Molecular Cell, *Volume 37*

Supplemental Information

The Histone Chaperone Nap1 Promotes Nucleosome Assembly by Eliminating Nonnucleosomal Histone DNA Interactions

Andrew J. Andrews, Xu Chen, Alexander Zevin, Laurie A. Stargell, and Karolin Luger

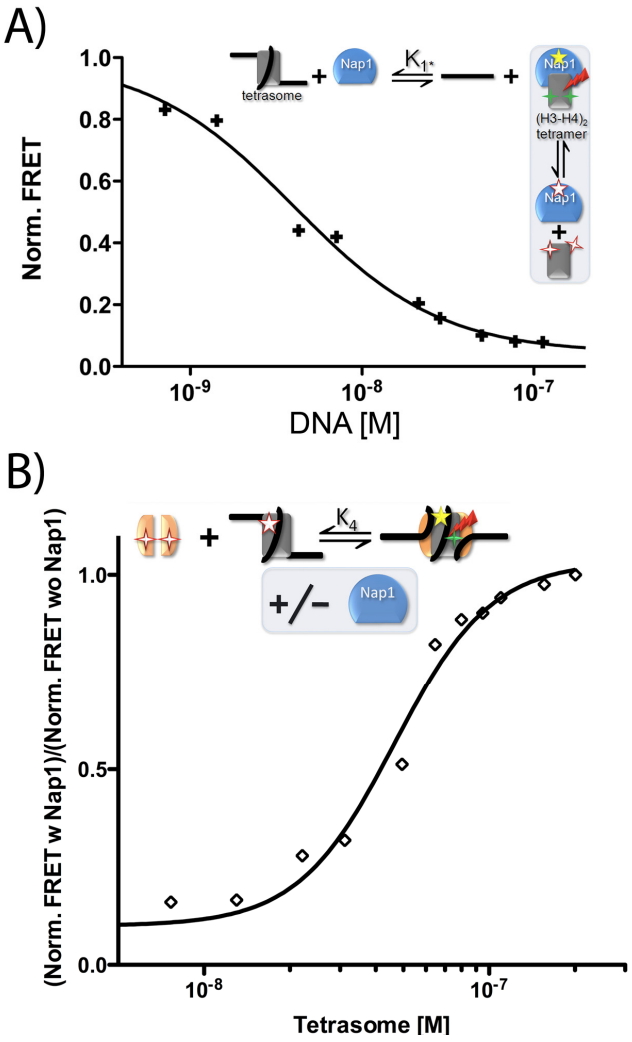

Figure S1: Measurement of the thermodynamic constants for nucleosome formation (pertains to Fig. 2).
A)The normalized FRET ratio between Nap1 and H3 as a function of DNA (601). This experiment assays for the competetion between Nap1 and DNA for $(H3\text{-}H4)_2$ tetramer. The binding constant for tetramer to DNA was obtained by fitting eq. 8 (supplemental information) to these data. The value of the obtained binding constant ($1.8\pm1.0\times10^{-9}$) is within error of that obtained from direct measurement.
B) Independent evidence that nucleosomes are formed in the competition assay shown in Fig. 2B. We monitored the FRET ratio between H2A and H4 under conditions where H2A-H2B is low (0.5 nM) and tetrasome (on a 5S 207 bp DNA fragment) is titrated into two cuvettes, one of which also contains Nap1 at 8×10^{-9} M concentration. A standard binding isotherm is fit to the difference between the two signals. The constant thus determined is within error of that obtained in the coupled equilibrium assay ($K_{app}=3.5\pm0.4\times10^{-8}$ M, $n_H=2.4\pm0.6$).

