A Systematic Mutational Analysis of a Histone H3 Residue in Budding Yeast Provides Insights into Chromatin Dynamics

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ABSTRACT

In previous work using the Saccharomyces cerevisiae model system, a mutant version of histone H3—H3-L61W—was found to confer a variety of abnormal growth phenotypes and defects in specific aspects of the transcription process, including a pronounced alteration in the distribution pattern of the transcription elongation factor Spt16 across transcribed genes and promotion of cryptic transcription initiation within the FLO8 gene. To gain insights into the contribution of the H3-L61 residue to chromatin function, we have generated yeast strains expressing versions of histone H3 harboring all possible natural amino acid substitutions at position 61 (H3-L61X mutants) and tested them in a series of assays. We found that whereas 16 of the 19 H3-L61X mutants support viability when expressed as the sole source of histone H3 in cells, all 19 confer abnormal phenotypes ranging from very mild to severe, a finding that might in part explain the high degree of conservation of the H3-L61 residue among eukaryotes. An examination of the strength of the defects conferred by each H3-L61X mutant and the nature of the corresponding substituted residue provides insights into structural features of the nucleosome required for proper Spt16—gene interactions and for prevention of cryptic transcription initiation events. Finally, we provide evidence that the defects imparted by H3-L61X mutants on Spt16—gene interactions and on repression of intragenic transcription initiation are mechanistically related to each other.

Within the eukaryotic cell nucleus, DNA is condensed through the formation of chromatin, a protein—DNA complex with the nucleosome as its fundamental unit. Nucleosomes are composed of ~147 base-pairs of DNA wrapped around the histone octamer, itself composed of a histone H3-H4 tetramer and two H2A-H2B dimers (Luger et al. 1997). In addition to directing DNA condensation, nucleosomes also are critically important for the regulation of processes that use DNA as template, including gene transcription. A variety of factors have been identified that can regulate specific aspects of transcription by affecting the dynamics of nucleosomes. These include enzymes that modify histone residues through the addition or removal of specific chemical groups, chromatin remodeling complexes that can alter DNA—histone interactions or the stoichiometry of canonical histones and histone variants within individual nucleosomes in an ATP-dependent manner, and histone chaperones that facilitate the assembly and disassembly of nucleosomes through mechanisms that do not rely on ATP hydrolysis (Campos and Reinberg 2009; Rando and Winston 2012).

FACT (FAcilitates Chromatin Transcription/Transactions) is a highly conserved histone chaperone complex with roles in several chromatin-based processes. In the budding yeast Saccharomyces cerevisiae, FACT is a heterodimer composed of the proteins Spt16 and Pob3 that can physically interact with nucleosomes with the assistance of the HMG protein Nhp6 (Brewster et al. 1998; Formosa et al. 2001; Ruone et al. 2003). FACT’s ability to interact with nucleosomes and alter their structure is used by cells to control chromatin environment dynamics during both the initiation and elongation phases of transcription (Reinberg and Sims 2006; Duina 2011; Winkler and Luger 2011; Formosa 2012). Arguably, the most extensively studied and better-understood aspect of FACT biology relates to its function during transcription elongation. Work by many groups during the course of the last few decades have demonstrated that FACT plays a dual role during elongation, altering nucleosome conformation ahead of
transcribing RNA polymerase II (Pol II) to facilitate Pol II progression across genes and assisting in the reassembly of nucleosomes after Pol II passage (Reinberg and Sims 2006; Duina 2011; Winkler and Luger 2011; Formosa 2012).

Several studies have provided insights into the mechanism of action of FACT in these processes. Some of these studies have culminated in a model whereby FACT—nucleosome interactions result in formation of so-called reorganized nucleosomes, in which FACT and all nucleosomal components are tethered together but whose histone subunits are more prone to dissociation during engagement with transcribing Pol II complexes (Formosa 2008; Xin et al. 2009). Other studies have supported a mechanism that involves specific interactions between FACT and H2A-H2B dimers as central drivers for alterations in DNA-histone interactions conducive to transcription elongation (Winkler et al. 2011; Hondele et al. 2013; Hsieh et al. 2013). FACT is thought to contribute to the maintenance of the epigenetic code across transcribed genes by reassembling nucleosomes using the same histone H3 and H4 subunits that were present on chromatin ahead of Pol II passage (Jamai et al. 2009).

