Genes to Cells

Global analysis for functional residues of histone variant Htz1 using the comprehensive point mutant library

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Histone variants perform unique functions and are deposited onto DNA by mechanisms distinct from those of canonical histones. The H2A variant, H2A.Z, also known as Htz1 in *Saccharomyces cerevisiae*, is not uniformly distributed across the genome but facilitates transcriptional activation at target gene promoters and anti-silencing at heterochromatin loci. Htz1 is also involved in DNA replication, DNA repair, chromosome segregation and cell cycle control. Its sequence identity to canonical H2A is only ~60%, and it is likely that the nonconserved residues are responsible for Htz1-specific functions. However, precise roles of these variant-specific residues are not well understood. To gain insights into the molecular basis underlying the functional differences between canonical and variant histones, 117 alanine-scanning point mutants of Htz1 were constructed for this study, and chemical genetic screens were carried out. Consequently, seven Htz1 residues that conferred one or more abnormal phenotypes when mutated were identified. Based on primary sequence and functional conservation between H2A and Htz1, two of these residues (F32 and I109) appear to have an Htz1-specific role, whereas the rest seem to have functions shared between H2A and Htz1. This study provides a useful resource for future investigations into functional convergence and divergence between canonical and variant histones.

Introduction

In eukaryotes, genomic DNA is organized into nucleosomes, the basic repeating unit of chromatin (Kornberg 1974). The nucleosome is composed of ~146 bp of DNA and two molecules each of canonical histones H2A, H2B, H3 and H4 (Arents & Moudrianakis 1993; Luger *et al.* 1997). The nucleosome structure is dynamically regulated and thought to play important roles in various DNA-templated reactions throughout the genome.

The exchange of canonical histones for histone variants is an important mechanism in the control of chromatin structure and function (Kamakaka & Biggins 2005; Sarma & Reinberg 2005; Talbert & Henikoff 2010). Histone variants have several characteristics distinct from canonical histones; for example, canonical histones are produced specifically in the S phase and are deposited onto newly synthesized DNA in a replication-dependent manner, whereas histone variants are synthesized throughout the cell cycle and are deposited in a replication-independent manner. Each histone variant is localized to specific chromatin loci and is thought to carry out particular functions; for instance, the centromeric histone H3 variant CenH3 (Cse4 in *Saccharomyces cerevisiae*) is localized to the centromere and is involved in kinetocore assembly (Allshire & Karpen 2008), whereas the histone H2A variant H2A.X is phosphorylated immediately after DNA damage near the sites of DNA double-strand breaks where it facilitates the assembly of DNA repair factors (van Attikum & Gasser 2009).

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What is the molecular basis underlying functional differences between canonical and variant histones? Canonical and variant histones differ in their expression patterns during the cell cycle and in their amino acid sequences, and the latter has been shown to be a critical determinant in some cases. In *Drosophila*, there are four amino acid changes between the canonical histone, H3.1, and the variant histone, H3.3. A variant-specific function of H3.3 is lost by substituting H3.3-specific residues with corresponding amino acids from H3.1 (Ahmad & Henikoff 2002), thus indicating that their functional differences are accounted for by the nonconserved amino acids. However, precise functional roles of variant-specific residues are not well understood.

H2A.Z (Htz1 in *S. cerevisiae*) is an H2A variant that is involved in the regulation of various DNA-templated processes (Zlatanova & Thakar 2008). Although H2A.Z is highly conserved across species, its sequence identity to canonical H2A is only ~60%, suggesting the presence of H2A.Z-specific conserved functions (Fig. 1A). In yeast, Htz1 is enriched at the

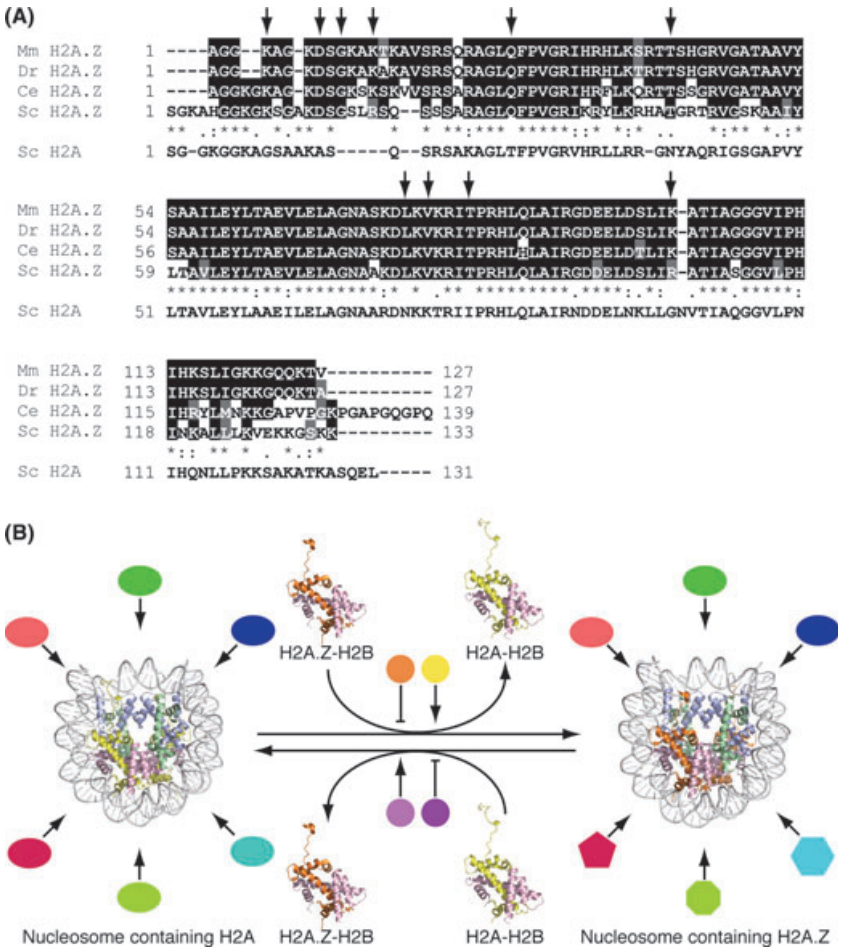

Figure 1 Study concept. (A) Sequence alignment of H2A.Z from various species with yeast H2A. Mm, *Mus musculus*; Dr, *Danio rerio*; Ce, *Caenorhabditis elegans*; Sc, *S. cerevisiae*. Clustal W2 and BoxShade 3.21 were used to perform the alignment (Larkin *et al.* 2007), and identical and similar residues are shaded in black and gray, respectively. In addition, sequence similarity between yeast H2A.Z and H2A is indicated below the alignment using the symbols 'asterisk,' 'colon' and 'dot' for identical, highly similar and weakly similar residues, respectively. Arrows denote the positions of those H2A.Z residues that are conserved across species but are not conserved in H2A. (B) A number of histone-binding proteins, shown in various shapes and colors, perform diverse cellular functions, including the H2A-H2A.Z exchange reaction, via nucleosome interactions. Many of these factors are unlikely to discriminate among different histone isoforms, but some (shown as polygons) are thought to interact with nucleosomes in a variant-specific manner.

promoter regions of thousands of genes and is involved in transcriptional activation (Li *et al.* 2005; Raisner *et al.* 2005; Zhang *et al.* 2005; Albert *et al.* 2007; Zlatanova & Thakar 2008). Htz1 is also found at heterochromatin loci such as the mating-type loci and telomeres and has been shown to prevent the spread of heterochromatin from these loci (Meneghini *et al.* 2003). Although the *HTZ1* gene is not essential, its disruption confers a slow growth phenotype, which is more pronounced at a lower temperature (Jackson & Gorovsky 2000). Moreover, it has been shown that the *htz1* deletion strain (*htz1Δ*) is sensitive to 6-azauracil (6AU) (Larochelle & Gaudreau 2003; Keogh *et al.* 2006), hydroxyurea (HU) (Kobor *et al.* 2004; Koc *et al.* 2004), methyl methanesulfonate (MMS) (Keogh *et al.* 2006), benomyl (Kobor *et al.* 2004; Krogan *et al.* 2004), and caffeine (Kobor *et al.* 2004), leading to the assumption that Htz1 is involved not only in transcription but also in diverse biological processes, such as DNA replication, DNA repair, chromosome segregation (Kobor *et al.* 2004; Krogan *et al.* 2004) and cell cycle control (Kobor *et al.* 2004; Dhillon *et al.* 2006).

It is likely that variant-specific amino acid residues of Htz1 mediate Htz1-specific functions by influencing nucleosomal stability through histone–histone or histone–DNA interactions, or by serving as binding sites for other molecules or as modification sites (Fig. 1A,B). In this respect, earlier studies have shown an important role for the C-terminal 37 amino acid region of Htz1 (Adam *et al.* 2001; Larochelle & Gaudreau 2003). The mutant in which the C-terminal region of H2A was replaced by the corresponding Htz1 sequence (AZ chimera) restored the slow growth phenotype of *htz1Δ* cells, whereas the mutant in which the N-terminal region of H2A was replaced by the corresponding Htz1 sequence (ZA chimera) did not (Adam *et al.* 2001). In addition, the ZA chimeric protein associated with target gene loci in a pattern distinct from wild-type Htz1 and more similar to that of H2A (Larochelle & Gaudreau 2003). However, it remains to be determined how these mutants behave in other functional assays, and a detailed structure-function analysis of Htz1 at the single amino acid level needs to be carried out.