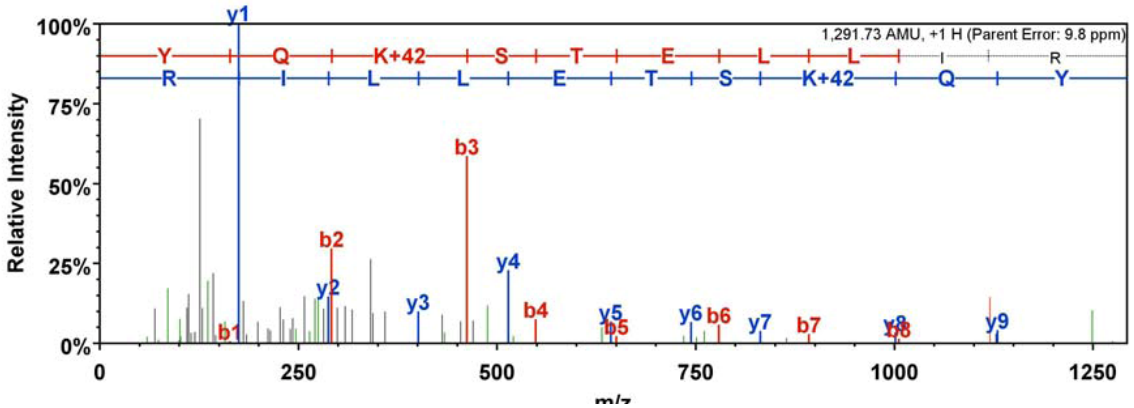

Figure S2: H3K56ac destabilizes tetrasome formation (pertains to Fig. 3). MS/MS spectrum of peptide YQK*STELLIR (amino acids 54-64 of H3) confirming acetylation on lysine 56. Confirmation is based on peptide mass (9.8 ppm mass error) and full coverage of theoretical y and b ions including the sequence region of acetylated K56. In addition, the starred peak at m/z 126 corresponds to an acetylated lysine immonium ion – NH3 which further confirms acetylation of K56.