A comprehensive model for the mode of action of FACT at transcribed genes requires an understanding of the physical and functional interplay between FACT and histones in vivo. Insights into these relationships have come in part from genetic studies in S. cerevisiae in which the effects of specific histone mutations on FACT function have been assessed. For example, recent work from the Formosa laboratory has shown that specific mutations predicted to disrupt the H2A-H2B dimer-H3-H4 tetramer interface suppress defects in FACT activity, a result consistent with the notion that FACT facilitates dimer-tetramer dissociation (McCullough et al. 2011). In another recent study, analyses of yeast cells expressing a histone H2B mutant lacking the HBR domain—a conserved region within the N-terminus of the protein—revealed a requirement for this domain in promoting FACT-mediated H2A-H2B eviction from nucleosomes (Zheng et al. 2014).

We have previously identified specific histone mutants—H3-L61W, H4-R36A, and H4-K31E—that markedly impair the ability of the FACT subunit Spt16 to properly associate with chromatin (Duina et al. 2007; Nguyen et al. 2013). These mutants cause decreased levels of Spt16 occupancy at the 5' regions of transcribed genes and increased levels at the 3' regions of the same genes, resulting in an overall shift in Spt16 distribution toward the 5' ends of genes. Because H3-L61, H4-R36 and H4-K31 are located near each other on the side of nucleosomes, we have proposed that these residues define a nucleosomal region important for proper Spt16—gene interactions, possibly by participating in physical interactions with Spt16 during transcription (Nguyen et al. 2013). In this study, we have carried out a systematic mutational analysis of the H3-L61 residue by generating and testing all possible natural amino acid substitutions at this residue (H3-L61X mutants) to determine its contribution in ensuring proper Spt16—chromatin interactions. As the H3-L61W mutant also causes cryptic intragenic transcription initiation at the FLO8 gene, we have tested the H3-L61X mutants for this defect and have uncovered a correlation between H3-L61X-mediated defects in repression of intragenic transcription initiation and impairments of Spt16—gene interactions. Our studies shed new light onto the contribution of the H3-L61 residue to chromatin dynamics, particularly in relation to FACT-mediated processes.

**MATERIALS AND METHODS**

**Yeast strains and growth media**

All strains used in this study, listed in Table 1, are GAL2* derivatives of the S288C strain background (Winston et al. 1995). Strains yADP83-yADP88 harbor plasmid pDM9, a centromeric URA3-marked plasmid containing the HHT1 and HHI1 genes described in a previous study (Duina and Winston 2004). Details for the generation of the H3-L61X mutants are provided below. Standard genetic techniques and details for media preparation have been presented previously (Rose et al. 1990). Drug-containing plates were prepared using the following concentrations: formamide, 3%; hydroxyurea, 150 mM; and caffeine, 15 mM.

**Generation of strains expressing H3-L61X mutants**

Polymerase chain reaction (PCR) products spanning the entire HHT2 gene and harboring specific mutations at the H3-L61—encoding codon were generated for each of the 19 H3-L61X—encoding genes using the strategy shown in Figure 1. Generation of a fragments (see Figure 1B) was accomplished using forward primer OAD20 and the appropriate reverse primer L61Xrev (where X is specific for each of the desired mutations—refer to Table 2 for a list of all primers used for these experiments). Generation of b fragments was accomplished using the corresponding forward primer L61Xfor and reverse primer OAD21. The template for these reactions was genomic DNA originating from an HHT2-wild-type strain. Amplification reactions were carried out using PrimeSTAR HS DNA Polymerase (Takara, Code No. R010A). In each case, the c fragments were obtained as a result of template-skipping events during PCR amplification reactions that included primers OAD20 and OAD21 (or primers OAD479 and OAD480), the appropriate set of fragments a and b (dissolved in melted low-melting point agarose) as templates, and PrimeSTAR HS DNA Polymerase.

The c fragments were then co-transformed along with the HIS3-marked plasmid pRS413 (Sikorski and Hieter 1989) into strain yADP106, which contains the URA3 and TRP1 genes in place of the HHT2 gene (see Figure 1A). Hisr transformants were then screened for 5-fluoroorotic acid (5-FOA) resistance and ability to grow on media lacking uracil and tryptophan, since these phenotypes indicate successful homologous recombination between the ends of the c fragments and regions flanking the URA3-TRP1 genes—the pRS413 plasmid was then lost through mitotic cell division (see Figure 1C). Integration candidates were then analyzed by sequencing both strands of fragments obtained from PCR reactions using the candidates’ genomic DNA as template and primers OAD476 and OAD477, which amplify a region that includes the entire integrated fragment as well as flanking sequences. For all H3-L61X strains used for subsequent experiments, sequencing of the corresponding OAD476-OAD477—amplified fragments revealed the expected mutations at the H3-L61X codon and no additional mutations. Strains yADP90-yADP105 were obtained through genetic crosses between the H3-L61X strains described above and yADP107. Strains yADP83-yADP88 were obtained through similar genetic crosses but with a version of yADP107 that harbors the pDM9 plasmid.

