The N-terminal acetylation of Sir3 stabilizes its binding to the nucleosome core particle

Nadia Arnaudo1, Israel S Fernández1, Stephen H McLaughlin1, Sew Y Peak-Chew1, Daniela Rhodes1,2 & Fabrizio Martino1

1Structural Studies Division, Medical Research Council–Laboratory of Molecular Biology, Cambridge, UK. 2Present address: School of Biological Sciences, Nanyang Technological University, Singapore. Correspondence should be addressed to F.M. (fabrizio@mrclmb.cam.ac.uk).

Received 28 March; accepted 25 June; published online 11 August 2013; doi:10.1038/nsmb.2641

The N-terminal acetylation of Sir3 is essential for heterochromatin establishment and maintenance in yeast, but its mechanism of action is unknown. The crystal structure of the N-terminally acetylated BAH domain of Saccharomyces cerevisiae Sir3 bound to the nucleosome core particle reveals that the N-terminal acetylation stabilizes the interaction of Sir3 with the nucleosome. Additionally, we present a new method for the production of protein–nucleosome complexes for structural analysis.

Silent information regulator (SIR) proteins belong to a family of chromatin-regulatory factors, such as heterochromatin protein 1 and Polycomb, that are thought to function by folding the chromatin fiber into a highly compacted, transcriptionally silent structure called heterochromatin. Heterochromatin has a crucial role in chromosome segregation, stabilization of repeated DNA sequences and cell-type determination. Despite their functional importance, it is not under -stood at the structural level how heterochromatin proteins recognize the nucleosome core particle (NCP), the basic unit of chromatin, and how they fold the chromatin fiber into higher-order structures that cause transcriptional gene silencing.

In budding yeast, heterochromatin is present at the mating-type loci and at subtelomeric regions, where it is established and maintained by the Sir complex formed by Sir2, Sir3 and Sir4. Sir3 has a crucial role in binding the NCP through its N-terminal bromo-adjacent homology (BAH) domain and possibly its C-terminal ATPases associated with diverse cellular activities (AAA+) domain. Notably, Sir3 is acetylated on its N terminus, and lack of this modification impairs the spreading of Sir3, and thus of silencing, along the chromatin fiber. The molecular mechanism behind the function of Sir3 N-terminal acetylation is unknown. Previous crystal structures of the BAH domain in complex with the NCP could not answer this question because they contained unacylated BAH. On a more general note, because >80% of eukaryotic proteins are N-terminally acetylated, it is important to understand how this post-translational modification affects protein structure and function.

To investigate whether the N-terminal acetylation of Sir3 regulates its binding to the NCP, we expressed a long version of the BAH spanning the first 380 amino acids of Sir3. This is the N-terminal fragment of Sir3 that best complements the silencing defect of a sir3Δ strain. We expressed the BAH both in insect cells in which Met1 was removed and in bacteria in which Met1 was cleaved but Ala2 not acetylated. MALDI analysis and SDS-PAGE confirmed that the two proteins are indistinguishable except for the N-terminal acetylation present only in the protein expressed in insect cells (Supplementary Fig. 1). Quantitative electrophoretic mobility shift assays (EMSAs) revealed that the N-terminal acetylation increases the affinity of the BAH for the NCP by at least a factor of 2 (Fig. 1a,b). To understand how N-terminal acetylation affects the chromatin binding properties of Sir3, we determined the crystal structure of the N-terminally acetylated BAH bound to the NCP. Because conventional matrix-based purification methods often cause sample heterogeneity and losses, we developed a new matrix-free method. In this method, the BAH–NCP complexes were precipitated with polyethylene glycol (PEG) and then resuspended in a buffer at a concentration suitable for crystallization trials with a final recovery of 90% of the initial complex. The purified complexes were highly homogeneous, as revealed by the presence of only one well-defined band in native gels (Supplementary Fig. 2a, lane 3). Notably, we isolated only BAH–NCP complexes and not free components, because no free NCPs or free BAH precipitates at the PEG concentration used (Supplementary Fig. 2a, lane 4, and Supplementary Fig. 2b, lane 2).

We analyzed the subunit composition of the purified BAH–NCP complex against known amounts of the NCP and the BAH. From this analysis, we estimated that the complex has a stoichiometry of two molecules of BAH per NCP (Supplementary Fig. 2c).

