spFRET studies of nucleosome dynamics modulated by histone modifications, histone variants and neighboring nucleosomes

Buning, R.

CHAPTER 4

Stability and dynamics of nucleosomes containing H2A or H2A.Z and their temperature response studied with spFRET

Ruth Buning, Anna Brestovitsky, Charles Ravarani, Philip A. Wigge, and John van Noort

Manuscript in preparation

Abstract

The incorporation of histone variants is one of the ways in which eukaryotic cells regulate their DNA activity. H2A.Z is a highly conserved histone variant involved in many transcription-related functions. In Arabidopsis Thaliana, H2A.Z plays an essential role in ambient temperature sensing. The mechanism by which H2A.Z does so remains however unresolved. Both enhanced and reduced nucleosome stability have been reported. Here we show that H2A.Z-containing nucleosomes are more stable than H2A-containing nucleosomes. We found in single-pair FRET experiments on individual nucleosomes that H2A.Z-containing nucleosomes have a lower unwrapping probability and are less susceptible to dissociation during gel electrophoresis, at low concentrations, and in the presence of surfaces. Ambient temperature changes between 7 and 37 °C have no detectable effect on the dynamics of H2A.Z-containing nucleosomes. Our results suggest that H2A.Z incorporation may act as a nucleosomal stability switch.

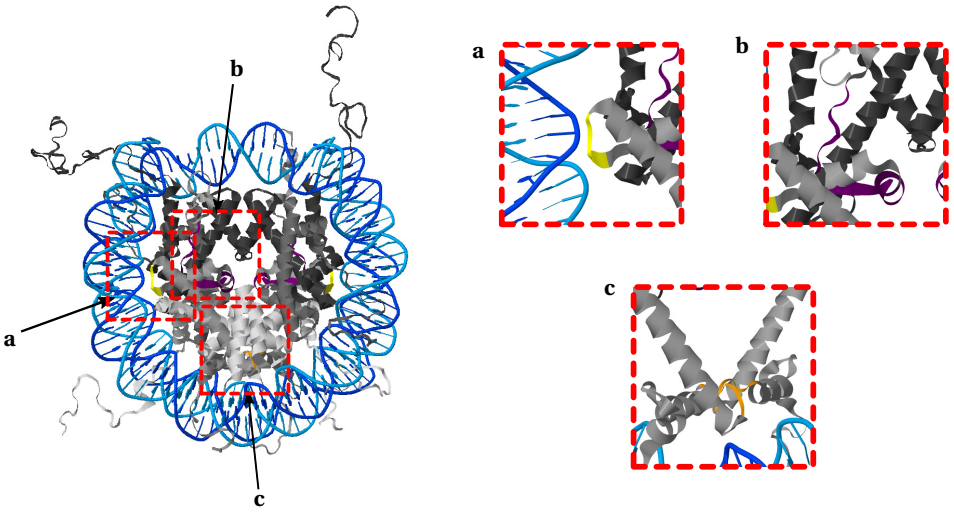


Figure 4.1 – Structural differences between H2A- and H2A.Z-containing nucleosomes. Crystal structure of the nucleosome (1KX5, [12]), with highlighted the regions where H2A and H2A.Z differ. Three regions show structural differences, which are conserved across species: **a (yellow):** H2A(Z)-DNA contact at the second minor groove; **b (purple):** H2A(Z) interface with H3; **c (orange):** interface between the two copies of H2A(Z). Left: full crystal structure. Right: Zooms of the marked regions, where for clarity only the first superhelical turn of the DNA is shown, and histones H2A and H3.

4.1 Introduction

The wrapping of eukaryotic DNA into nucleosomes and higher order chromatin structures restricts access of the cellular machinery to the DNA. The amount of DNA wrapping and dynamics regulates processes like transcription. DNA wrapping itself is in its turn regulated by ATP dependent remodeling of nucleosomes and/or chromatin, post-translational modifications to the histone proteins, or the incorporation of histone variants.

H2A.Z is an H2A-variant that is highly conserved among all eukaryotes. It is found to have multiple functions, for example in transcription regulation and progression through the cell cycle, and is essential for viability in many organisms [117]. Despite multiple reports on the behavior of H2A.Z-containing nucleosomes, no univocal view on the effect of H2A.Z on the accessibility of nucleosomal DNA has emerged yet. Both stabilization [118, 119] and destabilization [120, 121] have been reported.

The sequence and structure of H2A and H2A.Z are highly conserved among species. Three regions have been identified where H2A and H2A.Z differ [117, 122], indicated in figure 4.1. First, at the H2A(Z)-DNA contact in the second minor groove, there is a

conserved substitution of a Threonine (uncharged, polar) in H2A to a Lysine (positively charged) in H2A.Z. This additional charge in H2A.Z might render the DNA-histone contact more tight, decreasing the DNA unwrapping probability. Second, H2A and H2A.Z differ structurally at their interface with H3. This might result in a different dissociation constant of the H2A/H2B dimer from the tetramer (also called dimer loss). The third structural difference is at the H2A(Z)-H2A(Z) interface. This has been suggested to be the reason why a single nucleosome can only contain either $(H2A)_2$ or $(H2A.Z)_2$ [122], since there would be steric hindrance otherwise. Heterotypic nucleosomes have nevertheless been seen *in vitro* and *in vivo* [123].

Recently, Kumar et al. [124] found that H2A.Z is essential for ambient temperature sensing in Arabidopsis Thaliana. Wild type plants develop phenotypes specific for temperature changes between 12 and 27 °C, correlated with nucleosome and H2A.Z occupancy. H2A.Z knockouts, on the other hand, fail to produce the proper temperature response. Their phenotype at low temperature resembles the wild type phenotype at high temperature. In the same paper [124], Kumar et al. show that purified Arabidopsis nucleosomes containing H2A are more accessible to restriction enzymes than H2A.Z-containing nucleosomes. They suggest that temperature-dependent unwrapping of nucleosomal DNA in H2A.Z-containing nucleosomes might serve as a direct mechanism for temperature perception in plants.

We can estimate the effect of temperature change on the unwrapping probability P_{open} for the first contact point by a simple thermodynamic model assuming two states (open and closed):

$$P_{open} = e^{\frac{\Delta G}{k_B T}} \tag{4.1}$$

where ΔG is the binding energy per contact point, T the temperature and k_B the Boltzmann constant. The binding energy per contact point is only slightly more than one $k_B T$ [15], which allows frequent spontaneous transient unwrapping of the DNA. From equation 4.1 follows that, at ambient temperatures, the unwrapping probability ($0 < P_{open} < 1$) increases about 0.012 per 10 ° temperature increase. Such a small increase will probably not have a profound impact on gene regulation. Experimental data on the unwrapping probability of individual nucleosomes is required to exclude or identify the unwrapping of nucleosomal DNA as a temperature-sensing mechanism.

Here, we probe the effect of the H2A.Z histone variant on nucleosome dynamics and stability with single-pair Fluorescence (or Förster) Resonance Energy Transfer (spFRET) in nucleosomes reconstituted with Arabidopsis Thaliana histone octamers. We show that H2A.Z-containing nucleosomes are more stable than H2A-containing nucleosomes: their unwrapping probability is lower, and they are less susceptible to dissociating conditions like low concentrations and the presence of surfaces. Furthermore, we show that the stability of H2A.Z-containing nucleosomes is temperature

independent, excluding H2A.Z as direct ambient temperature sensor in Arabidopsis Thaliana.

