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Genetic interactions between *POB3* and the acetylation of newly synthesized histones

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Abstract

Pob3p is an essential component of the *S. cerevisiae* FACT complex (yFACT). Several lines of evidence indicate that the yFACT complex plays an important role in chromatin assembly including the observation that the *pob3* Q308K allele is synthetically lethal with an allele of histone H4 that prevents the diacetylation of newly synthesized molecules. We have analyzed the genetic interactions between the Q308K allele of *POB3* and mutations in all of the sites of acetylation that have been identified on newly synthesized histones. Genetic interactions were observed between *POB3* and sites of acetylation on the NH_2-terminal tails of H3 and H4. For histone H3, lysine residues 14 and 23 were particularly important when *POB3* activity is compromised. Surprisingly, synthetic defects observed when the *pob3* Q308K allele was combined with mutations of H4 lysines 5 and 12, were not phenocopied by deletion of *HAT1*, which encodes the enzyme that is thought to generate this pattern of acetylation on H4. Genetic interactions were also observed between *POB3* and sites of acetylation found in the core domain of newly synthesized histones H3 and H4. These include synthetic lethality with an allele of H4 lysine 91 that mimics constitutive acetylation. While the mutations that alter H4 lysines 5, 12 and 91 do not affect binding to Pob3p, mutation of histone H3 lysine 56 decreases the association of histones with Pob3p. These results support the model that the yFACT complex plays a central role in chromatin assembly pathways regulated by acetylation of newly synthesized histones.

Keywords

Chromatin; Chromatin assembly; Histone; Histone chaperone; Histone acetylation; FACT complex; HAT1

Introduction

The assembly of eukaryotic genomes into chromatin is essential for their packaging in nuclei. In addition to this structural function, the assembly of chromatin structure is also central to the propagation of the epigenetic information that is essential for proper regulation of nuclear processes such as transcription and replication. The assembly of chromatin structure is a complex process that must be precisely orchestrated (Corpet and Almouzni 2009; Polo and Almouzni 2006; Ransom et al. 2010). As might be expected, a large number

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of factors have been identified as being involved in the chromatin assembly process. These factors include histone chaperones, ATP-dependent chromatin remodelers and histone modifying enzymes (De Koning et al. 2007; Groth et al. 2007; Haushalter and Kadonaga 2003; Park and Luger 2008).

Histone chaperones are proteins that bind to histones and, in the process, shield the highly positively charged histones from non-specific or incorrect interactions with DNA (or other molecules in the cell) (Andrews et al. 2010; Eitoku et al. 2008; Park and Luger 2008; Rocha and Verreault 2008). Histone chaperones may also play a role in the assembly of chromatin structure but are not a permanent part of the finished structure.

One intriguing histone chaperone that has been implicated in nucleosome deposition is the FACT complex. The *S. cerevisiae* FACT complex (yFACT) is composed of three subunits Spt16p, Pob3p and Nhp6p (Brewster et al. 2001; Formosa et al. 2001). In some organisms, including humans, the Pob3p and Nhp6p polypeptides are combined in one open-reading frame known as SSRP1 (Orphanides et al. 1998). FACT plays multiple roles in nucleosome dynamics based on its ability to reorganize chromatin structure and has been shown to be important for the initiation and elongation of transcription and for DNA replication (Belotserkovskaya and Reinberg 2004; Formosa 2008; Reinberg and Sims 2006). A role for FACT in chromatin assembly is supported by both in vitro and in vivo evidence. In vitro, FACT complex components from several organisms have been shown to bind to both histone H2A/H2B and histone H3/H4 dimers (Belotserkovskaya et al. 2003; Orphanides et al. 1999; Stuwe et al. 2008). In addition, human FACT complex is capable of assembling nucleosomes in vitro (Orphanides et al. 1999). In vivo, mutations in yFACT subunits are synthetically lethal with deletions of the subunits of the Hir/Hpc replication-independent chromatin assembly complex (Formosa et al. 2002).

The middle region of the yFACT subunit Pob3p (Pob3-M) contains two pleckstrin homology folds. The surface of this region of the protein is highly conserved and appears to form a protein–protein interaction interface. This region of Pob3p is important for the role of yFACT in DNA replication, as it is the site of interaction with the ssDNA binding factor RPA. In addition, a genetic screen to identify *POB3* mutants that are defective in DNA replication identified a critical residue in this domain as all 23 mutants contained a change of the glutamine at residue 308 (Q308) to either lysine or arginine (VanDemark et al. 2006).

Importantly, the *pob3* Q308K allele has also provided a link between FACT activity and chromatin assembly through genetic interactions with mutations that effect the acetylation of newly synthesized histones (Biswas et al. 2008; Formosa et al. 2002; VanDemark et al. 2006). First, *pob3* Q308K mutants are sensitive to alterations in the levels of histone proteins. Intriguingly, the *pob3* Q308K allele shows a dramatic synthetic growth defect when combined with mutations in histone H4 that prevent the acetylation of lysines 5 and 12 (H4 K5,12R) which is the evolutionarily conserved pattern of acetylation found on newly synthesized molecules prior to chromatin assembly. In contrast, the *pob3* Q308K mutation, unlike other alleles of *POB3*, does not show any significant interaction with mutations that alter histone H4 lysine residues 8 and 16 (VanDemark et al. 2006).

In addition to histone H4 lysines 5 and 12, newly synthesized histones are also acetylated at a number of other lysine residues. Acetylation of the NH_2-terminal tail domain is a conserved feature of newly synthesized histone H3 in eukaryotes. However, a precise pattern of acetylation is not conserved as different sets of NH_2-terminal tail lysine residues are modified in different organisms (Benson et al. 2006; Kuo et al. 1996; Sobel et al. 1995; Tyler et al. 1999). In addition to the NH_2-terminal tails of histone H3 and histone H4, sites of acetylation have also been identified in the core domains of these histones. Newly

synthesized histone H3 is acetylated on lysine 56 (Masumoto et al. 2005; Ozdemir et al. 2005; Xu et al. 2005). Acetylation of this residue plays a role in replication-coupled chromatin assembly and promotes the interaction of histone H3/H4 complexes with histone chaperones involved in this process (Chen et al. 2008; Li et al. 2008). The acetylation of histone H4 lysine 91 (H4 K91) was also identified on soluble histones associated with a histone chaperone complex (Ye et al. 2005). This modification lies in a region of histone H4 that is involved in interactions between the histone H3/H4 tetramer and histone H2A/H2B dimers and may, therefore, play a role in regulating the formation or stability of histone octamers (Luger et al. 1997; Pinto and Winston 2000).

To further explore the link between the yFACT complex and the process of chromatin assembly, we have systematically combined mutations in all of the sites of acetylation on newly synthesized histones H3 and H4 with the *pob3* Q308K allele. We have identified a number of genetic interactions between *POB3* and sites of newly synthesized histone acetylation including synthetic lethality with specific alleles of histone H4 lysine 91. These genetic interactions do not appear to be a result of defects in the binding of the yFACT complex to histones containing these mutations. In addition, we have found that the genetic interactions between *pob3* Q308K and lysines 5 and 12 in the histone H4 NH_2-terminal tail are not mimicked by a deletion of the histone acetyltransferase *HAT1* suggesting that this enzyme is not solely responsible for the acetylation of these sites on newly synthesized histone H4. These results strongly support the model in which the yFACT complex plays a central role in histone deposition and nucleosome assembly.

Materials and methods

Plasmids

Plasmids pMP13, pMP14, pMP22, pMP110, pEE8, pEE9, pMP138, and pMP139 with histone H4(K5R), histone H4(K12R), histone H4(K8,16R), histone H4(K5,12R), his-tone H4(K91R), histone H4(K91Q), histone H3(K56R), histone H3(K56Q) mutations, respectively, were generated by site-directed mutagenesis of pMP3 and plasmids with histone H3 amino terminal lysine to arginine mutations have been described previously (Kelly et al. 2000). Mutant alleles were confirmed by DNA sequencing.

Strains

Yeast culture and genetic manipulation were done by standard methods. DNY214 was constructed as follows. TAP-tagged POB3 was amplified from genomic DNA of YML069W (Open Biosystems) and cloned into TOPO vector (Invitrogen) as per manufacturer's instructions. Site-directed mutagenesis of this plasmid was used to generate a Q308K allele. Linear fragment of TAP-tagged POB3 obtained by *Aat*II, *Not*I digestion of this plasmid was used to transform UCC1111 to generate DNY214 (*POB3*) and DNY215 (*pob3* Q308K) (Kelly et al. 2000). Plasmids and strains were confirmed by PCR followed by DNA sequencing. PCR and western blotting confirmed the presence of epitope tags. DNY216 and DNY217 strains were generated by deleting *HAT1* gene in DNY214 and DNY215, respectively, by transformation with *Eco*RI-, *Hae*II-digested linear DNA fragment *pHAT1::LYS2* (Parthun et al. 1996). Strains 8244-13-2 and 8244-18-4 with *POB3* WT and *pob3* Q308K, respectively, were generous gifts from Dr. Tim Formosa (VanDemark et al. 2006).

