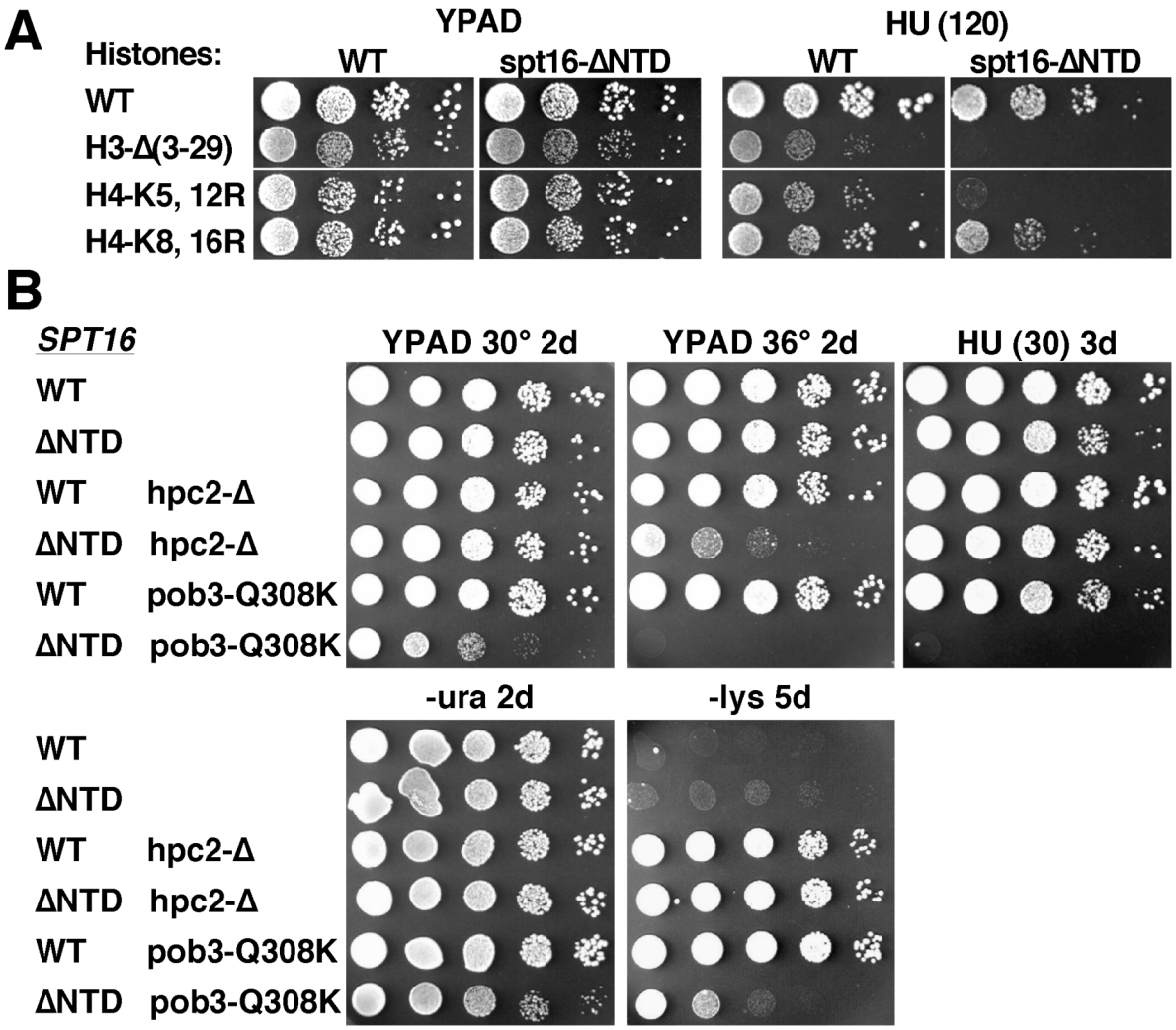

Fig S5 Histone mutations and Hir/Hpc mutations interact with *spt16-ΔNTD*
A) Strains 8252-1-1 (WT) and 8310-4-3 (*spt16-ΔNTD*) with both copies of the genes encoding H3 and H4 deleted from the genome and carrying a low-copy plasmid encoding the alleles of H3 and H4 indicated were constructed. Aliquots of 10-fold dilutions were spotted to rich medium without (YPAD) or with 120 mM HU and incubated at 30°. Deleting the N-terminal tail of H3 causes a severe growth defect, and this is enhanced by simultaneous deletion of the Spt16 NTD. Similar additive effects were observed with point mutations in the H4 tail, especially for the K5R, K12R combination, which affects residues that are modified during nucleosome deposition.
B) Strains 8277-26-1 (WT), 8137-10-3 (*spt16-ΔNTD*), 8281-2-3 (*hpc2-Δ*), 8281-8-4 (*hpc2-Δ spt16-ΔNTD*) 8151-1-2 (*pob3-Q308K*), and 8289-2-4 (*pob3-Q308K spt16-ΔNTD*), isogenic with the A364a genetic background, were constructed and aliquots of 10-fold serial dilutions were tested as indicated. Rows 1, 2, 5, and 6 here duplicate the data shown in Figure 3B of the main text. YPAD is rich medium, HU (30) indicates 30 mM hydroxyurea added to rich medium, -lys is synthetic medium lacking lysine. Growth on -lys medium indicates the Spt- phenotype. Slow growth of the double mutant in the bottom row on -lys reflects the slow growth of this strain even on rich medium, not an altered Spt- phenotype (compare with the same strain on YPAD at 30°).

Table S1 Yeast strains used

Strain	Back ground	Mating type	Genotype
8127-5-2	A364a	*MAT alpha*	*ura3-Δ0 leu2-Δ0 trp1-Δ2 his3 lys2-128∂*
8127-7-4	A364a	*MATa*	*ura3-Δ0 leu2-Δ0 trp1-Δ2 his3 lys2-128∂*
8137-10-3	A364a	*MATa*	*ura3 leu2 trp1-Δ2 his7 lys2-128∂ spt16-ΔN (469-1035; KanMX)*
8151-1-1	A364a	*MATa*	*ura3-Δ0 leu2-Δ0 trp1-Δ2 his7 lys2-128∂*
8151-1-2	A364a	*MATa*	*ura3-Δ0 leu2-Δ0 trp1-Δ2 his7 lys2-128∂ pob3-Q308K*
8252-1-1 DS1700	A364a	*MATa*	*ura3-Δ0 leu2-Δ0 trp1-Δ2 his3 lys2-128∂ hht1-hhf1-Δ(::LEU2) hht2-hhf2-Δ(::KanMX) DS1700 (YCp URA3 HHT2-HHF2)*
