

maltoside buffer, with 2 µl of ¹⁴C-labelled SAM (NEC-363 adenosyl-L-methionine, S-(methyl-¹⁴C)) and 40 µg of a mixture of soluble histones from calf thymus (H2A, H2B, H3 and H4; Sigma), 10 µg of recombinant *Xenopus* wild-type H3, or 10 µg of H3 deleted from the 26 first amino acids (*Δtail* H3) for 1 h at 30 °C.

Expression profiling

The *set1Δ* mutant and the isogenic UCC1001 strain were grown together in YPD medium at 30 °C to an optical density of 1.0 at 600 nm. Poly(A) RNA was isolated and reverse-transcribed incorporating amino-allyl dUTP. The resulting DNA probe was labelled with reactive Cy5 (mutant) or Cy3 (parent strain) dye and hybridized to a spotted complementary DNA microarray containing the yeast open reading frames as described previously^{17,18}. Microarrays were analysed using a GenePix4000A scanner and GENEPIX 3.0 software. Candidate genes regulated by Set1 were identified from duplicate experiments using a gene-specific error model described previously¹⁹. Genes reported to be activated by Set1 were downregulated in both profiles of the mutant *set1Δ* strain. Microarray data were verified by northern blot analysis. Using a 1.5-fold cut-off, there are 480 downregulated and 885 upregulated genes.

Northern blots and ChIPs

Northern blots and ChIPs were done exactly as described²⁰. We used 5 µl of tri-methylated K4 H3 antibody and 2 µl of di-methylated K4 H3 antibody. For the ChIP experiments, the cells were grown in minimal medium lacking inositol (–ino), diluted to an optical density of 0.15 at 600 nm in minimal medium lacking inositol or the same medium but supplemented with 100 mg l⁻¹ inositol (medium +ino), and grown to an optical density of 1.2 at 600 nm and then processed for chromatin preparation. Samples from medium without and with methionine were prepared in the same way except that the medium was supplemented with 30 mg l⁻¹ of methionine where appropriate. The constitutive genes were scored from medium with inositol (*PPH3*) and medium with methionine (*HAMI* and *NUP170*). Primers for the PCR analysis are described in Supplementary Fig. 4.

Antibodies

The di-methylated K4 H3 and the tri-methylated K4 H3 specific antibodies are available from Abcam (<http://www.abcam.com>).

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Competing interests statement

The authors declare competing financial interests: details accompany the paper on Nature's website (<http://www.nature.com/nature>).

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Acetylation of histone H4 by Esa1 is required for DNA double-strand break repair

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Although the acetylation of histones has a well-documented regulatory role in transcription^{1–4}, its role in other chromosomal functions remains largely unexplored. Here we show that distinct patterns of histone H4 acetylation are essential in two separate pathways of double-strand break repair. A budding yeast strain with mutations in wild-type H4 acetylation sites shows defects in nonhomologous end joining repair and in a newly described pathway of replication-coupled repair. Both pathways require the *ESA1* histone acetyl transferase (HAT), which is responsible for acetylating all H4 tail lysines, including ectopic lysines that restore repair capacity to a mutant H4 tail. Arp4, a protein that binds histone H4 tails and is part of the Esa1-containing NuA4 HAT complex, is recruited specifically to DNA double-strand breaks that are generated *in vivo*. The purified Esa1–Arp4 HAT complex acetylates linear nucleosomal arrays with far greater efficiency than circular arrays *in vitro*, indicating that it preferentially acetylates nucleosomes near a break site. Together, our data show that histone tail acetylation is required directly for DNA repair and suggest that a related human HAT complex may function similarly.

Four lysines, at positions 5, 8, 12 and 16, in the amino-terminal tail of histone H4 are reversibly acetylated *in vivo* in all eukaryotes⁵. The histone code hypothesis⁶ postulates that covalent modifications of histones such as acetylation, phosphorylation and methylation facilitate the binding of specific proteins to chromatin to alter transcription and possibly other events in DNA metabolism. HATs associate physically with the Ku70 DNA repair protein⁷ and with the ORC replication initiation complex^{8,9}, which suggests that histone acetylation may indeed be important in processes other than transcription.