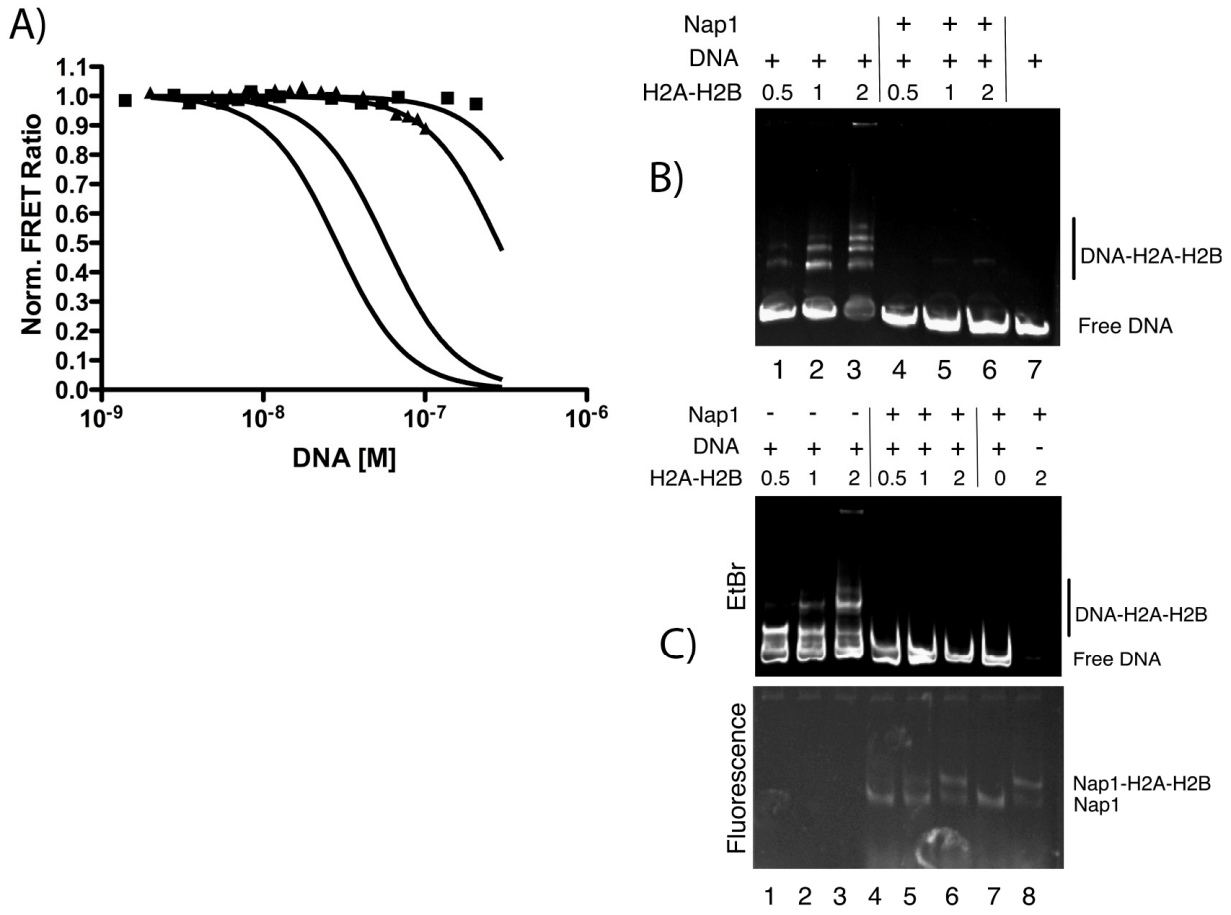

Figure S3: Nap1 disfavors the interaction between H2A-H2B dimer and DNA (pertains to Fig. 4).

A): The competition between Nap1, H2A-H2B, and DNA cannot be explained by simple binary interactions. Experimental data (obtained with the coupled equilibrium assay, measuring the competition of DNA and Nap1 for H2A-H2B) are the same as in Fig. 2E. Solid lines represent modeled curves, according to known binding constants. The two curves to the left are modeled data using the constants from the direct measurement of Nap1 to H2A-H2B and H2A-H2B to DNA (Table 1). The two curves to the right are simulated using constants determined by DNA interaction with the Nap1-H2A/H2B constant.

B, C) Nap1 removes H2A-H2B dimers from DNA. Histone-DNA and protein-protein complexes are analyzed by 5 % polyacrylamide gel electrophoresis. H2A-H2B was added to 1.5 µM DNA in molar ratios ranging from 0.5 to 2 at 0.2 M ionic strength (lanes 1-3). Nap1 was added in a fourfold excess to DNA (lanes 4-6). Lane 7: Nap1 and DNA; Lane 8 (only in B): Nap1-H2A-H2B. H2A and H2B do not enter the gel; Nap1 does not bind DNA. **B)** Ethidium bromide staining; **C)** The same experiment as in B), but with Nap1 labeled with Alexa-488. Upper panel: ethidium bromide staining, lower panel, viewed under UV light without staining. DNA-H2A-H2B