8277-26-1 Ura+	A364a	*MATa*	*ura3::YIplac211 (URA3) leu2 trp1 his7 lys2-128∂*
8281-2-3 Ura+	A364a	*MATa*	*ura3::YIplac211 (URA3) leu2 trp1 his7 lys2-128∂ hpc2-Δ(::TRP1)*
8281-8-4 Ura+	A364a	*MATa*	*ura3:YIplac211 (URA3) leu2 trp1 his7 lys2-128∂ spt16-ΔN (469-1035; KanMX) hpc2-Δ(::TRP1)*
8289-12-2	A364a	*MATa*	*ura3 leu2 trp1-Δ2 his3 lys2-128∂ spt16-ΔN (469-1035; KanMX)*
8289-2-4 Ura+	A364a	*MATa*	*leu2 trp1-Δ2 his7 lys2-128∂ pob3-Q308K(LEU2) spt16-ΔN (469-1035; KanMX)*
8310-4-3 DS1700	A364a	*MATa*	*ura3 leu2 trp1-Δ2 his3 lys2-128∂ hht1-hhf1-Δ(::LEU2) hht2-hhf2-Δ(::KanMX) spt16-ΔN (469-1035; KanMX) DS1700 (YCp URA3 HHT2-HHF2)*
8319-2-4 pCDC68	A364a	*MATa*	*ura3 leu2 trp1 his3 lys2-128∂ spt16-Δ3(::HIS3) pob3-Q308K pCDC68 (YEp URA3 Spt16)*
8324-1-4	A364a	*MAT alpha*	*ura3-Δ0 leu2-Δ0 trp1-Δ2 his3 lys2-128∂ pob3-Q308K(LEU2)*
8364-I260D-1c	A364a	*MATa*	*ura3 leu2 trp1 his7 lys2-128∂ spt16-I260D(KanMX) pob3-Q308K(LEU2)*
8364-I260D-2d	A364a	*MATa*	*ura3 leu2 trp1 his7 lys2-128∂ spt16-I260D(KanMX)*
8364-260-5a	A364a	*MAT alpha*	*ura3 leu2 trp1 his3 lys2-128∂ spt16-IIQ260DIR(KanMX)*
8364-260-8b	A364a	*MAT alpha*	*ura3 leu2 trp1 his3 lys2-128∂ spt16-IIQ260DIR(KanMX) pob3-Q308K(LEU2)*
8364-268-7d	A364a	*MATa*	*ura3 leu2 trp1 his7 lys2-128∂ spt16-DLR268KDD(KanMX) pob3-Q308K(LEU2)*
8364-268-8a	A364a	*MATa*	*ura3 leu2 trp1 his3 lys2-128∂ spt16-DLR268KDD(KanMX)*
8364-371-3c	A364a	*MATa*	*ura3 leu2 trp1 his7 lys2-128∂ pob3-Q308K(LEU2)*
8364-371-7c	A364a	*MAT alpha*	*ura3 leu2 trp1 his3 lys2-128∂ spt16-EFR371AAA(KanMX) pob3-Q308K(LEU2)*
8364-371-8b	A364a	*MAT alpha*	*ura3 leu2 trp1 his3 lys2-128∂ spt16-EFR371AAA(KanMX)*
8365-399-5c	A364a	*MATa*	*ura3-Δ0 leu2-Δ0 trp1-Δ2 his7 lys2-128∂ spt16-G399V(KanMX) pob3-Q308K(LEU2)*
8365-399-7b	A364a	*MATa*	*ura3-Δ0 leu2-Δ0 trp1-Δ2 his3 lys2-128∂ spt16-G399V(KanMX)*
DY150	W303	*MATa*	*ura3 ade2 trp1 can1 leu2 his3*
DY9999 pTF237	W303	*MATa*	*ade2 can1 his3 leu2 trp1 ura3 hht1-hhf1-Δ(::HIS3) hht2-hhf2-Δ(::KanMX) hta1-htb1-Δ(::NatMX) hta2-htb2-Δ(::HphMX) pTF237*

