“H2A.Z.2.2 is an alternatively spliced histone H2A.Z variant that causes severe nucleosome destabilization” by Bönisch et al.

SUPPLEMENTARY MATERIALS AND METHODS

Cell culture, transfection, FACS analysis and cloning

Cell lines were grown in DMEM medium (PAA) supplemented with 10% FCS (Sigma) and 1% penicillin/streptomycin or 50 µg/ml gentamicin (C127 cells) at 37°C and 5% CO_2. The following human cell lines were used in this study: HEK293 (embryonic kidney), HeLa (cervix carcinoma), HeLa Kyoto (cervix carcinoma), U2OS (osteosarcoma), hFB (fibroblasts), SK-N-SH (neuroblastoma), and the following mouse cell lines were used: NIH3T3 and C127. Human cell lines were transfected using FuGene HD (Roche Applied Science) and mouse C127 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Z.2.1 and Z.2.2 were cloned from HeLa cDNA into pT7blue3 (Novagen). For expression in human cells, Z.2.1, Z.2.2, Bbd and deletion or domain swap mutants were cloned into the pEGFP-C1 vector (Clontech) to generate N-terminally tagged proteins. For simplicity eGFP-tagged constructs are referred to as GFP-tagged throughout the text. Plasmids coding for GFP-H2A (H2A type 1, NP_003501.1) and GFP-Bbd (H2A.Bbd type 2/3, NP_542451.1) were kindly provided by Emily Bernstein. The plasmid coding for GFP-Z.1 was a gift from Sachihiro Matsunaga, the H1.0-GFP construct was kindly given by M.J. Hendzel (1) and the GFP-HP1α construct was provided by T. Misteli (2). Stable cell lines were selected with 600 µg/ml G418 (PAA) and individual cell clones sorted by using a FACSAria machine (Becton Dickinson). Expression levels of GFP-proteins were quantified by using a FACSCanto machine (Becton Dickinson). For expression in *E. coli*, Z.2.1, Z.2.2, Bbd and domain swap mutants were cloned into the pET-21a(+) vector (Novagen). Plasmids for expression of recombinant human H2A, H2B, H3 and H4 were kindly provided by Robert Schneider. Cloning and PCR amplification accuracy was verified by sequencing (MWG).

Histone extraction, RP-HPLC purification, cellular fractionation, MNase digestion, sucrose gradient fractionation and salt stability experiments

Acid extraction of histones was done as previously described (3). Histones from HEK293 cells were separated by RP-HPLC as previously described (4). Fractions were dried under vacuum and stored at -20°C.

Fractionation experiments were carried out as described previously (5) with minor changes. Briefly, 2 x 10^7 cells were resuspended in 1 ml buffer A supplemented with 0.1% NP40, incubated for 10 min and collected by centrifugation. The pellet was washed once with buffer A and incubated in buffer B for 30 min. The resulting chromatin pellet was washed once with buffer B and resuspended in SDS loading buffer (chromatin fraction). After sonification (Diagenode Bioruptor) and denaturation, nucleic acids were degraded by benzonase (VWR) treatment. All centrifugations were performed at 6.500 *g* for 5 min at 4°C except the final one (20.000 *g* for 20 min at 4°C). The soluble fraction was obtained by combining the supernatants of all centrifugations (incl. washing steps). Proteins were pelleted as described (6) and resuspended in SDS loading buffer. Identical cell equivalents of soluble and chromatin fractions were analyzed by immunoblotting.

