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A β -hairpin comprising the nuclear localization sequence sustains the self-associated states of Nucleosome Assembly Protein 1

Young-Jun Park, Steven J. McBryant, and Karolin Luger*

Howard Hughes Medical Institute And Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO 80523-1870

Summary

The histone chaperone Nucleosome Assembly Protein 1 (NAP1) is implicated in histone shuttling, nucleosome assembly and disassembly. Under physiological conditions, NAP1 dimers exist in a mixture of various high-molecular weight oligomers whose size may be regulated by the cell-cycle dependent concentration of NAP1. The functional and structural significance of the observed oligomers are unknown. We have resolved the molecular mechanism by which yeast NAP1 dimers oligomerize by applying x-ray crystallographic, hydrodynamic, and functional approaches. We find that an extended β hairpin that protrudes from the compact core of the yNAP1 dimer forms a stable β sheet with β hairpins of neighboring yNAP1 dimers. Disruption of the β hairpin (whose sequence is conserved among NAP1 proteins in various species) by the replacement of one or more amino acids with proline results in the complete loss of yNAP1 dimer oligomerization. The *in vitro* functions of yNAP1 remain unaffected by the mutations. We have thus identified a conserved structural feature of NAP1 whose function, in addition to presenting the nuclear localization sequence, appears to be the formation of higher-order oligomers.

Introduction

The nucleosome core particle (NCP) is the universally repeating unit in chromatin, consisting of an approximately equal mass of protein and DNA in a complex macromolecular assembly of 210 kDa¹. Nucleosome assembly is a stepwise process that starts with the association of histones H3 and H4 with the DNA, followed by the incorporation of H2A-H2B dimers to form the nucleosome². This process is orchestrated by a set of diverse chromatin assembly factors and chaperones^{3,4,5}.

Nucleosome assembly protein 1 (NAP-1, or NAP1) is a histone chaperone that binds H2A-H2B⁶ and linker histones⁷ (reviewed in⁸). Chromatin assembly activity has been demonstrated *in vitro*^{9,6,10}. Since H2A-H2B dimer deposition is likely reversible *in vivo*, NAP1 has been implicated in promoting chromatin fluidity through the removal of H2A-H2B dimers^{11,12,13,14,15}. We have shown previously that yNAP1 promotes nucleosome sliding through histone H2A-H2B dimer exchange, which under certain conditions results in histone variant incorporation into existing nucleosomes¹¹. NAP1 is a member of a growing family of related proteins whose members are implicated in transcription regulation, cell cycle regulation, apoptosis, histone modification, chromatin assembly, and histone shuttling⁸.

*Corresponding author: phone: 970-491-6405, Fax: 970-491-0494, email: kluger@lamar.colostate.edu.

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We have previously determined the crystal structure of yNAP1, and have demonstrated that the protein is an obligate dimer with an extensive dimerization interface¹⁶. Studies of NAP1 or NAP1/histone complexes revealed that NAP1 exists in multiple states of oligomers of varying size^{9,6,17,18,19}. Using sedimentation velocity experiments, oligomerization was shown to depend on the protein concentration and on the ionic strength of the buffer¹⁷. Similar experiments suggested that yeast NAP1 exists in a dimer-octamer equilibrium²⁰. Based on sedimentation data, a hexadecameric ring-shaped assembly was proposed for the largest observed yNAP1 complex, similar to the assemblies formed by the unrelated chaperone nucleoplasmin²¹. Since formation of higher order assemblies is concentration dependent, and since the nuclear concentration of NAP1 fluctuates in a cell-cycle dependent manner, it has been suggested that the oligomerization of yNAP1 dimers could exist at different times of the cell cycle^{22,20}.

Due to the strong propensity of yNAP1 dimers to form oligomers under physiological conditions, it is unknown whether dimeric and oligomeric forms of yNAP1 exhibit different functions. Furthermore, the molecular details of the interactions responsible for the oligomerization of yNAP1 dimers are unknown. Here we investigate yNAP1 oligomerization using x-ray crystallography, gel filtration, analytical ultracentrifugation as well as functional assays. We have identified an extended β -hairpin which encompasses the nuclear localization sequence as the site of intermolecular interactions through the formation of an extended β sheet, and have evidence that yNAP1 assemblies become more elongated with increasing size. We have disrupted the β -hairpin using site-directed mutagenesis, and find that this region is indeed responsible for the formation of oligomers, while it has no apparent effect on the various functions attributed to yNAP-1 *in vitro*.