**Figure 1** Sir3 N-terminal acetylation increases Sir3 BAH affinity for NCPs. (a) Native-PAGE of fluorescently labeled NCPs incubated with increasing concentrations of unacetylated (black, BAH) or N-terminally acetylated (red, Nt-Ac BAH) BAH. Asterisks indicate the titration point with the largest difference in substrate saturation between acetylated and unacylated BAH. (b) Quantification of Sir3 BAH binding to NCPs from a. The mean value (± s.d.) of the percentage of unbound NCPs from three independent experiments is plotted against BAH concentration. The concentration of BAH required for 50% binding to the NCP is indicated by a dashed line.
We solved the structure of the N-terminally acetylated BAH in complex with the NCP at 3.3-Å resolution (Supplementary Table 1) and superimposed it with the structure of unacetylated BAH bound to NCP (Fig. 2a,b). In addition to the multiple interactions involved in the recognition of the NCP by the BAH reported previously, we observe a set of additional interactions arising from the acetylated N terminus of Sir3 (Fig. 2c–e). As a result, loop 3 and helix 8 of Sir3 BAH are positioned closer to the histone core surface (Fig. 2b,c,e). In particular, the side chain of Asn80, located in loop 3, is within hydrogen-bonding distance of the side chains of histone H2B Arg93 and histone H4 Glu74 (Fig. 2c and Supplementary Fig. 3b,c). Asn80 is part of a tight network of interactions in which the position of the H2B Arg93 side chain is stabilized by H4 His75, whereas the H4 Glu74 side chain is held in place by a potential hydrogen bond with H4 Arg67 (Fig. 2c and Supplementary Fig. 3d). Loop 3 forms a pocket-like structure that holds histone H3 Lys79 in place as a lock holds a key (Fig. 2c). The presence of several acidic amino acids in loop 3 suggests that the side chain of H3 Lys79 can assume different orientations in the pocket, thus providing an explanation for the poor electron density observed for H3 Lys79. Well-defined experimental densities could be assigned to the other residues forming the pocket (Supplementary Fig. 3b–d).

Notably, the acetylated N terminus interacts with loop 3 and stabilizes its interactions with the core histones. In particular, Ala2 interacts with Val82 and Val83 and pushes loop 3 against the histone core (Fig. 2c). The position of Ala2 is determined by the interaction of the N-terminal acetyl group with the main chain amine of Leu143 and the aromatic ring of Trp142 (Fig. 2d and Supplementary Fig. 3a). The aromatic ring of Trp142 is rotated toward the methyl moiety of the acetyl group in a different conformation compared to that of the unacetylated BAH (Fig. 2d).

The interaction between the N-terminal acetyl group and loop 3 appears to generate a rotation of the whole BAH toward the surface of the NCP. As a consequence, helix 8 is positioned closer to the core histones as compared to its position in the unacetylated BAH (Fig. 2a,b). Helix 8 is crucial for silencing, and it has been proposed to bind nucleosomal DNA. In our structure, helix 8 is not facing DNA but histones H3 and H4 (Fig. 2a,b,c,e). In particular, BAH Lys202 is within hydrogen-bonding distance to H4 Glu63, whereas BAH Lys209 probably interacts with H4 Asp24 and H3 Glu73 (Fig. 2c,e). Notably, these interactions were not described for the previous unacetylated BAH–NCP structure because helix 8 was located too far from the histone core surface (Fig. 2c).

The new N-terminal acetylation-dependent interactions between the BAH and the NCP we observe correlate very well with in vivo data. Indeed, K209N and K202E in helix 8 have been identified as dominant negative mutations. Moreover, mutations of BAH Asn80, of H3 Lys79 and of the amino acids around H3 Lys79 cause severe silencing defects. Furthermore, H3 Lys79 is methylated in transcribed regions, and there is evidence that methylated H3 Lys79 prevents Sir3 mislocalization in vivo and also reduces Sir3 binding in vitro. Our structure suggests that methylation of H3K79 would create repulsion with the residues in the BAH loop 3, thus leading to the disruption of the BAH–NCP complex.

Our structure provides an interpretation for the effects of mutations at Ala2 that affect Sir3 function. It is notable that in all previous structures containing unacetylated BAH, the N terminus of Sir3 (from Ala2 to Gly10) was disordered. Instead, in our acetylated BAH–NCP complex, we observe a short helix (Asp9 to Thr4) that probably is necessary to ensure that the acetylated N terminus is in the correct position to interact with Sir3 loop 3 (Fig. 2d and Supplementary Fig. 3a). The short N-terminal helix is a crucial structural component of the N terminus because mutations in this helix cause a silencing phenotype. Yeast strains carrying the A2Q mutation exhibit a strong silencing defect at telomeres and HML that is consistent with the loss of Sir3 spreading. According to our structure, A2Q would be displaced because it is unacetylated, and the side chain of the unprocessed Met1 would clash with Trp142. Finally, the long side chain of glutamine would clash with the short N-terminal helix (Fig. 2d). Similarly, amino acids with a long side chain placed in position 2 would clash against the BAH N-terminal helix. Consistent with this interpretation, Ala2 mutations to lysine, glutamate or aspartate present strong phenotypes.