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Mutations of yeast histone H4 in which all four tail lysines are replaced by glutamines (mutant *hhf1-10* (4K → Q)) cause a pronounced defect in genome integrity and engage the RAD9 checkpoint^{10,11}. To determine whether the genomic instability observed in *hhf1-10* might result from endogenous damage or a failure of DNA repair, we tested the *hhf1-10* mutant for sensitivity to several DNA-damaging agents, including camptothecin (CPT) and methyl methane sulphate (MMS), which induce DNA double-strand breaks (DSBs), and ultraviolet radiation, which causes the formation of intrastrand photoproducts. The *hhf1-10* mutant was found to be markedly hypersensitive to both CPT and MMS (Fig. 1a), indicating the presence of a defect in DNA DSB repair. In contrast, the *hhf1-10* mutant was not even modestly hypersensitive to ultraviolet radiation (Fig. 1b), which causes DNA damage that is repaired through a separate pathway. These results show that the *hhf1-10* mutant is not globally defective in all DNA repair processes arising from an altered chromatin structure.

If lysine acetylation *per se* is required to repair damage caused by DSB-inducing agents, we reasoned that adding a single lysine to the *hhf1-10* tail, perhaps even in an ectopic location, might restore wild-type resistance to these agents, because a single lysine (but not a single arginine) is sufficient to reverse the RAD9 checkpoint arrest¹¹. Six mutants containing a single lysine were tested for CPT sensitivity: four mutants had a lysine in one of the natural positions in the H4 tail and two had an ectopic tail lysine. Adding a lysine into

five of the six positions in the H4 tail completely rescued the CPT hypersensitivity observed in the *hhf1-10* mutant (Fig. 1c), whereas lysine addition at position 16 only partially rescued hypersensitivity to DSB agents. Thus, a principal pathway for repair of DSB damage seems to require the presence of a single H4 tail lysine.

Critical to this interpretation is the prediction that the ectopic lysines in *hhf1-25* and *hhf1-35* will be capable of *in vivo* acetylation. To test this prediction, cell extracts from wild-type, *hhf1-10* (4K → Q), *hhf1-25* (4K → Q, + 1K) and *hhf1-35* (4K → Q, + 1K) strains were immunoblotted with an antibody against acetylated H4. Extracts from both *hhf1-25* and *hhf1-35*, but not the *hhf1-10* (4K → Q) control, showed a clear signal at the H4 position (Fig. 1d), indicating that the ectopic lysines are acetylated *in vivo*. This is consistent with the idea that lysine acetylation is required for the correct repair of DNA DSBs.

We reasoned that if *hhf1-10* is defective for DSB repair because lysine acetylation is blocked by mutation, then mutation of the HAT that acetylates the H4 lysines should also result in defective DSB repair. We tested mutants in several HATs that are not essential for DNA damage sensitivity including *HAT1*, *GCN5* and *SAS2*; none of these was hypersensitive to CPT (data not shown). We then examined the essential *ESA1* next because this HAT prefers H4 as a substrate *in vitro* and because loss of *ESA1* function has been reported to trigger the RAD9-dependent DNA damage checkpoint¹². We generated mutations in the chromosomal *ESA1* gene and scored colonies for both temperature-sensitive growth and CPT and MMS sensitivity at the permissive temperature. A subset of *esa1* mutants was specifically hypersensitive to CPT and MMS, whereas other temperature-sensitive mutants were not. Cells carrying the *esa1-L357H* allele did not grow at 37 °C but had wild-type resistance to CPT and MMS at 30 °C. By contrast, cells expressing the *esa1-1851* allele did not grow at 37 °C and were markedly hypersensitive to CPT and MMS at 30 °C (Fig. 2a). Thus, at the permissive temperature these two alleles separate the DSB repair function of *ESA1* from its essential function.

To determine whether the DSB repair defect in *esa1-1851* is reflected in the level of H4 acetylation, we examined the amounts of acetylated protein in both mutants by western blotting. The *esa1-1851* CPT hypersensitive mutant shows a loss of H4 acetylation even at the permissive temperature, whereas the *esa1-L357H* CPT resist-