4.2 Materials and Methods

4.2.1 Preparation of DNA constructs

A 155 bp DNA template containing a single 601 nucleosome positioning sequence was generated by PCR, separated on agarose gel and cleaned by Qiagen gel extraction kit. The forward and reverse primers were labeled at a single position with Cy3B and ATTO647N (via amino linker with 6-carbon spacer to the base, IBA)[1]. We generated three DNA constructs with a FRET pair at either of the nucleosome extremes (labels at position X or Z), or at a position 27 bp from one nucleosome end (Y) (see figure 4.2a). In all DNA constructs the donor and acceptor were separated by 76-81 bp (~24 nm). A DNA template without fluorescent labels was constructed as well to reconstitute unlabeled nucleosomes[2].

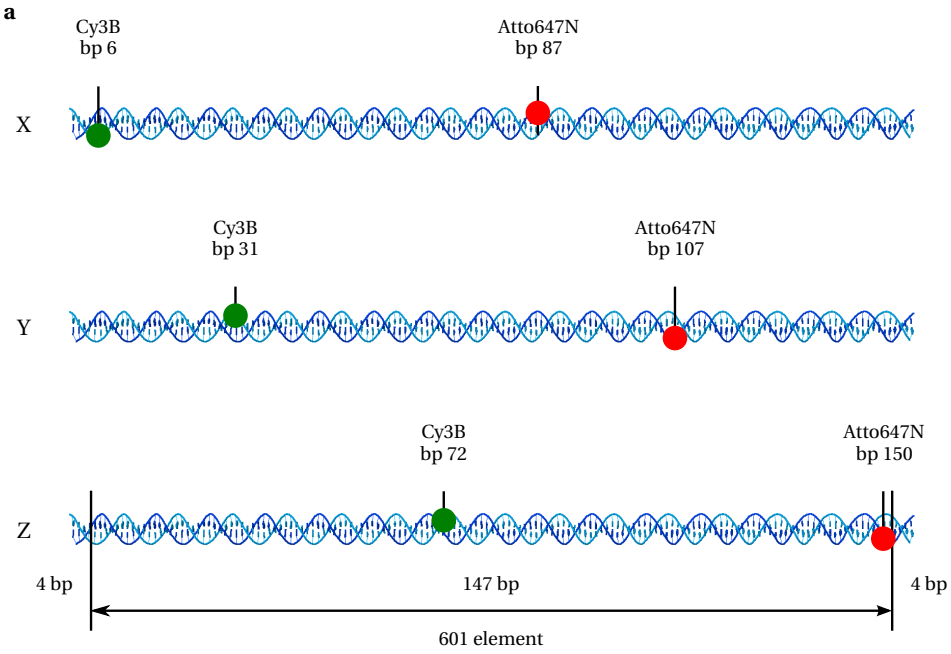
4.2.2 Preparation of histone octamers

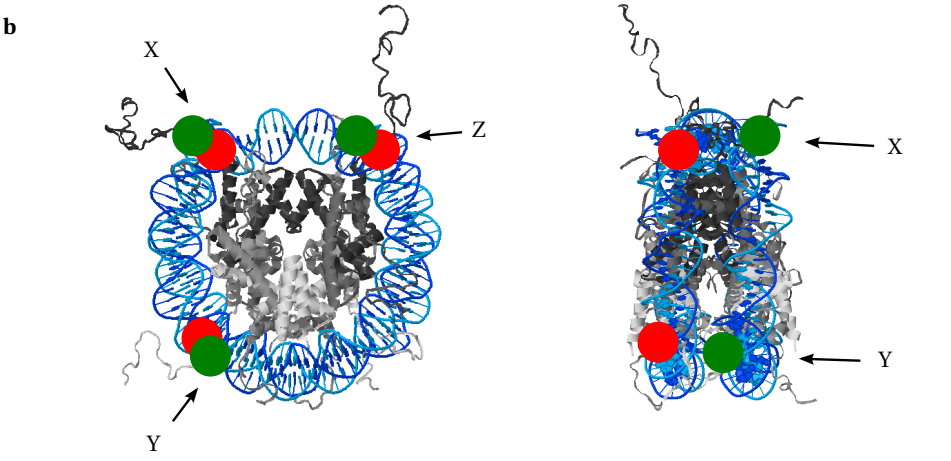
Wild type (WT) Xenopus laevis and Arabidopsis Thaliana H2A, H2A.Z, H2B, H3 and H4 histones were expressed in E. coli and purified as described previously [23]. Recombinant histone octamers were reconstituted from purified histones by refolding an equimolar mixture of each of the four denatured histone by dialysis against a buffer containing 2 M NaCl. The intact histone octamers were fractionated from histone tetramers and hexamers by size-exclusion chromatography as described [23].

[1] PCR primers were as follows (5'→3'), modified bases for either construct X, Y or Z indicated in bold: TTGGC**T**(Cy3B,X)GGAGAATCCCGGTGCCGAGGCCGC**T**(Cy3B,Y)CAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACG**T**(Cy3B,Z)ACGCGCTG and TTGGA**C**(ATTO647N,Z)AGGATGTATATATCTGACACGTGCCTGGAGACTAGGGAGTAA**T**(ATTO647N,Y)CCCCTTGGCGGTTAAAACG**C**(ATTO647N,X)GGGGGACAGC.

[2] PCR primers for the 155 bp non-labeled DNA construct were as follows (5'→3'): TTGGCTGGAGAATCCCGGT and TTGGACAGGATGTATATATCTGAC.

Figure 4.2 ***(facing page)*** – Constructs used for the experiments described in this chapter. **a:** The 155 bp DNA construct, indicating the position of the 601 positioning element and the fluorescent labels. Constructs X, Y and Z differ only in label positions. The donor and acceptor labels are separated by ~80 bp, preventing FRET in bare DNA. The labels are only several nm apart for fully reconstituted nucleosomes, allowing efficient FRET. **b:** Top and side view of the crystal structure of the nucleosome core particle (1KX5, [12]), consisting of 147 bp DNA wrapped around the histone octamer. The positions of the fluorescent labels for the different constructs X, Y and Z are indicated.


a
Cy3B
bp 6
Atto647N
bp 87
X
Cy3B
bp 31
Atto647N
bp 107
Y
Cy3B
bp 72
Atto647N
bp 150
Z
4 bp
147 bp
4 bp
601 element


b
X
Z
Y
X
Y

4.2.3 Nucleosome reconstitution

DNA and histone octamers containing either H2A or H2A.Z were mixed in various molar ratios in TE (1 mM EDTA, 10 mM Tris.HCl pH 8.0) and 2 M NaCl. Nucleosomes were reconstituted by salt gradient dialysis against 0.85, 0.65, 0.5 and finally 0 mM (figures 4.4 and 4.8) or 50 mM (other figures) NaCl, all buffered with TE in a total volume of 40 µl at a labeled DNA concentration around 200 nM. Competitor DNA, 147 bp unlabeled random sequence DNA (produced with PCR), was included in the reconstitution reaction at concentrations between 100 and 200 nM. After reconstitution, the products were transferred to non-stick tubes (Ambion), and BSA and Nonidet-P40 were added to concentrations of 0.1 mg/ml and 0.03 % w/v, respectively.