Phenotypic assays

As described previously, cultures were grown to mid-log phase in rich media and normalized by A_{600} (Kelly et al. 2000). Cells were pelleted, washed with distilled water and resuspended in 200 μl of water. Tenfold serial dilutions were spotted onto synthetic

complete (SC) media with or without DNA damaging agents. Hydroxyurea (HU) was used at 50 and 100 mM concentrations and methyl methane sulfonate (MMS) was at 0.01%. For telomeric silencing assays, the SC plates contained 5-FOA (0.1%). UV treatment was carried out at 200 μJ after the serially diluted cultures were spotted onto the plates. Unless otherwise indicated, the plates were incubated at 30°C and images were taken after 3 days. For temperature sensitivity assays, the plates were incubated at 37°C. At least two individual isolates of each strain were used in the assays.

Whole cell extracts

Whole cell extracts were prepared as described previously (Kushnirov 2000). Briefly, ~2.5 OD_{600} cells growing in log phase were collected by centrifugation and resuspended in 100 μl of distilled water. Equal volume of 0.2 M NaOH was added, and the mixture was incubated at room temperature for 5 min. The cells were then pelleted, taken up in 50 μl of SDS-PAGE sample buffer and boiled for 5 min. Cell debris was pelleted and the clear supernatant was analyzed by SDS-PAGE and western blotting with anti-TAP and anti-GAPDH antibodies.

Immunoprecipitation

The procedure was modified from Zhou et al. (2006). Cells growing in log phase in 200 ml cultures were pelleted and washed with distilled water. The cell pellet was taken up in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X100, 2 mM $MgCl_2$, 2 mM DTT) with protease inhibitor cocktail (Roche) added prior to use. The resuspended cells were ground in the presence of dry ice and total extracts were made by centrifugation of the cell lysates at 10,000×*g* for 10 min. The supernatant was transferred to fresh tubes and spun again at 10,000×*g* for 10 min. IgG-Sepharose (GE-Pharmacia) was added to the resulting supernatant and incubated at 4°C for 2 h. The beads were extensively washed with lysis buffer minus protease inhibitor cocktail and bound proteins were eluted with glycine buffer, pH 2.0. SDS-PAGE sample buffer was added to the eluates and boiled for 5 min. These were analyzed by SDS-PAGE followed by western blotting with anti-histone H4 and anti-TAP antibodies (Table 1).

Results

Genetic interactions between *POB3* and histone H4 lysines 5 and 12

To expand on the original observation made by the Formosa group, we determined whether the synthetic growth defect seen when the *pob3* Q308K allele was combined with the H4 K5,12R allele required alteration of both lysines 5 and 12 or whether a single mutation of either site was sufficient for this phenotype. Plasmids containing either an H4 K5R, H4 K12R or H4 K5,12R were introduced into strain 8244-18-4 (a generous gift from Dr. Tim Formosa). This strain contains the *pob3* Q308K allele and also contains a single copy of the genes encoding histones H3 and H4 present on a *URA3*-based plasmid. Cells were grown in the absence of selection for the *URA3*-based plasmid and then spotted onto synthetic media in the absence and presence of 5-FOA. A synthetic growth defect between the *pob3* Q308K allele and the histone H4 mutations will be indicated by their inability to lose the plasmid containing the wild type histone genes and their subsequent inability to grow in the presence of 5-FOA. As seen in Fig. 1a, the H4 K5R and H4 K12R alleles cause only a slight decrease in growth of the *pob3* Q308K strain. In addition, as reported previously, the H4 K5,12R allele causes a severe synthetic growth defect in combination with the *pob3* Q308K allele with very few cells surviving in the absence of the wild type histones. This result indicates that the complete pattern of acetylation on newly synthesized histone H4 is functionally redundant with a function of the yFACT complex.

To determine whether the genetic interactions between the *pob3* Q308K allele and the histone H4 mutants were strain specific or whether they would be observed in other strain backgrounds, we introduced a *pob3* Q308K allele into a strain background commonly used in our laboratory to study the effects of histone mutations (UCC111, originally derived from BY4705) (Kelly et al. 2000). This strain has a number of useful features. First, the *HHT1-HHF1* and *HHT2-HHF2* loci have been deleted and the cells contain a copy of *HHT2-HHF2* on an *ADE2*-based plasmid. Therefore, plasmids containing mutant alleles of the histones can be introduced and cells that have lost the wild type histone plasmid can be identified as red colonies. In addition, this strain has the *URA3* reporter gene inserted near the telomere of chromosome VII L. This provides a sensitive assay for mutations that de-repress sub-telomeric heterochromatin.

Plasmids containing the H4 K5R, H4 K12R, H4 K5,12R and H4 K8,16R alleles were introduced into a UCC1111 *pob3* Q308K derivative of UCC1111 (DNY215) and cells were grown in the absence of selection for the *ADE2*-based plasmid carrying the wild type *HHT2-HHF2* locus. Cells were then plated onto rich media (YPD) and red colonies were easily isolated from all strains. Isolates of each were then spotted in tenfold serial dilutions on a YPD plate to assess overall growth (Fig. 1b). The H4 K5,12R allele, but not the H4 K8,16R allele, causes a synthetic growth defect in the *pob3* Q308K mutant. However, the magnitude of this defect is much less severe than that seen in strain 8244-18-4. This result is consistent with yFACT and the acetylation of H4 lysines 5 and 12 functioning in overlapping pathways but indicates that the importance of these pathways to cell viability can be dependent on the strain background.

The relatively mild growth defect seen when the *pob3* Q308K and H4 K5,12R alleles are combined in the UCC1111 strain background allowed us to use this strain to test whether combining these mutations generated other synthetic phenotypes. In Fig. 2, combinations of *pob3* Q308K and H4 K5,12R alleles are tested for several phenotypes including, HU, MMS and UV sensitivity, telomeric silencing and temperature sensitive growth. These phenotypes were chosen because they monitor processes that are influenced by chromatin assembly and that are often found to be defective when yeast cells are lacking known chromatin assembly factors (Enomoto and Berman 1998; Enomoto et al. 1997; Formosa et al. 2002; Game and Kaufman 1999; Kaufman et al. 1998; Kelly et al. 2000; Monson et al. 1997; Qin and Parthun 2002; Tyler et al. 1999).

HU inhibits ribonucleotide reductase leading to decreased levels of dNTPs and replicational stress (Eklund et al. 2001; Koc et al. 2004). In the presence of either low or high levels of HU (50 and 100 mM, respectively), the H4 K5R, H4 K12R and H4 K5,12R alleles show normal levels of growth and the *pob3* Q308K allele on its own confers only a mild increase in sensitivity (Fig. 2a–c, top). When these mutations were combined, there was an increase in HU sensitivity. The H4 K5R and H4 K12R individually had equivalent effects on increasing the HU sensitivity of the *pob3* Q308K strain. The *pob3* Q308K/H4 K5,12R mutant showed little or no growth on either concentration of HU. However, when the effect of this combination of mutations on cell growth (SC plate) is taken into account, the H4 K5,12R allele does not significantly increase HU sensitivity relative to the H4 K5R and H4 K12R alleles. These results suggest that the function of yFACT in replication is functionally redundant with a pathway that is dependent on the presence of acetylation at either histone H4 lysine 5 or 12 and not on the diacetylation pattern seen on newly synthesized molecules.

The integrity of telomeric silent chromatin in yeast can be assayed in vivo by the use of marker genes inserted near the ends of chromosomes. The strains used here contain the *URA3* gene located near the left arm of chromosome VII L. When telomeric silent chromatin structure is intact, the *URA3* gene is transcriptionally silenced (telomeric silencing) and

mutations that disrupt this silent chromatin result in the expression of the *URA3* marker. Analyzing the growth of cells on media containing 5-FOA, which is toxic to cells expressing *URA3*, can sensitively monitor the expression level of the telomeric *URA3*. Interestingly, telomeric silencing has been a useful phenotype for the genetic characterization of factors involved in chromatin assembly. The *pob3* Q308K mutation caused a slight decrease in viability in the presence of 5-FOA indicating that yFACT may play a minor role in the formation or maintenance of telomeric silent chromatin structure (Fig. 2d, top). As *POB3* is an essential gene, it is difficult to determine the full contribution of the FACT complex to telomeric chromatin structure using hypomorphic alleles. The pattern of interactions between the *pob3* Q308K allele and the histone H4 K5 and K12 mutations was similar to that observed for MMS sensitivity. The H4 K5R allele did not affect telomeric silencing while the H4 K12R had a minor, but reproducible, effect. While the H4 K5,12R allele caused a telomeric silencing defect in *POB3* cells, the *pob3* Q308K/H4 K5,12R combination was the most sensitive to 5-FOA. However, the difference between the H4 K5,12R and H4 K12R alleles can largely be attributed to the impact of the former allele on overall cell growth.