For MNase digestion, chromatin was prepared from 5 x 10^7 HK cells stably expressing GFP-Z.2.2 as described above, resuspended in 500 µl EX100 (10 mM HEPES pH 7.6, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10 % (v/v) glycerol, 1 mM DTT, 1 x Roche protease inhibitors) and digested with 1.5 U MNase (Sigma) for 20 min at 26°C. The reaction was stopped by addition of EGTA to a final concentration of 10 mM and centrifuged (3.200 *g* for 20 min at 4°C). A gradient of 5%-35% sucrose in EX100 was prepared using a Gradient Master (Biocomp), 400 µl of the supernatant after MNase digest were loaded on top and ultracentrifuged (30.000 rpm for 18 h at 4°C using a Beckmann SW41 rotor). Afterwards, 500 µl fractions were manually taken from top and analyzed for DNA content after RNase A and Proteinase K digest by agarose gel electrophoresis. Purity of mononucleosome containing fractions was verified using DNA 1000 reagents (Agilent Technologies) with the 2100 Bioanalyzer (Agilent Technologies). Pure mononucleosome fractions were combined, concentrated by TCA precipitation and analyzed by immunoblot.
For salt stability assays (7), chromatin was prepared in the same manner as in fractionation experiments. After washing with buffer B, chromatin was incubated with incubation buffer (10 mM TrisHCl pH 7.5, 1 mM DTT, 1 mM EDTA, 1x protease inhibitors (Roche Applied Science), 0.1% Triton X-100) containing different salt concentrations ranging from 50 mM to 600 mM NaCl for 1 h at room temperature. Chromatin was pelleted, solubilized in the same manner as in fractionation experiments and analyzed by immunoblotting.

FRAP and exponential fitting

FRAP experiments were performed using an UltraVIEW VoX spinning disk microscope system (PerkinElmer) equipped with a heated environmental chamber and CO_2 perfusion as previously described (4) with the following changes. To determine short-term recovery kinetics, 2D time series were recorded for 2 min to 1 h with time intervals between 0.1 s and 1 min depending on the recovery kinetics of the construct. For long-term recovery kinetics, image z-stacks were recorded with intervals between 1 min and 5 min. The central 3-5 image planes were average projected for quantitative evaluation. To correct for cell-to-cell differences in bleaching depth, the normalized mean intensity values of the first postbleach values were linearly interpolated to determine an estimated value for the time point t = 0. This value was subtracted from all mean fluorescence values after previous double normalization to correct for potential gain or loss of total fluorescence, e.g. by import and bleaching-by-acquisition.
The normalized FRAP curves were further evaluated and quantified by a commonly used fitting procedure (8): A sum of exponential time dependencies can be used to describe the intensity I(t) of the fluorescence recovery.

$$I(t) = I_0 * (1 - \sum_i A_i * e^{-\alpha_i t})$$

To discriminate at least two different species within our sample, we restricted our self to the bi-exponential case, where $A_1 + A_2 = 1$. Therefore the parameters α_1, α_2 and A had to be determined from the individual recovery curves by a least-square optimization algorithm. This was carried out automatically by a self-made python (www.python.org) script, which applied the *leastsq* function from the *scipy.optimize* package (www.scipy.org). Only curves with a reasonable set of resulting parameters were taken into account for the final summary, wherein the exponents α are transformed into their corresponding, more intuitive half-times of recovery.

$$T_1 = \frac{\ln 2}{\alpha_1}$$

Most of the recovery data were described adequately by this bi-exponential characteristic, manifested in a close approximation by the fitted curve. An evaluation of the goodness of the fits was therefore not necessary. However, in some experiments where GFP alone was expressed, a single exponential curve already allowed a sufficiently close approximation. Applying the bi-exponential model to these data sets leads to two almost identical exponentials, showing the invalidity of the more complex model. This categorization allowed us therefore to distinguish between two types of complexity: One type with only a single mobile species and on the other hand the case, where at least two mobile species are apparent. Extension to three mobile species did typically not result in significant improvement of the fits.

Stable isotope labeling with amino acids in cell culture (SILAC) and mass spectrometry (MS) identification of H2A.Z-specific chaperone complexes

HeLa cells expressing GFP-Z.2.1 or GFP-Z.2.2 were SILAC labeled and nuclear extracts were prepared as described before (9). Nuclear extracts were diluted in incubation buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.25% NP40) supplemented with complete protease inhibitors w/o EDTA (Roche) and 0.5 mM DTT to a concentration of 1.5 µg/µl. 400 µl solution were incubated with GFP trap (Chromotek) for 3 h at 4°C. Beads were washed 3 times with 1 ml incubation buffer, combined and eluted by boiling in loading buffer. Samples were loaded on 1D NuPAGE gels (Invitrogen), lanes cut into 8 slices, in gel digested with trypsin and desalted by stage tipping. Mass spectrometry (MS) was performed on an LTQ Orbitrap mass spectrometer as described before (10). Peptides were separated online to the mass spectrometer by using an easy nano-LC system (Proxeon Biosystems) with a 15-cm fused silica emitter with an inner diameter of 75 µm packed in house with RP ReproSil-Pur C18-AQ 3 µm resin (Dr. Maisch). Peptides were eluted with a segmented gradient from 5% to 60% B with a constant flow of 0.25 ml/min (solvent B: 80% acetonitrile, 0.5% acetic acid) over 110 min.

