Published in final edited form as:

Nature. ; 483(7387): 104–107. doi:10.1038/nature10861.

Structural basis for recognition of H3K56-acetylated histone H3–H4 by the chaperone Rtt106

Dan Su^{1,*}, Qi Hu^{1,*}, Qing Li^{1,*}, James R. Thompson², Gaofeng Cui¹, Ahmed Fazly¹, Brian A. Davies¹, Maria Victoria Botuyan¹, Zhiguo Zhang¹, and Georges Mer¹

¹Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905, USA.

²Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota 55905, USA.

Abstract

Dynamic variations in the structure of chromatin influence virtually all DNA-related processes in eukaryotes and are controlled in part by post-translational modifications of histones^{1–3}. One such modification, the acetylation of lysine 56 (H3K56ac) in the amino-terminal α -helix (α N) of histone H3, has been implicated in the regulation of nucleosome assembly during DNA replication and repair, and nucleosome disassembly during gene transcription^{4–10}. In *Saccharomyces cerevisiae*, the histone chaperone Rtt106 contributes to the deposition of newly synthesized H3K56ac-carrying H3–H4 complex on replicating DNA⁵, but it is unclear how Rtt106 binds H3–H4 and specifically recognizes H3K56ac as there is no apparent acetylated lysine reader domain in Rtt106. Here, we show that two domains of Rtt106 are involved in a combinatorial recognition of H3–H4. An N-terminal domain homodimerizes and interacts with H3–H4 independently of acetylation while a double pleckstrin-homology (PH) domain binds the K56-containing region of H3. Affinity is markedly enhanced upon acetylation of K56, an effect that is probably due to increased conformational entropy of the α N helix of H3. Our data support a mode of interaction where the N-terminal homodimeric domain of Rtt106 intercalates between the two H3–H4 components of the (H3–H4)₂ tetramer while two double PH domains in the Rtt106 dimer interact with each of the two H3K56ac sites in (H3–H4)₂. We show that the Rtt106–(H3–H4)₂ interaction is important for gene silencing and the DNA damage response.

©2012 Macmillan Publishers Limited. All rights reserved

Correspondence and requests for materials should be addressed to G.M. (mer.georges@mayo.edu) or Z.Z. (zhang.zhiguo@mayo.edu).

*These authors contributed equally to this work.

Author Contributions Q.H. carried out the NMR spectroscopy experiments, prepared methylthiocarbonyl-aziridine and acetylated H3–H4, and performed the ITC measurements and analysis with assistance from G.M.; D.S. obtained crystals of the Rtt106 constructs, performed the X-ray diffraction measurements and solved the structures. Q.L. did the *in vivo* experiments. G.C. helped with the NMR experiments and ITC data analysis, A.F. and B.A.D. purified Rtt106 for initial crystal screening. J.R.T. worked on the refinement of crystal structures, M.V.B. contributed extensively to plasmid design, mutagenesis and protein preparations. G.M. and Z.Z. supervised the research. G.M. wrote the manuscript. All authors contributed in editing the manuscript.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Author Information The atomic coordinates and structure factors or NMR restraints of Rtt106DD, Rtt106PH, Rtt106PHL and Rtt106PH–acetyl-histamine have been deposited with the Protein Data Bank under accession codes 2LH0, 3FSS, 3TVV and 3TW1, respectively. The NMR chemical shifts of Rtt106 (residues 1–67) have been deposited in the Biological Magnetic Resonance Bank with the accession code 17832. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature.

To understand the mode of action of Rtt106, we characterized its three-dimensional (3D) structure and probed its association with histones. Rtt106 is modular (Supplementary Fig. 1), consisting of a homodimeric N-terminal domain (Rtt106DD; residues 1–42) and a double PH domain (Rtt106PH; residues 68–301) linked via a disordered region (residues 43–67) (Fig. 1a, b and Supplementary Figs 2 and 3). The 3D structure of Rtt106DD, determined using NMR spectroscopy, shows a previously undiscovered fold with each protomer adopting a V-shaped conformation consisting of two α -helices separated by a trans-proline residue (Fig. 1a, Supplementary Fig. 3 and Supplementary Table 1). The first and second α -helices of each protomer interact with the second and first α -helices of the other protomer, respectively, through extensive hydrophobic contacts (Fig. 1a). The 3D structure of Rtt106PH, determined by X-ray crystallography to a resolution of 1.4 Å (Fig. 1b and Supplementary Table 2), reveals similarity to the structure of Pob3, a protein thought to have a role in histone deposition¹¹ (Supplementary Fig. 4).

We performed isothermal titration calorimetry (ITC) measurements to probe the interaction of Rtt106DD–Rtt106PH (residues 1–301) with the histone H3–H4 complex reconstituted using non-acetylated H3 and H3 with an acetyl-lysine analogue chemically installed at position 56 (ref. 12). Rtt106 binds both non-acetylated and K56-acetylated H3–H4. However, acetylation results in enhanced affinity (Fig. 1c). In first approximation, the biphasic curve for the interaction of Rtt106 with non-acetylated H3–H4 in Fig. 1c can be interpreted as two concurrent binding isotherms in a two-site binding model with dissociation constants $K_{d1} = 0.4 \pm 0.2 \mu\text{M}$ and $K_{d2} = 1.5 \pm 0.2 \mu\text{M}$. Agreeing well with two binding sites, one from Rtt106DD and the other from Rtt106PH, Rtt106DD (residues 1–42) alone binds H3–H4 in an acetylation-independent manner. ITC data are consistent with a one-site binding model with a K_d of $0.6 \pm 0.1 \mu\text{M}$ (Fig. 1d), close to K_{d1} above. The reaction stoichiometry indicates that Rtt106DD, a dimer, binds two H3–H4 molecules, most likely in the form of an (H3–H4)₂ tetramer (Fig. 1d). Binding of Rtt106DD with H3–H4 was also demonstrated by NMR spectroscopy (Supplementary Fig. 5).