Exposure to UV radiation results in the generation of single strand DNA damage, primarily in the form of thymine dimers, while MMS treatment causes double strand DNA damage at high concentrations. Sensitivity to these treatments was assayed to determine the ability of cells containing the various combinations of *POB3* and histone H4 alleles to repair these forms of DNA damage. The *pob3* Q308K allele conferred a slight sensitivity to UV and MMS treatments, while none of the histone H4 alleles caused sensitivity to either agent (Fig. 2e, f, top). When these mutations are combined, the H4 K5R, H4 K12R and H4 K5,12R alleles had only a minor effect on UV sensitivity. The H4 K5R allele did not increase the MMS sensitivity of the *pob3* Q308K strain, while the H4 K12R allele causes a moderate increase in sensitivity. The *pob3* Q308K/H4 K5,12R mutant was highly sensitive to MMS. However, the limited range of response available with this assay makes it hard to conclude whether this is the result of an increase in MMS sensitivity or due to the effect of the H4 K5,12R allele on cell growth. Taken together, these results indicate that yFACT appears to play a relatively minor role in the repair of single and double strand DNA damage and that there is little functional overlap with pathways that are dependent on the acetylation of histone H4 lysines 5 and 12.

Temperature-sensitive growth defects were observed with combinations of the *pob3* Q308K and the histone H4 K5 and K12 mutations. Comparing growth on rich media (YPD) at 30 and 37°C, the *pob3* Q308K allele was partially temperature sensitive (Fig. 2g, h, top). Both the H4 K5R and H4 K12R alleles decreased the viability of the *pob3* Q308K mutant at the higher temperature with the H4 K12R allele having a more pronounced effect. In addition, the *pob3* Q308K/H4 K5,12R mutant was essentially inviable at 37°C.

By analyzing several phenotypes, we have observed that the genetic interactions between the *pob3* Q308K allele and mutations that alter histone H4 lysines 5 and 12 are not uniform. When assessing overall cell growth, on either synthetic or rich media, only the combined H4 K5,12R allele decreases the viability of the *pob3* Q308K mutant. For other phenotypes, such as HU sensitivity, equivalent defects were seen with either the histone H4 K5R or H4 K12R single mutants alone, while in other phenotypes, such as MMS and UV sensitivity and telomeric silencing, the genetic interactions were primarily seen with the H4 K12R allele. These results suggest that the interplay between yFACT function and the acetylation of the H4 is likely to be complex and may not only occur exclusively within the context of newly synthesized histones.

Genetic interactions between *POB3* and the histone acetyltransferase *HAT1*

The observation that the *pob3* Q308K mutation displays genetic interactions with a mutation that alters histone H4 lysines 5 and 12 to arginine but not with a comparable mutation of histone H4 lysines 8 and 16 suggests a connection between yFACT activity and the acetylation of newly synthesized histones. The type B histone acetyltransferase Hat1p is thought to be involved in generating this well-known pattern of acetylation on newly synthesized histone H4 molecules and, in particular, there is significant evidence that Hat1p is responsible for the acetylation of histone H4 lysine 12. First, yeast Hat1p has a substrate specificity consistent with a role in the acetylation of newly synthesized histone H4. The native complex isolated from yeast is only active on non-nucleosomal histone H4 molecules and specifically modifies lysine 12 (Parthun et al. 1996). In addition, the recombinant yeast Hat1p enzyme is also capable of acetylating lysine 5 (Kleff et al. 1995; Parthun et al. 1996). Second, yeast Hat1p, though predominantly nuclear, is also localized to the cytoplasm where preassembled histones are first found (Ai and Parthun 2004; Poveda et al. 2004). Third, when combined with specific mutations of the histone H3 NH_2-terminal tail, *hat1Δ* mutants show a defect in telomeric silencing that is phenocopied by an H4 K12R allele (but not a H4 K5R allele) (Kelly et al. 2000). In addition, Hat1p has been shown to be responsible for the increase in acetylation of histone H4 lysine 12 observed during the recombinational repair of a DNA double strand break (Qin and Parthun 2006). Finally, *HAT1* was found to be responsible for the peak of H4 lysine 12 acetylation that occurs in S phase following an HU-induced cell cycle block (Poveda and Sendra 2008). Therefore, we predicted that the genetic interactions observed when the *pob3* Q308K mutation is combined with histone H4 mutants (and, in particular, alleles that contain the H4 K12R mutation) would be phenocopied by a *hat1Δ* mutation.

To test this prediction, we introduced wild type, H4 K5R, H4 K12R and H4 K5,12R alleles of histone H4 into *hat1Δ* and *pob3* Q308K/*hat1Δ* mutants. If the genetic interactions seen when combining *pob3* Q308K with these histone H4 alleles are due to loss of Hat1p-mediated acetylation of newly synthesized molecules, then these phenotypes should be observed in a *pob3* Q308K/*hat1Δ* mutant in the presence of wild type histone H4. As seen in Fig. 2 (bottom panels), this is clearly not the case. For all of the phenotypes tested, deletion of *HAT1* did not result in a synthetic defect with *pob3* Q308K in the presence of wild type histones. In addition, deletion of *HAT1* in the *pob3* Q308K/H4 K5R background did not result in the same phenotypes observed in the *pob3* Q308K/H4 K5,12R background as would be expected if Hat1p was the primary enzyme involved in the acetylation of lysine 12 on newly synthesized histone H4.

The most surprising genetic interaction observed between *HAT1* and *POB3* was that in *pob3* Q308K cells, loss of *HAT1* actually suppressed the effect of mutating H4 lysine 12 to arginine. Using HU sensitivity as an example (Fig. 2b, c), the level of sensitivity of *pob3* Q308K/*hat1Δ*/H4 K12R cells was similar to *pob3* Q308K/*hat1Δ*/H4 WT cells and *pob3* Q308K/*hat1Δ*/H4 K5,12R cells were similar to *pob3* Q308K/*hat1Δ*/H4 K5R cells. This effect of the *hat1Δ* mutation was seen with all of the phenotypes we have examined and was only seen in a *pob3* mutant background. These results uncovered unanticipated genetic interactions between *POB3* and *HAT1* and suggest a number of possibilities. First, it is likely that other histone acetyltransferases contribute to the acetylation of newly synthesized histone H4 on lysines 5 and 12. In addition, when yFACT activity is compromised, a function of Hat1p other than its histone acetyltransferase activity can influence, in a negative fashion, the process of chromatin assembly.

Genetic interactions between *POB3* and sites of acetylation on the NH_2-terminal tail of histone H3

While the acetylation of the NH_2-terminal tail of newly synthesized histone H4 occurs in a pattern that is highly conserved across eukaryotic evolution, the acetylation of new histone H3 molecules occurs in a variety of patterns. For example, in *D. melanogaster*, of the five acetylatable lysines in the histone H3 NH_2-terminal tail (at positions 9, 14, 18, 23 and 27), newly synthesized molecules are modified at positions 14 and 23. In *T. thermophila*, the pattern has been found to be acetylation of lysines 9 and 14 (Sobel et al. 1995). In *S. cerevisiae*, a definitive pattern has not been determined. Newly synthesized histone H3 from yeast had the highest levels of acetylation on lysines 9 and 27. However, it is not known whether these modifications occur together as a pattern on the same histone H3 molecules. In addition, there were lower, but detectable, levels of acetylation on lysines 14, 18 and 23, as well (Kuo et al. 1996). Given that the *pob3* Q308K allele showed specific genetic interactions with mutations that altered the pattern of acetylation seen on newly synthesized histone H4, we reasoned that if genetic interactions occurred between *pob3* Q308K and mutations that altered specific lysine residues in the histone H3 NH_2-terminal tail this might suggest the presence of a critical pattern of acetylation on newly synthesized yeast histone H3.