We have established the strategies, termed 'GLobal Analysis of Surfaces by Point mutation' (GLASP) (Matsubara *et al.* 2007) and 'GLobal Analysis of Mutual interaction surfaces of multi-subunit protein complex by Point mutation' (GLAMP) (Sakamoto *et al.* 2009), which allow the comprehensive identification of functionally important residues of evolutionarily conserved, multi-functional proteins. For GLASP and GLAMP, a comprehensive alanine-scanning point mutant library is first constructed for all the exposed or buried residues of a protein of interest, respectively, and then genetic analyses are carried out using a collection of yeast strains carrying these point mutants. These strategies were initially applied to the four canonical histones and to the histone chaperone, CIA/ASF1, and a histone point mutant Global Library (histone-GLibrary) composed of 439 mutant strains and a CIA/ASF1-GLibrary were constructed, respectively (Matsubara *et al.* 2007; Natsume *et al.* 2007; Sakamoto *et al.* 2009; Sato *et al.* 2010). Subsequently, other research groups have also reported the use of similar approaches in the characterization of canonical histones (Dai *et al.* 2008; Nakanishi *et al.* 2008).

We have used these libraries in phenotypic and drug-sensitivity assays to identify histone and CIA/ASF1 residues involved in diverse cellular functions, including transcription initiation and elongation, DNA replication and DNA repair (Matsubara *et al.* 2007; Natsume *et al.* 2007; Sakamoto *et al.* 2009). Mapping these functional residues to the three-dimensional structure of the nucleosome and the CIA/ASF1–histone H3–H4 complex showed the convergence and divergence of nucleosomal sites involved in diverse cellular functions (Matsubara *et al.* 2007; Natsume *et al.* 2007; Sakamoto *et al.* 2009). In this study, GLASP and GLAMP were applied to histone variant Htz1, resulting in the successful identification of functional residues involved in diverse biological processes.

Results

Construction of a comprehensive point mutant library of *HTZ1*

In Fig. 1A, the positions of the H2A.Z residues that are well conserved across species but are not conserved in H2A are indicated with arrows. These residues are obviously good candidates for Htz1-specific functions. To determine which Htz1 residues are critical for its diverse cellular functions, 117 point mutant strains were constructed in which all the amino acid residues of Htz1, except the first Met and 15 Ala residues, were individually mutated to Ala. In yeast, although the deletion of *HTA1* and *HTA2*, the gene pair encoding histone H2A, is lethal, the deletion of *HTZ1* is not (Kolodrubetz *et al.* 1982; Jackson & Gorovsky 2000). Hence, the *htz1* point mutant

library was expected to show less severe phenotypes than an H2A point mutant library. Indeed, whereas three strains of the H2A point mutant library were inviable (Matsubara *et al.* 2007; Sakamoto *et al.* 2009), all strains from the Htz1 point mutant library were found to be viable. However, It is worth noting that certain *htz1* mutant strains showed growth defects of greater severity than the *htz1Δ* strain (see later).

htz1Δ and *htz1* point mutants do not exhibit the Spt⁻ phenotype or show 6AU sensitivity

Htz1 is predominantly localized to nucleosomes at promoter regions, particularly of inactive genes (Guillemette *et al.* 2005), and it is often involved in the transcriptional activation of inducible genes, such as *GAL1* and *GAL10* (Santisteban *et al.* 2000; Adam *et al.* 2001). Therefore, we investigated whether *HTZ1* mutations would also affect transcription initiation and elongation. Suppression of *Tγ* or *δ* insertion mutations (Spt⁻ phenotype) was used to study the role of Htz1 in transcription initiation. Insertion of a *Tγ* or *δ* transposable element (which itself contains signals for transcription initiation) into the promoter region of a gene such as *HIS4* and *LYS2* interferes with transcription of the adjacent gene, resulting in an amino acid auxotroph. Suppressor mutations that overcome this inhibition are therefore thought to affect transcription, particularly transcription start site selection of *Tγ*, *δ* or the adjacent gene, and, in fact, various genes generally involved in transcription initiation, such as those encoding histones H2A and H2B, TBP and SAGA, have been shown to confer the Spt⁻ phenotype when mutated (reviewed in Yamaguchi *et al.* 2001). *htz1Δ* and the 117 *htz1* point mutant strains were screened for the Spt⁻ phenotype, without success (data not shown). These results suggest that Htz1 is not involved in transcription initiation in a way that affects the Spt⁻ phenotype. Alternatively, considering the fact that *HTA1* mutations result in the Spt⁻ phenotype (Matsubara *et al.* 2007; Sakamoto *et al.* 2009), it is possible that the presence of two copies of the wild-type H2A genes might compensate for the loss of Htz1 function in transcription initiation.

Next, 6AU was used to determine whether Htz1 is involved in transcription elongation. Transcription elongation is inhibited by 6AU, which disrupts the activities of enzymes that are critical for the synthesis of purine and pyrimidine, and reduces the intracellular levels of ribonucleotide triphosphates (Exinger & Lacroute 1992). Genes whose mutations confer 6AU sensitivity are therefore thought to be involved in transcription elongation. The *htz1Δ* strain and the Htz1 point mutant library were screened for 6AU sensitivity, but none of the mutant strains were found sensitive to 6AU (data not shown). Contrary to our findings, other studies have reported that *htz1Δ* is sensitive to 6AU (Larochelle & Gaudreau 2003; Keogh *et al.* 2006). This discrepancy might be because of differences in genetic backgrounds or other experimental conditions; however, the exact reason is unclear. Taken together, these data did not show whether or how Htz1 is involved in transcription initiation and/or elongation.

HU-sensitivity analysis of the Htz1 point mutant library

Htz1 has also been implicated in DNA replication. *HTZ1* genetically interacts with replication factors such as *ORC2* and *ORC5* (Dhillon *et al.* 2006), and *htz1Δ* is sensitive to HU, a ribonucleotide reductase inhibitor that activates the S-phase checkpoint by reducing the levels of intracellular deoxyribonucleotide triphosphates and by stalling DNA replication forks (Kobor *et al.* 2004; Koc *et al.* 2004). Genes whose mutations confer HU sensitivity are therefore thought to be involved in DNA replication and cell cycle checkpoint (Alvino *et al.* 2007). To identify Htz1 residues that are critical for S-phase progression, the Htz1 point mutant library was screened for HU sensitivity. For this experiment, threefold serial dilutions of the 117 point mutant strains were spotted and grown for 2 days at 30 °C on synthetic complete medium lacking leucine (SC-Leu) plates with or without HU. The following four mutants were found to be sensitive to HU: *htz1*-Y65A, *htz1*-E69A, *htz1*-D98A and *htz1*-I109A (Fig. 2A). For the sake of brevity, point mutants that did not show abnormal phenotypes have been excluded from Fig. 2A and the subsequent figures. Whereas *htz1*-E69A, *htz1*-D98A and *htz1*-I109A showed HU sensitivity comparable to *htz1Δ*, *htz1*-Y65A was less sensitive to HU. Intriguingly, the growth defects exhibited by *htz1*-E69A and *htz1*-D98A were more severe than that of *htz1Δ* in the absence of any drugs (Fig. 2A), indicating that these Htz1 mutant proteins actively inhibit cell proliferation.