To interpret the biphasic ITC thermograms for the interaction of Rtt106 with K56-acetylated H3–H4, we considered a two-site binding model with an activation term that accounts for the effect of acetylation. The first dissociation constant $K_{d1ac} = 0.8 \pm 0.4 \mu\text{M}$ is similar to K_{d1} (Fig. 1c and Supplementary Fig. 6). The second dissociation constant, K_{d2ac} , is $0.08 \pm 0.06 \mu\text{M}$. In comparison to K_{d2} , the apparent gain in affinity for Rtt106PH is approximately 15–20-fold. These results indicate that the acetylated region of H3 is recognized by Rtt106PH but not by Rtt106DD. This was verified by monitoring NMR chemical shift perturbations in ¹H–¹⁵N heteronuclear single-quantum coherence (HSQC) correlation spectra of ¹⁵N-labelled Rtt106 (residues 1–67) and Rtt106PH upon titration with a non-labelled H3K56ac peptide (residues 51–61) (Fig. 1b). No binding to Rtt106 (residues 1–67) was observed upon addition of up to 15 molar excess of peptide (data not shown). In contrast, the H3K56ac peptide does specifically bind Rtt106PH as demonstrated by marked chemical shift changes ($\Delta\delta = 0.2$ p.p.m.) for 38 backbone amide signals of Rtt106PH (Fig. 1e and Supplementary Fig. 7). The affected residues are mapped to the second PH domain, specifically at the interface of the carboxy-terminal α -helix (α_5), two underlying β -sheets and the flexible tether connecting the two PH domains (Fig. 1b). Noticeably, this region differs from the binding sites of previously characterized PH domains¹³ (Supplementary Fig. 8). The K_d for the Rtt106PH–H3K56ac peptide interaction is $0.9 \pm 0.1 \text{ mM}$ (Supplementary Fig. 9). The markedly reduced affinity compared to that obtained for Rtt106DD–Rtt106PH (residues 1–301) and full-length H3–H4 is expected because the complete interaction is combinatorial with Rtt106DD and Rtt106PH both contacting H3–H4. Rtt106PH by itself has limited selectivity towards acetylation with only a twofold decrease in affinity for the non-acetylated H3K56 peptide ($K_d = 1.9 \pm 0.4 \text{ mM}$).

How then does K56 acetylation specifically enhance the affinity of H3–H4 for Rtt106? Recent biophysical studies have highlighted the structural heterogeneity of H3 α N helix in the context of (H3–H4)₂, suggesting that the structure and dynamics of α N could be readily altered by post-translational modifications¹⁴. K56, being the C-terminal residue of α N, contributes favourably to helical stability via charge interaction with the α -helix dipole. Therefore, in neutralizing the charge of K56, acetylation may increase the conformational entropy of α N, favouring interaction with Rtt106. This may be correlated to the observed entropy-driven increase in affinity upon acetylation (Supplementary Fig. 6).

In comparison to Rtt106PH (residues 68–301), the crystal structure of the longer Rtt106PHL (residues 68–315), with an α -helical extension from residues 302 to 311 (Supplementary Fig. 4), reveals a change in the conformation of K301 that leads to the identification of a K56/K56ac binding cleft in Rtt106 (Fig. 2a and Supplementary Table 2). Via an approximately 180° hinged motion, the side chain of K301 changes from being solvent-exposed in the structure of Rtt106PH to being partially buried in a cleft that is part of the H3K56ac peptide-binding region identified using NMR spectroscopy (Figs 1b, e and 2a). Noticeably, the ¹H–¹⁵N chemical shifts of several Rtt106PHL residues in the vicinity of the K301-occupied cleft match well the end-point chemical shifts of corresponding Rtt106PH residues upon titration with the H3K56ac peptide (Fig. 2b and Supplementary Fig. 10). With only one lysine (K56) in the H3K56ac and H3K56 peptides (Fig. 1b), the correspondence in NMR chemical shifts between the Rtt106PH–H3K56ac complex and Rtt106PHL strongly suggests that the K301-binding cleft is also the binding pocket for acetylated and non-acetylated K56.

The structural difference between Rtt106PH and Rtt106PHL likely reflects a dynamic exchange between an open and a closed state of the binding site. Consistent with dual states, H3K56ac peptide binding to Rtt106PHL occurs, but with lower affinity than for Rtt106PH (Supplementary Fig. 11). The relatively large crystallographic B-factor values for the C-terminal residues of Rtt106PHL (residue 299 and onwards) are also consistent with conformational flexibility (Supplementary Fig. 12). Moreover, in another crystal structure of Rtt106 (residues 65–320)¹⁵, there is no detectable electron density for residues 303–320 encompassing the helical extension of Rtt106PHL. Also supporting a two-state binding site with the open conformation favouring histone binding, four mutations at the C-terminal end of Rtt106PH markedly increased Rtt106PH affinity for the H3K56ac peptide (for example, $K_d = 0.4 \pm 0.1$ mM for K299A, Supplementary Table 3).

