Investigating epigenome dynamics the RITE way
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LIMITED EXCHANGE OF HISTONE VARIANT H2A.Z IN DINAMIC NUCLEOSOMES OF NON-REPLICATING CELLS

Marit Terweij, Tibor van Welsem, Iris J.E. Stulemeijer, Hiroyuki Arai, and Fred van Leeuwen

In preparation
ABSTRACT

H2A.Z is a major variant of H2A and has been implicated in a variety of key cellular processes. Deposition of this histone variant is controlled by the SWR1 and INO80 complexes. These nucleosome remodelers can exchange H2A for H2A.Z and vice versa and can thereby mechanistically uncouple deposition of histones from DNA replication. Several lines of evidence suggest that H2A.Z influences gene regulation by destabilizing chromatin. In vivo, H2A.Z is mostly located in promoter regions, where canonical histones are known to be highly dynamic, and loss of H2A.Z leads to reduced exchange of histone H3 in yeast. Furthermore, metazoan nucleosomes containing H2A.Z, especially those also containing the histone variant H3.3, are readily lost under standard experimental procedures in vitro. A nucleosome destabilizing effect has also been found in reconstituted nucleosomes. To understand the role of H2A.Z in nucleosome dynamics we determined the exchange of H2A.Z itself under physiological conditions in vivo by using Recombination-Induced Tag Exchange (RITE). Unexpectedly, deposition of new H2A.Z molecules peaked in cells undergoing replication. Replication-independent exchange of H2A.Z was equal to or even lower than that of H3. Since H2A.Z is unlikely to remain at the DNA when H3 has been evicted, these findings suggest that H2A.Z transiently leaves the chromatin and is re-deposited. Ectopically overexpressed H2A.Z protein was readily incorporated into chromatin and partially replaced resident H2A.Z, lending support for a recycling model for H2A.Z. Whereas new H2A.Z is assembled in a replication-dependent manner, resident H2A.Z in non-replicating cells is dynamic but reassembled. We propose that H2A.Z recycling can facilitate nucleosome dynamics and thereby modulate the binding of transcription factors, while ensuring that histone proteins carrying their epigenetic marks are maintained.
INTRODUCTION

The eukaryotic genome is packaged into chromatin. The basic unit of chromatin is the nucleosome, which consists of ~146 bp of DNA wrapped around an octamer of the canonical histone proteins H2A, H2B, H3, and H4. It is now well established that chromatin is a very dynamic structure that influences the gene expression profile of the cell (Boeger et al., 2003; Deal and Henikoff, 2010; Dion et al., 2007; Jamai et al., 2007; Verzijlbergen et al., 2010). There are several ways by which chromatin structure can be altered. Post-translational modifications (PTMs) on histone proteins, such as acetylation and methylation can form binding sites for the recruitment of other proteins, or they can prevent other proteins from binding (Armache et al., 2011; Avvakumov et al., 2011; Babiarz et al., 2006; Sampath et al., 2009).

Alongside the four canonical histones, there are a number of histone variants known that have special functions in the cell. A well-known example is histone H3.3, a variant of H3, which is incorporated in a replication-independent manner (Ahmad and Henikoff, 2002; Chow et al., 2005; Dunleavy et al., 2011; Mito et al., 2005). In this study we focused on the major variant of histone H2A, H2A.Z. This variant is highly conserved throughout evolution and has a role in diverse cell processes, such as chromosome segregation, heterochromatin silencing, and cell cycle progression (reviewed in (Zlatanova and Thakar, 2008)).