Although the crystal structure of the yeast nucleosome containing Htz1 has not been solved, a nucleosome structure composed of mouse H2A.Z and *Xenopus* H2B, H3 and H4 has been reported (Suto *et al.* 2000). This structure was therefore used as a

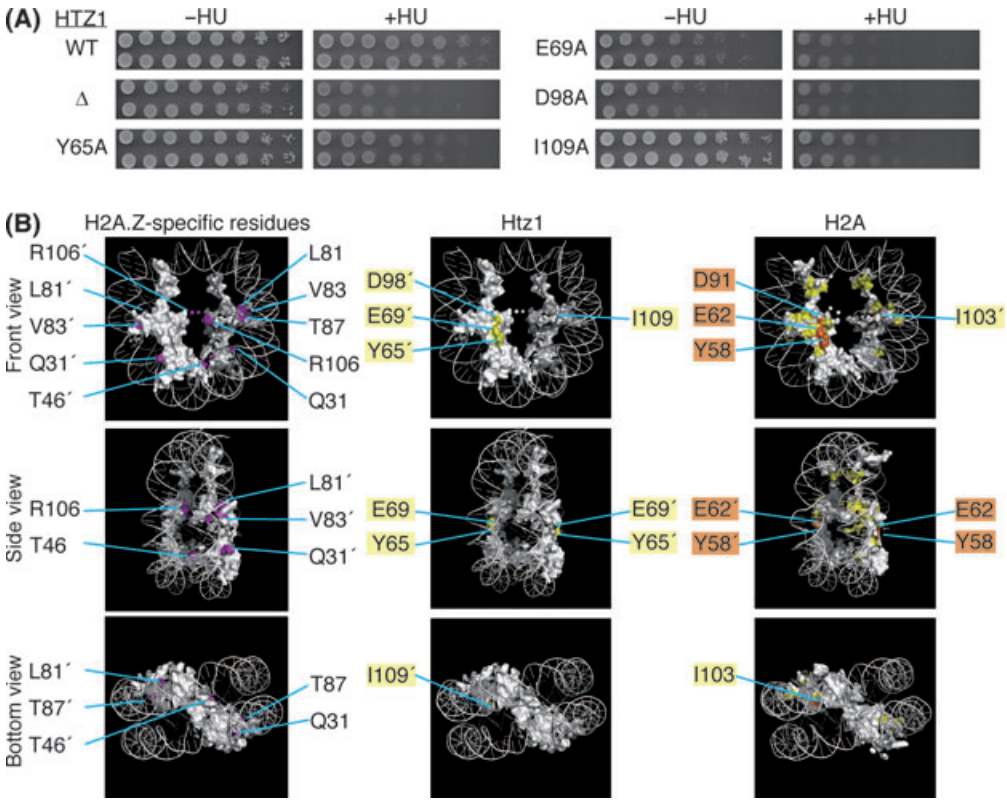

Figure 2 Identification of HU-sensitive *htz1* point mutants. (A) Threefold serial dilutions of the H2A.Z GLibrary were spotted in duplicate and grown for 2 days at 30 °C on SC-Leu medium plates with or without 100 mM HU. Only the *htz1* point mutants that were >10-fold more sensitive to HU than the wild-type strain are shown. (B) Structural mapping of variant-specific residues of yeast Htz1 (indicated in magenta) on the mouse H2A.Z-containing nucleosome (PDB ID, 1F66; Suto *et al.* 2000). Primes are used to distinguish amino acids from different copies of a histone pair. Histones H2B, H3 and H4 are not shown here for clarity. (C) Structural mapping of 'HU-sensitive residues' of Htz1 (left) and H2A (right). The left panel shows yeast Htz1 'HU-sensitive residues' (indicated in yellow) mapped on the mouse H2A.Z-containing nucleosome (PDB ID, 1F66), whereas the right panel shows yeast H2A 'HU-sensitive residues' and 'lethal residues' (indicated in yellow and orange, respectively) mapped on the yeast nucleosome (PDB ID, 1ID3; White *et al.* 2001). These residues are labeled using the yeast protein numbering system. Histones H2B, H3 and H4 are not shown here for clarity.

template to map the Htz1 residues identified earlier onto a three-dimensional structure. Contrary to the assumption that Htz1-specific residues might be important, the Htz1-specific residues identified in Fig. 1A and 'HU-sensitive residues' are not in close proximity to each other (compare Fig. 2B,C). All four 'HU-sensitive residues' are in fact positioned on the nucleosome surface, and two of the four residues (Htz1-E69 and Htz1-D98) are located in the acidic patch, a cluster of seven negatively charged amino acid residues derived from Htz1 and H2B. The acidic patch has been shown to interact with the positively charged N-terminal tail of H4 (Luger *et al.* 1997).

The results of the Htz1 point mutant library screen were compared with previously reported results from a histone H2A point mutant library (Matsubara *et al.* 2007; Sakamoto *et al.* 2009). Of the 112 histone H2A point mutants, 20 mutants show HU sensitivity, whereas of the 20 corresponding mutants of *HTZ1*, only I109A showed HU sensitivity (Fig. 6 and Table 1). Thus, it appears that the number of amino acid residues involved in the HU-sensitive phenotype is significantly smaller in Htz1 than in H2A. However, all the four Htz1 residues identified in the HU-sensitivity screen are conserved in primary sequence and position in canonical H2A as follows: Htz1-Y65 corresponds to H2A-Y58, Htz1-E69 to H2A-E62, Htz1-D98 to H2A-D91 and Htz1-I109 to H2A-I103 (Fig. 6 and Table 1). Of the four corresponding H2A mutant strains, Y58A, E62A and D91A

Table 1 The list of all the H2A point mutants showing abnormal phenotype(s) and of the corresponding *htzl* mutants

H2A		Htzl	
Residue	Phenotype s a h m b c†	Residue	Phenotype s a h m b c†
△	Lethal	△	– – h m b c
K4	s – – – – –	H5	– – – – – –
G5	s – – – – –	G6	– – – – – –
F26	s – – – – c	F32	– – – m – c
R30	– – h m – c	R36	– – – – – –
L35	s – – – – –	L41	– – – – – –
Y40	s – – – – –	G47	– – – – – –
I44	s – – m – –	V51	– – – – – –
L52	s – – – – –	L59	– – – – – –
V55	s – – – – –	V62	– – – – – –
L56	s – – – – –	L63	– – – – – –
E57	– – h m b c	E64	– – – – – –
Y58	Lethal	Y65	– – h m b c
L59	s – – – – –	L66	– – – – – –
E62	Lethal	E69	– – h m b c
I63	s – – – – –	V70	– – – – – –
L64	s – – – – –	L71	– – – – – –
E65	– a h m – c	E72	– – – – – –
L66	s – h m b c	L73	– – – – – –
G68	– – h m – –	G75	– – – – – –
D73	– a h m – –	D80	– – – – – –
N74	– a h m – –	L81	– – – m – –
K76	– – h m – –	V83	– – – – – –
R78	s – h m – c	R85	– – – – – –
I79	s – – – – –	I86	– – – – – –
R82	s – h m – –	R89	– – – – – –
H83	s – h m – –	H90	– – – – – –
L84	s – – – – –	L91	– – – – – –
L86	– a h m b –	L93	– – – – – –
I88	s – – – – –	I95	– – – – – –
N90	– – – m – –	G97	– – – – – –
D91	Lethal	D98	– – h m b c
E93	– – h m b c	E100	– – – – – –
L94	– – h m b c	L101	– – – – – –
G99	s – – – – –	R106	– – – – – –
I103	s – h m – c	I109	– – h m b c
G107	s – h m – c	G113	– – – m – –
L109	– – – m – –	L115	– – – – – –
P110	– – – m – –	P116	– – – – – –
I112	– a h m b –	I118	– – – – – –
H113	– a h m b c	N119	– – – – – –
N115	– – – m b c	A121	– – – – – –
L116	– a h m b c	L122	– – – – – –
L117	– – h m b c	L123	– – – – – –
S121	– – – m b –	E127	– – – – – –
S128	– – – m – –	–	– – – – – –
Q129	– – – m – –	–	– – – – – –
L131	– – – m – –	–	– – – – – –

†s, Spt$^-$; a, 6AU sensitive; h, HU sensitive; m, MMS sensitive; b, benomyl sensitive; c, caffeine sensitive.

were not viable, whereas I103A was viable and sensitive to HU. Considering the phenotypic difference of H2A and *HTZ1* null mutants, mutation of a functionally conserved residue of H2A should confer phenotypes equal to or more severe than the corresponding mutation in Htz1, and hence all the four 'HU-sensitive residues' of Htz1 can be assumed to be functionally conserved between H2A and Htz1. It cannot be excluded, however, that H2A-Y58, H2A-E62 or H2A-D91 might has yet another important, isoform-specific function and that its defect results in lethality.

MMS-sensitivity analysis of the Htz1 point mutant library

Htz1 is also thought to be involved in DNA repair. It is required for the activation of DNA damage checkpoint and for the phosphorylation of histone H2A during DNA double-strand break repair (Kalocsay *et al.* 2009), and *htz1Δ* is sensitive to DNA damaging agents such as Zeocin and MMS, which causes DNA lesions by generating alkylated bases and abasic sites. To identify Htz1 residues that are critical for DNA repair, the Htz1 point mutant library was screened for MMS sensitivity by growing the 117 mutant strains for 3 days at 30 °C on SC-Leu medium plates with or without MMS. The following seven mutants were found to be sensitive to MMS: F32A, Y65A, E69A, L81A, D98A, I109A and G113A (Fig. 3A). As four (Y65A, E69A, D98A and I109A) of the seven mutants also showed HU sensitivity, the 'HU-sensitive residues' appear to be a subset of the 'MMS-sensitive residues' (Fig. 6B).