To illustrate how Rtt106 may associate with (H3–H4)₂, we derived a structural model of the complex (Fig. 2c). In the model, in which the dyad symmetry axes of the Rtt106DD and (H3–H4)₂ structures coincide, Rtt106DD is placed in a positively charged cavity formed between the two H3–H4 subcomplexes¹⁶ and without any contact with the region of H3 encompassing K56, in accordance with experimental data. Furthermore, we verified by ITC that two different sets of mutations (D7K and E11K; and E29K, E32K and E33K) that reverse negatively charged surface areas of Rtt106DD without affecting the 3D structure disrupt binding to H3–H4 (Fig. 1d). We also note that removal of the dimeric domain renders Rtt106 non-functional *in vivo* (data not shown). In addition, in the model the flexible Rtt106 inter-domain linker (residues 43–67) has an appropriate length to position Rtt106PH in contact with the K56-containing region of H3.

The histone-binding surface of Rtt106PH was further validated by measuring the affinity of twenty Rtt106PH mutants for the H3K56ac peptide (Fig. 3a, Supplementary Table 3, Supplementary Fig. 9 and Supplementary Discussion). Several of the mutations that affect binding were then incorporated into full-length Rtt106 to assess interactions with intact histones *in vivo*. Wild-type and mutant Rtt106 proteins were produced from budding yeast,

isolated by tandem affinity purification and probed for association with histones by western blot. Whereas histone H3 co-purified with wild-type Rtt106, several surface mutations introduced in Rtt106 blocked (Y261A, F269A, Y291A and I294A) or diminished (I259A and Q288A) histone binding *in vivo* (Fig. 3b). Also consistent with the *in vitro* binding data (Fig. 3a and Supplementary Table 3), reduced amounts of H3 were detected with Rtt106 harbouring the Y297A mutation in the putative K56ac binding cleft (Supplementary Fig. 13a). These results indicate that the interaction interface identified *in vitro* from titration of Rtt106PH with an H3K56ac peptide is important for the proper interaction of Rtt106 and H3 *in vivo*.

Rtt106 is crucial for heterochromatin silencing in yeast in the absence of the Cac1 (also known as Rfl2) subunit of CAF-1, another histone chaperone implicated in K56ac-dependent replication-coupled chromatin assembly^{5,17}. Using the green fluorescent protein (GFP) as a reporter in a gene silencing assay¹⁸, we confirmed that there was significant loss of silencing of the GFP gene in *cac1Δrtt106Δ* cells (Fig. 4a and Supplementary Fig. 13b). GFP silencing was restored to almost the level in control W303-1A cells by expressing wild-type Rtt106 but not by expressing Rtt106 mutants (Y261A, F269A, Y291A and I294A) that are highly defective in H3 binding *in vivo*. Expression of Rtt106 mutants (I259A, Q288A and Y297A) that showed reduced H3K56ac binding *in vivo* slightly reduced GFP silencing in *cac1Δrtt106Δ* cells compared to wild-type Rtt106 expression (Fig. 4a and Supplementary Fig. 13b). These results show that the ability of Rtt106 to contact the K56-containing surface of H3 via the double PH domain is important for transcriptional silencing.

Rtt106 is also critical for maintenance of genomic stability in the absence of Cac1 (ref. 5). To test if the Rtt106 mutants that showed defects in H3 binding *in vivo* (Fig. 3b and Supplementary Fig. 13a) would have increased DNA damage sensitivity, yeast cells harbouring wild-type or mutant Rtt106 but lacking Cac1 were grown in media containing the genotoxic agents methyl methanesulphonate (MMS) or camptothecin (CPT). Rtt106 mutants severely defective for H3 interaction were more susceptible to MMS and CPT treatment than wild-type or Rtt106 mutants having little or no defect in H3 binding, indicating that the ability of Rtt106 to bind H3K56ac is connected to its role in preserving genomic integrity (Fig. 4b and Supplementary Fig. 13c).

In conclusion, our study supports a working model where direct binding of Rtt106 to H3K56-acetylated (H3–H4)₂ tetramers contributes to nucleosome assembly with implications for DNA replication, gene silencing and maintenance of genomic stability. Our findings strongly suggest that the preferential association of Rtt106 with acetylated (H3–H4)₂ originates primarily from a K56 acetylation-triggered increase in conformational entropy of H3 αN. This mode of specific association with a modified histone is fundamentally different from that of so-called histone mark reader domains¹⁹.

METHODS SUMMARY

Wild-type and mutant Rtt106 proteins were expressed in *Escherichia coli* as His₆-fusions and purified by immobilized metal affinity and gel filtration chromatography. Purification and reconstitution of H3–H4 followed established procedures²⁰. The homogeneous site-specific installation of an acetylated lysine analogue (methylthiocarbonyl-thiaLys) in place of H3K56 in H3–H4 was done as reported¹² (Supplementary Fig. 14). Protein labelling with selenomethionine (SeMet) for X-ray crystallography studies and with ¹⁵N, ¹⁵N/¹³C and ¹⁵N/¹³C/²H for NMR spectroscopy studies was achieved by growing *E. coli* cells in SeMet- and isotope-enriched media following standard procedures. All proteins were crystallized at 15 °C. X-ray diffraction data were collected on-site and at the Advance Photon Source (APS) synchrotron facility, Argonne National Laboratory. NMR experiments

were collected at 25 °C using a Bruker Avance 700 MHz spectrometer. The solution NMR structure of Rtt106 (residues 1–67) was determined using a simulated annealing-based protocol. Tandem affinity purification, gene silencing assays and DNA damage assays were done as reported^{5,17,18}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to N. Jurani, S. Macura and T. Burghardt for experimental advice, Y. Kim for assistance with X-ray data collection, Z. Zhang for suggestions on chemical synthesis, and K. Luger and A. Hieb for advice on the preparation of histones. We acknowledge the use of synchrotron beamlines 19BM and 19ID of the Structural Biology Center at Argonne National Laboratory's APS, supported by the US Department of Energy, Basic Energy Sciences, Office of Science, under contract no. W-31-109-ENG-38. This work was funded in part by National Institutes of Health grants to Z.Z. and G.M.