The MS was operated in data dependent mode. A full scan MS (m/z 300 - 1650) was acquired in the Orbitrap cell with a resolution of 60,000 at a theoretical m/z ratio of 400 after accumulation of 1,000,000 ions in the C-trap (maximum filling time of 1000 ms); the lock mass option was enabled to improve mass accuracy. The 5 most intense ions from the preview survey scan were isolated (target value of 5,000 ions at a maximum filling time of 150 ms) fragmented by collision-induced dissociation (collision energy 35 %) and measured in the ion trap concurrently to full scan acquisition in the Orbitrap. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged peptides were rejected. The dynamic exclusion list was set to a maximum of 500 entries with a maximum retention period of 90 s and a relative mass window of 5 ppm.

Raw data were analyzed using the MaxQuant software suite (11) (version 1.2.2.7) with the integrated Andromeda search engine (11) at default parameters using the IPI human database version 3.68 concatenated with a database containing common contaminants. For further analysis we removed contaminants and defined a ratio cutoff of 4 (Z.2.1 pull-down) and 0.25 (Z.2.2 pull-down). A complete list of all proteins identified is found in Supplementary Table S1.

EdU replication labeling, *in situ* extraction and fluorescence microscopy to assay cell cycle dependent GFP-Z.2.2 chromatin incorporation

C127 cells were pulse labeled for 25 min with 10 µM 5-ethynyl-2′-deoxyuridine (EdU, Baseclick) 48 h after transfection with GFP-Z.2.1 or -Z.2.2 plasmids, and immediately subjected to *in situ* extraction or fixation. For *in situ* extraction, GFP-Z.2.2 transfected cells were washed with PBS and incubated 15 sec in permeabilization buffer (0.1% Triton X-100, 150 mM NaCl in PBS). Thereafter, cells were fixed for 10 min at room temperature with 2% formaldehyde (Sigma) in PBS containing 0.1% Triton X-100. Cells expressing GFP-Z.2.1 were not *in situ* extracted and were fixed with 2% formaldehyde in PBS for 10 min at room temperature. All washing steps after fixation were performed with 0.02% Tween20 in PBS (PBST). After permeabilization with 0.2% Triton X-100 in PBS for 10 min, cells were blocked for at least 1 h in blocking buffer (2% BSA in PBST). Before EdU-detection, cells were incubated 1 h with GFP-booster (Chromotek) diluted in blocking buffer. Incorporated EdU was detected by incubating cells 30 min in 100 mM Tris-HCl pH 7, 4 mM $CuSO_4$, 20 µM azide dye Alexa Fluor 594 (Invitrogen) and 50 mM sodium ascorbate (adapted from (12)). Cells were counterstained with 200 ng/ml DAPI in PBST for 10 min and mounted on microscope slides in Vectashield mounting medium (Vector Laboratories).
Wide-field imaging was performed on a PersonalDV microscope system (Applied Precision) equipped with a 60x/1.42 PlanApo oil objective (Olympus), CoolSNAP ES2 interline CCD camera (Photometrics), Xenon illumination and appropriate filter sets. Image stacks were recorded with a z-distance of 200 nm and subjected to a constrained iterative deconvolution (enhanced ratio, 10 cycles, medium noise filtering, SoftWoRX, 3.7. imaging software package, Applied Precision).