4.2.4 Polyacrylamide gel electrophoresis

Nucleosome reconstitutions were analyzed with 5 % native polyacrylamide gel electrophoresis (PAGE). A sample of 2 µl of reconstitution product was loaded on the gel (37.5:1 acrylamide:bis (30 %, BioRad), 0.2 × TB, BioRad Mini-PROTEAN Tetra Cell (83 × 73 × 0.75 mm, figures 4.3 and 4.4) or Amersham Bioscience Hoefer SE 400 vertical gel slab unit (140 × 140 × 1.5 mm, figures 4.7 and 4.8)). The gel was run at 300 V at 7 °C for 90-120 minutes (figures 4.3 and 4.4) or at 200 V on ice for 30 minutes (other figures) to separate nucleosomes from free DNA. The fluorescence was imaged with a gel imager (Typhoon 9400, GE). Red: excitation at 633 nm, emission detected at 670 nm; Green: excitation at 532 nm, emission detected at 580 nm; FRET: excitation at 532 nm, emission detected at 670 nm (all 30 nm bandpass). Gel images were analyzed with ImageJ software to determine the relative intensities of the bands. Raw FRET efficiencies in the gel were calculated using equation 4.2 (see section 4.2.7). Gels with unlabeled nucleosomes were post-stained with ethidium bromide and imaged with an UV imager.

4.2.5 spFRET sample preparation

Nucleosomes in solution were diluted 100-300 × in a buffer containing 10 mM Tris.HCl pH 8.0, 0.1 mg/ml BSA, 0.03 % Nonidet-P40, 2 mM trolox, and 50 mM NaCl. The final concentration of labeled nucleosomes was 50-100 pM, estimated from the measured burst rate in the single-molecule experiments. Unlabeled nucleosomes were added in a 5-10 × excess to fluorescently labeled nucleosomes, both with the same histone composition. We used non-stick tubes (Ambion) for nucleosome dilutions. Microscope slides (#1.5, Menzel) were cleaned and passivated by coating with star PEG [64] as described in [34] to minimize interactions with the surface. We placed a 2-well culture insert (Ibidi), cleaned by sonication in ethanol, on the slide to get two confined chambers to prevent spreading of the sample over the entire slide. With the insert, two samples could be mounted on the setup simultaneously. First, we loaded 25 µl of

buffer containing only unlabeled nucleosomes in the wells. After incubating 5-10 minutes, we added 25 µl of buffer with both unlabeled nucleosomes and twice the final concentration labeled nucleosomes. Samples and slides were equilibrated on ice or at room temperature (depending on the measurement temperature) before mounting.

Nucleosomes in gel were imaged at single-molecule concentration by excising the nucleosome band from the gel. The gel slice was placed on an (untreated) glass cover slide. A drop of 30 µl buffer (10 mM Tris.HCl pH 8.0, 2 mM trolox) was used to match the refractive index of the gel and to prevent drying of the gel during the experiment.

After mounting or after changing the temperature of the setup, we allowed 30 minutes for temperature equilibration. To verify that this is sufficient, we monitored the photon statistics during the equilibration, which stabilized after 10-15 minutes.

4.2.6 Single-molecule fluorescence microscopy

Single molecules were imaged with a home-built confocal microscope as described in chapter 2 and [42]. Excitation sources at 515 nm and 636 nm were alternated at 20 kHz. The beams were focussed 25 µm (for in-solution measurements) or 50 µm (for in-gel measurements) above the glass-buffer interface. The excitation power was 12 µW for 515 nm excitation, and 8 µW for 636 nm excitation. The experiment was kept at a specific temperature by circulating thermostated water through a heating device mounted on the objective and the sample stage. In a typical experiment, data was collected for 30 minutes (unless stated otherwise) in which 2000-10000 bursts of fluorescence were detected.

4.2.7 Single-molecule data analysis

Bursts of fluorescence were detected using the method described in [66]. A burst was assigned if a minimum of 50 photons arrived subsequently, with a maximum interphoton time of 100 µs. Photon arrival times in the donor (D) and acceptor (A) channel were sorted according to excitation wavelength, resulting in four photon streams: D-emission upon D-excitation: $I_{D_{ex}}^{D_{em}}$, A-emission upon D-excitation: $I_{D_{ex}}^{A_{em}}$, D-emission upon A-excitation: $I_{A_{ex}}^{D_{em}}$, A-emission upon A-excitation: $I_{A_{ex}}^{A_{em}}$.

For each burst, we estimated the FRET efficiency by the sensitized-acceptor emission method [69, 70]. Following the definitions as proposed and discussed in detail by Lee et al. [31], we calculated the raw FRET efficiency E_{PR}^{raw} and label stoichiometry S^{raw} from the total number of photons in the burst for the different photon streams:

$$E_{PR}^{raw} = \frac{I_{D_{ex}}^{A_{em}}}{I_{D_{ex}}^{A_{em}} + I_{D_{ex}}^{D_{em}}} \tag{4.2}$$

$$S^{raw} = \frac{I_{D_{ex}}^{A_{em}} + I_{D_{ex}}^{D_{em}}}{I_{D_{ex}}^{A_{em}} + I_{D_{ex}}^{D_{em}} + I_{A_{ex}}^{A_{em}}} \tag{4.3}$$

Doubly labeled nucleosomes were selected for further analysis by taking only bursts with $0.2 < S^{raw} < 0.8$. Histograms of the FRET efficiencies of these bursts show the distribution of FRET efficiencies in the sample. Histograms were normalized to a total area below the curve of 1 to allow comparison of different measurements. The fraction without FRET (representing open or (partly) dissociated nucleosomes) was determined by taking the area below the histogram for $E_{PR}^{raw} < 0.3$.