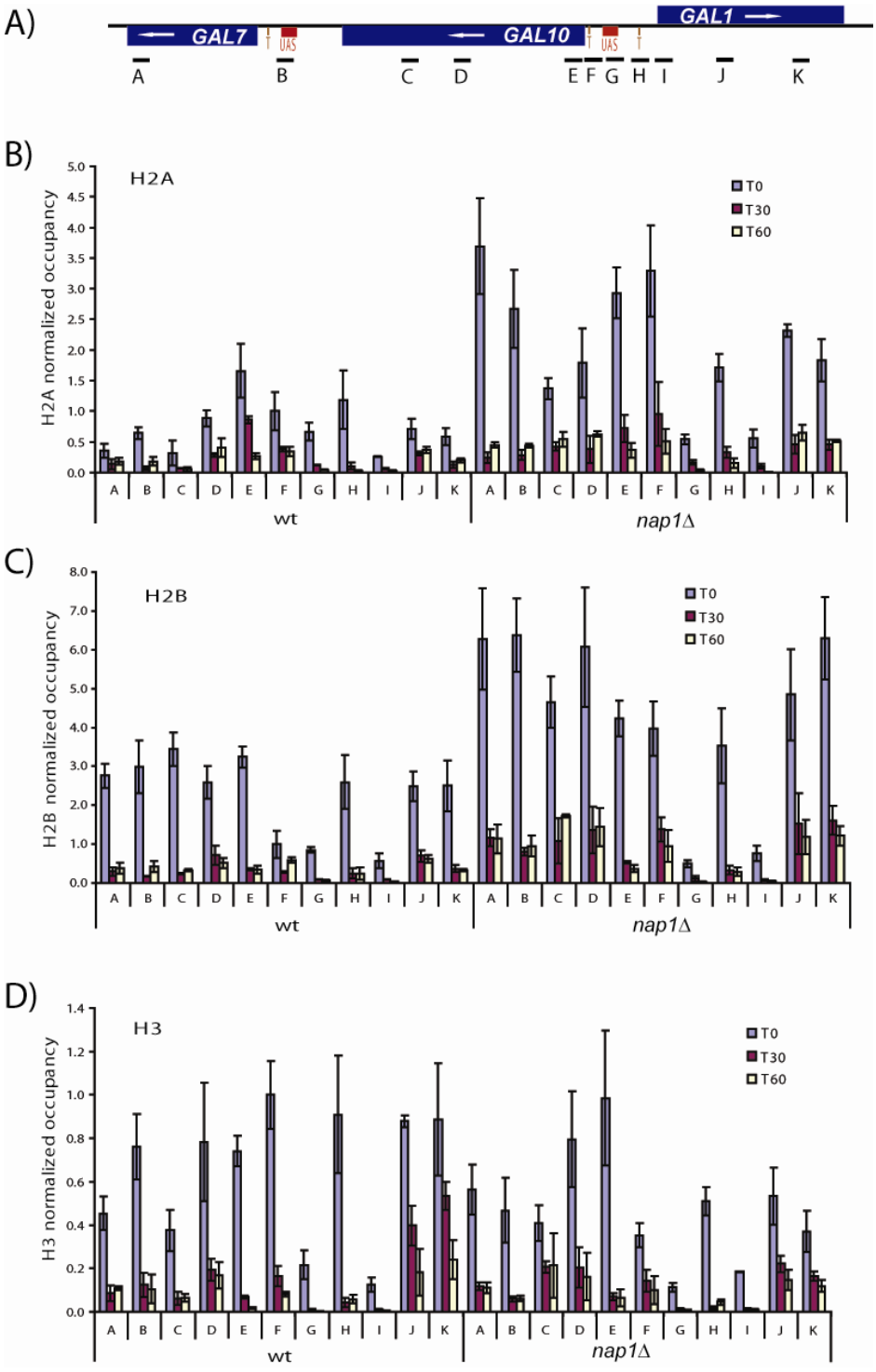

Figure S4: Deletion of *NAP1* alters histone occupancy *in vivo* (pertains to Fig. 5). Histones are evicted upon transcription activation in the wild type and *nap1Δ* strains. (A) Schematic of the *GAL* gene locus, showing the relative position of the coding sequence for *GAL1, GAL7* and *GAL10*. The location of the amplicons used for ChIP assays are indicated (A-K). (B-D) Changes in histone occupancy upon galactose induction for wild type (wt) and *nap1Δ* strains. Samples were taken at 0, 30, and 60 minutes after addition of galactose (T0, T30, T60), and analyzed as described in Fig. 5. Error bars indicate standard deviations from three independent biological replicates.

Hill coefficients and cooperativity

We have previously described the difficulties in interpreting the Hill coefficients in systems in which protein dimers and/or tetramers interact with each other (Andrews et al., 2008). As discussed in Andrews *et al.* it is unknown if the cooperativity observed in these experiments is 'true' cooperativity or the result of dimerization (or tetramerization) of the interaction partners.