Results

The structure of yNAP1_(74–365) reveals a highly defined β hairpin

The central domain of yeast NAP1 (residues 74 to 365; yNAP1_(74–365)) retains native-like activity in histone binding and nucleosome assembly^{9,23}. In contrast to wild type yNAP1 which crystallized at pH 4.85, crystals of NAP1_(74–365) were obtained at neutral pH in a tetragonal space group (P4₂2₁2). The refined structure of this truncated form of yNAP1 is for the most part identical to the previously published structure of full length yNAP1 (Figure 1A). The two structures superimpose with an rmsd of ~ 1 Å. Like full length yNAP1, yNAP1_(74–365) forms a stable homodimer. However, while the two monomers are related by crystallographic symmetry in full length yNAP1, the asymmetric unit for yNAP1_(74–365) encompasses the two monomers of the physiological dimer. The two independently built and refined monomers are identical and superimpose with a least square difference of 0.79 Å.

The only striking difference between the two structures is found in $\beta 5$ and $\beta 6$ (Fig. 1A). This region was structurally ill-defined and partially disordered in the previously published structure¹⁶. In yNAP1_(74–365) it forms a β -hairpin that extends over 11 amino acids for each β strand and is stabilized by nine hydrogen bonds (Figure 1B). Amino acids 295–298 form a typical β -turn. The entire hairpin is exceedingly well ordered, as signified by the highly defined electron density (Fig. 1B) and B-factors that are approximately 20 Å² lower than the average for the entire molecule. The β hairpin protrudes by a distance of 3.7 nm from either side of the compact core of the NAP1 dimer.

The high definition of this region in the present structure can be attributed to its strong contributions to crystal contacts (Figure 2A). A total of eight β -strands are arranged to form a consecutive antiparallel β -sheet that links four yNAP1 dimers. The β -hairpin interacts with the β -hairpin of a neighboring yNAP1 dimer through an extensive hydrogen bonding network over its entire length (Figure 2B). One additional yNAP1 dimer docks on either side of this dimer

of dimers. This interaction is slightly weaker due to a larger twist of the β -sheet imposed by lattice constraints. The distance of the adjacent β -strand backbones as well as the distances between facing oxygen and hydrogen atoms are within the canonical range.

The β -hairpin is implicated in the formation of yNAP1 oligomers

It is well established that interactions between exposed edges of β -sheets is an important mode of protein-protein interaction with potentially pathological consequences (for example, ²⁴). To test whether the β -hairpin of yNAP1 is responsible for oligomerization, single point mutations were designed to disrupt the β hairpin (Figure S1A). Arg-290, Arg-301, Thr-302 and Lys-305 were mutated to proline, an amino acid that has a low propensity to form β -sheets and that is generally occluded from naturally occurring β -strands ^{25,26}. A mutant containing all four point mutations (R290P, R301P, T302P, and K305P) was also prepared. All mutations were generated in the full length protein, with the exception of K305P, which was also mutated in yNAP1_(74–365). All mutants were expressed in soluble form, and were purified under identical conditions compared to their wild type counterparts (Figure S1B).

The oligomerization state of wild type and mutant NAP1 proteins was investigated by size exclusion chromatography. Since we have previously demonstrated yNAP1 oligomerization below 500 mM NaCl ¹⁷, and since the original crystals of yNAP1 were grown at a pH of 4.8 ¹⁶, we examined the elution profile of wild type yNAP1 (at a concentration of ~50 μ M) under three sets of conditions that varied in ionic strength and pH (Figure 3). At physiological pH and low salt, wild type NAP1(1–417) eluted as a broad peak at an apparent size that is indicative of a heterogeneous mixture of various oligomeric assemblies (Fig. 3A), as reported ¹⁷. At high salt (Fig. 3B), or under acidic buffer conditions (Fig. 3C), the protein eluted from the column at an apparent molecular weight of ~95 kDa, consistent with the dimeric form of a 417 amino acid protein. This finding suggests that oligomerization of NAP1 is sensitive to low pH, explaining our ability to crystallize a protein that under physiological conditions is present in various states of oligomerization.

In striking contrast, all mutants eluted as well-behaved apparent dimers under physiological conditions (Fig. 3D), indicating that disruption of the β -sheet by the substitution of a single residue with proline completely abolishes the propensity of yNAP1 to form oligomers. The minor differences in elution volumes between the different mutants may be explained by the degree of disruption of the β -strands. Since they extend prominently from the compact core of the protein (Fig. 1), the presence of residual β strands in some mutants, but not in others, could have a distinct effect on the hydrodynamic radius of the yNAP1 dimer.

The self-association states of wild type and mutant yNAP1 were further analyzed by analytical ultracentrifugation. Sedimentation velocity experiments were performed at ~3 μ M protein concentration under all solution conditions. This concentration is ~12-fold lower than that used for gel filtration. Boundaries were analyzed by the $G(s)$ method to obtain an integral distribution of sedimentation coefficients that are corrected for diffusion, and are accurate for even highly heterogeneous samples ²⁷. A vertical $G(s)$ plot is indicative of a homogeneous sample while a heterogeneous sample yields a plot with one or more regions of positive slope. For a protein that is >95% pure (as are the samples under investigation here), a significant slope in the $G(s)$ plot is diagnostic for the presence of protein oligomers, and the shape of the plot provides insight into the oligomerization process.