4.3 Results

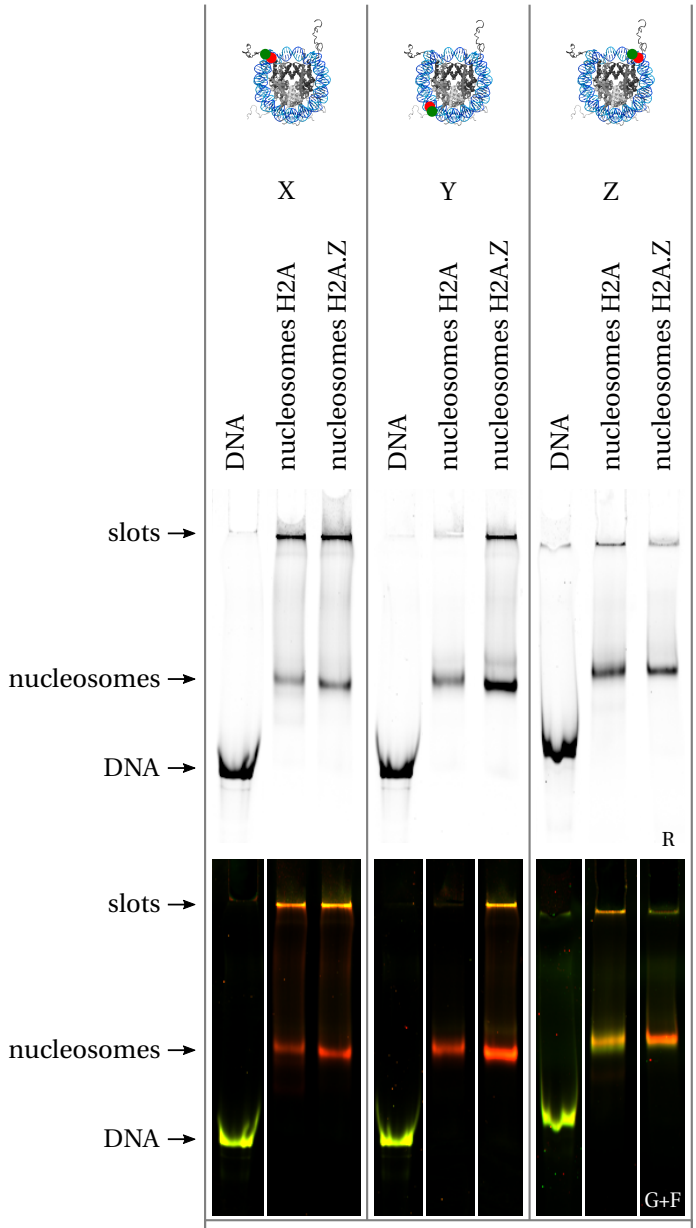
4.3.1 Gel electrophoresis reveals near 100 % reconstitution yield

Native gel electrophoresis separates free DNA from nucleosomes. Fluorescence images of polyacrylamide gels with reconstitution products, as in figure 4.3, show that the reconstitution efficiency is near 100 %. Besides a band shift of reconstituted nucleosomes with respect to bare DNA, fully reconstituted nucleosomes can also be distinguished based on their FRET efficiency, which is apparent in the fluorescence gel images as well. From observing the gel, some differences between H2A- and H2A.Z-containing nucleosomes already become clear:

H2A-containing nucleosomes run slightly higher than H2A.Z-containing nucleosomes, due to their weight and charge difference (for Arabidopsis Thaliana: 13.8 kDa and +8.4 for H2A, 14.3 kDa and +12.5 for H2A.Z, at pH=8.0 [125, 126]), possibly in combination with conformational differences like DNA breathing.

Conformational differences should be reflected in FRET differences. Indeed, H2A-containing nucleosomes show slightly less FRET than H2A.Z-containing nucleosomes, indicating a more compact conformation for H2A.Z-containing nucleosomes. The

Figure 4.3 ***(facing page)*** – Fluorescence images of 5 % polyacrylamide gels with reconstituted mononucleosomes. For every construct (X/Y/Z), a compilation of sections from the same gel is displayed (indicated by white space between the lanes). Top (R): acceptor fluorescence upon direct acceptor excitation. Bottom (G+F): false color overlay of donor and acceptor (FRET) fluorescence upon donor excitation. Reconstituted nucleosomes show FRET, in contrast to bare DNA. On the very bottom, the relative intensities of the bands in each lane from the direct acceptor excitation image are displayed, providing a measure for the relative concentrations of the different components (free DNA, nucleosomes, aggregates) present in the sample. Percentages are approximate within a few percent. Numbers corresponding to mononucleosome bands are highlighted, and the FRET efficiency calculated from the green and FRET intensities of the bands is indicated in the bottom row.



	relative intensities in %:								
slot	1	52	56	1	10	30	4	16	17
nucleosomes		46	42		87	68		80	80
DNA	99	2	2	99	3	2	96	4	3
E_{PR}^{raw}		.27	.28		.28	.35		.11	.16

FRET efficiency difference for the nucleosomes with the labels at position Y indicates that conformational differences between H2A and H2A.Z-containing nucleosomes in this gel are larger than the breathing of the first 30 bp of the nucleosomal DNA only [127].

While the fluorescent label positions X and Z are both at the second bp from the nucleosome exit, Z nucleosomes show significantly less FRET. Also, the nucleosome band has a small side band, that shows no FRET. We attribute this asymmetry to the non-palindromic 601 sequence, the only asymmetric component in the nucleosome constructs. Either both sides are not equally stable wrapped, or positioning is favored towards one of the DNA ends.

There is a significant amount of material left in the slots. Aggregates were however no problem for single-molecule experiments. Reconstitutions were spun down before the single-molecule measurements, and no signs of aggregates were present in the single-molecule data.

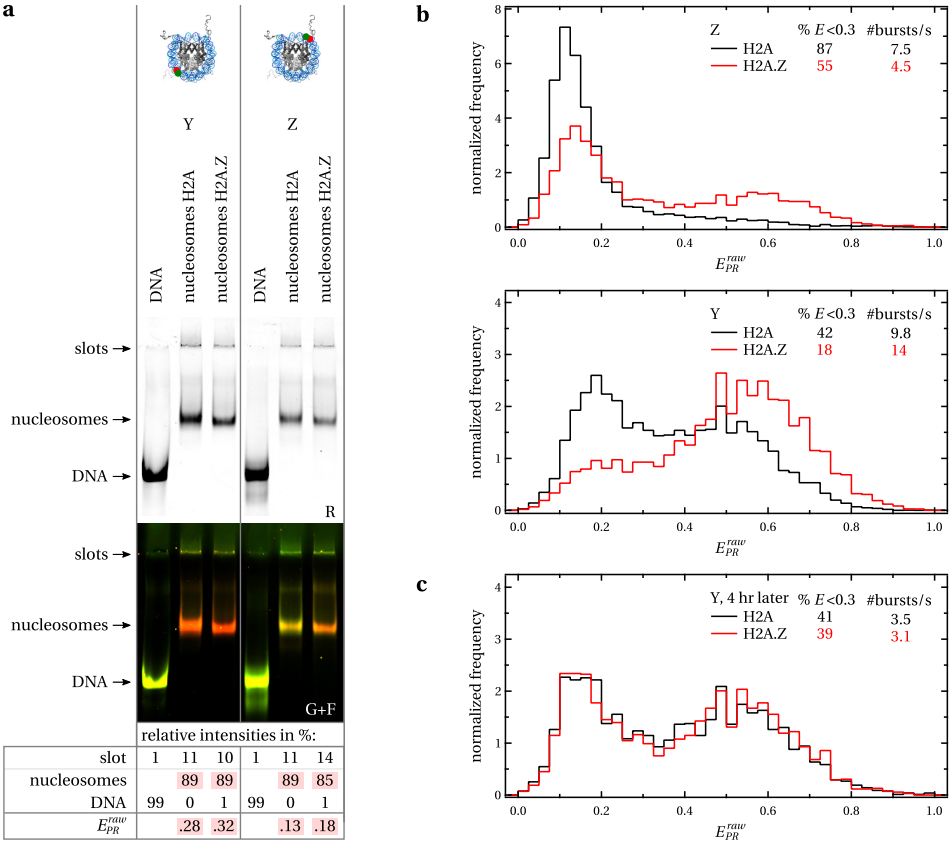
4.3.2 spFRET in gel shows H2A.Z-nucleosomes are more stable

Making use of the separating power of gel electrophoresis, we performed single-molecule FRET experiments inside the nucleosome band in a 5 % polyacrylamide gel. Figure 4.4a shows the fluorescence image of the gel used for these single-molecule experiments. The FRET efficiencies in the gel are reproducible within ~3 %. Figure 4.4b shows the FRET efficiency distributions of the nucleosome bands measured within two hours after gel imaging. Nucleosomes with labels at position Y show significantly more FRET than nucleosomes with labels at position Z. For both label positions, H2A.Z-containing nucleosomes show a larger high-FRET population, in accordance with the higher FRET efficiency measured from the gel image.