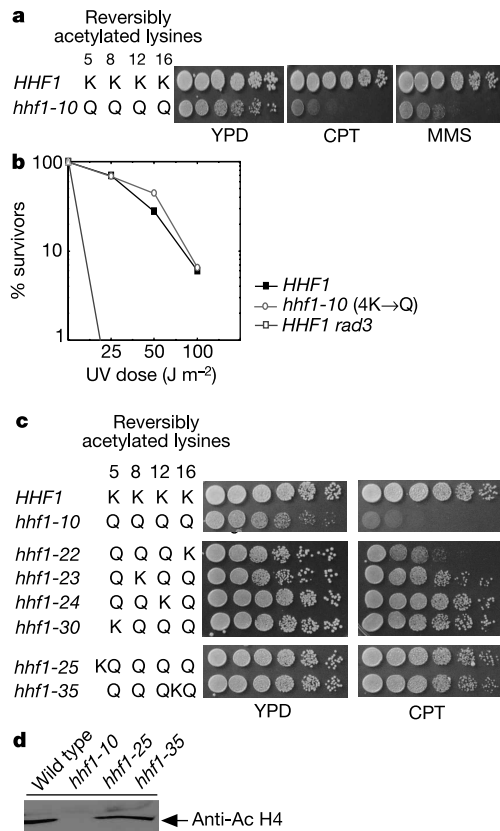


Figure 1 *hhf1-10* is hypersensitive to DSBs and addition of a single lysine to *hhf1-10* rescues CPT hypersensitivity. **a**, *HHF1* and *hhf1-10* strains were serially diluted on YPD, CPT (40 μg ml⁻¹) and MMS (0.030%) plates. **b**, *HHF1*, *rad3*, *hhf1-10* strains were irradiated using a Stratalinker ultraviolet (UV) source for various times and plated on YPD, incubated for 3 d and counted for surviving colonies. **c**, Strains were diluted on YPD and CPT (40 μg ml⁻¹) plates. **d**, The lysine inserted in *hhf1-25* and *hhf1-35* is acetylated *in vivo*. Cell extracts were resolved by SDS-PAGE and immunoblotted with antibody against acetylated H4 peptide.

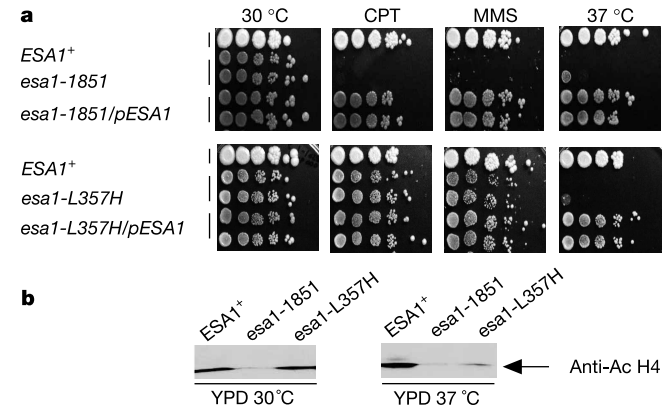


Figure 2 *ESA1* is required for H4 tail acetylation and for DSB repair. **a**, Histone H4 is hypoacetylated in *esa1* mutants. Cell extracts of *esa1* mutants grown at the permissive and non-permissive temperature were blotted with antibody against hyperacetylated histone H4. **b**, Conditional *esa1* alleles. The conditional *esa1-1851* allele gives rise to hypoacetylation of H4 at the permissive temperature and causes hypersensitivity to DSB-inducing agents. The *esa1-L357H* allele is equally sensitive to temperature, but shows no hypersensitivity to DSB agents or H4 hypoacetylation at the permissive temperature. *esa1-1851/pESA1* and *esa1-L357H/pESA1* are *esa1* mutants with a wild type plasmid re-introduced.

ant allele does not (Fig. 2b, left). At the nonpermissive temperature, H4 acetylation is diminished in both mutants, in agreement with reports that *Esa1* is responsible for most nucleosomal H4 acetylation *in vivo*¹³. Thus, the acetylation status of histone H4 at 30 °C correlates with cellular DSB repair capacity. These data also showed that *ESAI* is required both to acetylate the lysines in the H4 tail *in vivo* and for DSB repair. Similar to the *hhf1-10* mutant, the defect in the *esa1-1851* strain is very specific because we observed no marked hypersensitivity to ultraviolet damage (data not shown). Notably, the human homologue of *ESAI*, TIP60, also seems to be involved in DSB repair through an unknown mechanism¹⁴.