Analysis of full-length wild type NAP1 under moderate salt concentration (100mM NaCl) shows the characteristic $G(s)$ plot of a self-associating system, with solutes exhibiting sedimentation coefficients from ~5–8S (Fig. 4A). This result is in good agreement with a previous sedimentation velocity analysis of NAP1 ²⁰, and allows observation of the stable ~4.5S dimers and the larger oligomers. The 4.5S NAP1 homodimer (98,458 Daltons) exhibits

a frictional ratio (f/f_0) of 1.8, consistent with the asymmetric nature of the dimer¹⁶. In contrast, the $G(s)$ distributions of all mutants tested [NAP1(R290P), NAP1(K305P), and NAP1(R290, R301P, T302P, K305P)], sedimented as a single, homogeneous ~4.5 S species under all conditions tested (Fig. 4A). These distributions are nearly identical to the distribution of wild type NAP1 under high salt conditions (500mM NaCl; Figure 4B, and ¹⁷). This suggests that disruption of $\beta 5$ and $\beta 6$ does not affect the overall structure of the yNAP1 dimer. The $G(s)$ distributions of NAP1 mutants were unchanged by high salt.

To investigate the possibility that amino acids 1–70 and 370–417 (which are disordered in both structures) contribute to oligomerization, we analyzed the sedimentation behavior of yNAP1_(74–365) and found no differences compared to wild type under any condition tested (Fig. 4C, compare with 4A). Thus, neither the N- or C-terminus contributes to yNAP1 oligomerization under these conditions. As expected, the sedimentation coefficient distribution for the proline mutant (K305P) in the context of yNAP1_(74–365) shows a nearly vertical plot with a single, ~4.5 S component. However, f/f_0 is changed from 1.8 for wild type to 1.46 in the mutant (not shown), consistent with loss of frictional drag due to the loss of N- and C-termini. Together, these results show that disruption of the formation of higher order oligomers requires mutation of only one of the residues that contribute to the formation of $\beta 5$ and $\beta 6$ of NAP1. Further, it demonstrates that neither the N or C termini of NAP1 contribute significantly to the formation of the higher-order oligomers.

The β -hairpin is not required for yNAP1 activity *in vitro*

Most *in vitro* assays of yNAP1 activity were done under conditions where yNAP1 dimers form large macromolecular assemblages, and thus it is unknown whether the various activities of yNAP1 require oligomerization beyond the dimeric state. We tested the ability of all yNAP1 mutants to form histone H2A-H2B/NAP1 complexes, to assemble mono-nucleosomes *in vitro*, and to dissociate H2A-H2B dimers from assembled nucleosomes. Histone binding was analyzed by GST pulldown assays under increasingly stringent conditions (Fig. 5A). No differences were observed between wild type and mutant proteins, in that all complexes appeared to be stable up to the same ionic strength. GST alone did not interact with histones except for at the lowest ionic strengths (100 and 150 mM; Fig. 5A, lowest panel). To assess the nucleosome assembly activity of NAP1 mutants, histone octamer, DNA, and wild type or mutant NAP1 were mixed, and the assembled nucleosomes were resolved by native PAGE followed by SYBR-Gold staining. The NAP1 mutants showed only minor (if any) defects in this activity (Fig. 5B). The higher migrating bands are routinely observed with salt-dependent as well as chaperone-mediated assembly, and probably represent aggregated/misfolded nucleosomes. Finally, the ability of wild type and mutant yNAP1 to remove H2A-H2B dimers from the nucleosome was analyzed by monitoring the appearance of a (H3-H4)₂ – DNA band (in addition to a nucleosomal band) in a native gel (“S3” in Fig. 5C, lanes 2–4), with a concomitant appearance of a yNAP1 – H2A-H2B dimer band (“GST-S2” in Fig. 5C, lanes 10–12), as described earlier¹¹. These latter experiments were also performed with GST-yNAP1 to achieve better separation between the nucleosome and the yNAP1 – H2A-H2B dimer complex. Together, these data indicate that the β hairpin is not involved in the major yNAP1 *in vitro* functions identified previously¹⁶.

Discussion

We have shown that the large macromolecular assemblies previously observed for NAP1 under physiological conditions^{28 17 20 29} are the result of intermolecular interactions involving an extended β -hairpin that encompasses the nuclear localization sequence. The structural integrity of the β -hairpin is not required for histone binding, nucleosome assembly, and nucleosome disassembly *in vitro*, even though all 24 amino acids are conserved among different species.

We have thus identified a conserved structural feature of NAP1 whose function, in addition to presenting the nuclear localization sequence, appears to be the formation of higher-order oligomers, with as yet undetermined implications for its *in vivo* function.