The same nucleosome bands were measured again four hours later. In these four hours, the FRET distribution of H2A-containing nucleosomes has hardly changed, still ~40 % of the nucleosomes shows no FRET. The fraction without FRET for H2A.Z-containing nucleosomes, on the other hand, has doubled from ~20 to ~40 % to resemble the distribution of H2A-containing nucleosomes. The nucleosomes appear to dissociate inside the gel at a timescale of a few hours. This dissociation occurs more slowly in H2A.Z-containing nucleosomes, which apparently are more stable than H2A-containing nucleosomes. The dissociation seems to reach a steady state after 2 hours for H2A-containing nucleosomes and 6 hours for H2A.Z-containing nucleosomes.

4.3.3 FRET distributions are temperature independent

Since the nucleosomes used in this study dissociate inside a polyacrylamide gel within a few hours, we proceeded with single-molecule experiments in solution. This was



	relative intensities in %:					
slot	1	11	10	1	11	14
nucleosomes		89	89		89	85
DNA	99	0	1	99	0	1
E_{PR}^{raw}		.28	.32		.13	.18

Figure 4.4 – Single-molecule FRET experiments inside a polyacrylamide gel. **a:** Fluorescence images of a 5 % polyacrylamide gel with reconstituted nucleosomes with labels at positions Y or Z. Top (R): acceptor fluorescence upon direct acceptor excitation. Bottom (G+F): false color overlay of donor and acceptor (FRET) fluorescence upon donor excitation. The nucleosome bands are cut from the gel right after imaging and placed on the single-molecule fluorescence microscope. **b:** FRET efficiency distributions of nucleosomes in the gel showed in a. The fraction without FRET is much larger for Z nucleosomes than for Y nucleosomes. For both Z and Y nucleosomes, the H2A-containing nucleosomes show a larger population without FRET than the H2A.Z-containing nucleosomes. **c:** Same bands measured again 4 hours later. The fraction without FRET for H2A.Z-containing nucleosomes has increased to resemble the distribution of H2A-containing nucleosomes. Single-molecule data was collected for 15 minutes.

possible because the reconstitution yield was close to 100 %. In contrast to our observations inside the gel, on passivated slides and with optimal buffer conditions the fraction of nucleosomes without FRET remained relatively constant over more than six hours (figure 4.5).

A sample of H2A- and a sample of H2A.Z-containing nucleosomes in buffer were placed simultaneously on the single-molecule fluorescence microscope and measured subsequently at 7, 22, 37 and again at 7 °C. The result is shown in figure 4.5. There is a slight increase in the fraction of nucleosomes without FRET during the course of the measurement. The fraction size did not return to its initial value when resetting the temperature to 7 °C, indicating that the small change in distribution is temperature-independent and rather reflects nucleosome instability over time.

The single-molecule data of nucleosomes with labels at position X suggest small differences in stability between H2A- and H2A.Z-containing nucleosomes. H2A-containing nucleosomes show on average a slightly larger population without FRET, and also more variation in fraction size. The FRET distribution of H2A.Z-containing nucleosomes is more narrow and shows more intermediate values. Based on the almost equal number of bursts per second, this is not a concentration-dependent artifact.

4.3.4 spFRET measurements on nucleosomes depend critically on slide passivation

We repeated the experiments for nucleosomes with labels at position X described in section 4.3.3 and shown in figure 4.5b after approximately one and two weeks. During this period, the reconstitutions were stored at 4 °C. From these stored reconstitutions, a new sample was diluted on the day of measurement. The fraction of nucleosomes without FRET increased for both H2A- and H2A.Z-containing nucleosomes in a linear fashion, which extrapolates to a minimum value at the date of slide passivation (see figure 4.6). This observation suggests that the increase of nucleosomes without FRET is caused by a decrease in starPEG-coated slide quality. A decrease in quality of the starPEG coating after a few days is expected [64, 65]. While we did not observe an effect of the age of starPEG coated slides on single-molecule FRET experiments on nucleosomes with histones from chicken erythrocytes or recombinant Xenopus Leavis histones, the Arabidopsis Thaliana histones used in this study are apparently more susceptible to surface conditions. FRET-loss due to decreased surface quality is an undesired artifact, but it also enhances the initially small difference between H2A- and H2A.Z-containing nucleosomes, where H2A.Z-containing nucleosomes appear less susceptible to destabilization by surface interactions than H2A-containing nucleosomes.

The fact that the fraction without FRET for Y nucleosomes is after nine days still

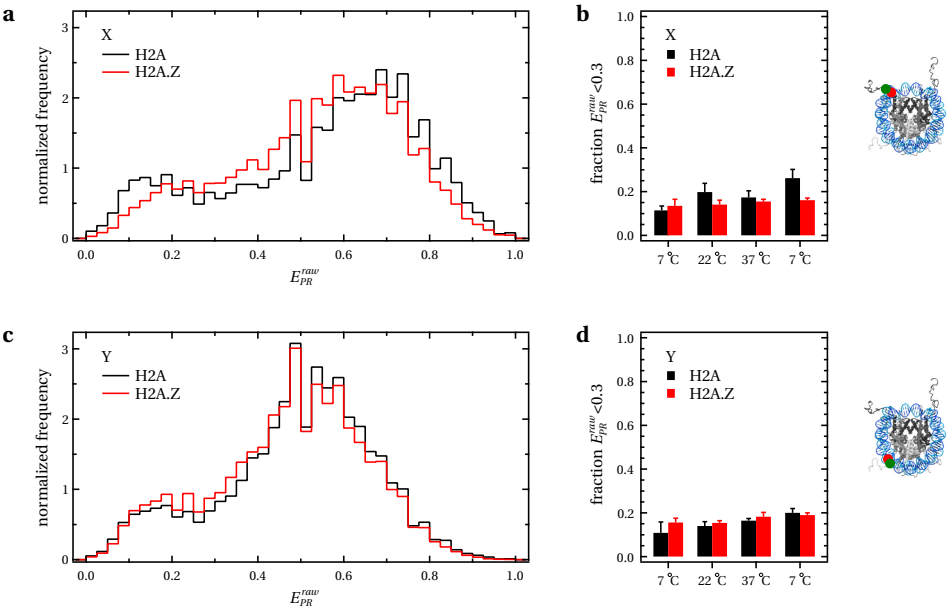


Figure 4.5 – FRET efficiency distributions at different temperatures of H2A- and H2A.Z-containing nucleosomes in solution with labels at position X (a, b) or at position Y (c, d). A sample of H2A- and a sample of H2A.Z-containing nucleosomes in buffer were placed simultaneously on the single-molecule fluorescence microscope and measured subsequently at 7, 22, 37 and again 7 °C. **a, c:** Combined histograms of the measurements at different temperatures. **b, d:** Fraction of bursts with FRET efficiency below 0.3 at different temperatures. Error bars represent standard deviations of the values for 100 s intervals of the 1800 s measurements.