Formosa and colleagues have previously demonstrated that *pob3* Q308K cells require the histone H3 NH_2-terminal tail, as the *pob3* Q308K mutation is synthetically lethal with a large deletion within this domain. In addition, the *pob3* Q308K allele was also shown to display a strong synthetic growth defect when combined with a deletion of the histone acetyltransferase *GCN5* (VanDemark et al. 2006). Importantly, Gcn5p was recently shown to play an important role in replication-coupled nucleosome assembly (Burgess et al. 2010). To determine whether synthetic growth defects between *pob3* Q308K and mutations that alter sites of acetylation on the histone H3 NH_2-terminal tail occurred in a specific pattern (or patterns) that would be indicative of a pattern of acetylation on newly synthesized yeast histone H3, we combined the *pob3* Q308K allele present in the 8244-18-4 strain background with a collection of histone H3 alleles that mutate all five of the lysine residues in the NH_2-terminal tail to arginine in every possible combination (i.e. all single, double, triple, quadruple and quintuple mutants).

As described above, the 8244-18-4 strain contains a single copy of the genes encoding histone H3 and histone H4 on a *URA3*-based plasmid. Following introduction of a plasmid containing a mutant histone H3 allele, the ability of this strain to survive with only the altered form of histone H3 is indicated by its ability to grow in the presence of 5-FOA. The data in Fig. 3a compare the viability of a wild type and a *pob3* Q308K strain when combined with specific histone H3 alleles. When all five lysine residues were changed to arginine there was a significant growth defect in the wild type strain that was exacerbated by the *pob3* Q308K mutation. Restoration of a single lysine residue at any position except 18 restored full viability to the wild type strain. Interestingly, viability is only restored in the *pob3* Q308K background when there is a single lysine residue at either position 14 or 23. This suggests that when yFACT activity is impaired, the ability to acetylate either histone H3 lysine 14 or lysine 23 is essential for viability. The importance of acetylation of histone H3 lysine 14 and 23 would be consistent with the involvement of these residues in chromatin assembly in other organisms.

A second pattern of genetic interactions was observed when all of the triple K to R alleles were examined. As expected from the previous results, mutants in which both lysine 14 and 23 are mutated were synthetically lethal with the *pob3* Q308K allele. However, there was one exception. When both histone H3 lysines 14 and 23 were changed to arginine, the presence of a lysine residue at positions 9 and 27 restored viability. This requirement for

histone H3 lysines 9 and 27 was only seen when both lysines 14 and 23 were altered as lysine 9 and lysine 27 could both be mutated to arginine if either position 14 or 23 could be acetylated. A potential role for histone H3 lysines 9 and 27 in histone deposition is consistent with the observation that these sites display the highest levels of acetylation on newly synthesized histones and are also sites modified by Gcn5p, a HAT that has been implicated in the acetylation of newly synthesized histone H3 (Burgess et al. 2010; Kuo et al. 1996). Experiments with histone H3 single and double lysine to arginine mutations gave consistent results with the histone H3 K14,23R allele showing the lowest level of viability in the *pob3* Q308K background (Supplemental Figure 1A and 1B).

As we had observed differences in the severity of the synthetic growth defects of the *pob3* Q308K and histone H4 mutations between the 8244-18-4 strain background and the UCC1111 background, we also examined the collection of H3 alleles in the UCC1111 strain. In this background, synthetic lethality is indicated by a lack of red colonies due to the inability to lose the *ADE2*-based plasmid containing the wild type histone H3/H4 genes. Consistent with the data described above, we were unable to isolate red colonies when the *pob3* Q308K allele was combined with a mutation that changed all five lysine residues into arginine (Fig. 3b). However, we were able to obtain red colonies from all of the histone H3 quadruple lysine mutants. This again indicates that the pattern of synthetic lethal interactions that are observed with *pob3* Q308K mutants can be strain specific and that, in general, these interactions are less severe in the UCC1111 background. As our results were not able to definitively identify a pattern of acetylation for the NH_2-terminal tail of histone H3, we have not performed a detailed phenotypic analysis of the interactions between a specific histone H3 allele and the *pob3* Q308K allele.

Genetic interactions between *POB3* and the acetylation of histone H3 lysine 56

Histone H3 lysine 56 acetylation is the modification that has been most directly linked to the process of histone deposition. The acetylation of histone H3 lysine 56 occurs in S phase and has been shown to be involved in replication-coupled chromatin assembly through enhancing the interaction of histone H3/H4 complexes with the CAF-1 histone chaperone complex (Chen et al. 2008; Li et al. 2008; Masumoto et al. 2005). Therefore, we examined whether genetic interactions existed between mutations that alter this site of modification and the *pob3* Q308K allele.

We introduced the plasmids containing H3 K56R and K56Q alleles into wild type and *pob3* Q308K strains (UCC1111 background). The H3 K56R allele was used to mimic the constitutively unacetylated state and the H3 K56Q allele was used to mimic constitutive acetylation at this site. As has been reported by others, in a *POB3* strain, the H3 K56R allele caused several dramatic phenotypes, such as growth defects, sensitivity to HU and MMS and defects in telomeric silencing (Fig. 4) (Celic et al. 2008; Driscoll et al. 2007; Han et al. 2007; Masumoto et al. 2005; Recht et al. 2006; Schneider et al. 2006). The H3 K56Q allele had little effect on most of these phenotypes. However, in our strain background, the histone H3 K56Q mutation does cause an increase in sensitivity to MMS and a defect in telomeric silencing (Fig. 4d, e).

When the H3 K56R allele was combined with the *pob3* Q308K mutation, there was little or no synthetic growth defect (at either 30 or 37°C, Fig. 4g, h) as the H3 K56R/*pob3* Q308K double mutant grew like the histone H3 K56R single mutant. In addition, there were no significant additive defects observed when these alleles were combined in HU or MMS sensitivity or for telomeric silencing (Fig. 4a–e). None of the combinations of mutations in histone H3 or *POB3* influenced sensitivity to UV radiation (Fig. 4f).

A complex pattern of interactions was observed when the H3 K56Q allele was combined with the *pob3* Q308K allele. Phenotypes that were impacted by the histone H3 K56Q mutation alone, MMS sensitivity and telomeric silencing, demonstrated synthetic defects when this histone H3 mutation was combined with the *pob3* Q308K allele (Fig. 4d, e). When assessing growth at 30°C or HU sensitivity, which was not affected by the H3 K56Q allele, we observed that this mutation did not alter the phenotype of the *pob3* Q308K single mutant (Fig. 4b, c, g). However, for growth at 37°C, which is also not affected by the H3 K56Q mutation, we observed a synthetic decrease in viability of the *pob3* Q308K/H3 K56Q double mutant (Fig. 4h). These results suggest that modulation of the acetylation state of H3 lysine 56 can have differential effects on nuclear processes. In addition, these results indicate that modification of this site has functional overlap with yFACT complex function.

Genetic interactions between *POB3* and the acetylation of histone H4 lysine 91

Histone H4 lysine 91 lays within an interface between histone H3/H4 tetramers and histone H2A/H2B dimers. The importance of this residue in histone octamer structure is suggested by its ability to form a salt bridge with a glutamic acid residue in histone H2B (E75 of yeast histone H2B) (Cosgrove et al. 2004; Luger et al. 1997). Hence, acetylation of this residue may play a role in regulating the interactions between histone H3/H4 tetramers and histone H2A/ H2B dimers and, thereby, influence the formation or stability of histone octamers during the chromatin assembly process (Ye et al. 2005). Given the evidence that the FACT complex can modulate the association of histone H2/H2B dimers with nucleosomes, we were interested in identifying genetic interactions between mutations that modify histone H4 K91 and the *pob3* Q308K allele (Reinberg and Sims 2006).

We introduced plasmids containing H4 K91R, H4 K91Q and H4 K91A alleles into *POB3* and *pob3* Q308K cells (UCC1111 background). As expected from previous results, we were able to isolate cells with these H4 mutants as the only source of histone H4 in the *POB3* strain (Supplemental Figure S2) (Ye et al. 2005). However, as seen in Fig. 5a, the *pob3* Q308K strain was not able to lose the wild type histone plasmid in the presence of the H4 K91A or H4 K91Q allele. This indicates that the *pob3* Q308K allele is synthetically lethal with alleles that either mimic the constitutive acetylation of histone H4 lysine 91 or remove the positive charge at this residue suggesting that when yFACT activity is compromised, the presence of a positive charge at histone H4 lysine 91 is essential for cell viability.