**Chromatin immunoprecipitation (ChIP) assays**

ChIP assays and real-time quantitative (q)PCR experiments to determine Spt16 occupancy at the different regions tested in this study were conducted using rabbit polyclonal antibodies specific for Spt16 (a gift from Tim Formosa) as previously described (Myers et al. 2011). The primers used for amplification of the specific genomic regions assayed by real-time qPCR were as follows: PMA1 5’ region, OAD394 and OAD395; PMA1 internal region, OAD416 and OAD417; PMA1 3’ region, OAD383 and OAD384; FBA1 5’ region, OAD419 and OAD420; FBA1 internal region, OAD421 and OAD422; FBA1 3’ region, OAD423 and OAD424; and nontranscribed region on
chromosome V (also referred to as NO ORF region), OAD377 and OAD378. The sequences for these primers have been described in previous studies (Myers et al. 2011; Nguyen et al. 2013).

Visualization of the nucleosomal region of interest and modeling of H3-L61X mutants

The yeast nucleosome structures shown in Figure 6 were obtained using the PyMOL Molecular Graphics System, Version 1.5.0.3 Schrödinger, LLC using structural information obtained in a previous study (White et al. 2001) and available at the Research Collaboratory for Structural Bioinformatics protein data bank (PDB ID:1id3). In each case, modeling of an amino acid substitution at position 61 of histone H3 was accomplished using the Mutagenesis function in PyMOL and using the rotamer with the highest percentage value.

RESULTS

Generation and initial functional evaluation of H3-L61X mutants

To investigate the effects of amino acid substitutions at position 61 of histone H3 on various cellular processes, we first generated haploid yeast cells carrying targeted mutations at the endogenous HHT1 locus (one of the two histone H3-encoding genes in the haploid yeast genome) using the strategy shown in Figure 1. These experiments gave rise to 19 yeast strains, each expressing a version of histone H3 with a different substituted amino acid at position 61 (referred herein as H3-L61X mutants), collectively representing all possible natural amino acid substitutions at this position. The presence of a wild-type copy of HHT1, the other gene encoding histone H3 in yeast, ensured that any strain expressing an H3-L61X mutant unable to support viability on its own could nevertheless be generated and analyzed. Each of the 19 mutant strains was then crossed with a strain deleted for HHT1 to yield spores whose sole source of histone H3 was derived from the H3-L61X encoding gene. Results from these experiments showed that 16 of the 19 H3-L61X mutants are able to support life when present as only source of histone H3 in cells (see Figure 4), and provided initial evidence that three mutants—H3-L61P, H3-L61R, and H3-L61D—are unable to do so, as viable hht1Δ hht2 double mutants were not recovered from their respective crosses. Subsequent viability tests confirmed that these three mutants are indeed unable to support life in the absence of a wild-type source of histone H3 (Figure 2).

Effects of H3-L61X mutants on Spt16—gene interactions

Previous studies have shown that the H3-L61W mutant perturbs association of the transcription elongation factor Sp16 across transcribed genes, causing a marked shift in its distribution toward the 3’ end of transcribed genes (Dunia et al. 2007; Nguyen et al. 2013). To investigate the effects of the newly generated H3-L61X mutants on Sp16—gene interactions, we performed ChIP assays to monitor the binding pattern of Sp16 across two constitutively transcribed genes in the context of the H3-L61X mutants [note that these and all subsequent experiments have been carried out using haploid strains

Table 1 Saccharomyces cerevisiae strains

| Strain   | Genotype                                                                 |
|----------|--------------------------------------------------------------------------|
| yADP83  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 HHT2 |
| yADP84  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2:::TRP1 |
| yADP85  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP86  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP87  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP88  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP89  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP90  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP91  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP92  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP93  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP94  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP95  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP96  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP97  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP98  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP99  | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP100 | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP101 | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP102 | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP103 | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP104 | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP105 | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP106 | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |
| yADP107 | MATA his3D200 leu2A1 ura3A lys2-1288 (hht1-hhtf1):::LEU2 CEN4-GAL1pr-FLO8-HIS3 hht2::HHT2 |