METHODS

Protein preparation

Constructs of Rtt106 encompassing amino acids 1–315, 1–301 (Rtt106DD–Rtt106PH), 1–67, 1–42 (Rtt106DD), 68–299, 68–301 (Rtt106PH) and 68–315 (Rtt106PHL) were cloned in a modified pET vector (Novagen) encoding an N-terminal His₆-tag and a tobacco etch virus (TEV) protease cleavage site. The wild-type proteins were overexpressed in *Escherichia coli* BL21(DE3) initially grown in LB broth at 37 °C to $D_{600\text{nm}}$ of ~0.8, then transferred to 15 °C, and after 45 min, induced by 1 mM isopropyl- β -D-thiogalactoside (IPTG) for 14 to 18 h. The cells were collected by centrifugation and resuspended in 50 mM sodium phosphate buffer, pH 7.5, 300 mM NaCl and 1 mM phenylmethanesulphonylfluoride, and lysed using an Emulsiflex C5 high-pressure homogenizer (Avestin). After centrifugation, the proteins were purified by affinity chromatography with Ni²⁺-loaded NTA resin (Qiagen) according to the manufacturer's recommended protocol. The His₆-tag was cleaved with TEV protease at 4 °C overnight and further purification was performed by size exclusion chromatography using preparative Superdex 75 or 200 columns (GE Healthcare). Cleavage of the His₆-tag leaves three residues (GHM) at the N terminus of each protein.

Point mutants of Rtt106 were made following the QuikChange site-directed mutagenesis protocol (Stratagene) and were verified by DNA sequencing. All mutants were purified as described for the wild-type protein.

The preparation of isotopically labelled Rtt106 (residues 1–67), Rtt106PH and Rtt106PHL followed similar steps as above except that instead of LB broth, M9 media containing 1 g l⁻¹ ¹⁵N NH₄Cl, 4 g l⁻¹ [¹²C₆]-D-glucose and 1 g l⁻¹ ¹⁵N Isogro (Isotec) (for ¹⁵N-labelled samples); and 1 g l⁻¹ ¹⁵N NH₄Cl, 2 g l⁻¹ [¹³C₆]-D-glucose and 1 g l⁻¹ ¹⁵N/¹³C Isogro (Isotec) (for ¹⁵N/¹³C-labelled samples) were used²¹. The procedure was similar for producing ²H/¹⁵N/¹³C-labelled samples but with the *E. coli* cells grown in culture media prepared with 99.9% D₂O.

For producing selenomethionine (SeMet)-enriched Rtt106PH and Rtt106PHL, a similar protocol as above was used but with protein overexpression in the methionine-auxotroph *E. coli* strain B834(DE3) (Novagen) grown in M9 media with SeMet and all amino acids except methionine²².

The preparation of histones H3 and H4 is based on a published protocol²⁰. Histones H3 and H4 from *Xenopus laevis* were overexpressed in *E. coli* BL21 (DE3) Rosetta pLysS, purified separately under denaturing conditions and then combined to reconstitute the (H3–H4)₂ tetramer. For each histone, cells were grown at 37 °C to $D_{600\text{ nm}}$ of 0.6–0.8, induced with 1 mM IPTG, collected after 3 h and lysed using an Emulsiflex C5 high-pressure homogenizer (Avestin). After centrifugation, the pellet was washed with 1 M L-arginine monohydrochloride, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5 three times and with 1 M L-arginine monohydrochloride, 5 mM 2-mercaptoethanol, 1 M guanidine hydrochloride, 10 mM Tris-HCl, pH 7.5 once. Next, the pellet was dissolved in 10 mM dithiothreitol, 7 M guanidine hydrochloride, 20 mM Tris-HCl, pH 7.5 and centrifuged. The supernatant was dialysed several times in water containing 5 mM 2-mercaptoethanol for a period of 3 days and then lyophilized. The lyophilized solid was dissolved in 10 mM dithiothreitol, 6 M urea, 20 mM Tris-HCl, pH 7.5 (buffer A) and residual solids were spun down. The supernatant was passed through a Resource S cation exchange column (GE Healthcare) using buffer A as running buffer and eluted with a 0 to 1 M NaCl gradient. Resulting solutions of H3 and H4 were mixed at equimolar ratio and dialysed in 2 M NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5 (refolding buffer). Refolded H3–H4 was purified by size-exclusion chromatography using a Superdex 200 column (GE Healthcare) and refolding buffer as the running buffer.

Incorporation of an acetyl-lysine analogue in H3–H4

For incorporation of an acetyl-lysine analogue at position 56 of histone H3, we closely followed a published protocol¹². The single cysteine (C110) in histone H3 was replaced by an alanine and K56 was replaced by a cysteine. C56 of H3 (K56C, C110A) or H3 (K56C, C110A)–H4 was alkylated with methylthiocarbonyl-aziridine (MTCA) to generate the acetylated lysine analogue methylthiocarbonyl-thiaLys (Supplementary Fig. 14).