There are at least two pathways by which cells can repair DSBs: homologous recombination and non-homologous end joining (NHEJ). Much evidence indicated that homologous recombination was not defective in the *hhf1-10* mutant (Supplementary Information Table 1). To examine NHEJ, we used a standard plasmid recircularization assay^{15–17}. In this assay, the DNA around the DSB is located in the *kan^r* gene and is therefore not homologous with sequences on any of the yeast chromosomes. Thus, the DSB is repaired solely through NHEJ. We found that the *hhf1-10* mutant was highly defective in NHEJ, achieving only 20% of the repair of the wild-type strain (Supplementary Information Fig. 1).

Unexpectedly, mutants containing a single lysine at position 5, 8, 12 or 16 all showed a defect in NHEJ similar to that of the *hhf1-10* mutant (data not shown), which indicates that acetylation of more than one lysine is required for NHEJ. Consistent with this, adding a single ectopic lysine to the *hhf1-10* mutant tail did not suppress the defect in NHEJ (data not shown), despite the fact that a single lysine could restore the main DSB repair pathway (Fig. 1c). This showed that there are two distinct repair pathways that are defective in *hhf1-10*: an NHEJ pathway that requires acetylation of more than one of the H4 tail lysines but has a minor role in the cellular resistance to CPT or MMS; and a distinct pathway that requires acetylation of any one of the H4 tail lysines and is the principal determinant of DSB repair capacity. Only this second pathway was restored by adding a single ectopic lysine. Because this pathway was important in the repair of CPT-induced damage, which results in DSBs specifically at

the replication fork¹⁸, and not that caused by ionizing radiation, which results in random DSBs, we called this pathway the ‘replication-coupled pathway’.

The *ESAI* gene is the primary HAT required for acetylation of the lysines in H4 (Fig. 2b); therefore, it might be required for one or both DSB repair pathways. To address this issue, we carried out NHEJ assays on the *esa1* temperature-sensitive mutants grown at the permissive temperature. Both mutants were defective for NHEJ, consistent with the requirement of *ESAI* for H4 acetylation (Supplementary Information Fig. 1). One of the *esa1* temperature-sensitive mutants was markedly hypersensitive to CPT, which strongly suggests that it also has a defect in the replication-coupled pathway.

To understand further the replication-coupled pathway, we carried out a genetic screen to identify genes whose overexpression restored camptothecin resistance to *hhf1-10*. A class of suppressors, represented by seven independent clones, carried the gene for the histone tail-binding protein Arp4 (Supplementary Information Fig. 1). This protein binds the wild-type histone H4 tail sequence *in vitro*¹⁹, but not an H4 tail in which the four conserved lysines are changed to glutamines (that is, *hhf1-10*)²⁰. We propose that overexpression of Arp4 drives its association with the mutant *hhf1-10* tail, partially restoring the DSB repair pathway. Notably, Arp4 and *Esa1* are both members of the NuA4 HAT complex²⁰ and thus independent lines of investigation have implicated the NuA4 HAT complex in DNA repair. The replication-coupled DSB repair pathway involves at least two components of NuA4, the direct histone tail-binding protein Arp4, and the only catalytic subunit of the HAT complex, *Esa1*. These results suggest a model in which the Arp4-containing complex binds to chromatin at a replication-coupled DSB to facilitate DNA repair.

To test this model directly, we examined the recruitment of Arp4 to the site of a specific DSB by *in vivo* crosslinking and chromatin immunoprecipitation (CHIP). A single DSB was created on chromosome III by inducing expression of the site-specific homothallic (HO) endonuclease in strains expressing a Myc-tagged Arp4 protein. We amplified DNA isolated from the anti-Myc antibody immunoprecipitates by polymerase chain reaction (PCR) using primers adjacent to the HO break site (0.6 kilobases), or primers located on a different chromosome as a control (Fig. 3a). Hdf2 (Ku80), a protein required for efficient NHEJ, is recruited to an HO-induced DSB by CHIP and serves as a positive control in these experiments²¹. As expected, there was a specific increase in amounts of Hdf2 near the DSB site 90 min after inducing the endonuclease