The discussion of the Hill coefficients listed in Table 1 is similarly complex. The observed Hill coefficient for K_4 is expected since two H2A-H2B dimers are added to one tetrasome to form the nucleosome. The cooperativity for nucleosome formation has also been observed in other studies of Nap1 mediated nucleosome assembly (Mazurkiewicz et al., 2006). However, the observed apparent cooperativity for the interaction of H2A-H2B with DNA (K_6) is unexpected. One possible explanation is that the H2A-H2B dimers interact with each other and the DNA in the absence of the tetrasome. Our finding that more than one dimer is bound to 207 base pairs of DNA (Fig. S3B, C) is consistent with this interpretation.

Nap1 mediated nucleosome formation as monitored by FRET between histones H3 and H2B

To demonstrate that nucleosomes were formed in the coupled equilibrium assay (Fig. 2D), the formation of histone octamer in the presence of Nap1 was monitored. Limiting amounts of fluorescently labeled H2A-H2B dimer (labeled on H2B at T112C, donor) were titrated with tetrasome in which H4 was labeled at position E63C (acceptor). This was done in parallel experiments in the presence and absence of Nap1, respectively. The difference in the FRET ratio between the two experiments was then calculated. The $K_{1/2}$ (or apparent K_4) can be estimated by fitting the data to a simple binding isotherm, or more accurately determined by using the more comprehensive fit (see data analysis).

Competition studies between Nap1 and DNA for tetramer.

We used a modified coupled equilibrium assay to measure the binding constant of $(H3\text{-}H4)_2$ tetramer to DNA in the presence of Nap1. In this experiment we monitored the FRET signal between Nap1 (acceptor) and H3 (donor) as a function of DNA (Fig. S1A). The binding constant obtained for tetramer to DNA by this method is within error of the direct measurement (K_3 in Table 1), suggesting that Nap1 and DNA are simply competing for tetramer and Nap1 has no effect of tetramer-DNA interaction.

Supplemental Methods

Fluorescence titrations – Fluorescence was measured using a Horiba Jobin Yvon Fluorolog-3 spectrofluorometer. Labeled protein was added to both the sample and the reference cuvette; non-labeled protein was added to the sample cuvette, and buffer was added to the reference. Varying incubation times (2-15 minutes)

confirmed that the fluorescence signal had reached equilibrium. The normalized fluorescence change was determined by equation 1, where R_{obs} is the observed

$$Norm.F.C._{obs} = \frac{R_{obs} - R_f}{R_f - R_i} \qquad \text{eq. 1}$$

ratio of the fluorescence signal (sample cuvette signal divided by the reference cuvette signal), R_i is ratio of the fluorescence initial, and R_f is the ratio of the fluorescence final. While the magnitude of the signal change was constant for each experiment, it varied from 10 to 30% between different experiments (i.e. labeled yNap1 binding H2A-H2B versus labeled H2A-H2B binding to yNap1) and with the label used (546 or 488 Alexa).

To confirm that the observed signal change is due to protein interactions we also monitored the normalized fluorescence ratio of protein titrated into its corresponding binding partner in either buffer or 5 M guanidium HCl. The presence of guanidium HCl did not alter the initial signal (pre-addition of the binding partner) nor did the signal change with the addition of μM concentrations of the binding partner in guanidium HCl. For FRET experiments the normalized FRET was calculated using equation 1 with R replaced by the FRET ratio. Coupled equilibrium experiments were done by placing labeled H2A/H2B in a four cuvette sample changer; 6-12x10^{-9} M Nap1 was subsequently added to each. In each cuvette either buffer; H3/H4; DNA; or H3/H4 and DNA (DNA was kept above 1x10^{-9} M) was added, respectively.