Combining the H4 K91R and *pob3* Q308K alleles resulted in viable cells (Fig. 5a). We then tested whether this combination of mutations generated synthetic phenotypes. The H4 K91R allele had no effect on any of the phenotypes examined (Fig. 5b–i). When the H4 K91R allele was combined with the *pob3* Q308K allele, there was an increased sensitivity to HU and MMS and a decrease in telomeric silencing (Fig. 5c–f). The H4 K91R/*pob3* Q308K combination also showed no effect on growth at 30°C, but there was a decrease in viability at 37°C (Fig. 5h, i). As seen with the histone H3/*pob3* Q308K mutant combinations, there was no increase in sensitivity to UV radiation (Fig. 5g). These results suggest that the ability to modulate the acetylation state of histone H4 lysine 91, or perhaps form a salt bridge between histones H4 and H2B, becomes important when yFACT activity is not optimal.

Potential effects of newly synthesized histone acetylation on Pob3p function

The genetic interactions observed between mutations in *POB3* and the sites of acetylation on newly synthesized histones H3 and H4 can be interpreted in two basic ways. First, these interactions may indicate that Pob3p and newly synthesized histone acetylation function in parallel pathways that function redundantly to accomplish proper chromatin assembly. Alternatively, Pob3p and newly synthesized histone acetylation may function in a common pathway and the alleles used in this study may have partial and additive effects on the

function of this pathway. In an effort to distinguish between these possibilities, we have explored a number of mechanisms that might underlie the genetic interactions observed.

Mutations that alter the sites of acetylation on newly synthesized histones could act by a number of mechanisms to decrease the effectiveness of a Pob3p-dependent chromatin assembly pathway. The most straightforward mechanism is that the histone mutations could lower the expression of Pob3p. Decreasing the level of Pob3p would then exacerbate the phenotypes that result from the Q308K mutation. To test this idea, we measured Pob3p levels by western blot using antibodies that recognize the TAP tag to visualize Pob3p that contains this tag. There is no significant decrease in Pob3p levels in any of the H3/H4 mutant strains and, in particular, no decreases in Pob3p levels that correlate with any of the synthetic phenotypes that were observed (Supplemental Figure 3A and 3B).

Another mechanism by which newly synthesized histone acetylation site mutants could influence Pob3p-dependent chromatin assembly is through directly affecting the interaction of Pob3p with histones H3 and H4. There is precedence for this type of mechanism as the acetylation of histone H3 lysine 56 has been shown to promote the binding of H3/H4 to the histone chaperones CAF-1 and Rtt106p (Li et al. 2008). Therefore, we analyzed mutations that alter the sites of newly synthesized histone acetylation to see if they disrupt the binding of these histones to Pob3p (both the WT and Q308K alleles).

The *POB3* and *pob3* Q308K strains used in this study contain a TAP tag fused to the COOH-terminus of the *POB3* open-reading frame. Whole cell extracts were made from cells containing either WT or mutant histones alleles and the TAP tag was used to isolate Pob3p and its associated proteins. These extracts are prepared at a low ionic strength and, therefore, the histones present represent the soluble pool. As seen in Fig. 6a, histone H4 (as a marker of histone H3/H4 complexes) co-purified with Pob3p and this association was not diminished by the *pob3* Q308K mutation. The H4 K5,12R and H4 K91R alleles also did not diminish the interaction between Pob3p and histone H4 (Fig. 6a, c). Therefore, these mutations did not generate a synthetic phenotype by decreasing the interaction between Pob3p and histones. The situation with the histone H3 K56 mutations was more complex. The H3 K56Q allele decreased the association of histones with FACT in the *POB3* strain, while the H3 K56R allele decreased the level of histone associated with the Q308K mutant of Pob3p (Fig. 6b). Interestingly, while the H3 K56Q allele did not cause a defect in most of the phenotypes we examined in the POB3 background (growth and HU and UV sensitivity), this allele caused a distinct defect in telomeric silencing and MMS sensitivity. Similarly, the H3 K56R allele displayed a number of synthetic phenotypes when combined with the pob3 Q308K mutation. These results suggest that a decreased association of the FACT complex with histones may underlie the phenotypes seen with the histone H3 lysine 56 mutations.

In an effort to better understand the involvement of the yFACT complex in histone deposition and chromatin assembly, we have systematically analyzed genetic interactions that exist between an essential component of this complex, *POB3*, and all of the sites of acetylation that have been identified on newly synthesized histones. The identification of multiple interactions as evidenced by the production of synthetic defects using a variety of phenotypes that monitor nuclear processes that can be influenced by chromatin assembly strongly supports the hypothesis that yFACT plays an important role in histone deposition.

Discussion

FACT is a multifunctional complex that modulates chromatin structure and influences both transcription and DNA replication (Belotserkovskaya and Reinberg 2004; Formosa 2008; Reinberg and Sims 2006). Biochemically, FACT has the ability to reorganize nucleosomes

without the concomitant hydrolysis of ATP. This reorganization can involve the removal of a histone H2A/H2B dimer from the histone octamer. In addition, FACT has been shown to physically interact with both histone H3/H4 and histone H2A/H2B complexes and to promote the assembly of nucleosomes (Belotserkovskaya et al. 2003; Orphanides et al. 1999; Stuwe et al. 2008). The in vivo significance of the histone chaperone/chromatin assembly factor activity of FACT has been supported by a number of genetic experiments. First, mutations of the essential subunits of yFACT can be synthetically lethal with the Hir/Hpc replication-independent chromatin assembly factor complex (Formosa et al. 2002). In addition, yFACT mutants that are defective in DNA replication have been found to be synthetically lethal in combination with an allele of histone H4 that cannot be acetylated in the pattern normally found on newly synthesized molecules (on lysines 5 and 12) (Formosa et al. 2002; VanDemark et al. 2006). We have expanded on this observation by showing that this synthetic lethality requires mutation of both histone H4 lysine 5 and lysine 12.

Despite the fact that the diacetylation of histone H4 lysines 5 and 12 may be a universally conserved characteristic of newly synthesized molecules, mutations that prevent this modification (such as H4 K5,12R) have relatively insignificant phenotypic consequences (Ma et al. 1998; Sobel et al. 1994, 1995). This has led to the proposition that this pattern of acetylation plays only a minor role in the process of chromatin assembly (Shibahara et al. 2000). The observation that an H4 K5,12R allele was lethal in a *pob3* Q308K mutant background suggested not only that yFACT might be involved in nucleosome deposition, but also that this diacetylation pattern may be a critical component of at least one chromatin assembly pathway (VanDemark et al. 2006). Whether the relevant histone deposition event is occurring in the context of DNA replication or another nuclear process requiring chromatin assembly, such as DNA repair or transcription, is not clear. This idea is based on the interpretation that the synthetic lethality displayed by the *pob3* Q308K/H4 K5,12R double mutant results from yFACT and histone H4 lysine 5/12 acetylation functioning in parallel, and functionally redundant, chromatin assembly pathways. An alternative interpretation is that yFACT and histone H4 lysine 5/12 acetylation both function in a common pathway of chromatin assembly and that mutations in each only partially disable the pathway. When the mutations are combined, the pathway is compromised to a greater extent and the cells are no longer viable. Distinguishing between these two possibilities is difficult when it is not possible to construct null alleles of the genes involved.

We have observed that the severity of the growth defect generated by combining the *pob3* Q308K and histone H4 K5,12R mutations appears to be strain dependent. Importantly, however, the pattern of mutations on the H4 tail that genetically interact with the *POB3* allele is consistent, strongly supporting the hypothesis that yFACT acts in a pathway that functionally overlaps with one involving newly synthesized histones. The fact that histone alleles can have varied effects in different strains has been reported previously. For example, a comprehensive analysis of histone mutations identified numerous histone alleles that demonstrated either lethality or other significant phenotype, in one strain background but produced little phenotypic effect in other backgrounds (Dai et al. 2008). The basis for these strain specific effects of histone alleles has not been identified.

Regardless of strain background, the growth defects observed when the *pob3* Q308K allele is combined with mutations in histone H4 require that both lysine 5 and lysine 12 are altered, strongly suggesting that the role of yFACT is linked to the acetylation of newly synthesized histones. However, the dramatic increase in sensitivity of the *pob3* Q308K strain to HU-induced replicational stress can be caused by a mutation of either histone H4 lysine 5 or lysine 12. This suggests a number of possibilities. First, in the context of replicational stress, the acetylation of either histone H4 lysine 5 or lysine 12 may be sufficient to support the assembly or reorganization of chromatin that is necessary to

promote DNA replication. Alternatively, in the presence of HU, the important functions of yFACT or histone H4 NH_2-terminal tail acetylation may not be related to the assembly of newly synthesized histones. Rather, these factors may be involved in events such as the transcriptional or check point responses to the double strand breaks produced by the collapse of replication forks or be directly involved in their repair (van Attikum and Gasser 2009).