In summary, our work provides a biochemical and structural explanation for the importance of Sir3 N-terminal acetylation in NCP recognition and in the establishment of gene silencing. We propose that the N-terminal acetylation stabilizes the interaction between Sir3 and the NCP by positioning Sir3 loop 3 and helix 8 within hydrogen-bonding distance to the histone core surface. This proposal is substantiated by the excellent correlation between genetic and structural data, hence affirming the biological relevance of the interactions.
described. On a more general note, our structure suggests that
the common N-terminal acetylation of proteins has a key role in the
stabilization of protein-protein interactions.

METHODS
Methods and any associated references are available in the online
version of the paper.

Accession codes. Coordinates and structure factors have been
deposited in the Protein Data Bank under accession code 4LD9.

Note: Any Supplementary Information and Source Data files are available in the online
version of the paper.

ACKNOWLEDGMENTS
We thank G. Murshudov, F. Long, R. Nicholls, P. Emsley and H. Powell for
troubleshooting and sharing tools for the software Refmac5, LbG, Prosmart, Coot and iMosflm. We thank M. Lamers and K. Nagai’s laboratory for critical
discussion of the results. We thank M. Yu for helping with the usage of the I24 beamlines at Diamond Light Source. F.M. is supported by the Swiss National
Fund (PBGEP3-123695), a European Molecular Biology Organization Long Term Fellowship (ALTF419-2009) and a Marie Curie Intra European Long Term Fellowship (FP7-PEOPLE-2009-IEF-251794). N.A. is supported by the EU FP7
Marie Curie Initial training Nucleosome 4D network (609511-238176). The project was supported by the UK Medical Research Council (MC-A025-5PJ80).

AUTHOR CONTRIBUTIONS
D.R., F.M. and N.A. designed the experiments. F.M. and N.A. purified all the
proteins, DNA and complexes used, prepared, optimized and cryoprotected the
crystals, solved the structure and wrote the manuscript. I.S.F. froze the crystals.
F.M., N.A. and I.S.F. collected the X-ray diffraction data. N.A. and S.H.M.
performed the binding experiments. S.Y.P.-C. performed the MALDI. D.R. revised
the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/
reprints/index.html.

1. Bühler, M. & Gasser, S.M. EMBO J. 28, 2149–2161 (2009).
2. Buchberger, J.R. et al. Mol. Cell. Biol. 28, 6903–6918 (2008).
3. Cubizolles, F., Martino, F., Perrod, S. & Gasser, S.M. Mol. Cell 21, 825–836
(2006).
4. Ehrentraut, S. et al. Genes Dev. 25, 1835–1846 (2011).
5. Onishi, M., Liu, G.G., Buchberger, J.R., Walz, T. & Moazed, D. Mol. Cell 28,
1015–1028 (2007).
6. Wang, X., Connelly, J.J., Wang, C.L. & Sternglanz, R. Genetics 168, 547–551
(2004).
7. Ruault, M., De Meyer, A., Loiodice, I. & Taddei, A. J. Cell Biol. 192, 417–431
(2011).
8. Sampath, V. et al. Mol. Cell. Biol. 29, 2532–2545 (2009).
9. van Welsem, T. et al. Mol. Cell. Biol. 28, 3861–3872 (2008).
10. Armache, K.J., Garlick, J.D., Canzio, D., Narlikar, G.J. & Kingston, R.E. Science
334, 977–982 (2011).
11. Wang, F. et al. Proc. Natl. Acad. Sci. USA 110, 8495–8500 (2013).
12. Arnesen, T. PLoS Biol. 9, e1000174 (2011).
13. Connelly, J.J. et al. Mol. Cell. Biol. 26, 3256–3265 (2006).
14. Park, J.H., Cosgrove, M.S., Youngman, E., Wolberger, C. & Boeke, J.D. Nat. Genet.
32, 273–279 (2002).
15. Martino, F. et al. Mol. Cell 33, 323–334 (2009).
ONLINE METHODS

Preparation and crystallization of BAH–NCP complexes. Sir3 BAH domain (1–380 or 1–229) was cloned in pOPIN-E vector (Oxford Protein Expression Facility) with a C-terminal His6 tag. The proteins were expressed in BL21(DE3) pLYS S E. coli at 20 °C overnight or in the SF21 insect cell line for 3 d after infection. Sir3 BAH was purified by chromatography on Ni-NTA resin followed by heparin, S/Q and Superdex 2000/60. The proteins were concentrated to 30 mg/ml in 300 mM NaCl, 50 mM Tris, pH 8, 1 mM EDTA and 1 mM TCEP and flash frozen in liquid nitrogen. Recombinant Xenopus core histones were expressed, purified and reconstituted with the Widom 601 DNA sequence into NCP by a previously described protocol16. The N-terminally acetylated Sir3 BAH domain and Cy5-labeled NCP was assembled by mixing of the BAH domain with NCP at 2 μM in a 2:1 molar ratio. The complex was then purified by differential precipitation with PEG 6000 (Hampton). BAH–NCP crystals were obtained from BAH fragments of different length, but only one, from amino acid 2 to 229, produced crystals that diffracted at resolution higher than 4 Å. Crystals of the complex were grown by vapor diffusion at 20 °C in MRC-MAXI plates by mixing of 0.5 μl of the complex (at 12 μM) with 0.5 μl of 50 mM MES, pH 6.5, 12% PEG 400, 12 mM MnCl2, 100 mM NaCl and 10 mM EDTA. After six weeks, crystals were soaked gradually in increasing concentrations of PEG 400 (up to 27%). The soaked crystals were then flash frozen in liquid nitrogen for data collection.