a The allele at this locus is either ura3-52 or ura3Δ.
generate PCR product b includes the analogous substitution(s) (but in the sense orientation, also indicated by a red circle) as that/those present in the reverse primer used for the generation of product a. PCR products a and b are then used as templates for the PCR reaction shown in diagram 2. Template-switching events during the PCR can produce DNA molecules that span both fragments a and b, which can then serve as templates for the generation of full length PCR product c as shown in diagram 3. (C) Strain yADP106 is co-transformed with PCR products c and with a plasmid harboring the HIS3 nutritional marker. Cells from the transformation reactions are plated on media lacking histidine, and resulting colonies are then screened for 5-FOA resistance and for a Ura-/Trp- phenotype to identify cells that have replaced the hht2 gene with the nutritional markers URA3 and TRP1 markers through homologous recombination to generate strain yADP106. (B) Two overlapping segments of the wild-type HHT2 gene are amplified by PCR as illustrated in diagram 1. The reverse primer (left-pointing arrow) used to generate PCR product a includes substitution(s) of one, two, or three nucleotides (red circle) within the HHT2 coding region. As previously shown in Figure 3, all 16 viable H3-L61X mutants cause statistically significant defects in Spt16 interactions at PMA1 and, with the exception of the H3-L61F mutant, at FBA1 as well. Interestingly, the mutant that confers the strongest defects in these assays is H3-L61W, which is the mutant that was identified and tested in our original studies (Duina and Winston 2004; Duina et al. 2007). As previously shown for H3-L61W (Duina et al. 2007; Nguyen et al. 2013), the H3-L61X mutants cause an overall shift in Spt16 occupancy toward the 3' ends of the PMA1 and FBA1 genes (see Figure 3, C and F). These results underscore the importance of the H3-L61 residue in contributing to proper Spt16—gene interactions. The defects conferred by the H3-L61X mutants on Spt16—gene interactions range from very mild to severe, and their relative degree of severity is comparable at the PMA1 and the FBA1 genes (compare Figure 3, C and F), suggesting that the mechanism that underlies Spt16—histone H3 interactions is similar at these genes and that specific H3-L61X mutants interfere with this mechanism similarly at both genes. Since previous work has shown that H3-L61W, the most defective mutant, causes relatively minor perturbations in nucleosome occupancy at the PMA1-LEU1 region (Duina et al. 2007), we consider it unlikely that the H3-L61X mutants mediate their effects on Spt16—gene interactions through alteration in overall abundance of nucleosomes across genes.

An analysis of the results from these experiments offers insights into the features of the H3-L61 residue important for promoting proper Spt16—gene interactions. First, its hydrophobic nature appears to be critical as hydrophilic amino acid substitutions at position 61 are among the ones causing the strongest defects. This finding is consistent with the observation that whereas the H3-L61F mutant causes minor defects in Spt16 interactions with PMA1 and FBA1, H3-L61Y causes much more pronounced perturbations, possibly due to the hydrophilic nature of the hydroxyl group on tyrosine. Second, the finding that substitutions at position 61 with either isoleucine or valine—both of which might be expected to be well tolerated in place of leucine—cause strong defects in these assays could indicate that the precise structural conformation adopted by H3-L61 is required for proper Spt16—gene interactions and that isoleucine and valine—both C-beta branched amino acids—are unable to adopt such conformation due their more limited rotational freedom across the polypeptide backbone. Third, the size of the amino acid side chain at position 61 also seems to be relevant as a substitution with the hydrophobic but much bulkier amino acid tryptophan causes the strongest perturbations in Spt16—gene interactions. Attributes shared between leucine and the substitutions that confer the smallest

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**Figure 1** Strategy for the generation of yeast strains expressing histone H3-L61X mutant proteins from the endogenous HHT2 locus. (A) Top: the haploid yeast genome harbors two sets of divergently transcribed genes encoding histones H3 and H4 (histone H4 is encoded by HHT1 and HHF2 and histone H3 is encoded by HHT1 and HHT2—the open reading frames are represented by rectangles and the direction of transcription for each gene is indicated by the corresponding arrow). Bottom: the HHT2 gene was replaced with the nutritional markers URA3 and TRP1 through homologous recombination to generate strain yADP106. (B) Two overlapping segments of the wild-type HHT2 gene are amplified by PCR as illustrated in diagram 1. The reverse primer (left-pointing arrow) used to generate PCR product a includes substitution(s) of one, two, or three nucleotides (red circle) within the HHT2 coding region. As previously shown in Figure 3, all 16 viable H3-L61X mutants cause statistically significant defects in Spt16 interactions at PMA1 and, with the exception of the H3-L61F mutant, at FBA1 as well. Interestingly, the mutant that confers the strongest defects in these assays is H3-L61W, which is the mutant that was identified and tested in our original studies (Duina and Winston 2004; Duina et al. 2007). As previously shown for H3-L61W (Duina et al. 2007; Nguyen et al. 2013), the H3-L61X mutants cause an overall shift in Spt16 occupancy toward the 3' ends of the PMA1 and FBA1 genes (see Figure 3, C and F). These results underscore the importance of the H3-L61 residue in contributing to proper Spt16—gene interactions. The defects conferred by the H3-L61X mutants on Spt16—gene interactions range from very mild to severe, and their relative degree of severity is comparable at the PMA1 and the FBA1 genes (compare Figure 3, C and F), suggesting that the mechanism that underlies Spt16—histone H3 interactions is similar at these genes and that specific H3-L61X mutants interfere with this mechanism similarly at both genes. Since previous work has shown that H3-L61W, the most defective mutant, causes relatively minor perturbations in nucleosome occupancy at the PMA1-LEU1 region (Duina et al. 2007), we consider it unlikely that the H3-L61X mutants mediate their effects on Spt16—gene interactions through alteration in overall abundance of nucleosomes across genes.