The results of the Htz1 point mutant library screen were compared with previously reported results from the histone H2A point mutant library (Matsubara *et al.* 2007; Sakamoto *et al.* 2009). Of the 112 histone H2A point mutants, 29 mutants show MMS sensitivity, whereas of the 29 corresponding mutants of *HTZ1*, only I109A and G113A conferred MMS sensitivity (Fig. 6 and Table 1), indicating that a point mutation in *HTZ1* tends to result in a less severe phenotype than a corresponding mutation in H2A. However, six of the seven Htz1 residues identified in the MMS-sensitivity screen are conserved in primary sequence and position in canonical H2A as follows: Htz1-F32 corresponds to H2A-F26, Htz1-Y65 to H2A-Y58, Htz1-E69 to H2A-E62, Htz1-L81 to H2A-N74, Htz1-D98 to H2A-D91, Htz1-I109 to H2A-I103, and Htz1-G113 to H2A-G107 (Fig. 6 and Table 1). Of the seven corresponding H2A mutant strains, Y58A, E62A and D91A were inviable

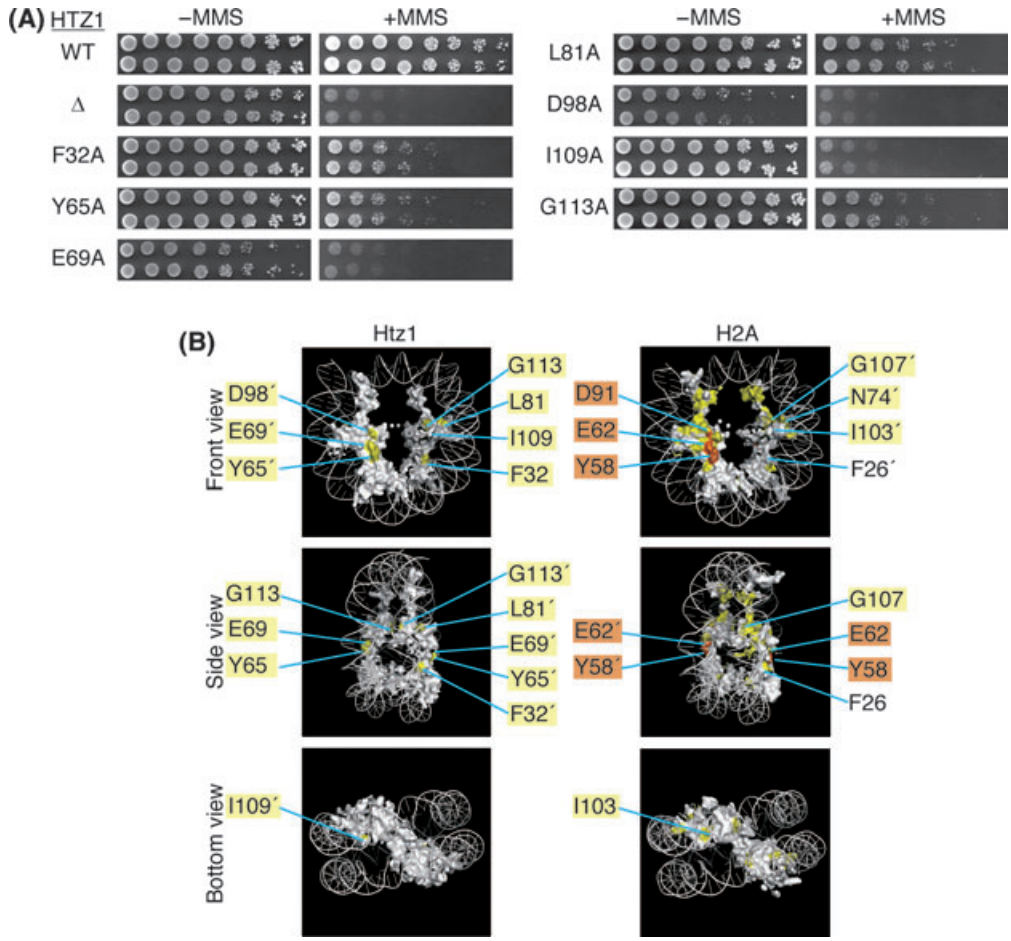

Figure 3 Identification of MMS-sensitive *htz1* point mutants. (A) Threefold serial dilutions of the H2A.Z GLibrary were spotted in duplicate and grown for 3 days at 30 °C on SC-Leu medium plates with or without 0.016% (v/v) MMS. Only the *htz1* point mutants that were >10-fold more sensitive to MMS than the wild-type strain are shown. (B) Structural mapping of 'MMS-sensitive residues' of Htz1 (left) and H2A (right). The left panel shows yeast Htz1 'MMS-sensitive residues' (indicated in yellow) mapped on the mouse H2A.Z-containing nucleosome (PDB ID, 1F66), whereas the right panel shows yeast H2A 'MMS-sensitive residues' and 'lethal residues' (indicated in yellow and orange, respectively) mapped on the yeast nucleosome (PDB ID, 1ID3). These residues are labeled using the yeast protein numbering system. Primes are used to distinguish amino acids from different copies of a histone pair.

whereas N74A, I103A and G107A were viable and sensitive to MMS, indicating that these six residues, including the nonconserved H2A-N74/Htz1-L81 residue, are conserved between H2A and Htz1 at the functional level. Remarkably, however, the remaining H2A mutant F26A was viable and insensitive to MMS, in contrast with the corresponding *htz1* mutant, F32A, suggesting that Htz1-F32 plays a variant-specific role in DNA repair.

Except for the cluster of Htz1-Y65, Htz1-E69 and Htz1-D98, the other MMS-sensitive residues, Htz1-F32, Htz1-L81, Htz1-I109 and Htz1-G113, are separated spatially from each other (Fig. 3B). However, in the canonical nucleosome, H2A-N74, the counterpart of Htz1-L81, is part of a larger cluster of 'phenotypic residues' ('MMS-sensitive residues' + 'lethal residues'), suggesting that Htz1-L81 might be functionally linked to Htz1-Y65, Htz1-E69 and Htz1-D98.

Benomyl-sensitivity analysis of the Htz1 point mutant library

Htz1 might also play a role in mitosis. *HTZ1* interacts genetically with kinetocore components and *htz1Δ* is defective in chromosome segregation and is sensitive to the microtubule-destabilizing drug benomyl

(Kobor *et al.* 2004; Krogan *et al.* 2004). To determine the Htz1 residues that are critical for mitosis, the Htz1 point mutant library was screened for benomyl sensitivity by growing the 117 mutant strains for 3 days at 30 °C on SC-Leu medium plates with or without benomyl. The following four mutants were found to be sensitive to benomyl: Y65A, E69A, D98A and I109A (Fig. 4A). Interestingly, the 'benomyl-sensitive residues' were identical to the 'HU-sensitive residues' and were found to be a subset of the 'MMS-sensitive residues' (Fig. 6B).

The results from the Htz1 point mutant library were compared with results from the histone H2A point mutant library (N. Sano, unpublished data). Of the 112 histone H2A point mutants, 11 mutants show benomyl sensitivity, whereas none of the 11 corresponding *HTZ1* mutants exhibited sensitivity to the drug (Fig. 6 and Table 1). However, all the four 'benomyl/HU-sensitive residues' of Htz1 were conserved in primary sequence and position between H2A and Htz1, and of the four corresponding H2A mutant strains, three (Y58A, E62A, and D91A) were inviable, as described previously. Interestingly, however, the remaining H2A mutant, I103A, was viable and insensitive to benomyl in contrast to the corresponding *htz1* mutant, I109A, which suggested that Htz1-I109 plays a variant-specific role in mitosis. When viewed in 3D, Htz1-I109 was separated spatially from the other 'benomyl-sensitive residues' (Fig. 4B). Additionally, in the canonical nucleosome, there is no 'benomyl-