The abundance of H2A.Z in promoter regions points to a role for H2A.Z in gene regulation, one possible function being poising genes for activation (Guillemette et al., 2005). H2A.Z in promoter regions is predominantly located in the nucleosomes flanking the nucleosome depleted region (NDR) around the transcription start site (TSS) (Albert et al., 2007; Guillemette et al., 2005; Petter et al., 2011). The incorporation of H2A.Z has been suggested to be replication-independent (Skene and Henikoff, 2013). H2A.Z is loaded into chromatin by the SRCAP and p400/Tip60 complexes in mammalian cells. Histone acetylases Tip48 and Tip49 promote this activity by acetylation of nucleosomal H2A (Choi et al., 2005). In yeast, the SWR1 complex incorporates H2A.Z-H2B dimers by replacing H2A-H2B (Altaf et al., 2010; Luk et al., 2010; Morillo-Huesca et al., 2010). This deposition of H2A.Z by SWR1 is stimulated by acetylation of the tails of histones H4 and H2A by the NuA4 histone acetyltransferase complex (Altaf et al., 2010; Keogh et al., 2006). In subtelomeric regions, acetylation of lysine 16 in the H4 tail by SAS-I is required for H2A.Z deposition (Shia et al., 2006). Surprisingly, a recent study showed that the SWR1 complex can also perform replacement in the opposite direction, replacing H2A.Z-H2B with H2A-H2B, when stimulated by acetylation of lysine 56 on histone H3 (H3K56ac) (Watanabe et al., 2013). Another complex related to the SWR1 complex, the INO80 complex, can also remove H2A.Z from chromatin (Papamichos-Chronakis et al., 2011). H2A.Z can be acetylated on its N-terminal tail by the NuA4 and SAGA complexes in yeast (Babiarz et al., 2006; Ishibashi et al., 2009; Keogh...
et al., 2006; Mehta et al., 2010; Millar et al., 2006). This acetylation occurs mostly on promoter-located H2A.Z. Indeed, the INO80 complex is found mostly in coding regions and in cells with a deficient INO80 complex, unacetylated H2A.Z accumulates in coding regions, suggesting that the INO80 complex removes unacetylated H2A.Z from these regions (Papamichos-Chronakis et al., 2011). Structural studies have shown that nucleosomes containing H2A.Z are less stable than their H2A-containing counterparts in vitro (Suto et al., 2000). Double variant nucleosomes, containing H2A.Z as well as H3.3 seem to be particularly unstable (Henikoff, 2009; Jin and Felsenfeld, 2007; Jin et al., 2009). Since H3.3 is also located at promoter regions (Henikoff, 2009), it has been suggested that these unstable double variant nucleosomes actually occupy the NDR, but this goes undetected because the unstable nucleosomes are lost during conventional biochemical fractionation techniques (Henikoff, 2009). In vivo, unstable nucleosomes containing H2A.Z and H3.3 may be easier to remove from the chromatin, thereby revealing transcription factor binding sites and facilitating transcription regulation.

Together, these findings describe a strong association between the presence of H2A.Z and nucleosome dynamics. However, little is known about the dynamics of H2A.Z in vivo. Here, we used Saccharomyces cerevisiae as a model organism to study the exchange of H2A.Z under physiological conditions and endogenous expression levels. To discriminate between old, existing histones and newly synthesized ones we used Recombination-Induced Tag Exchange (RITE) (Terweij et al., 2013; Verzijlbergen et al., 2010), a genetic pulse-chase assay (Fig. 1). Although H2A.Z can be exchanged by replication-independent mechanisms, the majority of new H2A.Z deposition occurred in cells that undergo replication. In non-replicating, G1-arrested cells, H2A.Z exchange was equal to or lower than H3 exchange. Disassembly of nucleosomes occurs by first evicting H2A-H2B (or H2A.Z-H2B) dimers and then removing of H3-H4 tetramers. Thus, the observations made in this study suggest that evicted old H2A.Z molecules are reassembled while evicted histone H3 proteins are replaced by new ones. A recycling model of H2A.Z was supported by misregulated overexpression of H2A.Z. The extra H2A.Z proteins were readily incorporated into chromatin in non-replicating cells and replaced part of the resident H2A.Z, indicating that H2A.Z-containing nucleosomes are dynamic structures. Our findings show that in a dynamic nucleosome, the different histones can have different fates when evicted. In nucleosomes where H3 is evicted and replaced by new H3 protein, evicted H2A.Z can be recycled. This process uncouples destabilization of nucleosomes from replacement of modified histones by new naïve ones and may thereby help to preserve epigenetic signals present on H2A.Z. Interestingly, H2A.Z is overexpressed in several types of cancer. It will be interesting to determine if this tips the balance from recycling to exchange and thereby alter the fate of evicted histones and promoting the erosion of epigenetic states.
MATERIALS AND METHODS