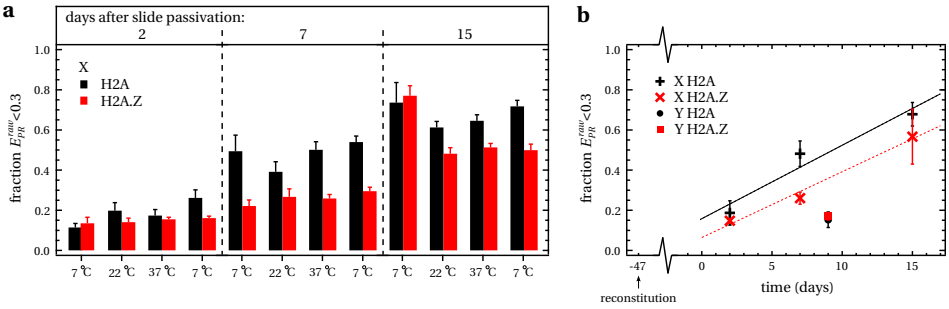


Figure 4.6 – Loss of FRET in time. **a:** Fraction of bursts with FRET efficiency below 0.3 at different temperatures. The experiment is repeated twice after several days, in which the fraction without FRET increased for both H2A- and H2A.Z-containing nucleosomes. Error bars represent standard deviations of the values for 100 s intervals of the 1800 s measurements. **b:** Fraction of bursts with FRET efficiency below 0.3 as a function of number of days after slide passivation. Data points represent averages of the four measurements at different temperatures shown in a, error bars the standard deviations, dashed lines linear fits. Reconstitution occurred 48 (H2A) or 46 (H2A.Z) days before slide passivation.

much lower than for X nucleosomes, suggests that the loss of FRET in X nucleosomes is not caused by full dissociation into free DNA and histones.

4.3.5 H2A.Z nucleosomes are more stable during extended periods of storage

To test whether nucleosomes destabilized during storage or measurement, we performed gel electrophoresis with nucleosomes after 76 days of storage at 4 °C (figure 4.7). Stored nucleosomes still show near 100 % reconstitution yield. The amount of free DNA is negligible; besides nucleosome bands there is only material in the slots. While bands with H2A.Z-containing nucleosomes are still nice and sharp, the H2A-containing nucleosome bands appear different: more smeared and shifted upwards. Whether this degradation of H2A-containing nucleosomes has happened during storage or is an artifact caused by the gel, is not entirely clear, but it shows again a different behavior for H2A- and H2A.Z-containing nucleosomes, where H2A.Z-containing nucleosomes are more stable.

We also loaded nucleosome samples recovered from the setup after the last single-molecule experiment (15 days after slide passivation, figure 4.6) on the gel. The samples that were run on the gel after single-molecule experiments show significant amounts of free DNA, indicating full dissociation of (part of the) nucleosomes. The nucleosomes that were not fully dissociated show the same gel pattern as the stored nucleosomes. Whether the dissociation happened during the single-molecule experi-

ment or during gel electrophoresis is not clear, because the reduced nucleosome concentration of single-molecule samples, despite loading of the maximum amount of material in the gel, makes nucleosomes unstable in gel electrophoresis conditions, as discussed in chapter 2.

4.3.6 Xenopus H2A.Z nucleosomes also more stable

In addition to experiments with nucleosomes reconstituted with Arabidopsis Thaliana (AT) histones, we performed experiments with nucleosomes reconstituted with Xenopus Leavis (XL) histone octamers. Xenopus Leavis histones are commonly used in *in vitro* studies. The XL nucleosomes appeared less sensitive to disrupting conditions like eppendorf tube or glass surfaces and gel electrophoresis. Despite the limited dataset for XL nucleosomes, and the multitude of factors that influence nucleosome stability as described in chapter 2, our data show that the difference between H2A- and H2A.Z XL nucleosomes follows the same trend as observed for AT nucleosomes.

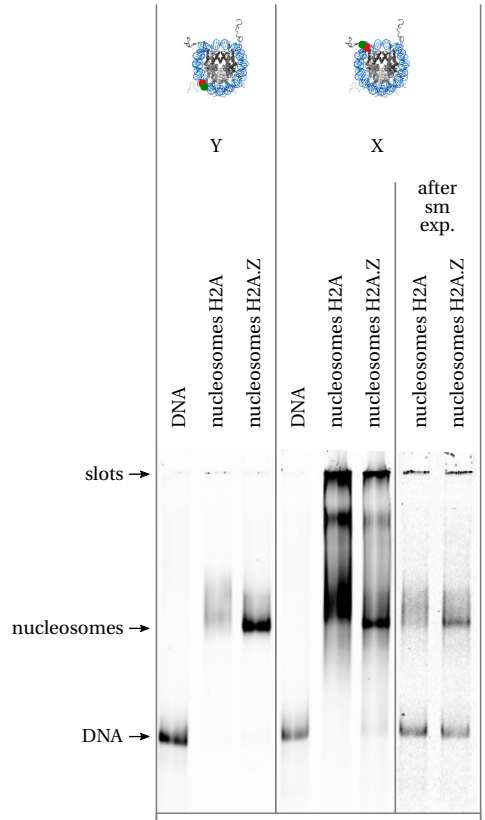
We obtained fully reconstituted nucleosomes with XL histones containing either H2A or H2A.Z, as verified by gel electrophoresis (see figure 4.8a). The XL nucleosomes run faster than AT nucleosomes due to their weight and charge difference (for Xenopus Leavis: 14.0 kDa and +15.4 for H2A, 13.5 kDa and +12.5 for H2A.Z; for Arabidopsis Thaliana: 13.8 kDa and +8.4 for H2A, 14.3 kDa and +12.5 for H2A.Z (pH=8.0) [125, 126]). Also, conformational differences can give rise to variations in running speed: a more compact structure will experience less gel resistance and hence run faster. The FRET efficiency of XL nucleoosmes is much higher than of AT nucleosomes, reflecting a more compact conformation and/or higher stability.

Three independent single-molecule measurements inside a polyacrylamide gel reveal FRET distributions of H2A- and H2A.Z-containing nucleosomes. Although the fraction of nucleosomes without FRET shows a correlation with the local nucleosome concentration (see figure 4.8b), these measurements suggest that H2A.Z-containing nucleosomes are more stable than H2A-containing nucleosomes for XL as well as for AT.