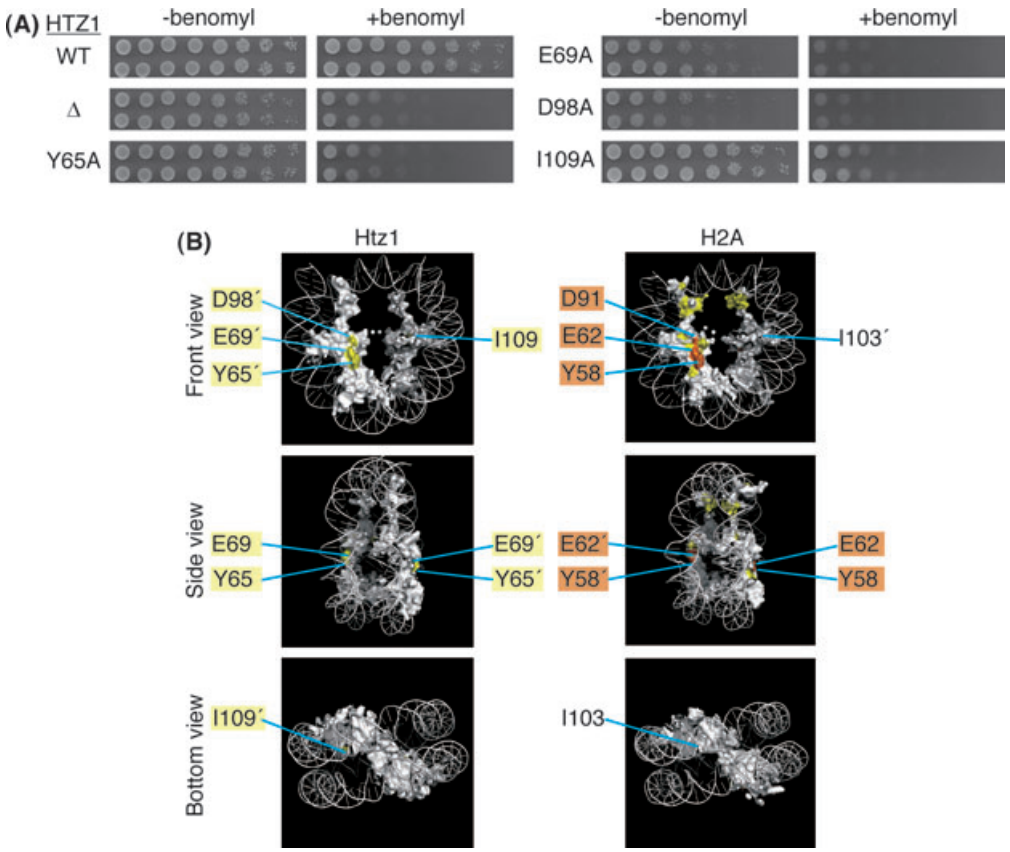

Figure 4 Identification of benomyl-sensitive *htz1* point mutants. (A) Threefold serial dilutions of the H2A.Z GLibrary were spotted in duplicate and grown for 3 days at 30 °C on SC-Leu medium plates with or without 10 μg/mL benomyl. Only the *htz1* point mutants that were >10-fold more sensitive to benomyl than the wild-type strain are shown. (B) Structural mapping of 'benomyl-sensitive residues' of Htz1 (left) and H2A (right). The left panel shows yeast Htz1 'benomyl-sensitive residues' (indicated in yellow) mapped on the mouse H2A.Z-containing nucleosome (PDB ID, 1F66), whereas the right panel shows yeast H2A 'benomyl-sensitive residues' and 'lethal residues' (indicated in yellow and orange, respectively) mapped on the yeast nucleosome (PDB ID, 1ID3). These residues are labeled using the yeast protein numbering system. Primes are used to distinguish amino acids from different copies of a histone pair.

sensitive residue' around H2A-I103, the counterpart of Htz1-I109 (Fig. 4B), which supports the idea that Htz1-I109 has a variant-specific role.

Caffeine-sensitivity analysis of the Htz1 point mutant library

Htz1 has also been implicated in cell cycle control, as it has been shown to be involved in transcriptional activation of G1/S cyclin genes such as *CLN2* and *CLB5* (Dhillon *et al.* 2006). Moreover, *htz1Δ* exhibits slow cell cycle progression and is sensitive to caffeine (Kobor *et al.* 2004), a drug that overrides the cell cycle checkpoint by inhibiting ATM and other protein kinases (Sarkaria *et al.* 1999; Zhou *et al.* 2000). To identify the Htz1 residues that are critical for cell cycle control, the Htz1 point mutant library was screened for caffeine sensitivity by growing the 117 mutant strains for 5 days at 30 °C on SC medium plates with or without caffeine. The following five mutants were found to be sensitive to caffeine: F32A, Y65A, E69A, D98A, and I109A (Fig. 5A). Four of these, Y65A, E69A, D98A and I109A, were also involved in HU-, MMS-, and benomyl-sensitive phenotypes. Thus, the 'HU-sensitive residues' and the 'benomyl-sensitive residues' are a subset of the 'caffeine-sensitive residues,' which are themselves a subset of the 'MMS-sensitive residues' (Fig. 6B).

The caffeine sensitivity of the H2A point mutant library was also examined, as this has not been investigated previously. Of the 112 H2A point mutants, the following 14 mutants were found to be sensitive to caffeine: F26A, R30A, E57A, E65A, L66A, R78A, E93A, L94A, I103A, G107A, H113A, N115A, L116A and L117A (Fig. 5B).

The results from the Htz1 point mutant library were then compared with the results from the histone H2A point mutant library. Of the 14 *HTZ1* mutants that corresponded to the H2A caffeine-sensitive mutants, only F32A and I109A showed caffeine sensitivity (Fig. 6 and Table 1), indicating that a point mutation in *HTZ1* tends to result in a less severe phenotype than a corresponding mutation in H2A. However, all five Htz1 residues identified in the caffeine-sensitivity screen were conserved in primary sequence and position in canonical H2A as follows: Htz1-F32 corresponds to H2A-F26, Htz1-Y65 to H2A-Y58, Htz1-E69 to H2A-E62, Htz1-D98 to H2A-D91, and Htz1-I109 to H2A-I103 (Fig. 6 and Table 1). Of the five corresponding H2A mutant strains, Y58A, E62A and D91A were inviable whereas F26A and I103A were viable and sensitive to caffeine, indicating that these residues are functionally conserved between H2A and Htz1. Thus, the aforementioned screen did not show amino acid residues that play an Htz1-specific role in cell cycle control.

When viewed in 3D, Htz1-F32 and -I109 are separated spatially from each other and from the cluster of Htz1-Y65, Htz1-E69 and Htz1-D98 (Fig. 5C). As mentioned earlier, H2A-F26 and H2A-I103, the H2A residues corresponding to Htz1-F32 and Htz1-I109, conferred sensitivity to caffeine when mutated individually. Moreover, the mutation of nearby residues such as H2A-R30, H2A-E58 and H2A-G107 also conferred caffeine sensitivity (Fig. 5C), suggesting that H2A-F26/Htz1-F32 and H2A-I103/Htz1-I109 might contribute to cell cycle control by distinct mechanisms.

Discussion

To gain insights into the molecular basis underlying functional differences between canonical and variant histones, we constructed the H2A.Z GLibrary and examined functional roles of its individual amino acid residues in transcription initiation and elongation, DNA replication, DNA repair, chromosome segregation and cell cycle control by using the Spt^- phenotype and sensitivity to 6AU, HU, MMS, benomyl and caffeine as indicators. In consequence, seven residues were identified as being involved in one or more of these processes: Htz1-F32, Htz1-Y65, Htz1-E69, Htz1-L81, Htz1-D98, Htz1-I109 and Htz1-G113. Immunoblot analysis showed that mutations of these residues did not have a substantial effect on Htz1 protein expression levels (A. Kawano, data not shown), suggesting that these mutations affected Htz1 functions during or after incorporation into the nucleosome.

The chemical genetic screens identified a relatively small number of 'phenotypic residues,' of which only L81 and G113 appeared to be involved in a single process (DNA repair), whereas the other five residues appeared to be involved in multiple processes (Fig. 6). Particularly, mutation of Y65, E69, D98 or I109 resulted in sensitivity to HU, MMS, benomyl and caffeine, the same spectrum of abnormal phenotypes observed in *htz1Δ*, suggesting that these four residues, which we call common functional residues, are involved in fundamental aspects of Htz1 functions (Fig. 6C). 'Phenotypic residues' are likely to exert their functions (i) by influencing nucleosomal stability through histone–histone or histone–DNA interactions, or (ii) by serving as binding sites for other