The intermolecular antiparallel β -sheet interactions observed in the crystal lattice resemble those seen in amyloid fiber structures^{30,31}. An extensive network of hydrogen bonds maintains the intermolecular contacts between individual NAP1 dimers. About 510 Å² of surface area is buried between two β -hairpins of adjacent yNAP1 dimers in the crystal lattice, an area that is much smaller than the area buried by the interaction of two α 2 helices to form the yNAP1 dimer¹⁶. This is consistent with the finding that the interface formed between yNAP1 dimers is destabilized at moderate levels of chaotropic agents without a measurable loss of secondary structure, while complete unfolding is required to disrupt the yNAP1 dimer interface¹⁷. The disordered N and C terminal regions, comprising about 30% of the total mass of yNAP1, have no effect on the dimer-oligomer equilibrium, consistent with a recent report for *Xenopus laevis* NAP1²⁹.

In yeast, NAP1 is a mostly cytoplasmic protein³². It participates in the active transport of histones from the cytoplasm to the nucleus by forming a ternary complex with H2A-H2B dimers and a member of the karyopherin family of proteins (Kap114). Kap114 recognizes the nuclear localization sequence (NLS) of histones and yNAP1, presumably in a non-canonical manner³². The NLS has been located to the equivalent of β 5 of yNAP1 β hairpin in a variety of species^{33,34,32,35}. Although the interaction interface between yNAP1 and histones remains to be characterized, we have evidence that the β hairpin does not contribute to the histone interaction (A.J. Andrews and K. Luger, unpublished results). This is consistent with the earlier result that yNAP1 persists as a ternary complex with histones and the NLS-interacting protein Kap114 in the nucleus³⁴.

We hypothesize that the nuclear localization of yNAP1 may be regulated by altering the accessibility of the nuclear export sequence (as suggested earlier,¹⁶) and NLS. Our structural studies suggest that the oligomeric version of yNAP1 is incapable of interacting with karyopherins, whereas the NLS is highly accessible in the dimeric form of yNAP1. The nuclear localization of NAP1 is regulated through cell-cycle specific phosphorylation of residues located in the immediate vicinity of the NES and NLS in *Drosophila* and presumably also in other species³⁶. Although the sites of phosphorylation have not yet been identified in yeast, it is possible that phosphorylation in and around the β hairpin has the potential to disrupt oligomerization, thus regulating interaction with karyopherins and nuclear transport.

Materials and Methods

Protein expression, purification and activity

Wild type and mutant yeast NAP-1 were expressed and purified as described before²³. For the histone exchange assay, NCPs were reconstituted with CPM labeled DNA or H2B T112C. Histone binding, nucleosome assembly, and histone exchange assays were performed as described^{23,11}. GST-NAP1 pulldown assays were performed as follows: 0.25 nmoles of GST-tagged wild type or mutant NAP1 was immobilized on 50 μ l of Glutathione Sepharose 4B resin. The resin was then mixed with 100 pmoles of refolded H2A-H2B dimer and incubated for 3 hours. Unbound H2A-H2B was removed by washing three times with HEPES buffer (20mM HEPES, 0.5 mM EDTA, 10% glycerol, 0.05% Nonidet p-40, 5 μ M ZnSO₄, 2.5 mM MgCl₂, and 100mM to 450 mM KCl). GST alone was used as a control to test the non-specific binding of H2A-H2B. The results were analyzed by 15% SDS PAGE and stained with coomassie brilliant blue.

Nucleosome assembly was assayed by incubating 2.5 μM of NAP1 (wild type or mutant, respectively) with recombinant *Xenopus laevis* histone octamer (0.1–0.4 μM) and 0.1 μM of a 146 bp DNA fragment derived from the 5S rRNA gene. The reaction (in 10 mM HEPES at pH 7.5, 100 mM NaCl, 5 mM MgCl_2 , 0.5 mM EGTA, and 0.2 mM PMSF) was allowed to proceed for 1 hour at 4°C, and the resulting nucleosomes were analyzed by native PAGE and SYBR-Gold staining.

H2A-H2B dimer dissociation from the nucleosome was analyzed by electrophoretic mobility shift assay (EMSA). CPM labeled DNA or CPM-labeled H2A-H2B dimer containing nucleosomes were prepared as described before ¹¹. Fluorescently labeled nucleosomes on the gels were visualized without staining on a transilluminator at 365nm.

Structure determination

Recombinant yNAP-1_(74–365) was crystallized at 4°C by sitting drop vapor diffusion. yNAP1_(74–365) at 15 mg/ml protein, in 20 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl was combined in a 1:1 ratio with reservoir solution containing 40% MPD, 0.1M Sodium citrate and 0.2M Ammonium acetate at pH 7.5. Crystals (average size $0.06 \times 0.03 \times 0.03$ mm) were obtained after about 20 days at 4 C. Diffraction data were collected at beamline 5.0.2 at the Advanced Light Source. Data were processed with Denzo, and reduced with SCALEPACK ³⁷. The structure was solved by a molecular replacement ³⁸, using PDB code 2ayu as an initial search model. The model was built with O ³⁹, and refined using CNS ³⁸. All of the images were prepared using PYMOL ⁴⁰. Structure-superpositions were carried out using LSQMAN ⁴¹. The buried surface area for the complex was calculated using AREAIMOL.