MTCA was prepared as reported previously¹². Briefly, to pre-cooled diethyl ether (30 ml) in a round-bottom flask (–80 °C, maintained with dry ice and acetone), aziridine (100 μ l, 1.93 mmol), triethylamine (0.27 ml, 1.93 mmol) and methyl chlorothiolformate (0.16 ml, 1.89 mmol) were added with stirring. The reaction was allowed to proceed for 3 h before diluting the reaction mixture with diethyl ether:H₂O (15 ml:10 ml). The mixture was next shaken in a separation funnel and the organic layer isolated and washed as follow: 0.01 M HCl (5 ml, twice), H₂O (5 ml, twice) and brine (10 ml, twice). The organic layer was dried over anhydrous MgSO₄ and then concentrated using a rotary evaporator. The product, MTCA (100–200 mg yield), was verified by NMR spectroscopy (Supplementary Fig. 14). Aziridine was purchased from ChemService. All other chemicals were purchased from Sigma-Aldrich. Commercial reagents were used as received without further purification.

Resource S-purified H3 mutant (K56C, C110A) was dialysed extensively in water with 5 mM 2-mercaptoethanol and then in water alone. Next, it was lyophilized and then dissolved in 100 mM ammonium bicarbonate, pH 8.0, to a final concentration of ~2 mg ml⁻¹. MTCA was added to a final concentration of 50–200 mM. The reaction proceeded for ~200 min at room temperature. The modified product was verified by mass spectrometry, lyophilized and subsequently used for reconstitution with H4 as explained above (Supplementary Fig. 14). Alternatively, the acetylation reaction was carried out on refolded H3 (K56C, C110A)–H4. After purification with Superdex 200, H3 (K56C, C110A)–H4 was dialysed in 100 mM ammonium bicarbonate, pH 8.0, 300 mM NaCl and concentrated to 2–10 mg ml⁻¹. 100 mM of MTCA was added and the reaction was left at room temperature for ~200 min. Acetylated H3–H4 obtained from either method was then extensively dialysed in the final buffer of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl for ITC experiments.

Isothermal titration calorimetry

Measurements were carried out at 10 °C using a VP-ITC titration calorimeter (MicroCal). All proteins were prepared in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl. Rtt106DD, Rtt106 (residues 1–67) or Rtt106DD–Rtt106PH (residues 1–301) in the calorimeter injection syringe at concentrations ranging from 0.5 mM to 0.74 mM were delivered as a series of 5- to 8- μ l injections every 5 min to the reaction cell containing non-acetylated or K56-acetylated H3–H4 at concentrations of 20 μ M or 30 μ M. Measurements were paired with control experiments for heat of mixing and dilution. Data were analysed with Levenberg–Marquardt nonlinear regression using different models programmed in Origin 7.0 and in-house software. Data were also simulated using Mathematica (Wolfram Research).

Crystallization, data collection and structure determination

Crystals of SeMet-labelled Rtt106PH were grown at 15 °C by vapour diffusion of hanging drops by mixing 1 μ l of Rtt106PH at 30 mg ml^{−1} in 20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 10% glycerol with 1 μ l of reservoir solution containing 4% (v/v) Tacsimate, pH 5.0, 12% PEG 3350. The crystals were cryoprotected by transfer to reservoir solution supplemented with 20% glycerol for 10–15 min, and were quick-frozen in a cryoloop (Hampton Research) with liquid nitrogen.

Single anomalous diffraction data were collected for Rtt106PH at APS 19BM beamline, Argonne National Laboratory. Processing of diffraction images and scaling of the integrated intensities were performed using HKL3000 (ref. 23). Crystals were of the space group *C*2 with one molecule of Rtt106PH in the asymmetric unit and a Matthews coefficient of 2.32 Å³ Da^{−1}. The four Se atom positions were determined using SHELXD²⁴, followed by density modification with RESOLVE²⁵ and initial automatic model building using ARP/wARP²⁶. Model correction and refinement were undertaken using the programs COOT²⁷ and REFMAC5 (ref. 28). Resolution is 1.4 Å. For the Ramachandran geometry, 91.5% of all dihedral angles are located in most favoured regions and 8.5% in additionally allowed regions.

A complex of Rtt106PH and acetyl-histamine (AHN) was prepared by soaking the centred monoclinic crystals of SeMet-labelled Rtt106PH for 5 min in a 1 M solution of AHN (Sigma) prepared in the mother liquor and by flash-freezing in liquid nitrogen. Diffraction data were collected at 100 K using a Rigaku Microfocus 007 generator and Rigaku R-Axis IV⁺⁺ area detector. Data processing and scaling of the integrated intensities were performed using HKL2000 (ref. 29). The structure was solved by molecular replacement using PHASER³⁰ with the crystal structure of Rtt106PH as a search model. Model correction and refinement were undertaken using the programs COOT²⁷ and PHENIX³¹. Resolution is 1.8 Å. For the Ramachandran geometry, 92% of all dihedral angles are located in most favoured regions and 8% in additionally allowed regions.

Crystals of SeMet-labelled Rtt106PHL (residues 68–315) were grown at 15 °C by hanging drop vapour diffusion after mixing 1 μ l of Rtt106PHL at 30 mg ml^{−1} in 20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 10% glycerol with 1 μ l of reservoir solution containing 8% (v/v) Tacsimate, pH 5.0, 20% PEG 3350. The crystals were cryoprotected as for Rtt106PH above.

Diffraction data were collected at 100 K at the APS 19ID beamline, Argonne National Laboratory. Processing of diffraction images and scaling of the integrated intensities were performed using HKL3000 (ref. 23). Crystals were of the space group *P*2₁ with two molecules of Rtt106PHL in the asymmetric unit and a Matthews coefficient of 2.86 Å³ Da^{−1}.

The structure was solved by molecular replacement using PHASER³⁰ with the crystal structure of Rtt106PH as a search model. Model correction and refinement were undertaken using the programs COOT²⁷ and PHENIX³¹. Resolution is 2.6 Å. For the Ramachandran geometry, 86.8% of all dihedral angles are located in most favoured regions and 12.9% in additionally allowed regions.