Strains and growth conditions

Yeast strains were cultured under standard conditions (van Leeuwen and Gottschling, 2002). Yeast strains used in this study are detailed in Table 1. UCC7179 was derived from BY4705a (van Leeuwen and Gottschling, 2002). NKL6061 was derived from a cross between BY4727 (Brachmann et al., 1998) and the arg4::KanMX deletion mutant from the MATA knock-out collection. Strains were grown in YEPD (1% yeast extract, 2% bacto peptone, 2% glucose) in shaking flasks at 30°C. To select for Hygromycin resistance, cells were grown in YEPD containing 200 µg/ml Hygromycin. For RITE+ experiments, strains were grown in YEPraf (1% yeast extract, 2% bacto peptone, 3% raffinose) pre- and post-switch, and strains were released (4h and G1 samples) in YEPtrgal (1% yeast extract, 2% bacto peptone, 3% galactose) in shaking flasks at 30°C.

Whole-cell extracts in SUMEB

Samples of 2x10^8 cells were harvested and washed with 10 mM Tris pH 8, 1 mM EDTA (TE) containing 0.2 mM PMSF. Cell pellets were stored at -80°C until further processing, but at least thirty minutes. Whole-cell extracts were prepared in SUMEB

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*(Brachmann et al., 1998; Terweij et al., 2013)
(1% SDS, 8 M Urea, 10 mM MOPS pH 6.8, 10 mM EDTA, 0.01% bromophenol blue) containing protease inhibitors (1 mM PMSF, 1 mM DTT, 5 mM benzamidine, 1 µg/ml pepstatin, 1 µg/ml leupeptin) by glass bead disruption in a multivortex. The resulting lysate was incubated for 10 minutes at 65°C and subsequently clarified by centrifuging 5 minutes at 14000 rpm. Samples were stored at -20°C. Prior to gel separation, samples were incubated for 5 min. at 65°C and centrifuged for 1 min. at 14000 rpm at room temperature.

**Chromatin Association Assay (CAA)**

Samples of 2x10^8 cells were harvested in 50-ml tubes and paralyzed with 0.1% final concentration sodium azide. Samples were centrifuged for 2 min at 3000 rpm at room temperature. Samples were washed once with 25 ml H_2O at room temperature and centrifuged for 2 min. at 3000 rpm at room temperature. Cells were resuspended in 0.5 ml pre-spheroplast buffer (100 mM PIPES KOH pH 9.4, 10 mM DTT) and incubated for 30 min. at 30°C while shaking. Cells were centrifuged for 2 min. at 3000 rpm at room temperature and resuspended in 0.3 ml spheroplast buffer (50 mM KPO_4, 0.6 M sorbitol, 10 mM DTT, 0.5 mM PMSF) with 27 units of Lyticase. Cells were incubated 30 min. at 30°C, shaking. Formation of spheroplast was determined by microscopy and was at least 95%. Cells were centrifuged 3 min. at 3000 rpm at room temperature and resuspended in 150 µl lysis buffer (20 mM PIPES pH 6.8, 150 mM NaCl, 0.4 M sorbitol, 50 mM KOAc, 3 mM MgAc_2, 0.5 mM PMSF, Protease Inhibitor Cocktail mini EDTA free (Roche), 1 tablet/5 ml lysis buffer) using a wide-bore tip and transferred to a 1.5-ml tube. Samples were kept on ice from this step onwards. Cells were centrifuged for 2 min. at 4000 rpm at 4°C. Supernatant was discarded and cells were resuspended in 75 µl lysis buffer using a wide-bore tip. To lyse the cells, 0.1% final concentration of Triton-X100 was added and mixed by flicking the tube. Samples were incubated on ice for 5 min. A whole-cell extract sample was taken and samples were centrifuged for 5 min. at 14000 rpm at 4°C. The supernatant (cytoplasm) was transferred to a new 1.5-ml tube. The pellet (nuclei) was washed with 200 µl lysis buffer with 0.1% final concentration Triton-X100 (wide-bore tip) and centrifuged for 5 min. at 14000 rpm at 4°C. The supernatant was discarded. Nuclei were resuspended in 75 µl lysis buffer (wide-bore tip) and Triton-X100 was added to 1% final concentration to lyse the nuclei. Samples were incubated for 5 min. on ice. Samples were centrifuged for 15 min. at 12000 rpm at 4°C. The supernatant (nucleoplasm) was transferred to a new 1.5-ml tube. The pellet (chromatin) was resuspended in 2x Sample Buffer (wide-bore tip) and subsequently sonicated for 3 min. in a Bioruptor with 30 sec. on/off cycles at high power. Samples were incubated for 10 min. at 95°C. To whole-cell extract, cytoplasm, and nucleoplasm samples 1x Sample Buffer was added and samples were incubated for 10 min. at 95°C. Samples were stored at -20°C. Prior to gel separation, samples were incubated for 5 min. at 95°C.
Protein detection by immunoblot and antibodies