Size-exclusion chromatography

Gel filtration chromatography was done on an AKTA FPLC using a Superdex S-200 16/60 (120 mL) column (Amersham Biosciences) as described ¹⁷. Following equilibration in either 100 or 500 mM NaCl chromatography buffer, 1 mL of 5mg/ml samples were applied and run at 1 mL/min and 4 C. The elution solution used in the experiments contained 100 or 500 mM NaCl in 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA. For the low pH experiment, 100 mM NaCl in 20 mM Bicine, pH 5.6, 1 mM dithiothreitol, 1 mM EDTA was used. Peak fractions were checked by SDS-PAGE (data not shown).

Analytical Ultracentrifugation (AUC) analysis

Sedimentation velocity experiments were performed in a Beckman XL-series analytical ultracentrifuge using the absorbance optical system. 400 μL of sample and 420 μL of reference (buffer) were loaded into two-sector, charcoal-filled Epon centerpieces (12mm pathlength) assembled with quartz windows and centrifuged in an AN60Ti 4-hole rotor at 23°C. All samples were prepared to absorbance values at 230 nm of 0.5, which results in an absorbance signal of 0.6 in the AUC. NAP1 concentrations were determined by absorbance spectrometry using a calculated molar extinction coefficient of 36,100 $\text{M}^{-1} \text{cm}^{-1}$ at 276 nm ⁴². Velocity data were edited and analyzed using the boundary analysis method of Demeler and van Holde ⁴³ as implemented in UltraScan version 7.1 for MS Windows. This analysis yields the diffusion-corrected, integral distribution of S ($G(s)$). Sedimentation coefficients (s) are reported in units of Svedbergs (S), where $1 \text{ S} = 1 \times 10^{-13} \text{ s}$, and are corrected to that of water at 20°C ($s_{20,w}$). The partial specific volume ($v\text{-bar}$) of NAP1 (0.7174 cm^3/g at 20°C) was calculated from the primary amino acid sequence, and solvent densities (ρ) were calculated within UltraScan. For these experiments, we assumed no significant change in the ϵ_{276} due to the change in oligomeric state of the proline substitutions mutants. Frictional ratios (f/f_0) were calculated using the observed, experimental sedimentation coefficients and the known molecular weight of NAP1 within the simulations menu in UltraScan. f/f_0 values account for

the geometric symmetry/asymmetry of the molecule as well as the frictional drag imparted by unstructured or loosely structured domains in the molecule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Luger K, Maeder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 1997;389:251–259. [PubMed: 9305837]
2. Kleinschmidt JA, Seiter A, Zentgraf H. Nucleosome assembly in vitro: separate histone transfer and synergistic interaction of native histone complexes purified from nuclei of *Xenopus laevis* oocytes. *Embo J* 1990;9:1309–18. [PubMed: 2323341]
3. Krude T. Chromatin. Nucleosome assembly during DNA replication. *Curr Biol* 1995;5:1232–4. [PubMed: 8574576]
4. Haushalter KA, Kadonaga JT. Chromatin assembly by DNA-translocating motors. *Nat Rev Mol Cell Biol* 2003;4:613–20. [PubMed: 12923523]
5. Philpott A, Krude T, Laskey RA. Nuclear chaperones. *Semin Cell Dev Biol* 2000;11:7–14. [PubMed: 10736259]
6. Ito T, Bulger M, Kobayashi R, Kadonaga JT. Drosophila NAP-1 is a core histone chaperone that functions in ATP-facilitated assembly of regularly spaced nucleosomal arrays. *Mol Cell Biol* 1996;16:3112–24. [PubMed: 8649423]
7. Shintomi K, Iwabuchi M, Saeki H, Ura K, Kishimoto T, Ohsumi K. Nucleosome assembly protein-1 is a linker histone chaperone in *Xenopus* eggs. *Proc Natl Acad Sci U S A* 2005;102:8210–5. [PubMed: 15928086]
8. Zlatanova J, Seebart C, Tomschik M. Nap1: taking a closer look at a juggler protein of extraordinary skills. *Faseb J* 2007;21:1294–310. [PubMed: 17317729]
9. Fujii Nakata T, Ishimi Y, Okuda A, Kikuchi A. Functional analysis of nucleosome assembly protein, NAP-1. The negatively charged COOH-terminal region is not necessary for the intrinsic assembly activity. *J Biol Chem* 1992;267:20980–20986. [PubMed: 1400414]
10. Chang L, Loranger SS, Mizzen C, Ernst SG, Allis CD, Annunziato AT. Histones in transit: cytosolic histone complexes and diacetylation of H4 during nucleosome assembly in human cells. *Biochemistry* 1997;36:469–80. [PubMed: 9012662]
11. Park YJ, Chodaparambil JV, Bao Y, McBryant SJ, Luger K. Nucleosome assembly protein 1 exchanges histone H2A-H2B dimers and assists nucleosome sliding. *J Biol Chem* 2005;280:1817–25. [PubMed: 15516689]
12. Okuwaki M, Kato K, Shimahara H, Tate S, Nagata K. Assembly and disassembly of nucleosome core particles containing histone variants by human nucleosome assembly protein I. *Mol Cell Biol* 2005;25:10639–51. [PubMed: 16287874]
13. Levchenko V, Jackson B, Jackson V. Histone release during transcription: displacement of the two H2A-H2B dimers in the nucleosome is dependent on different levels of transcription-induced positive stress. *Biochemistry* 2005;44:5357–72. [PubMed: 15807529]
14. Lorch Y, Maier-Davis B, Kornberg RD. Chromatin remodeling by nucleosome disassembly in vitro. *Proc Natl Acad Sci U S A* 2006;103:3090–3. [PubMed: 16492771]
15. Walfridsson J, Khorosjutina O, Matikainen P, Gustafsson CM, Ekwall K. A genome-wide role for CHD remodelling factors and Nap1 in nucleosome disassembly. *Embo J*. 2007
16. Park YJ, Luger K. The structure of nucleosome assembly protein 1. *Proc Natl Acad Sci U S A* 2006;103:1248–53. [PubMed: 16432217]