Expression and purification of recombinant human histone proteins in *E.coli*, *in vitro* octamer and mononucleosome reconstitution and MNase digestion of recombinant mononucleosomes
Histones were expressed, purified and assembled into octamers as described (13). DNA for mononucleosome assembly was obtained from a pUC18 plasmid containing 25 repeats of the 601 nucleosome positioning sequence (14) kindly donated by Daniela Rhodes. After AvaI digestion, monomeric DNA was purified by gel electrophoresis and electroeluted using the Elutrap system (Whatman). Assembly of nucleosomes was performed by salt gradient deposition (13,15). For H2A and Z.2.1 nucleosomes, respective octamers and DNA were mixed in a 1:1 ratio; for Z.2.2 nucleosomes, Z.2.2-H2B dimers, $(H3\text{-}H4)_2$ tetramers and DNA were mixed in a 2:1:1 ratio. Assembly of histones on DNA was evaluated by EMSA using 5% native PAGE or native 1.5% agarose gels. Incubation of mononucleosomes for 1 h at 37°C (15) did not change position as evaluated by 5% native PAGE (data not shown). To analyze the histone content of nucleosomes after assembly, the corresponding band was excised from native 1.1% agarose gels, nucleosomes were electroeluted using the Elutrap system and protein content was analyzed by 18% SDS-PAGE after Benzonase treatment by Coomassie staining or immunoblot.
Equal amounts of nucleosomes (1 µg) were digested with different amounts of MNase (Sigma) for 10 min at 37 °C in MNase digest buffer (13.85 mM TrisHCl ph 7.5, 67 mM KCl, 10.75% Glycerol, 1 mM DTT, 5 mM $CaCl_2$). The reaction was stopped by addition of nine volumes of 5 mM EGTA. DNA was deproteinized by phenol/chloroform extraction, ethanol precipitated and analyzed using DNA 1000 reagents (Agilent Technologies) with the 2100 Bioanalyzer (Agilent Technologies).

Single molecule Förster resonance energy transfer (smFRET)

Single molecule burst analysis:

To gain information on salt dependent nucleosome stability single-molecule Förster resonance energy transfer (smFRET) measurements of dual labeled nucleosomes freely diffusing through the focal volume of a confocal microscope were performed. To this end a dual labeled 159 bp DNA was prepared using dye-labeled primers (IBA), a DNA template containing the 601 (14) sequence and six additional bases on each side together with dNTPs (Finnzymes) and the Phusion DNA-polymerase (Finnzymes) in a PCR reaction. Dye labels were at position 65 (donor dye, Tamra) and position 20 on the reverse strand (acceptor dye, Alexa647). A mixture of this labeled DNA and unlabeled DNA (molar ratio of 1:50) was used for mononucleosome assembly (see above).

It is well known, that ultra-low concentrations of nucleosomes are prone to become instable in typical experimental geometries (16). To minimize such effects measurements were performed using a 1:250 mixture of double labeled to unlabeled nucleosomes at a total concentration of 25 nM in commercially available TE buffer (Sigma-Aldrich, pH 7.6) containing 10 mM DTT and 0 mM, 300 mM, 400 mM, 500 mM, 600 mM and 700 mM NaCl, respectively. The samples were incubated at the respective salt concentration for 1 hour at 21°C before a drop of 20 µl was put onto cover slips (Marienfeld) for data collection. The cover slips were cleaned with 2% Hellmanex III (Hellma) and water prior to silanization for 15 min with 2% (3-aminopropyl)triethoxysilane (Sigma-Aldrich) in Acetone, and coating with 40 mg/100 µl polyethylene glycol (mPEG-SVA MW 5000, Laysan Bio Inc.) in ddH_2O for 1 h.

The confocal measurements were performed on a custom built experimental setup using pulsed interleaved excitation (PIE) (17) with lasers at 532 nm (Pico-TA-Picoquant, power before the objective 80µW) and 640 nm (LDH-D-C-640, Picoquant, power before the objective 80 µW) at a repetition rate of 26.66 MHz. The fluorescence was separated for polarization and color and detected on four avalanche-photo-diodes (green channel AQRH-14, red channel AQR-16, Perkin Elmer). Photon arrival times were recorded using four single-photon-counting-modules (Becker&Hickl SPC-150) and data was processed using custom software written in MATLAB (MathWorks). Since the anisotropy of the molecules was not of importance for this study, photons of identical wavelength but different polarization were merged into one detector channel. Data were collected for 10 min and the collected photons were sorted into three different channels, namely donor detection after direct excitation (green), acceptor detection after direct excitation (red) and acceptor detection after donor excitation (fret). Labeled complexes diffusing through the focal volume of the microscope resulted in bursts of detected photons. An all photons burst search (APBS) was applied with the criteria of detecting at least 3 photons within 500 µs with a total of at least 60 photons per burst (18). From the photon bursts, the Stoichiometry (S) and FRET Efficiency (E) were calculated (including the predetermined correction factor) according to:

$$E = \frac{GR}{GR + \gamma * GG} \text{ , and } S = \frac{GR + \gamma * GG}{GR + \gamma * GG + RR}$$