Prior to immunoblotting, 3-8 µl of lysate was separated on a 16% polyacrylamide gel. Separated proteins were transferred to a 0.45 µm nitrocellulose membrane for one (H3 and H2B) or 1.5 (when Pgk1 was detected on the same membrane) hours at 0.1 A. Membranes were blocked with PBS containing 2% or 5% Nutrilon (Nutricia) for one hour, first antibody incubations were done either for two hours at room temperature or overnight at 4°C in TBST with 2% Nutrilon. After washing three times in TBS containing 10% Tween-20 (TBST), secondary antibody incubation was performed in TBST with 2% Nutrilon and LI-COR Odyssey IRDye 800CW or IRDye 700CW antibody at 1:10,000 for 45 minutes at room temperature in the dark followed by two times 10-minute wash in TBST and one time 10-minute wash in PBS. Membranes were scanned using a LI-COR Odyssey IR Imager (Biosciences) and analyzed using the Odyssey LI-COR software package version 3.0 or the image studio software package. Antibodies used in this study are Pgk1 (A-6457, Invitrogen), histone H2B (39238, Active Motif), histone Htz1 (Active motif), HA (12CA5), V5 (R960-25, Invitrogen), histone H3 and LoxP (Verzijlbergen et al., 2010).

Chromatin Immunoprecipitation (ChIP)