Data analysis — Binary affinity measurements were done using concentrations of labeled protein (P) at least 5 to 10-fold below the $K_d^{app.}$. $K_d^{app.}$ was determined by fitting eq. 2 derived from scheme 1 to the normalized fraction change (F.C.)

$$P + nL \rightleftharpoons P(L)_n \qquad \text{Scheme 1}$$

$$Norm.F.C. = f.c._{\max}\left(\frac{L_t^{n_H}}{L_t^{n_H} + K_d^{n_H}}\right) \qquad \text{eq. 2}$$

observed as a function of L, where L_t is the total concentration of protein titrated, n_H is the Hill coefficient, and K_d is the apparent dissociation constant. The n_H was assumed to be 1 unless the data dictated otherwise. When the n_H was determined not to be equal to one, the linearized form of equation 2 was used *(eq.3)*, where f is equal to the normalized f.c. divided by the normalized f.c. max.

$$Log[f/(1-f)] = n_H Log[L] + b \qquad \text{eq. 3}$$

Chaperone-based assay to measure nucleosome thermodynamics (coupled equilibrium assay). The chaperone based assay for nucleosome thermodynamics is a coupled equilibrium assay that works by monitoring the displacement of H2A-H2B from Nap1. The displacement H2A-H2B from Nap1 is observed by monitoring how the FRET signal between yNap1 and H2A-H2B changes as a function of another protein or protein-DNA complex (scheme 2). For these experiments, Nap1 is kept close to the

scheme 2

$$AB + C \overset{K_1}{\rightleftharpoons} A + B + C \overset{K_2}{\rightleftharpoons} BC + A$$

$K_d^{Nap\text{-}H2A/H2B}$ (8 nM, K_1) and H2A/H2B is ~0.5 nM or less. The letter designation in scheme 2 changes depending on what is being tested. To determine the K_d of H3-H4 to Nap1 (or K_1) by this method, Nap1 is "B", H2A-H2B is "A" and H3-H4 is "C" in scheme 2. For this scheme the fraction bound AB (Fb^{AB}) is equal to eq. 4 and the fraction bound of AB in the presence of C ($(H3\text{-}H4)_2$ tetramer (Fbi^{AB}) is equal to eq. 5. The free concentration of B or Nap1 (B_{free}) is equal to eq. 6, where the concentration of Nap1-$(H3\text{-}H4)_2$ tetramer (BC) is equal to eq. 7.

$$Fb^{AB} = B_{total}/(B_{total} + K_1) \qquad \text{eq. 4}$$

$$Fbi^{AB} = B_{free}/(B_{free} + K_1) \qquad \text{eq. 5}$$

$$B_{free} = B_{total} - BC \qquad \text{eq. 6}$$

$$BC = \frac{(C + B + K_2) - \sqrt{(C + B + K_2)^2 + 4BC}}{-2} \qquad \text{eq. 7}$$

To determine K_2 by this method the FRET signal is monitored a function of tetramer and eq. 8 is fit to the data. In eq. 8, S_{obs} is the FRET signal observed, S_f is the FRET signal final, and S_i is the FRET signal initial. The K_d obtained using this competition-based assay is within error to that obtained by direct measurements (Table 1, Fig 1).

$$S_{obs} = S_i + (S_f - S_i)(1 - (Fbi^{AB}/Fb^{AB})) \qquad \text{eq. 8}$$

To apply this approach to measure the affinity of H2A-H2B to DNA or to tetrasome, the letter designation for Nap1 is A, H2A-H2B is B, and C is tetrasome or DNA. Eq. 8 is fit to the experimental data. The difference in this analysis is that newly formed species as the result of the titration is less than the observed constant and therefore the fraction of Nap1-H2A-H2B (Fb^{AB}) is equal to eq. 9 and fraction of Nap1-H2A-H2B in the presence of DNA or tetrasome (Fbi^{AB}) is equal to eq. 10.