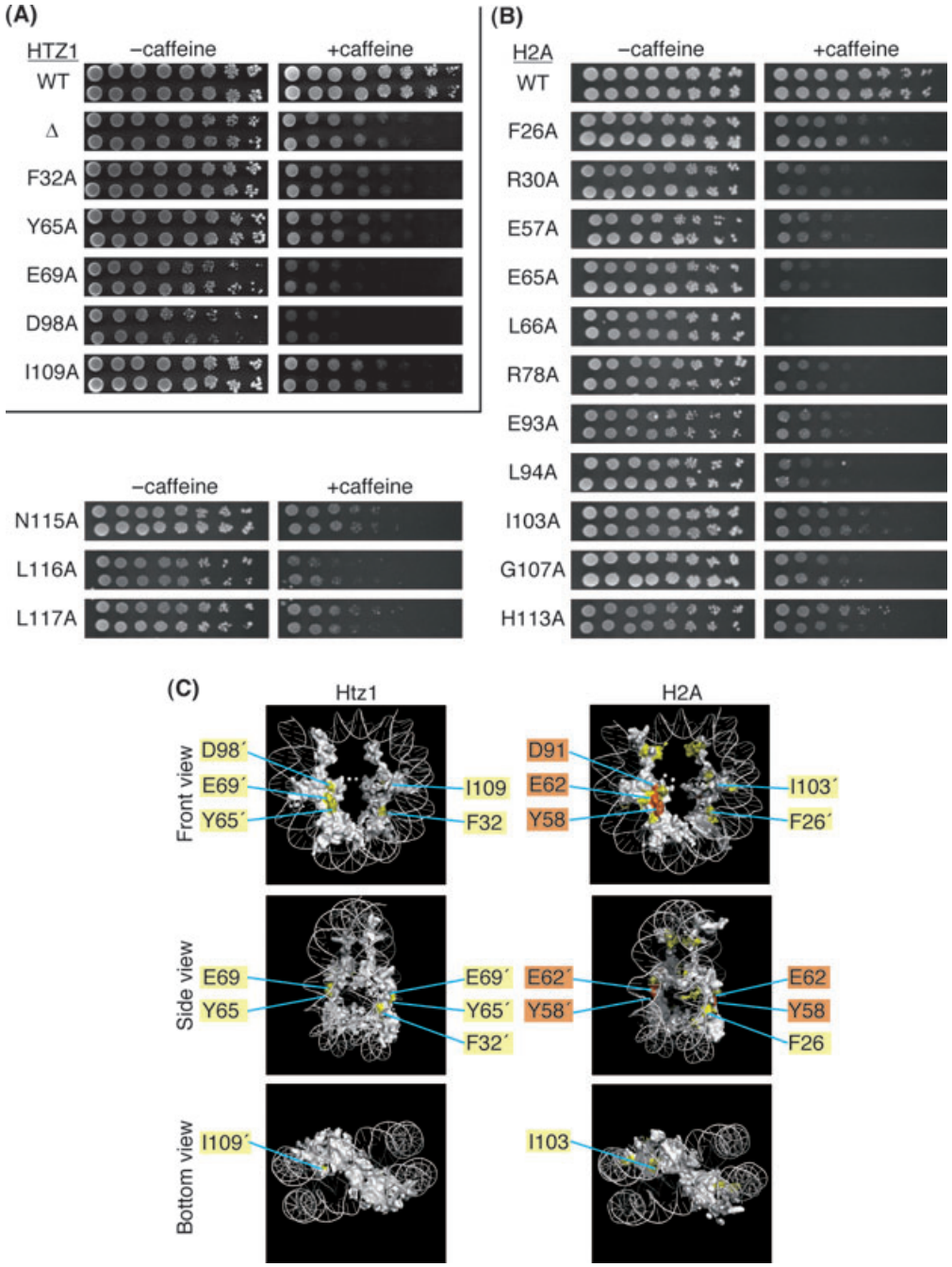

Figure 5 Identification of caffeine-sensitive *htz1* point mutants. (A) Threefold serial dilutions of the H2A.Z GLibrary were spotted in duplicate and grown for 5 days at 30 °C on SC-Leu medium plates with or without 9 mM caffeine. Only the *htz1* point mutants that were >10-fold more sensitive to caffeine than the wild-type strain are shown. (B) Threefold serial dilutions of the H2A GLibrary were spotted in duplicate and grown for 4 days at 30 °C on SC medium plates with or without 9 mM caffeine. Only the H2A point mutants that were >10-fold more sensitive to caffeine than the wild-type strain are shown. (C) Structural mapping of 'caffeine-sensitive residues' of Htz1 (left) and H2A (right). The left panel shows yeast Htz1 'caffeine-sensitive residues' (indicated in yellow) mapped on the mouse H2A.Z-containing nucleosome (PDB ID, 1F66), whereas the right panel shows yeast H2A 'caffeine-sensitive residues' and 'lethal residues' (indicated in yellow and orange, respectively) mapped on the yeast nucleosome (PDB ID, 1ID3). These residues are labeled using the yeast protein numbering system. Primes are used to distinguish amino acids from different copies of a histone pair.

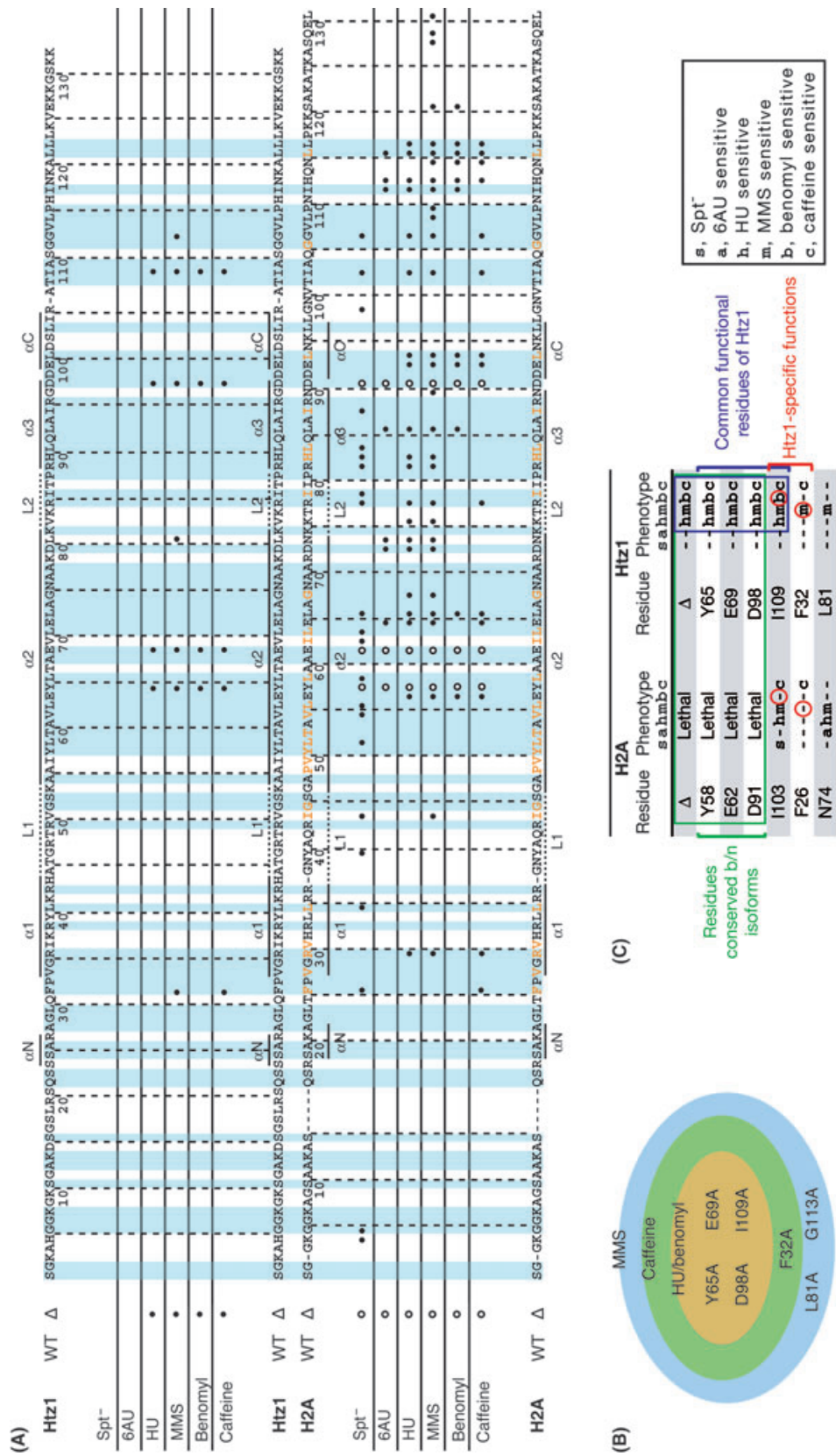

Figure 6 Summary of the results obtained from the six genetic screens of the histone variant H2A.Z GLibrary. (A) Sequence alignment of yeast Htz1 and H2A was carried out using Clustal W2 (Larkin *et al.* 2007). Buried residues of H2A are indicated in orange, and residues conserved between Htz1 and H2A are shaded. Positions of 'Spt⁻-phenotypic or drug-sensitive residues' and 'lethal residues' are indicated with filled circles and open circles, respectively. Shown to the left are phenotypes of the deletion mutants. (B) A Venn diagram of Htz1 mutants showing drug sensitivity. (C) Summary and comparison of the phenotypes of the seven Htz1 mutants identified and of the corresponding H2A mutants.

molecules or as modification sites. In the first case, a point mutant will behave similarly to *htz1*Δ, whereas in the second case, the spectrum of its abnormal phenotypes will be determined by the molecular functions of an affected interactor(s); multiple processes will be impaired by a point mutation if the residue interacts with a single interactor performing multiple functions or with multiple interactors each having a different function. If binding to Chz1, the Htz1-specific histone chaperone, is weakened by a mutation, for example, dynamic exchange between H2A and Htz1 will be impaired, and consequently multiple processes will be affected. It is therefore not surprising that many of the 'phenotypic residues' were found associated with multiple abnormal phenotypes.

Many of the identified residues also appeared to be functionally conserved between the isoforms, and only two (Htz1-F32 and -I109) seemed to be functionally distinct from the corresponding H2A residues, based on the assumption that mutation of a functionally conserved residue of H2A should confer phenotypes equal to or more severe than the corresponding mutation in Htz1 (Fig. 6C). These results might be accounted for as follows: (i) Single point mutations tend to impair protein functions only partially. Because deletion of *HTZ1* results in relatively mild phenotypes, many of its point mutations that do, in fact, affect Htz1-specific functions may not have been counted as positive in our assays, leading to the identification of only a small number of Htz1-specific 'phenotypic residues.' (ii) It is not surprising that mutations of Htz1-Y65, -E69, and -D98 produced noticeable phenotypes, considering that the corresponding mutations in H2A (Y58, E62, and D91) caused lethality. Such 'core' residues are likely to be identified as functionally conserved 'phenotypic residues.'