NMR spectroscopy

NMR experiments were conducted at 25 °C using a Bruker Avance 700 MHz spectrometer equipped with a cryogenic probe. The Rtt106DD, Rtt106 (residues 1–67), Rtt106PH and Rtt106PHL protein samples (wild-type and mutants) were at concentrations of ~0.6 mM in 20 mM sodium phosphate buffer, pH 6.9, 30 mM NaCl and 5 mM dithiothreitol. 95% of backbone carbon and nitrogen resonances of Rtt106PH and 98% of all resonances of Rtt106 (residues 1–67) were assigned from regular and transverse relaxation-optimized spectroscopy (TROSY)-based experiments³² using ²H/¹³C/¹⁵N-labelled or ¹³C/¹⁵N-labelled Rtt106 samples. NMR data were processed with NMRPipe/NMRDraw³³ and analysed with SPARKY (Goddard, T. D. & Kneller, D.G., <http://www.cgl.ucsf.edu/home/sparky/>). The solution structure of Rtt106 (1–67) homodimer was calculated and refined using the program CNS version 1.2, using a simulated annealing protocol for torsion angle dynamics³⁴. A total of 1,150 distance constraints derived from 3D ¹⁵N-resolved nuclear Overhauser enhancement spectroscopy (NOESY), 3D ¹³C-resolved NOESY, 2D NOESY and 2D ¹³C filtered–edited NOESY spectra were used in the structure calculations. Also included were 48 hydrogen bond distance constraints derived from hydrogen–deuterium exchange measurements and NOESY data, and 112 dihedral angles derived from chemical shift index analysis of ¹³Cα, ¹³Cβ, ¹³CO and ¹Hα atoms. The 20 lowest energy conformers from 200 refined structures were selected to represent the NMR ensemble. For the well-folded part of the molecule (residues 6–42), 94.3% of all dihedral angles are located in most favoured regions and 5.4% in additionally allowed regions of the Ramachandran plot.

The interactions of non-acetylated histone H3 (H3K56) and H3K56ac (residues 51–61 for both) peptides with wild-type Rtt106PH were quantified by recording a series of ¹H–¹⁵N HSQC spectra of ¹⁵N-labelled Rtt106PH at increasing concentrations of the peptides. The mutated Rtt106PH proteins were similarly titrated with the H3K56ac peptide. The dissociation constants (*K_d*s) were estimated from the NMR chemical shift perturbations of five to eight non-overlapping ¹H–¹⁵N resonances by nonlinear least-squares fitting of the following equation:

$$\frac{\Delta\delta}{\Delta\delta_{\max}} = 0.5 \left[\left(1 + \frac{K_d}{C_p} + M \right) - \sqrt{\left(\left(1 + \frac{K_d}{C_p} + M \right)^2 - 4M \right)} \right]$$

where *M* is the molar ratio of H3K56 or H3K56ac peptide to Rtt106PH, *C_p*, the concentration of Rtt106PH and Δδ, the normalized chemical shift change calculated as:

$$\Delta\delta = \sqrt{(\delta_{\text{HN}})^2 + (\delta_{\text{N}})^2}$$

where δ_{HN} and δ_N denote the amide hydrogen and nitrogen atoms chemical shift differences, respectively, between the free and peptide-bound states for Rtt106PH. Δδ_{max} is the normalized difference in chemical shifts of the free and peptide-saturated Rtt106PH.

Molecular illustrations

Molecular illustrations were prepared with PyMOL (<http://www.pymol.org/>) and MOLMOL³⁵.

Yeast strains, plasmids and antibodies

All budding yeast strains used in this study were derived from the parental W303 background strain (*leu2-3, ura3-1, his3-11, trp1-1, ade2-1, can1-100*) and are listed in Supplementary Table 4. Rtt106 constructs were tagged at their C termini with the tandem affinity purification (TAP) tag according to published procedures³⁶. Full-length Rtt106 containing its endogenous promoter and its C-terminal TAP tag was cloned into pRS313 vector and the resulting plasmid was used as a template to make Rtt106 mutants using the QuikChange site-directed mutagenesis kit (Stratagene). Mutant strains were constructed in the W303 background strain by standard yeast cloning methods³⁷. Antibodies used in this study were produced as described previously³⁸.

Binding of Rtt106 with H3 in yeast cells using tandem affinity purification

To test the effect of Rtt106 mutations on H3 binding, wild-type and mutant Rtt106 proteins were purified from yeast cells using the TAP tag procedure and co-purified proteins were detected by western blotting with antibodies against calmodulin-binding peptide (CBP) and histones H3 and H3K56ac as described previously⁵.

Assay for silencing at the *HMR* locus using the *GFP* reporter

The silencing assay was performed as described previously¹⁷. Briefly, exponentially growing wild-type or mutant cells were collected, washed with PBS, resuspended in SCM-TRP media, and analysed by flow cytometry.

Assay for the sensitivity towards DNA-damaging agents

To analyse the sensitivity of *cac1Δ* yeast cells harbouring wild-type or mutant Rtt106 to different DNA-damaging agents, tenfold serial dilutions of freshly grown yeast cells were spotted onto selective media SCM-HIS containing different concentrations of methyl methanesulphonate (MMS: 0.001, 0.005 and 0.01% (v/v)) or camptothecin (CPT: 0, 1, 2.5 and 5 $\mu\text{g ml}^{-1}$). Plates were incubated at 30 °C for 3 days and then photographed.