For ChIP, cells were grown to mid-log phase in YEPD with 200 µg/ml Hygromycin for pre-switch strains, or YEPD for post-switch strains. Samples of 1-3x10⁹ cells were taken, fixed for 10 minutes in 1% formaldehyde and washed with cold TBS. Pellets were stored in 12 ml flat bottom-tubes or 2 ml screw-cap tubes at -80°C until further processing. Cells in 12 ml tubes were disrupted in 300 µl breaking buffer (100 mM Tris pH 7.9, 20% glycerol, protease inhibitor cocktail EDTA-free (Roche)) with 400 µl glass beads in a multivortex for 20 minutes at 4°C. Cells in screw-cap tubes were disrupted in 200 µl breaking buffer with 500 µl glass beads in a bead beater in a 4°C cold block for 2 minutes. Lysis was at least 70%, as determined by microscopy. Lysates were transferred to 1.5 or 2 ml tubes and 1 ml FA buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, protease inhibitor cocktail EDTA-free) was added. The mixture was centrifuged for 1 minute at 14000 rpm at 4°C, and the pellet was washed once more with FA buffer. The pellet was resuspended in 450 µl FA, divided over two 1.5 ml tubes and sonicated for 6-7 minutes in a Bioruptor (Diagenode) with 30 second on-off cycles on high power. Lysates were cleared by centrifugation for 5 minutes at 4°C at 14000 rpm. Supernatant containing chromatin was transferred to 1.5 ml tube and 1 ml FA was added to samples of the 12 ml tubes. Screw-cap tube chromatin samples were treated with Micrococcal Nuclease (MN) to generate mononucleosomes. For these samples, 800 µl final buffer (15 mM Tris pH 7.4, 50 mM NaCl, 1.5 mM CaCl₂, 5 mM β-mercaptoethanol, 5 mM MgCl₂) was added. Samples were incubated with 30 units of MN (Fermentas) at 37°C for 20 minutes. The reaction was stopped by adding EGTA and EDTA to a final concentration of 10 mM.
and placing tubes on ice. The chromatin solution was centrifuged for 15 minutes at 14000 rpm at 4°C; the supernatant was transferred to a new 1.5 ml tube and stored at -20°C. Magnetic dynabeads coupled with Protein G (Dynal) were incubated in PBS containing 5 mg/ml BSA with antibody for at least four hours at 4°C. 200 (for 12 ml tube samples) or 400 (for screw-cap samples) µl soluble chromatin was added to prepared dynabeads and incubated rotating overnight at 4°C. Then 1 ml of FA buffer was added and samples were incubated rotating for 5 minutes at RT. The samples were washed twice with each of the buffers FA, FA-HS (50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate), RIPA (10 mM Tris pH 8, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA). Finally, the samples were washed once with TE (10 mM Tris pH 8, 1 mM EDTA). 100 µl elution buffer (50 mM Tris pH 8, 10 mM EDTA, 1% SDS) was added to the samples and incubated for 10 minutes at 65°C. Subsequently the samples were centrifuged 1 minute at 14000 rpm and 80 µl supernatant was collected. 70 µl TE was added to samples and cross links were reversed in 0.625 mg/ml ProtK and 3 µg/ml RNaseA incubated for 1 hour at 50°C and subsequently overnight at 65°C. For input samples, 20 (for 12 ml tube samples) or 40 (for screw-cap samples) µl chromatin solution was combined with 60 µl elution buffer and 70 µl TE, and treated in the same manner as IP samples to reverse cross links. DNA was purified by using the High Pure PCR Product Purification Kit (Roche).

Reverse transcription
Total yeast mRNA was prepared from 4x10^7 using the RNeasy kit (Qiagen) according to the manufacturer's manual using mechanical disruption of the cells. mRNA was treated with DNAse and cDNA was made as described previously (Verzijlbergen et al., 2010).

Quantitative PCR
Quantitative real time-PCR (qPCR) was performed with SYBRgreen master mix (Applied Biosystems or Roche) according to the manufacturer's manual. IP and cDNA samples were diluted 10 times, and input samples were diluted 100 times before analyzing by qPCR on a 7500 Fast Real-Time PCR system (Applied Biosystems) or LightCycler 480 II (Roche). qPCR primers are shown in Table 2.

FACS analysis of cell cycle progression
The DNA content was measured using SYTOXGreen in flow cytometry using a 530/30 filter on a FACS calibur (Becton-Dickinson) as described previously (Haase and Reed, 2002; van Welsem et al., 2008) For FACS analysis of DNA content samples of 1x10^7 cells were collected, centrifuged for 10 sec. at full speed, resuspended in 1 ml 70% ethanol, and stored at -20°C until further processing. For each measurement 100,000 cells were counted. Analysis was performed using FCS express 2.
RESULTS