$$Fb^{AB} = A/(A + K_1) \quad \text{eq. 9}$$

$$Fbi^{AB} = \frac{AK_2}{K_1K_2 + AK_2 + CK_1} \quad \text{eq. 10}$$

Modeling/calculating constants using equations derived from the thermodynamic scheme from figure 1. We can calculate the fraction of nucleosomes by solving the scheme shown in figure 1. The concentration of nucleosome in this scheme is equal to eq. 11, where the concentration of tetrasome is equal to eq. 12.

$$[Nucleosome] = ([H2A - H2B][tetrasome])^{n_H 4}/K_4^{n_H 4} \qquad \text{eq. 11}$$

$$[tetrasome] = \frac{([DNA] + [(H3 - H4)_2] + K_3) - \sqrt{([DNA] + [(H3 - H4)_2] + K_3)^2 - 4[DNA][(H3 - H4)_2]}}{-2}$$

eq. 12

$$[Nap1 \bullet H2A - H2B \bullet DNA] = \left(\frac{([Nap1 \bullet H2A - H2B][DNA]_{free})^{n_H 5}}{K_5^{n_H 5}} \right) \quad \text{eq. 13}$$

The next major complex we need to solve for is the Nap1-H2A-H2B-DNA complex. The concentration of the Nap1-H2A-H2B-DNA complex is equal to eq 13, where the concentration of Nap1-H2A-H2B is equal to eq. 14 and the concentration of free DNA is equal eq 15. Given that in our experimental conditions H2A-H2B is at low concentrations (<<1 nM) we solved for the fraction of H2A-H2B that exist as nucleosomes.

$$[Nap1 \bullet H2A-H2B] = ([Nap1][H2A-H2B])/K_2 \quad \text{eq. 14}$$

$$[DNA]_{free} = [DNA]_{total} - [tetrasome] \quad \text{eq. 15}$$

The total H2A-H2B concentration is equal to eq. 16. The fraction of nucleosome can be calculated by using eq. 17.

$$[H2A-H2B]_{total} = [H2A-H2B]_{free} + [Nap1 \bullet H2A-H2B] + [Nap1 \bullet H2A-H2B \bullet DNA] + 2[nucleosome] \quad \text{eq. 16}$$

$$F_{nuc} = [nucleosome]/[H2A-H2B]_{total} \quad \text{eq. 17}$$

It is important to note that free H2A-H2B cancels out of this equation. The F_{nuc} (F_X) can be used to calculate the binding constant for H2A-H2B to tetrasome (in the H2B H3 FRET experiment)

$$S_{obs} = S_i + (S_m - S_i)F_X \quad \text{eq. 18}$$

by using eq. 18, where S_{obs} is the normalized FRET signal with Nap1 is divided by the normalized FRET without Nap1, S_i is the normalized FRET signal with no tetrasome added, and S_{max} is the maximum signal change. This method can also be used to calculate the loss of Nap1-H2A-H2B

$$F_{Nap1-H2A-H2B} = [Nap1 \bullet H2A-H2B]/[H2A-H2B]_{total} \quad \text{eq. 19}$$

as a function of tetrasome by eq. 19 (coupled assay data), where the concentration Nap1 bound to H2A-H2B is equal to eq. 14 and total H2A-H2B is equal to eq. 16.

Gel shift assays – H2A-H2B-DNA complexes were analyzed by electrophoretic mobility shift assays under native conditions. 1.5 μM DNA was mixed with various concentrations of H2A-H2B with and without fluorescently Nap1. After incubation for 1 hr at 25 °C, the samples were loaded onto a 5% acrylamide, 0.2x Tris-borate with EDTA (TBE) gel, and electrophoresed for 50 min at 150 V and viewed by UV and/or stained with EtBr.

Supplemental References

Andrews, A.J., Downing, G., Brown, K., Park, Y.J., and Luger, K. (2008). A thermodynamic model for Nap1-histone interactions. J Biol Chem *283*, 32412-32418.

Mazurkiewicz, J., Kepert, J.F., and Rippe, K. (2006). On the mechanism of nucleosome assembly by histone chaperone NAP1. J Biol Chem *281*, 16462-16472.