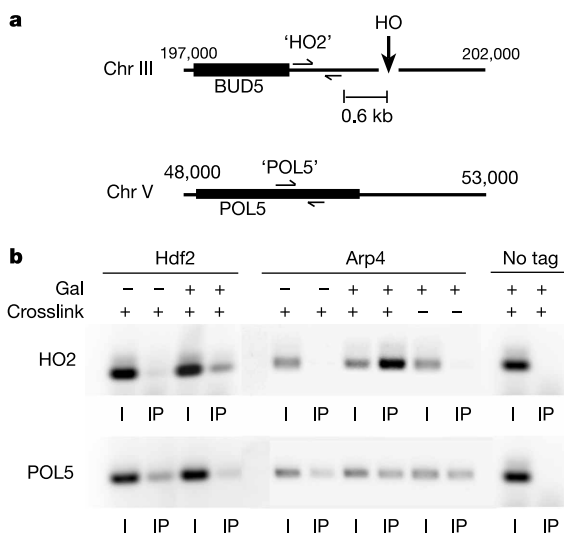


Figure 3 Arp4 is recruited to a DSB site *in vivo*. **a**, Location of the primers used in the chromatin immunoprecipitation (CHIP) assay relative to the HO cleavage site. **b**, CHIP experiments were carried out on strains containing Myc-tagged Arp4, Myc-tagged Hdf2, or no tag. Strains were grown in galactose for 45 or 90 min to induce expression of HO endonuclease. After induction, a substantial increase was observed in the localization of Arp4 and Hdf2 at DNA adjacent to the break site (HO2), but not to a distant site (POL5). I, input; IP, immunoprecipitate.

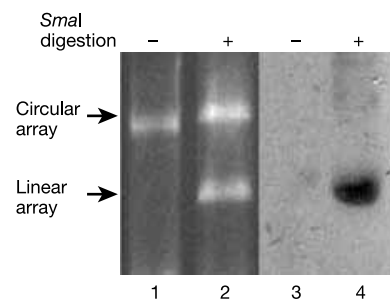


Figure 4 NuA4 preferentially acetylates linear nucleosomal arrays. Nucleosomes were reconstituted on covalently closed circular plasmid DNA and a portion was then linearized by digestion with *SmaI* endonuclease. Histone acetylation by NuA4 was assayed using either the circular nucleosomal template alone (*SmaI* –) or an equal mixture of circular and linear templates in a competition reaction (*SmaI* +). The reactions were separated by agarose gel electrophoresis and visualized with ethidium bromide for plasmid DNA (lanes 1 and 2) or by fluorography for histone acetylation (lanes 3 and 4). The locations of the circular and linear nucleosomal arrays are marked.

(Fig. 3b). There was also an increase in Arp4 crosslinking near the HO break site after endonuclease induction but no increase in crosslinking at the control site. A no-crosslinking control indicated that the Arp4 association with the break site occurs *in vivo* (Fig. 3b). These results show that within 90 min Arp4 is recruited specifically to a chromosomal locus in response to a DNA DSB and suggest that Arp4 and the associated HAT, Esa1, probably have a direct role in the repair process.

Because these results strongly implicated the NuA4 complex in the replication-coupled DSB repair pathway, we examined whether purified NuA4 complex might recognize a DSB in the context of chromatin *in vitro*. Nucleosomes were first reconstituted onto a covalently closed circular DNA template to form a nucleosomal array. A portion of this material was then linearized by digestion with *Sma*I, which created a DSB and a template with free chromatin ends. These circular and linear nucleosomal templates were then assayed in competition HAT reactions using purified NuA4 and [³H]acetyl-coenzyme A (CoA). These assays showed that purified NuA4 complex has a marked preference for nucleosomal histones in the linear array template and negligible activity on the circular array template (Fig. 4, lanes 2 and 4 contained equal amounts of circular and linear nucleosomal arrays present in the same reaction). A clear preference for the linear array could be seen in lane 4, as this form shows a much greater incorporation of [³H]acetate from [³H]acetyl-CoA than does the circular array. Thus, the NuA4 complex has an inherent ability to recognize a DSB in the context of chromatin. This is consistent with the observation that NuA4 can acetylate the interior portion of a linear array only if drawn there by an activator, but it can act on the ends without an activator²².

Our data indicate that the acetylation of histone H4 participates directly in one or more steps required to repair broken chromatin. First, Arp4, a histone tail-binding protein that is sensitive to mutations in H4 lysines, is specifically recruited to the site of a DSB *in vivo*. Second, NuA4, a HAT complex that acetylates histone H4 lysines, preferentially acetylates linear chromatin templates *in vitro*. Third, northern blot analysis of the expression of DNA damage repair genes, including *RAD50*, *MRE11*, *XRS2*, *DNL4*, *RNR1* and *RNR3*, shows that the amounts of their messenger RNAs are not altered in either *hhf1-10* or the *esal* mutant strains (data not shown). Last, comparison of the transcription profiles of *hhf1-10* and *hhf1-25* using whole-genome oligonucleotide microarrays (Affymetrix) shows that none of the genes involved in any DNA DSB repair pathway has substantially decreased expression (Supplementary Information Table 2).