FRET efficiency distributions of XL-H2A.Z-containing nucleosomes in solution at 7, 22, and 37 °C are shown in figure 4.8c. The FRET efficiency of the high FRET population is higher than for AT nucleosomes, indicating a more compact conformation for XL nucleosomes. Though the same sample is measured at increasing temperatures, the distribution is constant and is thus temperature-independent.

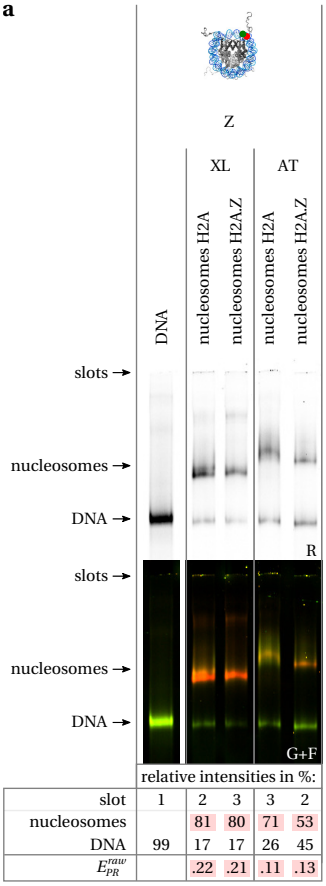
4.4 Discussion

This is the first time that temperature dependent stability of H2A.Z-containing nucleosomes is measured at the single-molecule level. We have reconstituted nucleosomes

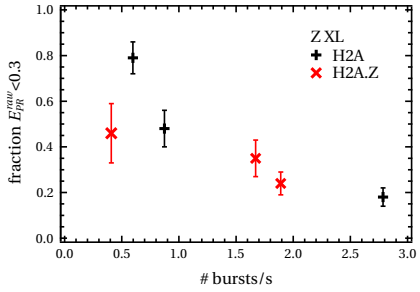


	relative intensities in %:							
slot	1	3	1	2	49	52	13	23
nucleosomes		96	98		50	45	54	56
DNA	99	1	1	98	1	3	33	21

Figure 4.7 – Fluorescence image (direct acceptor excitation) of polyacrylamide gel with nucleosomes, 76 days after reconstitution. The two lanes on the right show the sample that was used for the last single-molecule experiments (15 days after slide passivation). The contrast of these lanes is increased in order to make the 100 times less concentrated material visible. The bands of H2A-containing nucleosomes are smeared compared to gel images made shortly after reconstitution, whereas the bands of H2A.Z-containing nucleosomes are still sharp. Nucleosome samples that have been measured in the single-molecule fluorescence microscope contain a significant amount of free DNA, indicating full dissociation.



relative intensities in %:					
slot	1	2	3	3	2
nucleosomes		81	80	71	53
DNA	99	17	17	26	45
E_{PR}^{raw}		.22	.21	.11	.13



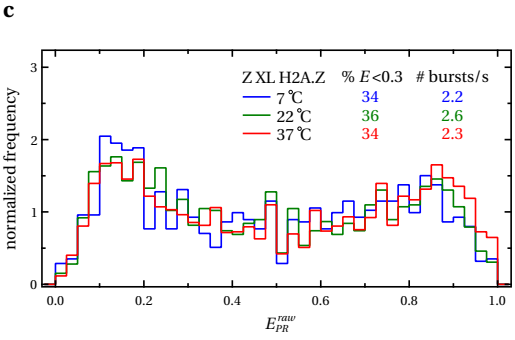


Figure 4.8 – Nucleosomes reconstituted with Xenopus Leavis histones. **a:** Fluorescence images of a 5 % polyacrylamide gel with reconstituted nucleosomes with labels at position Z. Nucleosomes with either Xenopus Leavis (XL) or Arabidopsis Thaliana (AT) histones. Displayed is a compilation of two sections from the same gel, indicated by whitespace between the lanes. Top (R): acceptor fluorescence upon direct acceptor excitation. Bottom (G+F): false color overlay of donor and acceptor (FRET) fluorescence upon donor excitation. **b:** Fraction of nucleosomes without FRET as a function of number of bursts per second for three independent single-molecule measurements of XL nucleosomes inside a polyacrylamide gel at 22 °C. Error bars represent standard deviations of the values for 200 s intervals of the 1800 s measurements. **c:** FRET efficiency distributions of XL nucleosomes in solution. The same sample is measured at three different temperatures. Single-molecule data was collected for 10 minutes.

with Arabidopsis Thaliana histone octamers containing either H2A or H2A.Z with practically 100 % reconstitution yield. We have investigated their FRET efficiencies in a polyacrylamide gel, and FRET distributions with spFRET inside the gel and in solution at different ambient temperatures.

The nucleosomes containing Arabidopsis Thaliana histone octamers used in this study were very sensitive to disrupting conditions. At single-molecule concentrations, the nucleosomes lose FRET in gel, upon exposure to surface, and in suboptimal buffer conditions. Nevertheless, H2A.Z nucleosomes consistently have a larger high-FRET population and are less susceptible to disrupting conditions than H2A-containing nucleosomes. The ensemble FRET (in gel) of H2A-containing nucleosomes is lower (figures 4.3 and 4.4a), they lose FRET more rapidly in gel (figure 4.4b), and are more susceptible to surface interactions and degradation during storage than H2A.Z-containing nucleosomes (figures 4.6 and 4.7). Not only Arabidopsis Thaliana, but also Xenopus Leavis histone octamers show that H2A.Z-containing nucleosomes are slightly more stable (figure 4.8).

FRET can be lost due to increased DNA breathing, dimer loss, or full dissociation into free DNA and histones, schematically depicted in figure 4.9. Comparison of the data for nucleosomes with labels at position X, Y or Z gives some insight into the possible mechanisms causing increase of the population without FRET. Nucleosomes with labels at position X or Z lose FRET for DNA unwrapping of 30 bp or more or (irreversible) dimer loss. Nucleosomes with labels at position Y lose FRET only for unwrapping of more than 60 bp, possibly accompanied by dimer loss. All constructs lose FRET upon complete dissociation.

While the ensemble FRET efficiency in gel is the same for H2A- and H2A.Z-containing nucleosomes with labels at position X and H2A-containing nucleosomes at position Y, the H2A.Z-containing nucleosomes with labels at position Y have a higher ensemble FRET efficiency. This indicates that the population of nucleosomes where more than 60 bp is unwrapped is larger in H2A-containing nucleosomes than in H2A.Z-containing nucleosomes. Z-labeled nucleosomes show a much lower FRET efficiency than X-labeled nucleosomes. We attribute this asymmetry to the non-palindromic 601 sequence, the only asymmetric component in the nucleosome constructs. Asymmetric unwrapping of 601-based nucleosomes has been reported before [128]. Asymmetry of the 601 sequence is not always found in spFRET experiments with single nucleosomes. For example, the data from Koopmans et al. [42] show equal distributions X- and Z-labeled nucleosomes with in-gel spFRET. These experiments were performed with histone octamers purified from chicken erythrocytes, from which the energy landscape for DNA unwrapping could differ from Arabidopsis Thaliana histones such that any differences caused by 601 asymmetry are not detectable.