Hat1p is the catalytic subunit of a highly conserved type B histone acetyltransferase complex that is thought to be responsible for the acetylation of the NH_2-terminal tail of newly synthesized histone H4 (Parthun 2007). Therefore, it was surprising that not only did a *hat1Δ* not mimic an H4 K5,12R allele when combined with a *pob3* Q308K allele but that it specifically suppressed the phenotypes that resulted from mutation of histone H4 lysine 12 to arginine. This suggests that when histone H4 lysine 12 cannot be acetylated, the presence of Hat1p can be detrimental. This effect may be linked to the histone chaperone activity of the Hat1p-containing complex. In both cytoplasmic and nuclear extracts, the Hat1p-containing complex is stably associated with histones H3 and H4 (Ai and Parthun 2004; Ejlassi-Lassallette et al. 2011; Mosammaparast et al. 2002; Poveda et al. 2004). One possibility is that the proper dissociation of the Hat1p/histone H3–H4 complex may be triggered by the acetylation of histone H4 lysine 12 by Hat1p. In this scenario, the presence of an arginine residue at position 12 would prevent this dissociation and the inappropriate presence of Hat1p would interfere with downstream events in the chromatin assembly process.

While most organisms display a discreet pattern of acetylation on the NH_2-terminal tail of histone H3, the modification state of newly synthesized histone H3 molecules in *S. cerevisiae* is less clear. Direct sequencing of the histone H3 NH_2-terminal tail following pulse labeling with ^{3}H-lysine indicated that there were varied levels of acetylation on five lysine residues (positions 9,14, 18, 23 and 27) (Kuo et al. 1996). Of these, the highest levels of modification were found on lysines 9 and 27. Moderate levels of acetylation were found on lysines 14 and 23 and trace levels were seen on lysine 18. An S phase-specific peak of acetylation on lysine 9 has also been identified (Adkins et al. 2007). Our results suggest that in the presence of the *pob3* Q308K mutation, which sensitizes cells to alterations in the pattern of acetylation on newly synthesized histone H3, lysines 14 and 23 (individually) are the most critical for viability. In addition, the combination of lysines 9 and 27 is also important when both histone H3 lysines 14 and 23 are altered. Based on these data, it is tempting to speculate that there are discreet pools of newly synthesized histone H3 molecules in *S. cerevisiae* that contain distinct patterns of acetylation and that these pools of histones can be targeted to specialized pathways of chromatin assembly.

The most extreme genetic interaction that we have identified is between *pob3* Q308K and mutations that alter histone H4 lysine 91 to either glutamine or alanine. The pattern of synthetic lethality observed between the *POB3* and H4 K91 alleles indicates that mutations at histone H4 lysine 91 that destabilize the histone octamer are not tolerated when yFACT activity is compromised (Ye et al. 2005). While histone H4 lysine 91 acetylation has been observed on soluble histones that are associated with a histone chaperone complex, it is not known whether this modification is specific to newly synthesized histones or whether it can also occur on histones following their assembly into chromatin. Therefore, it is not clear whether the genetic interactions that exist between POB3 and histone H4 K91 mutations are indicative of defects in chromatin assembly. An alternative is that histone H4 lysine 91 may be modified as nucleosomes become disassembled during the process of transcription as a mechanism for keeping chromatin in an open configuration. Synthetic lethality between *pob3* Q308K and H4 K91Q or H4 K91A alleles could then be a reflection of cumulative defects in transcription elongation through chromatin.

POB3 is the second gene reported to show strong synthetic growth defects in combination with mutations at histone H4 lysine 91. The first is *UBP8* (Dai et al. 2008). Ubp8p is responsible for the deubiquitylation of histone H2B lysine 123, which plays an important role in the transcription cycle (Henry et al. 2003). The molecular basis for the synthetic growth defects seen in *ubp8*Δ/H4 K91 mutants is not clear. However, histone H4 lysine 91 was recently identified as a site of ubiquitylation in mammalian cells opening up the possibility that ubiquitylation of histone H2B lysine 123 may functionally overlap with the ubiquitylation of histone H4 lysine 91 (Yan et al. 2009).

The mechanisms by which acetylation influences the process of chromatin assembly are not clear. For the case of histone H3 lysine 56 acetylation, one mechanism appears to be through modulating the interaction between newly synthesized histones and their cognate histone chaperones (Li et al. 2008). Our results suggest that this site of modification may also influence the binding of newly synthesized histones to the FACT complex, as well. However, the sites of acetylation on newly synthesized histone H4, which show similar patterns of genetic interaction with *POB3* mutations, do not impact the histone–FACT complex interaction suggesting that other mechanisms are also involved.

We have identified genetic interactions between the yFACT component *POB3* and all of the sites of acetylation on newly synthesized histones. Taken together, these observations support the hypothesis that the yFACT complex is involved in the process of nucleosome assembly. In addition, the complexity of the interactions seen between *POB3* and sites of new histone acetylation indicate that there are multiple, overlapping pathways by which chromatin can be assembled and that the requirements for specific sites of acetylation may differ between these pathways. Future studies will be aimed at identifying the precise steps in chromatin assembly that are influenced by yFACT and the acetylation of newly synthesized histones.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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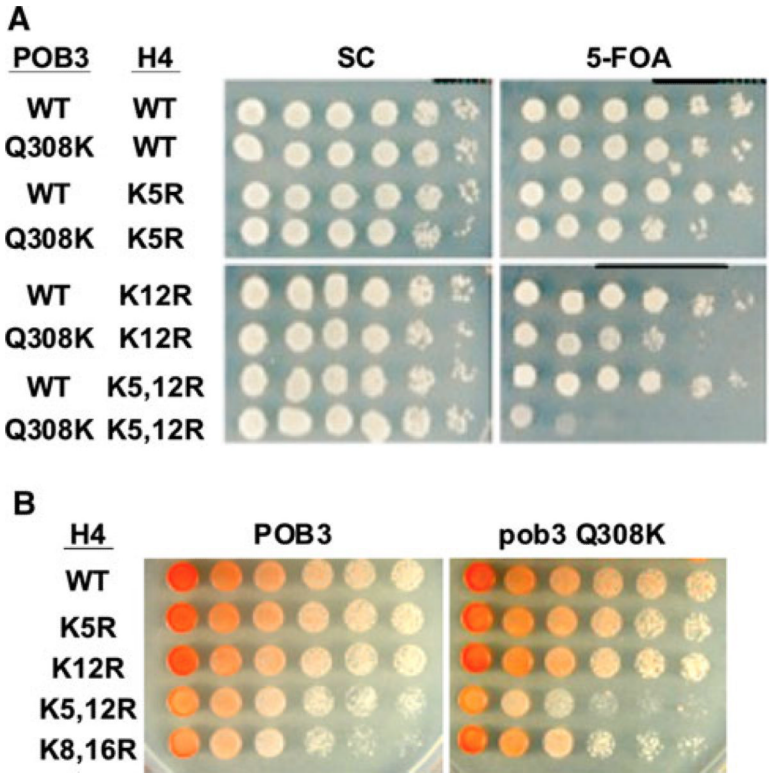

Fig. 1.

Genetic interactions between *POB3* and histone H4 lysines 5 and 12. **a** Strains 8244-13-2 or 8244-18-4, which contain the *POB3* or *pob3* Q308K alleles, respectively, also contain a wild type copy of the *HHT2-HHF2* loci on a *URA3*-based plasmid. Plasmids containing the indicated alleles of histone H4 (TRP1-based) were introduced into these strains and cells were grown overnight in synthetic complete (SC) media lacking tryptophan. Cells were harvested and tenfold serial dilutions were prepared and spotted on SC plates in the absence or presence of 5-FOA. **b** Plasmids containing the indicated alleles of histone H4 were introduced into strains DNY214 and DNY215 (*POB3* and *pob3* Q308K, respectively). Following plasmid shuffle, tenfold serial dilutions were spotted on plates containing rich media (YPD)