17. McBryant SJ, Peersen OB. Self-Association of the Yeast Nucleosome Assembly Protein 1. *Biochemistry*. 2004In Press
18. Ishimi Y, Hirosumi J, Sato W, Sugasawa K, Yokota S, Hanaoka F, Yamada M. Purification and initial characterization of a protein which facilitates assembly of nucleosome-like structure from mammalian cells. *Eur J Biochem* 1984;142:431–9. [PubMed: 6468372]
19. Ishimi Y, Sato W, Kojima M, Sugasawa K, Hanaoka F, Yamada M. Rapid purification of nucleosome assembly protein (AP-I) and production of monoclonal antibodies against it. *Cell Struct Funct* 1985;10:373–82. [PubMed: 2417731]
20. Toth KF, Mazurkiewicz J, Rippe K. Association states of nucleosome assembly protein 1 and its complexes with histones. *J Biol Chem* 2005;280:15690–9. [PubMed: 15687486]
21. Dutta S, Akey IV, Dingwall C, Hartman KL, Laue T, Nolte RT, Head JF, Akey CW. The crystal structure of nucleoplasmin-core: implications for histone binding and nucleosome assembly. *Mol Cell* 2001;8:841–53. [PubMed: 11684019]
22. Rodriguez P, Munroe D, Prawitt D, Chu LL, Bric E, Kim J, Reid LH, Davies C, Nakagama H, Loebbert R, Winterpacht A, Petrucci MJ, Higgins MJ, Nowak N, Evans G, Shows T, Weissman BE, Zabel B, Housman DE, Pelletier J. Functional characterization of human nucleosome assembly protein-2 (NAP1L4) suggests a role as a histone chaperone. *Genomics* 1997;44:253–265. [PubMed: 9325046]
23. McBryant SJ, Park YJ, Abernathy SM, Laybourn PJ, Nyborg JK, Luger K. Preferential binding of the histone (H3-H4)₂ tetramer by NAP1 is mediated by the amino-terminal histone tails. *J Biol Chem* 2003;278:44574–83. [PubMed: 12928440]
24. Siepen JA, Radford SE, Westhead DR. Beta edge strands in protein structure prediction and aggregation. *Protein Sci* 2003;12:2348–59. [PubMed: 14500893]
25. Minor DL Jr, Kim PS. Measurement of the beta-sheet-forming propensities of amino acids. *Nature* 1994;367:660–3. [PubMed: 8107853]
26. Richardson JS, Richardson DC. Natural beta-sheet proteins use negative design to avoid edge-to-edge aggregation. *Proc Natl Acad Sci U S A* 2002;99:2754–9. [PubMed: 11880627]
27. Demeler B, van Holde KE. Sedimentation velocity analysis of highly heterogeneous systems. *Anal Biochem* 2004;335:279–88. [PubMed: 15556567]
28. Ito T, Tyler JK, Bulger M, Kobayashi R, Kadonaga JT. ATP-facilitated chromatin assembly with a nucleoplasmin-like protein from *Drosophila melanogaster*. *J Biol Chem* 1996;271:25041–8. [PubMed: 8798787]
29. Friedeberg C, Scarlett G, McGeehan J, Abu-Daya A, Guille M, Kneale G. Identification of a structural and functional domain in xNAP1 involved in protein-protein interactions. *Nucleic Acids Res* 2006;34:4893–9. [PubMed: 16982648]
30. Nelson R, Sawaya MR, Balbirnie M, Madsen AO, Riekel C, Grothe R, Eisenberg D. Structure of the cross-beta spine of amyloid-like fibrils. *Nature* 2005;435:773–8. [PubMed: 15944695]
31. Sawaya MR, Sambashivan S, Nelson R, Ivanova MI, Sievers SA, Apostol MI, Thompson MJ, Balbirnie M, Wiltzius JJ, McFarlane HT, Madsen AO, Riekel C, Eisenberg D. Atomic structures of amyloid cross-beta spines reveal varied steric zippers. *Nature*. 2007
32. Mosammaparast N, Ewart CS, Pemberton LF. A role for nucleosome assembly protein 1 in the nuclear transport of histones H2A and H2B. *Embo J* 2002;21:6527–38. [PubMed: 12456659]
33. Dong A, Zhu Y, Yu Y, Cao K, Sun C, Shen WH. Regulation of biosynthesis and intracellular localization of rice and tobacco homologues of nucleosome assembly protein 1. *Planta* 2003;216:561–70. [PubMed: 12569397]
34. Mosammaparast N, Del Rosario BC, Pemberton LF. Modulation of histone deposition by the karyopherin kap114. *Mol Cell Biol* 2005;25:1764–78. [PubMed: 15713633]
35. Rodriguez P, Pelletier J, Price GB, Zannis-Hadjopoulos M. NAP-2: histone chaperone function and phosphorylation state through the cell cycle. *J Mol Biol* 2000;298:225–38. [PubMed: 10764593]
36. Li M, Strand D, Krehan A, Pyerin W, Heid H, Neumann B, Mechler BM. Casein kinase 2 binds and phosphorylates the nucleosome assembly protein-1 (NAP1) in *Drosophila melanogaster*. *J Mol Biol* 1999;293:1067–84. [PubMed: 10547286]
37. Otwinowski, Z.; Minor, W. Processing of X-ray diffraction data collected in oscillation mode. In: Carter, CW.; Sweet, RM., editors. *Methods Enzymology*. 276. Macromolecular Crystallography, part A, Academic Press; New York: 1997.