The exact nature of the repair defect in the replication-coupled pathway remains to be determined. An intriguing possibility is the poorly understood role of the recombination-mediated restart of stalled replication forks, which is well documented in bacterial cells²³ but poorly understood in eukaryotes. The histone code hypothesis⁶ predicts that modifications such as acetylation and phosphorylation are likely to function as signals for recruiting specific repair complexes, rather than as simple modifiers of the charge on chromatin. The involvement of Arp4, which binds only to acetylated H4 tails²⁰, in the repair of DSBs supports the histone code hypothesis⁶. The code may be complex because H2A phosphorylation also seems to be involved in NHEJ²⁴. Our data show that histone acetylation is required for DNA repair in addition to its well-established role in transcription, and suggest that histone acetyltransferases are likely to function in other aspects of DNA metabolism such as recombination and replication. □

Methods

Drug sensitivity

To determine sensitivity to CPT and MMS, yeast strains were grown overnight, diluted to an absorbance at 600 nm (A_{600}) of 0.2–0.3 and grown to $A_{600} \approx 1.0$ before being plated on yeast peptone dextrose (YPD) medium at tenfold dilutions. We incubated all plates for 2–3 d at 30 °C.

NHEJ assays

Yeast strains were grown overnight, diluted to $A_{600} \approx 0.2$ – 0.3 and grown to $A_{600} \approx 0.6$ – 0.7 before being transformed with digested or undigested plasmid DNA as described¹⁷. Plasmid DNA was digested with *Hind*III for 3 h and extracted with phenol before transformation.

Western blots

We grew 10-ml cultures of the histone mutant strains to $A_{600} \approx 1.2$ – 1.6 and lysed them in HSB buffer (45 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA and 0.5% Nonidet P-40) plus 20 mM sodium butyrate by bead lysis homogenization. Total protein concentration of each of the samples was determined by the Bio-Rad protein assay and the amount of protein was normalized and loaded onto a 15% agarose gel. We carried out western blotting using an antibody against hyperacetylated histone H4 (a gift from D. Allis).

Chromatin immunoprecipitation

We carried out CHIP assays as described²¹. Fifty-microlitre cultures of Arp4-Myc, Hdf2-Myc and untagged strains were grown overnight to log phase, and expression of HO endonuclease was induced for 90 min. Cultures were then crosslinked for 60 min and extracts prepared for immunoprecipitation with antibodies against Myc. DNA isolated from the immunoprecipitates was used in PCR reactions containing the primers shown in Fig. 3a.

Detection of NuA4 targeting by fluorography

Fractions of NuA4 eluted from a Superose 6 column were purified and assayed for their ability to acetylate nucleosomal substrates as described²⁵. We carried out nucleosome reconstitution and HAT assays by a protocol modified from ref. 22. The plasmid pG5E4T (ref. 26), was used for reconstitution and NuA4 acetyltransferase assays. Circular plasmid DNA in 2 M NaCl was mixed with a 1:1 molar ratio of core histones purified from HeLa cells and reconstituted *in vitro* by step dilution as described²⁷. The efficiency of reconstitution was determined by comparing the electrophoretic mobility of the nucleosomal array with that of naked DNA separated on 1.2% agarose, 1 × tris acetate EDTA (TAE) gels for 240 V h⁻¹ and then stained with ethidium bromide. Reconstituted templates were linearized with *Sma*I for 1 h at 37 °C and then chilled on ice. To some reactions, we added an equal volume of uncut reconstituted template. Each reaction was incubated together with 1 μl of NuA4 complex and 0.25 μCi of [³H]acetyl-CoA (Amersham) and incubated for 15 min at 30 °C. The whole reaction was separated on a 1.2% agarose, 1 × TAE gel for 240 V h⁻¹. The gel was then fixed by agitation for 1 h in 10% acetic acid and 10% methanol, developed with EN3HANCE for 3 h (NEN Life Science Products), rinsed for 1 h in distilled deionized water, dried and exposed to film.

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Competing interests statement

The authors declare that they have no competing financial interests.

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