			(YCp URA3 HHT2-HHF2, HTA1-HTB1)
DY10003 pTF237	W303	*MATa*	*ade2 can1 his3 leu2 met15 trp1 ura3 spt16-11 hht1-hhf1-Δ(::HIS3) hht2-hhf2-Δ(::KanMX) hta1-htb1-Δ(::NatMX) hta2-htb2-Δ(::HphMX) pTF237 (YCp URA3 HHT2-HHF2, HTA1-HTB1)*
DY10890	W303	*MATa*	*ade2 can1 his3 leu2 lys2 trp1 ura3 pob3-Q308K*
DY11923	W303	*MAT alpha*	*ade2 can1 his3 leu2 trp1 ura3 spt16-ΔN(469-1035; KanMX)*
DY12431 pJW4	W303	*MATa*	*ade2 can1 his3 leu2 trp1 ura3 pob3-Q308K spt16-ΔN(469-1035; KanMX) pJW4 (YCp URA3 POB3)*
8264-17-3 pTF237	W303	*MATa*	*ade2 can1 his3 leu2 trp1 ura3 hht1-hhf1-Δ(::HIS3) hht2-hhf2-Δ(::KanMX3) pob3-Q308K hta1-htb1-Δ(::NatMX) hta2-htb2-Δ(::HphMX) pTF237 (YCp URA3 HHT2-HHF2, HTA1-HTB1)*
8368-3-2	W303	*MATa*	*ade2 can1 his3 leu2 trp1 ura3 spt16-ΔN(469-1035; KanMX)*
8407-10-2	W303	*MAT alpha*	*ade2 can1 his3 leu2 trp1 ura3 spt16-ΔN(469-1035) hht1-hhf1-Δ(::HIS3) hht2-hhf2-Δ(::KanMX) hta1-htb1-Δ(::NatMX) hta2-htb2-Δ(::HphMX) pTF237 (YCp URA3 HHT2-HHF2, HTA1-HTB1)*
7784-1-1 pTF125	S288c	*MATa*	*leu2-Δ1 trp1-Δ63 ura3-52 his4-912∂ lys2-128∂ spt16-Δ(::TRP1) pTF125 (YEp URA3 SPT16)*

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