References

1. Luger K. Dynamic nucleosomes. *Chromosome Res.* 2006; 14:5–16. [PubMed: 16506092]
2. Kouzarides T. Chromatin modifications and their function. *Cell.* 2007; 128:693–705. [PubMed: 17320507]
3. Corpet A, Almouzni G. Making copies of chromatin: the challenge of nucleosomal organization and epigenetic information. *Trends Cell Biol.* 2009; 19:29–41. [PubMed: 19027300]
4. Chen CC, et al. Acetylated lysine 56 on histone H3 drives chromatin assembly after repair and signals for the completion of repair. *Cell.* 2008; 134:231–243. [PubMed: 18662539]
5. Li Q, et al. Acetylation of histone H3 lysine 56 regulates replication-coupled nucleosome assembly. *Cell.* 2008; 134:244–255. [PubMed: 18662540]
6. Williams SK, Truong D, Tyler JK. Acetylation in the globular core of histone H3 on lysine-56 promotes chromatin disassembly during transcriptional activation. *Proc. Natl Acad. Sci. USA.* 2008; 105:9000–9005. [PubMed: 18577595]
7. Xie W, et al. Histone H3 lysine 56 acetylation is linked to the core transcriptional network in human embryonic stem cells. *Mol. Cell.* 2009; 33:417–427. [PubMed: 19250903]

8. Das C, Lucia MS, Hansen KC, Tyler JK. CBP/p300-mediated acetylation of histone H3 on lysine 56. *Nature*. 2009; 459:113–117. [PubMed: 19270680]
9. Tjeertes JV, Miller KM, Jackson SP. Screen for DNA-damage-responsive histone modifications identifies H3K9Ac and H3K56Ac in human cells. *EMBO J*. 2009; 28:1878–1889. [PubMed: 19407812]
10. Yuan J, Pu M, Zhang Z, Lou Z. Histone H3–K56 acetylation is important for genomic stability in mammals. *Cell Cycle*. 2009; 8:1747–1753. [PubMed: 19411844]
11. VanDemark AP, et al. The structure of the yFACT Pob3-M domain, its interaction with the DNA replication factor RPA, and a potential role in nucleosome deposition. *Mol. Cell*. 2006; 22:363–374. [PubMed: 16678108]
12. Huang R, et al. Site-specific introduction of an acetyl-lysine mimic into peptides and proteins by cysteine alkylation. *J. Am. Chem. Soc*. 2010; 132:9986–9987. [PubMed: 20608637]
13. Blomberg N, Baraldi E, Nilges M, Saraste M. The PH superfold: a structural scaffold for multiple functions. *Trends Biochem. Sci*. 1999; 24:441–445. [PubMed: 10542412]
14. Bowman A, Ward R, El-Mkami H, Owen-Hughes T, Norman DG. Probing the (H3–H4)₂ histone tetramer structure using pulsed EPR spectroscopy combined with site-directed spin labeling. *Nucleic Acids Res*. 2010; 38:695–707. [PubMed: 19914933]
15. Liu Y, et al. Structural analysis of Rtt106p reveals a DNA binding role required for heterochromatin silencing. *J. Biol. Chem*. 2010; 285:4251–4262. [PubMed: 20007951]
16. White CL, Suto RK, Luger K. Structure of the yeast nucleosome core particle reveals fundamental changes in internucleosome interactions. *EMBO J*. 2001; 20:5207–5218. [PubMed: 11566884]
17. Huang S, et al. Rtt106p is a histone chaperone involved in heterochromatin-mediated silencing. *Proc. Natl Acad. Sci. USA*. 2005; 102:13410–13415. [PubMed: 16157874]
18. Huang S, Zhou H, Tarara J, Zhang Z. A novel role for histone chaperones CAF-1 and Rtt106p in heterochromatin silencing. *EMBO J*. 2007; 26:2274–2283. [PubMed: 17410207]
19. Dhalluin C, et al. Structure and ligand of a histone acetyltransferase bromodomain. *Nature*. 1999; 399:491–496. [PubMed: 10365964]
20. Luger K, Rechsteiner TJ, Richmond TJ. Preparation of nucleosome core particle from recombinant histones. *Methods Enzymol*. 1999; 304:3–19. [PubMed: 10372352]
21. Botuyan MV, et al. Structural basis of BACH1 phosphopeptide recognition by BRCA1 tandem BRCT domains. *Structure*. 2004; 12:1137–1146. [PubMed: 15242590]
22. Hendrickson WA, Horton JR, LeMaster DM. Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure. *EMBO J*. 1990; 9:1665–1672. [PubMed: 2184035]
23. Minor W, Cymborowski M, Otwinowski Z, Chruszcz M. *HKL-3000*: the integration of data reduction and structure solution—from diffraction images to an initial model in minutes. *Acta Crystallogr. D*. 2006; 62:859–866. [PubMed: 16855301]
24. Sheldrick GM. A short history of *SHELX*. *Acta Crystallogr. A*. 2008; 64:112–122. [PubMed: 18156677]
25. Terwilliger TC. SOLVE and RESOLVE: automated structure solution and density modification. *Methods Enzymol*. 2003; 374:22–37. [PubMed: 14696367]
26. Perrakis A, Morris R, Lamzin VS. Automated protein model building combined with iterative structure refinement. *Nature Struct. Biol*. 1999; 6:458–463. [PubMed: 10331874]
27. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D*. 2004; 60:2126–2132. [PubMed: 15572765]
28. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr. D*. 1997; 53:240–255. [PubMed: 15299926]
29. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol*. 1997; 276:307–326.
30. Storoni LC, McCoy AJ, Read RJ. Likelihood-enhanced fast rotation functions. *Acta Crystallogr. D*. 2004; 60:432–438. [PubMed: 14993666]
31. Adams PD, et al. *PHENIX*: building new software for automated crystallographic structure determination. *Acta Crystallogr. D*. 2002; 58:1948–1954. [PubMed: 12393927]