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With the exception of the H3-L61I and H3-L61V mutants, there appears to be a general correlation between impaired growth at 37°C and perturbations in Spt16—gene interactions (Figure 4: compare growth at 37°C of the weakest Spt16—gene interaction mutants H3-L61M, -L61F, and -L61C with the other H3-L61X mutants). The observation that H3-L61I and H3-L61V mutants do not show a temperature sensitive phenotype at 37°C might indicate that although the structural rigidity imparted by isoleucine and valine at position 61 of histone H3 interferes with Spt16—gene interactions, it does not cause cells to become sensitive to elevated temperatures. Conversely, disruptions of Spt16—gene interactions through introduction of polarity/charge or bulk at position 61 also cause cells to display a temperature sensitive phenotype at 37°C. A general correlation between cold sensitivity and defects in Spt16—gene interactions can also be seen, as H3-L61X mutants that cause moderate to strong perturbations in Spt16—gene interactions show a range of sensitivities to the low temperature of 14°C (Figure 4).

Sensitivities of H3-L61X mutants to the drugs formamide, hydroxyurea, and caffeine range from none to very strong and they do not show a clear correlation with impairment in Spt16—gene interactions (Figure 3 and Figure 4). In most cases, H3-L61X mutants that show sensitivity to one drug also show sensitivity to the other two drugs suggesting that these sensitivities share a common underlying mechanism.

### Assessment of effects of H3-L61X mutants on phenotypes indicative of transcription and chromatin defects

We continued the characterization of the newly generated H3-L61X mutants by testing them for phenotypes associated with defects in transcription and chromatin function. In one set of experiments, H3-L61X mutant cells carrying the lys2-128 allele were assayed for growth on medium lacking lysine as the ability to grow under these conditions reflects the viability of cells in which histone H3 interferes with Spt16—gene interactions through introduction of polarity/charge or bulk at position 61. A general correlation between cold sensitivity and defects in Spt16—gene interactions can also be seen, as H3-L61X mutants that cause moderate to strong perturbations in Spt16—gene interactions show a range of sensitivities to the low temperature of 14°C (Figure 4).

| Name | Sequence (5’ to 3’)* |
|------|----------------------|
| OAD20 | GCGTTCAATTTCGCGCCAATGTG |
| OAD21 | GCGCTGATCAGTCAGGGCTG |
| OAD476 | GAAACTTGGGCGAGGCTCTA |
| OAD477 | CTCGCCGATCAACCCAGTACT |
| OAD480 | CTAGTGTCTCGCCGTGTAT |
| L61Mfor | CCAAAAATCTACTGAATGCTGAGTTCAGTAGATTTTTGG |
| L61Mrev | GGAACCTTTCGATCATCGTGTAGATTTTTGG |
| L61Prev | GGATTTCCGATGAGTTCAGTAGATTTTTGG |
| L61Cfor | CCAAAAATCTACTGAATGCTGAGTTCAGTAGATTTTTGG |
| L61Pfor | CCAAAAATCTACTGAATGCTGAGTTCAGTAGATTTTTGG |
| L61Hfor | CCAAAAATCTACTGAATGCTGAGTTCAGTAGATTTTTGG |
| L61Efor | CCAAAAATCTACTGAATGCTGAGTTCAGTAGATTTTTGG |
| L61Vfor | CCAAAAATCTACTGAATGCTGAGTTCAGTAGATTTTTGG |

* The lowercase letters represent nucleotides that differ from those present in the wild-type HHT2 gene that alter the wild-type H3-L61X-encoded codon (TTG) to a different one encoding the corresponding H3-L61X mutant.

### Analysis of growth phenotypes of H3-L61X mutants

Previous analyses have shown that the H3-L61W mutant displays several abnormal growth phenotypes (Duina and Winston 2004; Duina et al. 2007). To more fully characterize the newly generated histone H3 mutants and to assess possible correlations between growth defects and the defects in Spt16—gene interactions as determined by the ChIP experiments described in the previous section, we tested the ability of the viable H3-L61X mutants to grow under a variety of conditions using serial dilution spot test assays (Figure 4).