38. Brunger AT, Adams PD, Rice LM. New applications of simulated annealing in X-ray crystallography and solution NMR. *Structure* 1997;5:325–336. [PubMed: 9083112]
39. Jones TA, Zou JY, Cowan SW, Kjeldgaard M. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Cryst* 1991;A47:110–119.
40. DeLano, WL. *The PyMOL User's Manual*. DeLano Scientific; San Carlos, CA, USA: 2002.
41. Kleywegt GJ. Use of non-crystallographic symmetry in protein structure refinement. *Acta Cryst* 1996;D52:842–857.
42. Edelhoch H. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry* 1967;6:1948–54. [PubMed: 6049437]
43. Demeler B, Saber H, Hansen JC. Identification and interpretation of complexity in sedimentation velocity boundaries. *Biophys J* 1997;72:397–407. [PubMed: 8994626]

Figure 1. Overall structure of full-length NAP1 (1-417) and NAP1 (74-365)
(A) The dimer structure of yNAP1₍₇₄₋₃₆₅₎ is shown as a ribbon diagram. Subdomains A, B, C and D are shown in blue, yellow, green, and red, respectively (see 16 for details). Helices (α1-α8) and strands (β1-β6) are indicated. The structure of full length yNAP1(1-417) (pdb access code 2ayu) is superimposed (grey). **(B)** Detailed view of the β hairpin of yNAP1₍₇₄₋₃₆₅₎, including 3.2 Å electron density contoured at 2 sigma.

Figure 2. NAP1 forms a crystal lattice via the β hairpin motif

(A) Overview of the crystal lattice of yNAP1_(74–365) dimers. Four yNAP1 dimers related through crystallographic symmetry are shown in grey, red, blue, and grey, respectively. Interactions are mediated primarily by anti parallel $\beta - \beta$ interactions. (B) Stereo diagram of the β sheet connecting four yNAP1 dimers. Coloring is as above.

Figure 3. Size-exclusion chromatography of wild type and mutant yNAP1

All proteins (1ml of 5mg/ml or more) were equilibrated with the indicated buffer at 4°C before loading. Wild type yNAP1 (A–C) was injected onto the column equilibrated with buffer as indicated. (D) Mutant yNAP1 proteins were run in 20 mM Tris pH 7.5 (4 C) with 100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol (as in Figure 3A). Blue: yNAP1 R290P; red: K305P; yellow: yNAP1 R290P, R301P, T302P, K305P NAP1. Each peak fraction was analyzed by SDS PAGE (data not shown). The elution positions of the calibration standards are indicated (Note 98 kDa yNAP1 dimer eluates at apparent molecular mass of ~300 kDa because of ellipsoid shape¹⁷).