Global exchange of H2A.Z is similar to that of H3 and peaks in replicating cells

To determine the stability of H2A.Z in the yeast epigenome, we made use of RITE to simultaneously monitor old histones and newly synthesized ones. RITE enables conditional switching of epitope tags by controlling Cre-recombination in a hormone-dependent manner (Fig. 1). A RITE cassette was introduced at the HTZ1 gene, encoding H2A.Z in budding yeast. As a comparison, the same cassette was introduced at HHT2, the sole gene encoding H3 in strains where HHT1 has been deleted. A RITE cassette was used that results in a switch from a V5 tag (V5, old tag, pre-switch) to an HA-6xHIS tag (HAH, new tag, post-switch) (Fig. 1), based on previous optimizations (Terweij et al., 2013). Strains lacking H2A.Z are viable but are sensitive to caffeine and somewhat cold-sensitive (Morillo-Huesca et al., 2010). Strains carrying RITE tags on H2A.Z behaved like wild-type strains when grown in the presence of caffeine or at low temperature, showing that the RITE tags on H2A.Z did not functionally compromise H2A.Z (Fig. 2). Similarly, RITE tags on the essential H3 protein did not affect the growth of yeast cells under the conditions examined (Fig. 2). To determine the global levels of H2A.Z exchange (loss of old, gain of new), an arrest-release protocol was used (Fig. 3A): cells were arrested by nutrient starvation, incubated in the presence of estradiol to activate Cre-recombinase and induce the tag-switch in the genome, and finally released into fresh media to let them

Table 2

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**Figure 1.** Outline of Recombination-Induced Tag Exchange (RITE). Following integration of a RITE cassette behind the gene of interest (*GENE*), recombination between LoxP sites is induced by Cre recombinase, causing a permanent switch from V5 (old tag) to HA-6xHIS (HAH; new tag) in the coding sequence for the protein of interest. S, spacer; L, LoxP recombination sites (which are also part of the coding sequence); T<sub><i>ADH1</i></sub>, <i>ADH1</i> terminator; HphMX, Hygromycin resistance cassette.

**Figure 2.** Growth of strains expressing RITE tags on histone H3 or H2A.Z. Strains were spotted in a 10-fold dilutions series on YEPD or YEPD containing 10 mM caffeine. Plates were grown at 30°C or 23°C for three days. A <i>htz1Δ</i> strain was used as control. Strains are listed in Table 1.

re-enter the cell cycle. The release was performed in the absence and presence of alpha factor to obtain cells going through S-phase and non-replicating cells arrested in G1, respectively. Global histone exchange was analyzed at two release time points using immunoblot analysis using whole-cell extracts. The trend of exchange of H2A.Z was similar to that of H3 (Figs. 3B-D). Exchange was low in starvation, higher in G1 arrest, and highest in replicating cells. However, unexpectedly, the levels of exchange of H2A.Z in G1 were lower than that of H3. Arrest and release of the cells was confirmed by FACS (Fig. 4) and was similar for H2A.Z- and H3-RITE
Figure 3. Global exchange of histone H2A.Z compared to that of H3. A) Experimental set up, see text. B) Representative Anti-LoxP immunoblot of H2A.Z exchange (NKI8053). Old H2A.Z (V5 tag) and new H2A.Z (HAH tag) can be distinguished due to their different mobility on polyacrylamide gels. An antibody against the spacer-LoxP sequence simultaneously detects old and new RITE-tagged proteins. Histone H2B was used as loading control. C) Representative anti-LoxP immunoblot of H3-RITE tagged proteins during a switch (NKI8050). H2B was used as loading control. D) Quantification of H3 and H2A.Z exchange during switch experiments. Average of seven biological replicates ± SEM.