The high-FRET fraction of both H2A- and H2A.Z-containing nucleosomes decreases inside a polyacrylamide gel within a few hours. Both H2A- and H2A.Z-con-

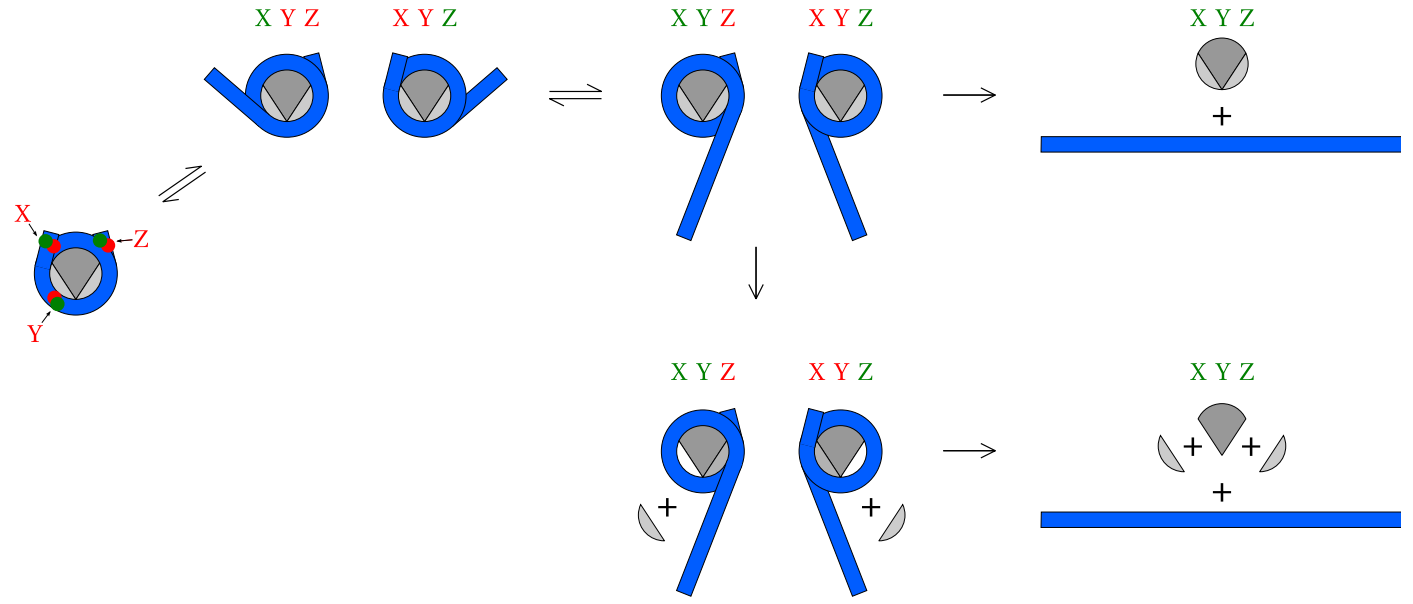


Figure 4.9 – Schematic overview of possible nucleosome conformations. Blue: DNA; dark grey: $(H3/H4)_2$ tetramer; light grey: H2A/H2B dimer. X, Y and Z indicate the positions of the fluorescent labels. Red: high FRET; green: no FRET.

taining nucleosomes reach the same distribution between FRET and no FRET, but H2A.Z-containing nucleosomes reach this distribution more slowly. This suggests that (at least) two populations are present, and the observed distribution is a superposition of the distributions of these two populations. The first population is constant over time and exists of intact nucleosomes with a certain unwrapping probability. The second population changes from high FRET to complete loss of FRET in a few hours, and exists of nucleosomes that dissociate irreversibly. This last population might consist of nucleosomes that initially are not properly folded and therefore lose their dimers or completely dissociate more easily, depending on whether they contain H2A or H2A.Z.

The nucleosomes used in this spFRET study lose their FRET mainly due to interactions with the microscope slide surface. The fraction of X-labeled nucleosomes without FRET increases from about 20 to 70 % within 15 days after slide passivation. This means that the quality of the slides diminishes rapidly. It is also clear that H2A.Z-containing nucleosomes suffer less from the surface interactions than H2A-containing nucleosomes. Comparison with Y-labeled nucleosomes shows however that while the no-FRET population of X-labeled nucleosomes is about doubled in 9 days, the no-FRET population of Y-labeled nucleosomes is still below 20 % (figure 4.6). Therefore, the loss of FRET in X-labeled nucleosomes is due to an increased DNA unwrapping probability or possibly (irreversible) dimer loss and not due to full dissociation into free DNA and histones.

The experimentally found differences between H2A- and H2A.Z-containing nucleosomes are of two kinds: decreased unwrapping probability and decreased dissociation probability for H2A.Z-containing nucleosomes. The decreased unwrapping probability is in agreement with the expectation based on the substitution of a Threonine in H2A to a Lysine in H2A.Z at the second minor groove of the nucleosomal DNA. Threonine is an uncharged polar amino-acid, while lysine is positively charged, resulting in an electrostatically less strong DNA-histone binding at the second contact point for H2A. A similar increase in unwrapping probability at the first DNA-histone contact point has been seen for nucleosomes where a lysine at H3 K56 has been acetylated, which also removes a positive charge [50] (see chapter 3 in this thesis). The structural difference between H2A- and H2A.Z-containing octamers at the H2A(Z)/H3 interface is probably the reason for a decreased probability for dimer loss in H2A.Z-containing nucleosomes. The third structural difference between H2A- and H2A.Z-containing nucleosomes is the H2A(Z)-H2A(Z) interface. It is possible that the H2A(Z)-H2A(Z) interactions at this interface are stronger in H2A.Z-containing nucleosomes, thus decreasing the probability for loss of one of the dimers.

The thermodynamically expected temperature effect of a few percent with 30 ° temperature change lies within the measurement uncertainty, and may be too small to have consequences for gene manipulation. We have found no influence of temperature on the unwrapping or dissociation probability of H2A.Z-containing nucleosomes

between 7 and 37 °C. Thus we exclude H2A.Z as a direct temperature sensor responsible for ambient-temperature dependent changes in phenotype seen in Arabidopsis Thaliana [124]. H2A.Z could still be indirectly involved in the ambient temperature sensing pathway via for example remodeling by an ambient temperature dependent factor.

4.5 Conclusions

We have directly measured the temperature dependent stability of H2A- and H2A.Z-containing nucleosomes at the single-molecule level. Although the nucleosomes containing Arabidopsis Thaliana histone octamers were very susceptible to dissociating conditions like dilution to single-molecule concentrations and the presence of surfaces, we clearly showed that H2A- and H2A.Z-containing nucleosomes behave differently. H2A.Z-containing nucleosomes are more stable and less susceptible to dilution- or surface-induced dissociation. The unwrapping probability is constant in the ambient temperature range. Thus alternative explanations need to be explored to explain the role of H2A.Z in temperature sensing *in vivo*.

Acknowledgements

We thank Prof. Dr. Jürgen Groll (Universität Würzburg) for providing the NCO-starPEG material. This work is part of the research program of the Foundation for Fundamental Research on Matter (FOM), which is part of the Netherlands Organization for Scientific Research (NWO).