Figure 4. Analytical ultracentrifugation of wild type and mutant yNAP1

(A) Equivalent concentrations of wild type and mutant NAP1 were sedimented in buffer containing 100mM NaCl at 45k RPM in an analytical ultracentrifuge measuring in the absorbance mode at 229 nm. Boundaries were analyzed using the vanHolde–Weischet method and the resulting integral distributions of $S(G(s))$ [corrected for water at 20 °C($S_{20,w}$)] over the boundary are shown. (B) Equivalent concentrations of WT NAP1 and the proline mutants were sedimented in buffer containing 500mM NaCl at 50k RPM. $G(s)$ plots for the samples are shown. (C) Equivalent concentrations of NAP74-365 [WT and (305P)] were sedimented at 45k RPM in buffer containing 100mM NaCl. $G(s)$ plots for the samples are shown.

Figure 5. The *in vitro* activities of yNAP1 remain unchanged upon disruption of the β hairpin
(A) Histone binding was analyzed by GST-NAP1 pulldown assays. 0.25 nmoles of purified full length GST-NAP1(wt), GST-NAP1(R290P), GST-NAP1(K305P) or GST alone were incubated with 100 pmoles H2A-H2B dimer. Bound proteins were analyzed by 15% SDS PAGE and stained with coomassie brilliant blue. Note that recombinant *Xenopus laevis* H2A and H2B run in the exact same position under these conditions and weak non-specific interactions between H2A-H2B and GST was only observed at the lowest salt conditons. **(B)** Nucleosome assembly activity of wild type and mutant NAP1. Mono-nucleosome assembly reactions were carried out by incubating 0.1 μ M DNA with increasing amounts of histone octamer (0.1, 0.2, 0.3, 0.4 μ M), either in the absence or presence of NAP1. Lanes 1, 6, 11 and 16: DNA alone. Lane 2–5: without NAP1. Lane 7–10: wild type NAP1. Lane 12–15:

NAP1_{R290P}. Lane 17–20: NAP1_{K305P}. The formation of mononucleosomes was analyzed by native PAGE (5% polyacrylamide, 0.2x TBE), stained with SYBR-Gold. The gel on the left (lanes 1–10) was stained somewhat shorter than the gel on the right, explaining the differences in DNA and nucleosome intensity. (C) yNAP1 induced H2A-H2B dimer dissociation from the nucleosome was analyzed by electrophoretic mobility shift assay. Fluorescently labeled nucleosomes were reconstituted with recombinant *Xenopus laevis* histones using CPM labeled DNA (DNA*, lanes 1–8) or CPM-labeled H2A-H2B dimer (H2B*, lanes 9–12). Gels were viewed on a transilluminator (365nm) without staining (* indicates fluorescently labeled nucleosomal component). The labeled nucleosomes were incubated without yNAP1 (lanes 1, 5 and 9), with wild type NAP1 (lane 2), NAP1_{R290P} (lane 3), NAP1_{K305P} (lane 4), wild type GST-NAP1 (lanes 6 and 10), GST-NAP1_{R290P} (lanes 7 and 11), or GST-NAP1_{K305P} (lanes 8 and 12). Samples were incubated at 4° C for 10 hours and analyzed by 5% native PAGE. The nucleosome species N1 (centrally positioned octamer), S1 (nucleosome in which the histone octamer had shifted by 10 base pairs on the DNA, and S3 (tetrasome), are indicated (also see reference ¹¹). GST-yNAP1 – H2A-H2B dimer complex (GST-S2) are shown in as NCP (H2B*). Note that the presence of more than one band of GST-S2 is likely due to the frequently described dimerization of GST.

Table 1
Data collection and refinement statistics for yNAP1_(74–365)

	Native
Data collection	
Space group	P4 ₂ 2 ₁ 2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	125, 125, 146.7
α , β , γ (°)	90, 90, 90
Wavelength	1.1
Resolution (Å)	50–3.2
<i>R</i> _{merge} ^a	0.07 (0.38)
<i>I</i> / σ <i>I</i> ^a	14 (3.5)
Completeness (%) ^a	99 (92)
Redundancy ^a	5 (5)
Refinement	
Resolution (Å)	50–3.2
No. reflections ^b	17067
<i>R</i> _{work} / <i>R</i> _{free}	0.26/0.30
No. atoms	
Protein	4468
Ligand/ion	0
Water	0
<i>B</i> -factors	
Protein	85
Ligand/ion	N/A
Water	N/A
R.m.s deviations	
Bond lengths (Å)	0.0091
Bond angles (°)	1.5369

^aValues in parentheses are for the highest resolution shell.

^bReflections of $|F_{\text{obs}}| > 1.0$