In the following sections, the possible roles of key Htz1 residues will be discussed one by one from structural and functional viewpoints.

Insights into the common functional residues of Htz1

Similar to *htz1Δ*, four (Y65A, E69A, D98A and I109A) of the seven mutants showed sensitivity to HU, MMS, benomyl, and caffeine (Fig. 6), suggesting that these mutations affect fundamental roles of Htz1. However, a closer examination of the data suggested that this might not be entirely accurate. In the case of Htz1-Y65, the Y65A mutant strain was less sensitive to HU and MMS than the *htz1Δ* strain (Figs 2 and 3) whereas *htz1*-Y65A and *htz1Δ* showed a comparable level of benomyl and caffeine sensitivity (Figs 4 and 5), indicating that the Y65 residue is particularly important for the control of chromosome segregation and cell cycle. Regarding Htz1-E69 and Htz1-D98, the respective mutant strains showed greater growth defects than the *htz1Δ* strain in the absence of any drugs (Fig. 2), indicating that these Htz1 mutants actively inhibited cell proliferation. It is possible that although H2A can be substituted for Htz1 to some extent in the *htz1Δ* strain, these functionally impaired mutants remain bound to chromatin and prevent H2A from acting in place of Htz1. In addition, the mutation of Htz1-I109 resulted in drug sensitivity comparable to that for *htz1Δ*. It is therefore possible that Htz1-Y65, Htz1-E69, Htz1-D98 and Htz1-I109 in fact play distinct roles at the molecular level.

Htz1-Y65 and -E69

Although precise structural information for the nucleosome containing yeast Htz1 is not available, the crystal structure of the mouse H2A.Z-containing nucleosome is known (Suto *et al.* 2000). Hence, the possible molecular functions of individual Htz1 residues are discussed, mainly based on a combination of the structural data from the mouse H2A.Z-containing nucleosome and from the yeast nucleosome containing H2A (White *et al.* 2001). In our previous studies (Matsubara *et al.* 2007; Sakamoto *et al.* 2009), histone residues located within 4 Å of each residue of the nucleosome were extracted and classified as 'interacting residues.' According to this data set (Table 2), H2A-Y58, which corresponds to Htz1-Y65, interacts with two H2A residues (H2A-L59 and H2A-E62) and three H2B residues (H2B-L109, H2B-H112 and H2B-A113) (Fig. 7A,C). Similarly, H2A-E62, corresponding to Htz1-E69, interacts with three H2A residues (H2A-Y58, H2A-I63 and H2A-L66) and one H2B residue (H2B-L109) (Fig. 7D). Interestingly, H2A-Y58 and H2A-E62, both of which confer lethality when mutated, are in close proximity to each other and to H2B-L109, the only 'lethal residue' of H2B (Table 2). As all the three residues are located at the H2A-H2B dimer interface, it is reasonable to assume that these residues are important for dimer formation and that their mutations result in lethality by inhibiting dimer formation. As the spatial arrangement of these residues is conserved in the mouse H2A.Z-containing nucleosome (Table 2), Htz1-Y65 and Htz1-E69 are also likely to play key roles in Htz1-H2B dimer formation.

Table 2 List of Htz1- or H2A-interacting residues†

Phenotypic residues‡	Accessible surface area (%)§	Interacting residues‡¶															
		H2A or H2A.Z					H2B					H3	H4	H2A′ or H2A.Z′	H2B′	H3′	H4′
yHtz1-F32-mc	N.D.		P33	L63	E64	T67	Y40	Y43		V47	V69						
mH2A.Z-F27	0		P28	L58	E59	T62	Y37	Y40		V44	V66						
yH2A-F26-sc	0	T25	P27		E57-hmbc	A60	Y40	Y43-m	I44	V47-m							
yHtz1-Y65-hmbc	N.D.	L66	E69-hmbc				L109	H112	A113	E116							
mH2A.Z-Y60	9	L61	E64				L106	H109	A110	E113							
yH2A-Y58-l	12	L59-s	E62-l				L109-l	H112-m	A113								
yHtz1-E69-hmbc	N.D.	Y65-hmbc	V70				L109										
mH2A.Z-E64	29	Y60	V65				L106										
yH2A-E62-l	24	Y58-l	I63-s	L66-shmbc			L109-l										
yHtz1-L81-m	N.D.	A77	D80	H90													
mH2A.Z-L76	50	A72	D75	H85													
yH2A-N74-ahm	48	A70	D73-ahm	H83-shm													
yHtz1-D98-hmbc	N.D.		D99	E100	L101												
mH2A.Z-D93	27		E94	E95	L96												
yH2A-D91-l	18	L66-shmbc	D92	E93-hmbc	L94-hmbc												
yHtz1-I109-hmbc	N.D.	Q92	I95	T108	A110		I64										
mH2A.Z-I104	6	Q87	I90	T103	A105		I61										
yH2A-I103-shmc	6	Q85	I88-s	T102	A104		I64										Y98-hm
yHtz1-G113-m	N.D.	R89	G102	V114												T58	
mH2A.Z-G108	23	R84	G107	V109												T58	
yH2A-G107-shmc	21	R82-shm	G106	V108												T58-m	

†Shown are intranucleosomal interactions involving seven 'phenotypic residues' inferred from structural data.
‡l, lethal; a, 6AU sensitive; s, Spt$^-$; h, HU sensitive; m, MMS sensitive; b, benomyl sensitive; c, caffeine sensitive.
§N.D., not determined.
¶Residues found at the corresponding positions are aligned on the same column. A shaded background indicates residues conserved between yH2A and yHtz1 or mH2A.Z.

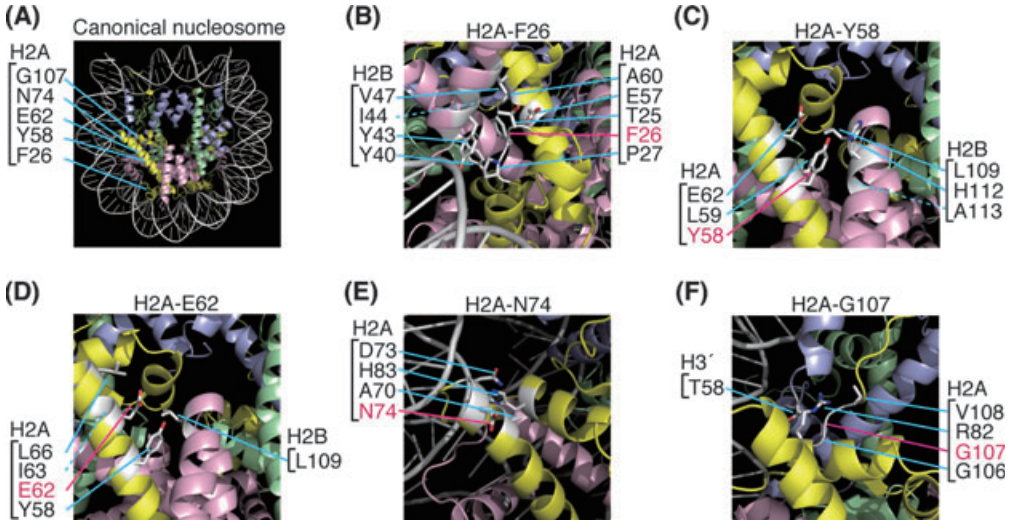

Figure 7 Molecular environment surrounding H2A residues homologous to 'drug-sensitive residues' of Htz1. (A) Relative positions of H2A-F26, -Y58, -E62, -N74, and -G107 within the yeast nucleosome (PDB ID, 1ID3; White *et al.* 2001). (B–F) Close-up views of these residues. In each panel, the residue of interest is labeled in red, and the surrounding residues that are thought to interact directly with the residue of interest are labeled in black. Solid and dotted lines indicate the positions of the residues whose side chains are visible and invisible on the illustrations, respectively.

Htz1-D98

In contrast to Htz1-Y65 and Htz1-E69, Htz1-D98 is unlikely to contribute to the stabilization of the internal structure of the nucleosome. This is because H2A.Z-D93, the mouse counterpart of yeast Htz1-D98, only interacts with its adjacent residues in the mouse H2A.Z-containing nucleosome (Table 2). Instead, Htz1-D98 and perhaps Htz1-E69 are located in the acidic patch, a cluster of seven negatively charged residues derived from Htz1 and H2B, which is thought to interact with the positively charged N-terminal tail of H4 in the nucleosome (Luger *et al.* 1997). Moreover, according to the structure of the Htz1-H2B dimer complexed with the histone chaperone Chz1 (Zhou *et al.* 2008), Htz1-Y65, Htz1-E69 and Htz1-D98 serve as a binding site for Chz1, raising an alternative possibility that their mutations might prevent Htz1 from being deposited onto DNA.