Results from these experiments show that most of the mutants display growth defects at high and low temperatures, as well as other mutant phenotypes. With the exception of the H3-L61I and H3-L61V mutants, there appears to be a general correlation between impaired growth at 37°C and perturbations in Spt16—gene interactions (Figure 4: compare growth at 37°C of the weakest Spt16—gene interaction mutants H3-L61M, -L61F, and -L61C with the other H3-L61X mutants). The observation that H3-L61I and H3-L61V mutants do not show a temperature sensitive phenotype at 37°C might indicate that although the structural rigidity imparted by isoleucine and valine at position 61 of histone H3 interferes with Spt16—gene interactions, it does not cause cells to become sensitive to elevated temperatures. Conversely, disruptions of Spt16—gene interactions through introduction of polarity/charge or bulk at position 61 also cause cells to display a temperature sensitive phenotype at 37°C. A general correlation between cold sensitivity and defects in Spt16—gene interactions can also be seen, as H3-L61X mutants that cause moderate to strong perturbations in Spt16—gene interactions show a range of sensitivities to the low temperature of 14°C (Figure 4).

Sensitivities of H3-L61X mutants to the drugs formamide, hydroxyurea, and caffeine range from none to very strong and they do not show a clear correlation with impairment in Spt16—gene interactions (Figure 3 and Figure 4). In most cases, H3-L61X mutants that show sensitivity to one drug also show sensitivity to the other two drugs suggesting that these sensitivities share a common underlying mechanism.
conditions—a phenotype referred to as Spt— is indicative of perturbations in transcription and chromatin structure (Winston 1992). As shown in Figure 5, with the exception of H3-L61F, all other H3-L61X mutants display a range of Spt phenotypes, thus underscoring the importance of L61 of histone H3 in maintaining chromatin integrity. The strength of the Spt phenotypes conferred by the H3-L61X mutants does not correlate with the strength of the defects they impart on Spt16— gene interactions (for example, compare the Spt phenotypes of the H3-L61C and H3-L61T mutants in Figure 5 with the perturbations they impart on Spt16— gene interaction in Figure 3), suggesting that the two phenotypes are not mechanistically related to each other.

In a second set of experiments we made use of the FLO8-HIS3 reporter gene, which contains the HIS3 coding region fused downstream from a cryptic transcription initiation site within the FLO8 coding region (Cheung et al. 2008), to monitor for intragenic transcription initiation events. Since FLO8-HIS3 is under the control of the inducible GAL1 promoter, intragenic transcription initiation events can be easily monitored by assaying cells—otherwise deleted for the endogenous HIS3 gene—for growth on media lacking histidine in the context of high transcription levels (i.e., in the presence of galactose) or in the context of little to no transcription (i.e., in the presence of glucose). As previously reported (Myers et al. 2011; Nguyen et al. 2013), the H3-L61W mutant confers a strong His phenotype on media containing either glucose or galactose (Figure 5). Although a few H3-L61X mutant strains behave indistinguishably from wild-type cells on media lacking histidine, most show His phenotypes ranging from mild to strong (Figure 5). Members of this latter class of mutants display stronger growth on galactose-containing medium (SC-his GAL) compared to glucose-containing medium (SC-his), suggesting that the chromatin defects leading to intragenic transcription initiation imparted by these H3-L61X mutants are more pronounced in the context of high levels of transcription (Figure 5). Interestingly, we see a general correlation between H3-L61X mutants’ ability to confer growth on SC-his GAL medium and the degree of the defects they impart on Spt16— gene interactions as assessed by the ChiP experiments shown in Figure 3. For example, the eight mutants that confer the strongest defects in Spt16— gene interactions (H3-L61I, Y, E, H, V, T, K, and W) are also the ones that show the most robust growth on SC-his GAL plates. Taken together, these results suggest that the defects in repression of intragenic transcription initiation and in Spt16— gene interactions caused by mutations at position 61 of histone H3 are in some manner mechanistically linked to each other.