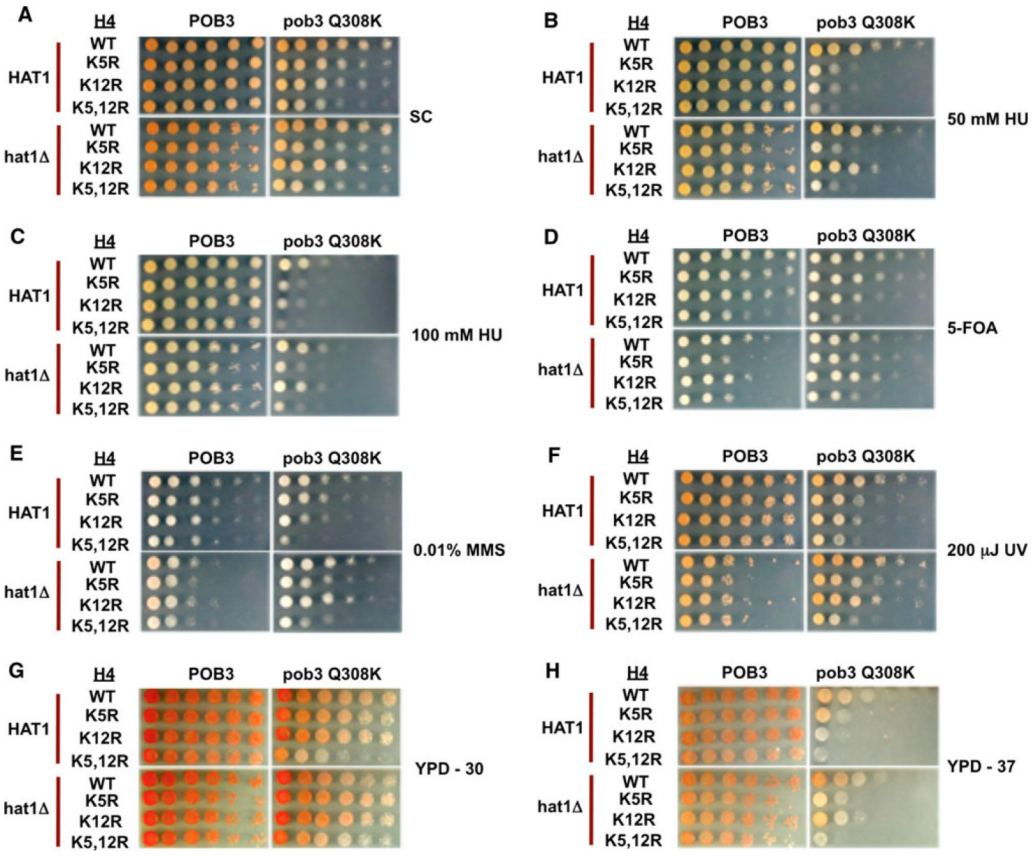

Fig. 2.

Genetic interactions between *POB3* and the histone acetyltransferase *HAT1*. Plasmids containing the indicated alleles of histone H4 were introduced in strains with the indicated alleles of *POB3* and *HAT1*. Following plasmid shuffle, tenfold serial dilutions were spotted on plates containing the indicated agent or subjected to the indicated treatment

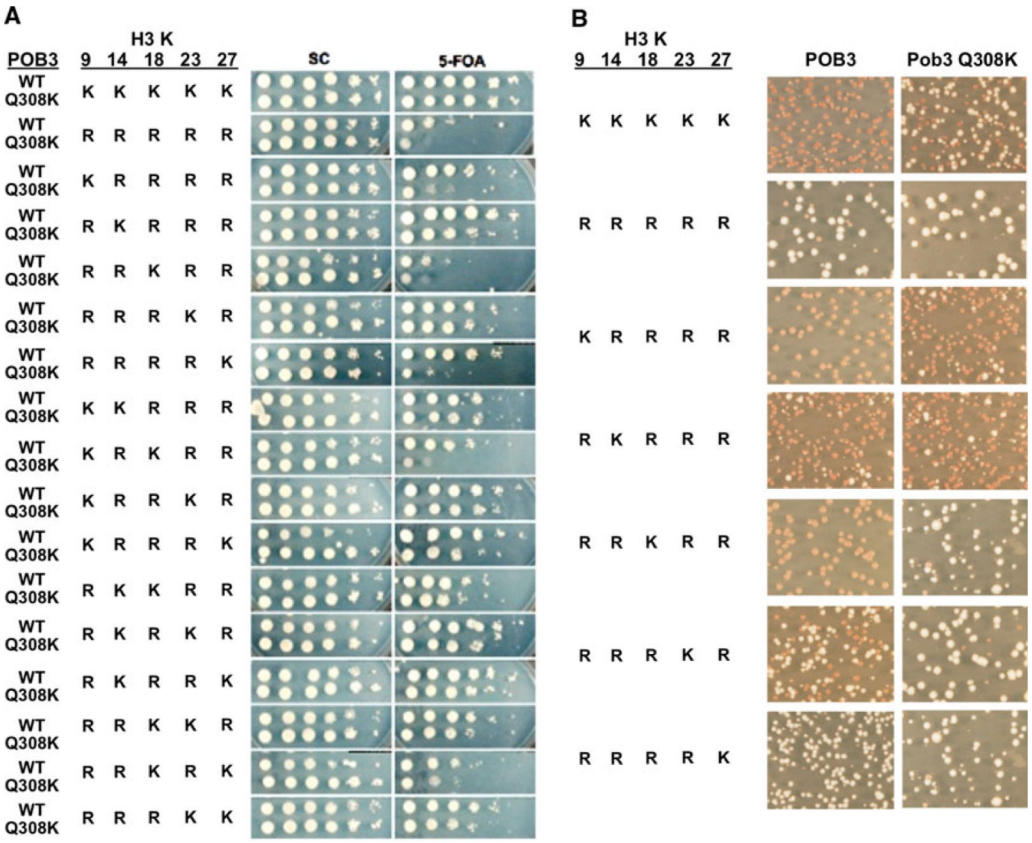

Fig. 3.

Genetic interactions between *POB3* and the sites of acetylation on the histone H3 NH_2-terminal tail. **a** Plasmids containing the indicated alleles of histone H3 were introduced into strains 8244-13-2 or 8244-18-4 and the cells were analyzed as described in Fig. 1a. **b** Plasmids containing the indicated alleles of histone H3 were introduced into strains DNY214 and DNY215 as described for Fig. 1. Cells were grown extensively in SC-Trp media and then plated on rich media (YPD). Plates were photographed after 3 days growth at 30°C

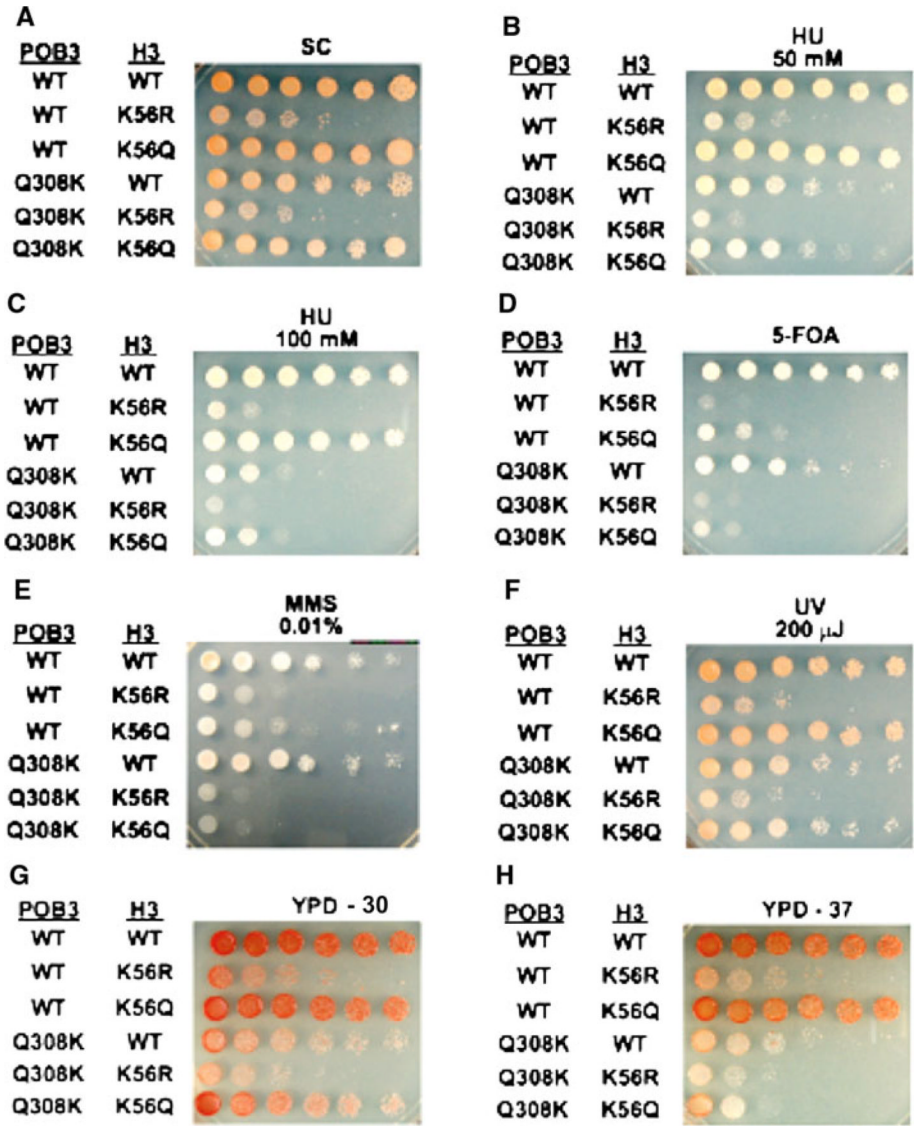

Fig. 4.