Differential PEG precipitation. The BAH–NCP complexes were incubated with 5% PEG 6000 for 5 min, and centrifugation was carried out at 10,000 g for 10 min. The pellet was resuspended in 20 mM TEA, pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM TCEP at a final concentration of 12 μM, and analyzed on both native polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE to assess, respectively, the purity and the composition of the sample.

MALDI-ISD analyses. Sir3 BAH expressed in baculovirus and E. coli were dia-lyzed in 20 mM ammonium bicarbonate. One microliter of proteins followed by 1 μl of 2,5-DHB (2,5-dihydroxybenzoic acid, saturated solution in 10% acetonitrile and 0.1% trifluoroacetic acid) matrix solution were deposited and mixed on the MALDI target. All MALDI-MS experiments were carried out on an Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics). In-source decay (ISD) analyses were acquired in positive reflectron mode, ISD spectra were externally calibrated with a standard-peptide mixture and consisted of [M+H]+ 904.4861, 1,296.6848, 1,645.8962, 2,093.0862, 2,932.5879 and 3,657.9294. FlexAnalysis 3.0 and Biotools 3.2 (Bruker Daltonics) were used for analysis and fragments assignment. Fragments were assigned with mass accuracies of <0.5 Da. The MALDI-ISD experiment confirmed the presence of N-terminal acetylation only in Sir3 BAH expressed with the baculovirus system. Indeed, in Sir3 BAH expressed in bacteria, the Met1 was removed, but Ala2 was not acetylated.

Electrophoretic mobility shift assays. Cy5-labeled NCPs (0.2 μM) were incubated with increasing concentrations of unacetylated (expressed in E. coli) or acetylated (expressed in baculovirus) Sir3 BAH domain in 10 mM TEA, pH 7.4, 1 mM EDTA, 50 mM NaCl and 1 mM TCEP. Samples were then analyzed on 5% native PAGE run in 0.2× TB buffer, and fluorescence was detected with the Typhoon system (GE Healthcare Life Sciences).

The affinity of Sir3 BAH for NCP was determined by measurement of the disappearance of the band corresponding to the NCP substrate. The binding analysis was repeated in triplicate and the measurements plotted with the corresponding errors. The binding curve shows that 50% of substrate saturation is reached at 0.4 μM of acetylated BAH compared to 0.8 μM of unacyetylated BAH.

X-ray data collection, processing, model building and refinement. X-ray diffraction data were collected at the I24 beamline equipped with a Pilatus3-6M detector, at the Diamond Light Source, Didcot, UK, and processed with XDS17. The structure was solved by molecular replacement using PHASER from the CCP4 suite18 and a search model containing two rigid bodies: (i) the Sir3 BAH (PDB 2FL7 (ref. 19)) deleted for both the N terminus (aa 1–11) and loop 3 (aa 75–83) and (ii) the NCP containing the Widom 601 DNA sequence (PDB 3LZ0 (ref. 20)). The model was refined with Phenix21, and additional model building was done with Coot. In parallel, the data set was refined with Refmac5, with restrains on α-helices and DNA base-pairing from the PROSMART and LIBG tools22. Both Phenix and Refmac5 gave a similar solution with equivalent statistics. All molecular graphics were prepared with PyMOL (http://www.pymol.org/).

16. Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F. & Richmond, T.J. Nature 389, 251–260 (1997).
17. Kabsch, W. Acta Crystallogr. D Biol. Crystallogr. 66, 133–144 (2010).
18. Winn, M.D. et al. Acta Crystallogr. D Biol. Crystallogr. 67, 235–242 (2011).
19. Hou, Z., Darzer, J.R., Fox, C.A. & Keck, J.L. Protein Sci. 15, 1182–1186 (2006).
20. Vasudevan, D., Chua, E.Y. & Davey, C.A. J. Mol. Biol. 403, 1–10 (2010).
21. Afonine, P.V. et al. Acta Crystallogr. D Biol. Crystallogr. 68, 352–367 (2012).
22. Nicholls, R.A., Long, F. & Murshudov, G.N. Acta Crystallogr. D Biol. Crystallogr. 68, 404–417 (2012).