DISCUSSION

In this work, we have carried out a systematic mutational analysis of the H3-L61 residue to gain insights into its contribution to chromatin function. Our finding that substitutions of H3-L61 to each of the remaining 19 natural amino acids confer defects in one or more of the
phenotypes we have assayed highlights the importance of H3-L61 in maintaining chromatin integrity and is consistent with the high degree of evolutionary conservation of this residue across eukaryotic organisms (we note that this level of conservation is not unique to the L61 residue as most other histone H3 residues are also highly conserved). An evaluation of the nature of the defects conferred by each of the H3-L61X mutants indicates that features of H3-L61 important for maintaining proper nucleosome functions include its hydrophobicity, its flexibility across the polypeptide backbone, and its lack of excessive bulk. The aliphatic nature of H3-L61 does not appear to be critical for these functions as a mutation to the aromatic amino acid phenylalanine did not cause strong defects in our assays. We also provide evidence that H3-L61X-dependent defects in Spt16 gene interaction and in repression of intragenic transcription initiation are mechanistically linked to each other.

In previous work, we showed that an H4-R36A mutant perturbs Spt16 association across genes in a fashion similar to that seen in the context of H3-L61W, and genetic evidence indicated that these two

**Figure 4** Phenotypes of cells expressing H3-L61X mutants subjected to a variety of growth conditions. Cells expressing the indicated histone H3 proteins as sole source of histone H3 were grown overnight on YPD medium and subsequently collected and spotted on the indicated plates (for each condition tested, all mutants were spotted on the same plate) in a 10-fold dilution series, with the most concentrated spots (left-most spot in each panel) consisting of ~120,000 cells. Plates were placed at the indicated temperatures (note that the drug-containing plates were incubated at 30°C), and photographed after the following approximate incubation times: YPD 30°C, 2 d; YPD 37°C, 2 d; YPD 14°C, 7 and 10 d, as indicated; YPD + formamide (Form), 3 d; YPD + hydroxyurea (HU), 3 d; YPD + caffeine (Caff), 5 d. The YPD 14°C plate is shown at two different incubation times to allow for assessment of subtle cold-sensitive phenotypes (e.g., for the H3-L61H mutant). Mutants are presented in the same order as that shown in Figure 3. The growth patterns shown in this figure were closely reproduced in an independent experiment for all samples except for H3-L61G, which showed modestly stronger growth on the YPD 30°C plate. Additional growth experiments showed that the variable growth phenotype conferred by H3-L61G is an inherent property of this mutant. The strains used in these experiments were yADP89-yADP105. YPD, yeast extract peptone dextrose.

**Figure 5** Effects of H3-L61X mutants on growth phenotypes indicative of transcription and chromatin defects. Cells expressing the indicated H3-L61X mutants were treated as described in Figure 4, except that the most concentrated spots consisted of ~240,000 cells. Plates were placed at 30°C and photographed after the following approximate times of incubation: SC, 2 d; SC-lys, 2 d; SC-his, 3 d; SC GAL, 3 d; SC-his GAL, 3 d. The growth patterns shown in this figure were closely reproduced in an independent experiment for all samples except for H3-L61G, which, as was the case in the experiments described in Figure 4, showed some variability: this mutant showed modestly stronger growth on the SC and SC GAL plates and weaker growth on the SC-his plate. The strains used in these experiments were yADP89-yADP105. SC, SC-lys, and SC-his plates contained glucose and SC GAL and SC-his GAL plates contained galactose as carbon and energy source.
histone mutants affect Spt16–gene interactions through a common mechanism (Nguyen et al. 2013). H4-R36 and H3-L61 are adjacent to each other on the side of the nucleosome, with the former being located on the surface making direct contacts with DNA and the latter being buried into the particle ((Luger et al. 1997; Luger and Richmond 1998) and Figure 6A). How do these histone residues contribute to proper Spt16–chromatin interactions, and how might they be functionally linked? Based on our original studies, we considered a model in which H4-R36 is part of a nucleosomal surface that interacts with Spt16, and mutations that perturb this surface can lead to abnormal Spt16 interactions across genes. In the context of this model, we envisioned two possible mechanisms by which H3-L61W could affect H4-R36 function: (i) the π electrons of the aromatic ring of the tryptophan residue could interfere with the ability of H4-R36 to form ionic bonds with other factors, or (ii) the integrity of the hydrophobic microenvironment normally occupied by H3-L61 is required for proper positioning of H4-R36 for interactions with other factors, and the tryptophan residue in H3-L61W disrupts this microenvironment through its bulky nature. The results from the present study do not support the first mechanism, as the associated predictions that substitutions of H3-L61 with the aromatic amino acid phenylalanine should cause strong perturbations in Spt16–gene interactions while substitutions with aliphatic hydrophobic residues (e.g., alanine and isoleucine) should not, were not substantiated. Our results are instead consistent with the second mechanism, as H3-L61X mutants (modeled in Figure 6B) that are predicted to disrupt the H3-L61 microenvironment through the introduction of polarity/charge or bulk or by interfering with H3 polypeptide flexibility all cause strong perturbations in Spt16–gene interactions. The H3-L61X mutants’ effects on Spt16–chromatin interactions are likely to be for the most part specific, as previous work has shown that H3-L61W, the strongest H3-L61X mutant, does not significantly affect Pol II– and Spt6–gene interactions and only modestly affects Spt6–gene interactions (Duina et al. 2007; Lloyd et al. 2009). The isolation of Spt16 mutants that can suppress H3-L61W–dependent defects in Spt16–chromatin interactions (Duina et al. 2007; Myers et al. 2011) further points to a specific relationship between Spt16 and the H3-L61 residue.