Genetic interactions between *POB3* and the acetylation of histone H3 lysine 56. Plasmids containing the indicated alleles of histone H3 were introduced into strains DNY214 (*POB3*) and DNY215 (*pob3* Q308K). Cells were then analyzed as described in Fig. 2

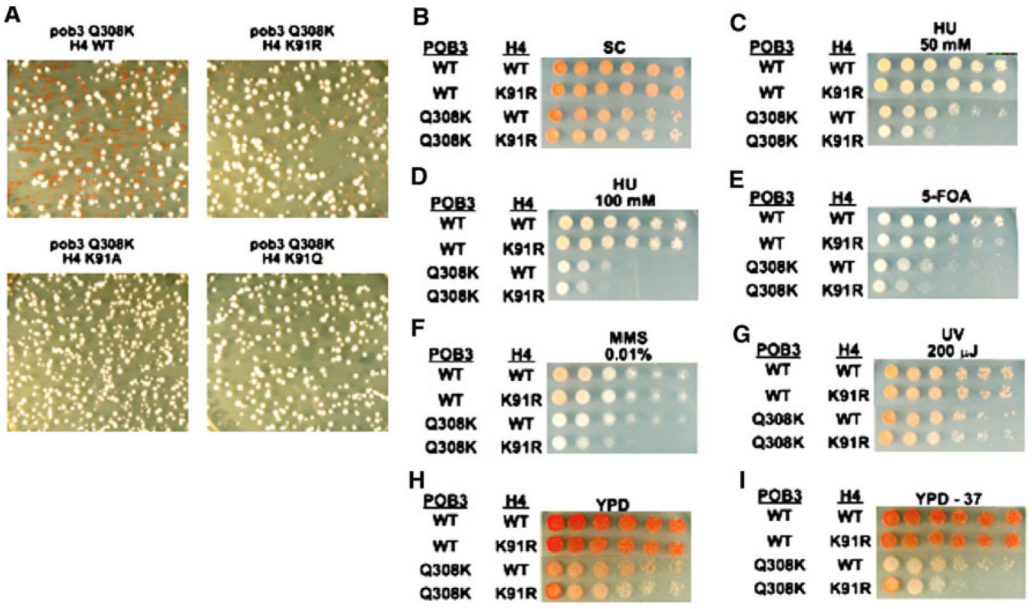

Fig. 5.

Genetic interactions between *POB3* and the acetylation of histone H4 lysine 91. **a** Plasmids containing the indicated alleles of histone H4 were introduced into strains DNY214 (*POB3*) and DNY215 (*pob3* Q308K) and analyzed as described in Fig. 3b. **b–i** Strains with the indicated alleles of histone H4 and *POB3* were analyzed as described in Fig. 2

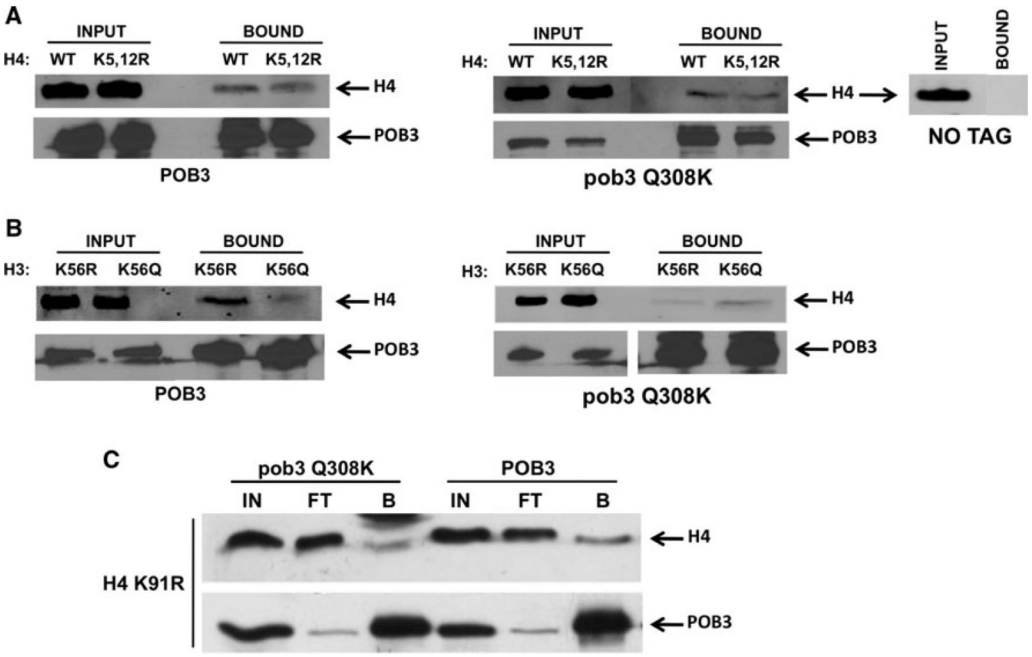

Fig. 6.

Impact of histone H3/H4 mutations on Pob3p-histone interactions. **a** Extracts (INPUT) from either *POB3* (*left*) or *pob3* Q308K strains (*middle*) containing a TAP tag on the COOH-terminus of Pob3p or an untagged control strain (UCC1111, *right*) and the indicated alleles of histone H4 were immunoprecipitated using IgG-Sepharose beads. After washing, the beads were incubated in 1 × SDS-PAGE load dye and the proteins bound to the beads (BOUND) analyzed by SDS-PAGE and western blotting against the indicated proteins. **b** Identical to (**a**) but with the indicated alleles of histone H3. **c** Same as in (**a**) with the input (IN) unbound (FT) and bound (**b**) fractions shown

Table 1

Yeast Strains used in this study

Strain	Genotype	Reference or source
UCC1111	*MAT α ade2::his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL (VII-L) hhfl-hht1::LEU2 hhf2-hht2::MET15/pRS412 (ADE2 CEN ARS)-HHF2-HHT2*	Kelly et al. (2000)
ASY50	*MAT α ade2::his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL (VII-L) hhf1-hht1::LEU2 hhf2-hht2::MET15 hat1::LYS2/pRS412 (ADE2 CEN ARS)-HHF2-HHT2*	Kelly et al. (2000)
DNY214	*MAT α ade2::his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL (VII-L) hhf1-hht1::LEU2 hhf2-hht2::MET15/pRS412 (ADE2 CEN ARS)-HHF2-HHT2 POB3-TAP Tag (TRP1)*	This study
DNY215	*MAT α ade2::his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL (VII-L) hhf1-hht1::LEU2 hhf2-hht2::MET15/pRS412 (ADE2 CEN ARS)-HHF2-HHT2 pob3Q308K-TAP Tag (TRP1)*	This study
DNY216	*MAT α ade2::his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL (VII-L) hhf1-hht1::LEU2 hhf2-hht2::MET15 hat1::LYS2/pRS412 (ADE2 CEN ARS)-HHF2-HHT2 POB3-TAP Tag (TRP1)*	This study
DNY217	*MAT α ade2::his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL (VII-L) hhf1-hht1::LEU2 hhf2-hht2::MET15 hat1::LYS2/pRS412 (ADE2 CEN ARS)-HHF2-HHT2 pob3Q308K-TAP Tag (TRP1)*	This study
8244-13-2	*MATα ura3-Δ0 leu-Δ0 trp1-Δ2his3 lys2-128∂ hht1-hhf1-Δ(::LEU2) hht2-hhf2-Δ(::KanMX) DS1700 (YCp URA3 HHT2-HHF2)*	VanDemark et al. (2006)
8244-18-4	*MATα ura3-Δ0 leu-Δ0 trp1-Δ2his3 lys2-128∂ hht1-hhf1-Δ(::LEU2) hht2-hhf2-Δ(::KanMX) pob3-Q308K DS1700 (YCp URA3 HHT2-HHF2)*	VanDemark et al. (2006)