32. Ferentz AE, Wagner G. NMR spectroscopy: a multifaceted approach to macromolecular structure. *Q. Rev. Biophys.* 2000; 33:29–65. [PubMed: 11075388]
33. Delaglio F, et al. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR.* 1995; 6:277–293. [PubMed: 8520220]
34. Brünger AT, et al. *Crystallography & NMR system*. A new software suite for macromolecular structure determination. *Acta Crystallogr. D.* 1998; 54:905–921. [PubMed: 9757107]
35. Koradi R, Billeter M, Wüthrich K. MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* 1996; 14:51–55. [PubMed: 8744573]
36. Puig O, et al. The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods.* 2001; 24:218–229. [PubMed: 11403571]
37. Thomas BJ, Rothstein R. Elevated recombination rates in transcriptionally active DNA. *Cell.* 1989; 56:619–630. [PubMed: 2645056]
38. Zhou H, Madden BJ, Muddiman DC, Zhang Z. Chromatin assembly factor 1 interacts with histone H3 methylated at lysine 79 in the processes of epigenetic silencing and DNA repair. *Biochemistry.* 2006; 45:2852–2861. [PubMed: 16503640]

Figure 1. 3D structures of Rtt106 dimeric and double PH domains and their interaction with histones

a, NMR structure of Rtt106 dimeric region (residues 1–67) with the hydrophobic residues constituting the dimerization interface in stick representation. Two protomers are in blue and green. Residues 43–67 are disordered and omitted for clarity. **b**, Crystal structure of Rtt106 double PH domain (Rtt106PH, residues 68–301) with H3K56ac peptide-binding surface in flesh. **c**, ITC results (top, raw titration data; bottom, integrated heat measurements) for the interaction of Rtt106DD–Rtt106PH (residues 1–301) with non-acetylated (H3–H4)₂ (black) and K56-acetylated (H3–H4)₂ (red). For the former interaction, a two-site binding model (dissociation constants K_{d1} and K_{d2}) was used. For the latter, an activation step accounting for the effect of acetylation was incorporated in the two-site binding model (K_{d1ac} and K_{d2ac}). K_d s are reported with s.d. determined by nonlinear least-squares analysis. The light blue envelop represents simulated data for K_{d2ac} varying from 0.01 to 0.1 μM and $K_{d1ac} = 0.4 \mu\text{M}$. **d**, ITC data for the interaction of Rtt106DD (residues 1–42) with (H3–H4)₂. Stoichiometry n and K_d are indicated. Data for two mutant forms of Rtt106DD, D7K, E11K and E29K, E32K, E33K, are also shown. **e**, ^1H – ^{15}N HSQC spectra of H3K56ac peptide-bound (red) versus free (black) Rtt106PH. Perturbed signals are labelled on the spectra.

Figure 2. Identification of a K56ac-binding cleft in Rtt106 and model of Rtt106 in complex with K56-acetylated (H3-H4)₂

a, Binding cleft and the side chain of K301 (red) in Rtt106PH and Rtt106PHL. **b**, Chemical shift changes in Rtt106PH ^1H - ^{15}N HSQC spectra upon titration with the H3K56ac peptide (from red to purple signals) are compared to the chemical shifts of free Rtt106PHL (black signals) with respect to seven residues in the vicinity of the binding cleft. A214, E215, K216 and I217 belong to the disordered loop adjacent to the binding cleft. **c**, Structural model of Rtt106 (residues 1–301) in complex with the (H3-H4)₂ tetramer. Atomic coordinates of (H3-H4)₂ are from the structure of budding yeast nucleosome core particle (PDB access code 1ID3)¹⁶.

Figure 3. Effects of Rtt106PH mutations on H3K56ac interaction

a, Surface representation of Rtt106PH with the H3K56ac peptide-binding region in orange. The upper box represents a mainly hydrophobic area of the binding site whereas the lower box highlights the K56/K56ac binding cleft. Affinities of Rtt106PH mutants for the H3K56ac peptide were measured and K_d s are reported in Supplementary Table 3. Mutated residues that totally abolish, decrease, have no effect or enhance binding are labelled in red, blue, black and white, respectively. **b**, Wild-type (WT) and mutant tandem affinity purification (TAP)-tagged full-length Rtt106 were purified from yeast cells and analysed by western blot using indicated antibodies. CBP, calmodulin-binding peptide tag. Rtt106 mutated outside the binding site (T232A) was used as control.

Figure 4. Effects of Rtt106 mutations on HMR silencing and genome stability

a, Schematic of the GFP-based gene silencing reporter assay. The *GFP* gene (*P_{URA3}-GFP*) at the silent mating type locus *HMR* (*hmr::GFP*) is controlled by the *URA3* gene promoter, silencers E and I, and *a2* gene. Gene silencing is reported as percentage of yeast cells expressing GFP. One representative of three independent experiments is shown. Silencing is observed in W303-1A control cells, but not in cells lacking the silent information regulator gene *SIR3*. Expression of wild-type (WT) Rtt106 in *cac1*Δ*rtt106*Δ cells restores silencing. Expression of Rtt106 mutated in the H3K56ac-binding surface does not or only partially restores silencing. **b**, Mutations in the H3 binding sites of Rtt106 enhance the DNA-damage sensitivity of *cac1*Δ mutant cells. Cells of the indicated genotypes were spotted onto media lacking histidine (SCM-HIS) for plasmid selection, without or with MMS or CPT for DNA damage assessment.