Most H3-L61X mutants cause decreased levels of Spt16 occupancy at the 5’ ends of PMA1 and FBA1 while simultaneously elevating the levels of Spt16 occupancy at the 3’ ends of the same genes. In addition, in this study we have provided evidence that the defects in Spt16–gene interactions imparted by H3-L61X mutants are correlated with defects in repression of intragenic transcription initiation. Currently, the molecular mechanism that underlies this relationship is unknown. One possibility is that the primary defect conferred by the H3-L61X mutants is to directly interfere with the ability of Spt16 to dissociate from the 3’ ends of genes at the end of the transcription process. Since this 3’ trapping may be occurring throughout the genome (we have previously shown that H3-L61W affects interactions between Spt16 and all other transcribed genes assayed—a total of six genes (Duina et al. 2007)), it could lead to reduced levels of Spt16 available for recruitment to transcribed genes, resulting in lower Spt16 occupancy levels at 5’ ends of genes as well as in defects in Spt16-mediated transcription-coupled nucleosome reassembly over the bodies of genes, which would ultimately lead to intragenic transcription initiation. Alternatively, it is possible that the H3-L61X mutants directly affect chromatin environments over transcribed genes in ways that cause cryptic intragenic transcription initiation, for example, by interfering with proper transcription-coupled nucleosome reassembly, which in turn could in some manner cause a shift in Spt16 distribution toward the 3’ ends of genes.

We note, however, that cryptic intragenic transcription initiation per se is not sufficient for causing a 3’ shift of Spt16 across genes, since in previous work we have isolated several Spt16 mutants that cause strong intragenic transcription initiation but that do not confer significant defects in Spt16 association across the PMA1 gene (Myers et al. 2011). We previously disfavored the possibility that the H3-L61W–dependent defects in Spt16–gene interactions and in repression of intragenic transcription initiation are functionally related to each other in light of the fact that among several Spt16 mutants we

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**Figure 6** Location of the H3-L61 and H4-R36 residues in the nucleosome and modeling of the H3-L61X mutants. (A) Side-view of the yeast nucleosome core particle with the DNA shown in olive green and the histones color-coded as indicated in the figure. The H3-L61 and H4-R36 residues are shown in stick representation and color-coded as indicated. The blue box represents the region analyzed in panel B. (B) Close-up views of the nucleosomal region indicated by the blue box in panel A showing the wild-type structure (top-left image with blue border) and models of the H3-L61X substitutions. The images with purple borders correspond to the H3-L61X mutants able to confer viability when expressed as a sole source of histone H3 in cells and are shown in the same order as that used for the experiments shown in Figure 3, Figure 4, and Figure 5. Models of the H3-L61X mutants unable to support viability in the absence of wild-type histone H3 (see Figure 2) are framed by orange borders. The H3-L61X models were generated using PyMOL and show the orientation of the substituted residues based on the rotamers with greatest frequencies found in other proteins as calculated by the software program.
isolated, Spt16-E735G was among the strongest suppressors of the H3-L61W-dependent defect in Spt16 distribution across PMA1, but was the weakest suppressor of the intragenic transcription initiation defect at FLO8-HIS3 (Myers et al. 2011). However, Spt16-E735G can cause intragenic transcription initiation at FLO8-HIS3 in a histone H3 wild-type background (Myers et al. 2011), and therefore the apparent lack of suppression of the H3-L61W-dependent defect in repression of intragenic transcription could be attributed to Spt16-E735G’s own ability to promote intragenic transcription initiation.

In conclusion, our systematic mutational analysis has shed new light on the contribution of the H3-L61 residue to proper chromatin function. Future studies will focus on further characterizing the nature of the nucleosomal region involved in controlling Spt16 interactions with transcribed genes. It will also be of interest to determine the functional outcomes of Spt16 trapping at the 3′ ends of genes due to histone mutants, in particular in relation to possible effects on nucleosome composition and on transcription termination. Collectively, these and related studies will contribute to our understanding of the mechanisms that govern interactions between Spt16 and nucleosomes during transcription, and possibly, during other chromatin processes that involve Spt16 function, such as DNA replication and repair.

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