Htz1-I109

H2A-I103, corresponding to Htz1-I109, has notable differences from H2A-Y58, H2A-E62 and H2A-D91 in the point that the H2A-I103A mutant is viable and sensitive to HU, MMS and caffeine (Fig. 6A). H2A-I103A is also different from *htz1*-I109A in that H2A-I103A does not show benomyl sensitivity (Fig. 6A). This phenotypic difference might be explained by assuming the existence of a chromosome segregation factor that selectively interacts with Htz1 in an I109-dependent manner. This idea is consistent with the fact that the primary sequences around Htz1-I109 and H2A-I103 are quite different from each other. A possible next step in our research is to identify such interacting molecules by using a genetic or biochemical approach.

Insights into the other functional residues of Htz1

Next, we focus on Htz1-F32, Htz1-L81 and Htz1-G113; the residues whose mutations resulted in sensitivity to only one or two of the drugs tested. The modifiable residues of Htz1, whose mutations did not cause any appreciable phenotypes, are also discussed.

Htz1-F32

Htz1-F32 and its corresponding H2A residue, H2A-F26, conferred distinct phenotypes when mutated. Specifically, the *htz1*-F32A strain was sensitive to MMS, but H2A-F26A was not (Fig. 6A), suggesting that the Htz1 mutation might influence the interaction of Htz1 with a DNA repair factor or the structural integrity of the nucleosome itself during DNA repair. As H2A-F26 is almost completely buried in the nucleosome (Fig. 7B and Table 2), Htz1-F32 may also be inaccessible to external protein factors

and is more likely to contribute to DNA repair by stabilizing the internal structure of the nucleosome. As shown in Table 2, there are several differences between the 'interacting residues' of H2A-F26 and Htz1-F32. Specifically, Htz1-L63 and H2B-V69 seem to interact with Htz1-F32 but not with H2A-F26, whereas H2A-T25 and H2B-I44 appear to interact only with H2A-F26. Moreover, H2A-A60/Htz1-T67, one of the common 'interacting residues' of H2A-F26/Htz1-F32, is not conserved in sequence between H2A and Htz1. These differences might account for the functional difference between H2A-F26 and Htz1-F32.

Htz1-L81 and -G113

Both Htz1-L81 and Htz1-G113 conferred MMS sensitivity when mutated (Fig. 6A). As the corresponding mutations in H2A, H2A-N74A and H2A-G107A, also resulted in MMS sensitivity (Fig. 6A), this phenotype appears to have been caused by a defect in the functions shared between H2A and Htz1. According to the crystal structure of the H2A-containing nucleosome, H2A-N74 interacts with three H2A residues (H2A-A70, H2A-D73 and H2A-H83) (Fig. 7E and Table 2), and H2A-G107 interacts with three H2A residues (H2A-R82, H2A-G106 and H2A-V108) and one H3 residue (H3′-T58) (Fig. 7F and Table 2). Coincidentally, most of these interacting residues, namely H2A-D73, H2A-R82 and H2A-H83, and H3′-T58, also conferred MMS sensitivity when mutated (Matsubara *et al.* 2007; Sakamoto *et al.* 2009; see also Table 1). This cluster of 'MMS-sensitive residues' might represent a binding site for a DNA repair factor or might be involved in structural changes associated with DNA repair. The surface areas of H2A-N74 and -G107 that are accessible to solvent are 48.0% and 20.6%, respectively, and hence these residues can function as both internal and external residues (Table 2).

A recent study found that Htz1 was recruited specifically to the sites of DNA double-strand breaks and that SUMOylation of Htz1-K126 and Htz1-K133 was required for its recruitment (Kalocsay *et al.* 2009). Contrary to this finding, the present data showed that the *htz1*-K126A and *htz1*-K133A strains were insensitive to MMS (Fig. 6A). One possible explanation is that these residues are functionally redundant. Whether *htz1*-L81A, *htz1*-G113A or other mutations resulting in MMS sensitivity affect the SUMOylation status of Htz1 is an interesting and unresolved issue.

Modifiable residues of Htz1

It is worth noting that the mutation of any of the known modification sites of Htz1 did not cause any appreciable phenotypes. Essentially identical observations were obtained for canonical histones (Matsubara *et al.* 2007; Sakamoto *et al.* 2009). Extensive acetylation of the four lysine residues in the N-terminal tail of Htz1 (K3, K8, K10 and K14) by NuA4 is thought to inhibit heterochromatin spreading and to promote transcription of its target genes (Babiarz *et al.* 2006; Millar *et al.* 2006). In addition, SUMOylation occurs on two lysine residues at the C-terminus of Htz1 (K126 and K133), as described earlier (Kalocsay *et al.* 2009). The data in the current study can be explained by assuming that multiple Htz1 lysine residues are modified simultaneously and exert redundant functions. This idea is consistent with an emerging view that the histone modification system is complex and proof against point mutations (Hayashi *et al.* 2009).

Conclusions and perspectives

In this study, four functional residues of Htz1 (Y65, E69, D98 and I109) were identified as being important in four different biological processes, DNA replication (HU), DNA repair (MMS), chromosome segregation (benomyl) and cell cycle control (caffeine). In addition, a residue (Htz1-F32) involved in DNA repair and cell cycle control and two residues (Htz1-L81 and -G113) specifically involved in DNA repair were discovered. Furthermore, comparison of 'phenotypic residues' of H2A and Htz1 showed that certain residues, such as Htz1-F32 and Htz1-I109, exert Htz1-specific functions, as mutations of the corresponding H2A residues did not result in identical phenotypes.

Last but not least, we propose three perspectives on the study of the H2A.Z GLibrary. First, the analysis of extragenic suppressors for mutations of the Htz1-specific functional residues will lead to further understanding of Htz1-specific roles at the molecular level. Second, the common functional residues of Htz1 are thought to contribute to a fundamental aspect of the nucleosome that is conserved between H2A and Htz1, such as nucleosomal stability. As mutational analysis of such residues is difficult to perform using H2A because of lethality issues, Htz1 will be a valuable alternative tool providing a better understanding of the nucleosomal structure and function. Third, our strategy can be applied to other conserved histone variants such as CenH3, leading

toward a comprehensive understanding of the functional convergence and divergence between canonical and variant histones.

Experimental procedures

Yeast strain and plasmids

A wild-type yeast strain FY119 (MATα,*his4-912δ*,*lys2-128δ*,*leu2Δ1*, *trp1Δ63*, *ura3-52*) was obtained from F. Winston (Harvard University). An *htz1* deletion mutant was created on the FY119 background using a one-step gene replacement strategy with an *htz1::TRP1* fragment. The deletion mutant was then transformed with pRS315 (*LEU2*) carrying wild-type *HTZ1* or one of the *htz1* point mutants. The yeast strain FY406 and plasmids carrying wild-type *HTA1* or one of *hta1* point mutants were described previously (Matsubara *et al.* 2007; Sakamoto *et al.* 2009).

Mutagenesis

Alanine substitution mutations were introduced into pRS315 (*LEU2*) *HTZ1* using one of two different site-directed mutagenesis methods: the Kunkel's method (Kunkel *et al.* 1987) or an inverse PCR-based method (Weiner *et al.* 1994). The sequences of the oligonucleotide primers used for mutagenesis will be provided on request.

Media

Synthetic Complete (SC) medium with or without leucine contained 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, 0.5% ammonium sulfate and 2% (w/v) agar, and the medium was supplemented with adenine and the appropriate amino acids. SC-Leu medium with or without lysine was used for the Spt$^-$ phenotype assay. For drug sensitivity assays, the following reagents were added to the SC-Leu medium where indicated: 1 mg/mL 6AU, 100 mM HU, 0.016% (v/v) MMS, 10 μg/mL benomyl and 9 mM caffeine.

Spt$^-$ phenotype and drug sensitivity assays

Threefold dilutions of strains with the indicated genotypes were spotted onto SC-Leu medium with or without lysine (for the Spt$^-$ phenotype assay), or SC-Leu medium with or without the appropriate drugs (for drug sensitivity assays). Each spot contained approximately 1×10^5, 3×10^4, 1×10^4, 3×10^3, 1×10^3, 3×10^2, 1×10^2 and 3×10^1 cells, respectively. The plates were then incubated at 30 °C for 3–4 days for the Spt$^-$ phenotype assay, 2 days for the HU-sensitivity assay, 3 days for the MMS- and benomyl-sensitivity assays, and 5 days for the caffeine-sensitivity assay. These experiments were carried out in duplicate and were repeated several times.

In silico structural analysis

Molecular graphics were prepared with the program PyMOL using Protein Database (PDB) files of the *Xenopus* nucleosome containing mouse H2A.Z (PDB ID, 1F66; Suto *et al.* 2000) and the yeast nucleosome (PDB ID, 1ID3; White *et al.* 2001). The accessible surface area of each residue was calculated as previously described (Samanta *et al.* 2002). In the present study, 'interacting residues' were defined as surrounding residues located within 4 Å from any side-chain atoms (but the β-carbon) of a residue of interest. In the case of glycine, the α-carbon was used instead as the reference point, and 'interacting residues' were defined as those located within 4 Å from the α-carbon.

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