Where GR are the red photons after green excitation, RR are the red photons after red excitation, GG are the green photons after green excitation and y is a factor correcting for the different efficiencies of the red and green detection channels (19). Multi-molecular events were removed from the data as described below using $TDS < 1$ and $TDS_{red\text{-}PIE} < 0.6$ for all complexes not showing molecular dynamics. Remaining

donor-only and acceptor-only bursts were removed using a stoichiometry threshold (S = 0.15 – S = 0.55).
Due to the chosen labeling positions on the nucleosome, closed nucleosomes have the donor and acceptor dyes positioned adjacent to each other leading to a high FRET state with an efficiency of ~80% while open or incomplete nucleosomes show a very low FRET signal. The fraction of closed nucleosomes was quantified for each salt concentration by analyzing how many of the detected fluorescence bursts have $E >$ 40% (Supplementary Figure S6A). The data was normalized to the fraction of closed molecules at 0 mM NaCl to allow for a comparison of the salt dependence for the three investigated samples (H2A, Z.2.1, Z.2.2).

Removal of multi-molecular events
Nucleosomes are prone to become instable at low concentrations as well as when interacting with surfaces. Thus in order to avoid artifacts the duration of the experiment has to be minimized. To address this difficulty relatively high nucleosome concentrations were used in the experiments to ensure that the occurrence of multi-complex bursts is not negligible. For a homogeneous population with only a single FRET species this is not a problem, however if several FRET states exist, multi-molecule events of different species will alter the determined FRET values. Moreover, impurities such as complexes labeled with only donor or acceptor observed simultaneously with double-labeled complexes will also lead to changes in FRET efficiencies. However, as two independently diffusing complexes do not enter and exit the excitation volume exactly at the same time it is possible to differentiate these multi-molecular events from single molecule events and to exclude them from further analysis. Independently diffusing molecules involved in a multi-molecule event will yield different values for the mean-macro-time (i.e. the time where 50% of the respective photons have arrived) for all photons of a burst, as compared to that for the photons of one color. We therefore calculated the characteristic Time-Deviation-Signal (TDS) defined as

$$TDS = \left((D_{total} - D_{green}) + \left|T_{total} - T_{green}\right|\right) * (1-P) + \left((D_{total} - D_{fret}) + \left|T_{total} - T_{fret}\right|\right) * P * \gamma$$

(1)

where D_x is the burst duration, T_x is the mean-macro-time, y is a factor correcting for the different efficiencies of the red and green detection channels and P is the proximity ratio given by the number of photons in the burst as $P = GR/(GR+GG)$.

In eq. 1 we compute the TDS of the green and red channels simultaneously and adjust the relative value to the percentage of photons detected. In addition, multi-molecule events containing low (0%) FRET and donor only complexes (which cannot be found using eq. 1) can also be determined using a PIE setup and defining:

$$TDS_{red-PIE} = (D_{total} - D_{red}) + \left|T_{total} - T_{red}\right| \quad \textbf{(2)}$$

To demonstrate the capabilities of this analysis scheme a sample containing Z.2.1 nucleosomes (TE pH 7.6, 0 mM NaCl) as well as impurities of donor only and acceptor only complexes was measured. In order to stress the discussed effects for this control experiment, the concentration of labeled complexes was increased to ~150 pM. By using thresholds of $TDS < 1$ and $TDS_{red-PIE} < 0.6$ in the TDS parameter space we are able to remove most of the observed trailing (i.e. events with high S and medium to low E) caused by multi-molecule events as well as photo-physics and receive a distribution showing populations of distinct FRET efficiencies and stoichiometries (Supplementary Figure S6B).

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