Differential mRNA stability is another possible confounder when comparing protein stability by RITE. If, following a Cre-mediated switch at the DNA level, the mRNA encoding the old tag is stable, newly synthesized proteins will still carry an old tag, thereby leading to underestimation of the amount of new protein synthesis. RT-qPCR analysis showed that the differences in protein stability between H3 and H2A.Z were not caused by differences in mRNA levels between \textit{HHT2} and \textit{HTZ1} (Fig. 5). Following induction of the tag switch in starved cells, the old mRNA of \textit{HHT2} as well as \textit{HTZ1} was degraded during starvation and reduced to background levels before the time of release (Fig. 5). Thus, analysis of H2A.Z dynamics by RITE revealed two unexpected findings. First, exchange of H2A.Z is low in G1 cells and predominantly occurs in replicating cells, despite the fact that this histone variant can be deposited into and removed from nucleosomes by replication-independent mechanisms. Second, H2A.Z dynamics is lower than expected since many lines of evidence point to a role for H2A.Z in nucleosome destabilization.
Global exchange of bulk H2A.Z reflects exchange changes in chromatin

Although histones not incorporated into chromatin are typically rapidly degraded (Keogh et al., 2006; Kobor et al., 2004; Morillo-Huesca et al., 2010; Singh et al., 2010; Wang et al., 2009), bulk H2A.Z exchange in the cell may not reflect exchange in chromatin if H2A.Z is evicted but retained in the cell in a soluble form. We first determined the chromatin-bound and soluble pools of H3 and H2A.Z using a chromatin association assay (CAA) involving a stepwise fractionation of a whole-cell extract (Fig. 6A). Wild-type H3 and H2A.Z as well as their RITE-tagged versions
were predominantly recovered in the chromatin fraction (Figs. 6A-C), confirming that the soluble pool of histones is low and that immunoblot analysis of bulk histones in whole-cell extracts reports on global changes in chromatin-bound histones. Second, we examined the exchange of H2A.Z in chromatin at specific loci by chromatin immunoprecipitation (ChIP). Three previously used reporter loci

**Figure 6.** Wild-type and RITE-tagged histone H3 and H2A.Z are predominantly bound to chromatin. A) Immunoblots of a Chromatin Association Assay using a WT strain (NK16061). H3, H2B, Hmo1, and Sir2 were used as controls for chromatin bound proteins; Pgk1 is a cytoplasmic protein; RNA polymerase II (PolII) has been reported to be present both in the cytoplasm and bound to DNA. WCE, whole-cell extract; cytopl, cytoplasm; wash1, wash step of the nuclear pellet; NP, nuclear pellet; NS, nuclear supernatant; wash2, wash step of chromatin; chrom, chromatin. B) Immunoblot comparison of H3 and H2A.Z RITE strains (NKI2176; NKI8050; NKI8058; NKI8001; NKI8053; NKI8059). Fractionation of each histone was determined in a WT strain and V5- and HAH-RITE strains. SUME, whole-cell extract using SUME lysis buffer. C) Comparison of H2B fractionation in a WT strain and H3- and H2A.Z-RITE strains.
were selected: PTC6 and RSA4 (high H2A.Z occupancy) and ABP1 (intermediate H2A.Z occupancy) and SPA2 (low H2A.Z occupancy) (Albert et al., 2007; Kobor et al., 2004). H2A.Z levels in our RITE strains were confirmed by ChIP analysis on log-phase cells (Fig. 7). Next we determined local histone exchange during the arrest-switch-release protocol. H2A.Z exchange peaked in replicating cells and was lower than histone H3 exchange in G1-arrested cells (Fig. 8). These results are generally consistent with the bulk levels of exchange observed by immunoblot analysis (Fig. 3). The relative stability of H2A.Z when compared to histone H3 is unexpected based on the intimate connection of H2A.Z with nucleosome instability. H2A.Z destabilizes nucleosomes in vitro and promotes H3 exchange in vivo. Furthermore, H2A-H2B or H2A.Z-H2B dimers are typically more easily lost from nucleosomes than H3-H4 tetramers under in vitro conditions and elegant assembly studies have shown that H2A-H2B dimers are unlikely to remain bound to DNA in the absence of H3-H4 tetramers. Importantly, the majority of H2A.Z in yeast is localized in dynamic nucleosomes, i.e. nucleosomes in which histone H3 undergoes high levels of histone exchange. The relative stability of H2A.Z in non-replicating cells that we observed here suggests that histone H3 and H2A.Z might have different fates when evicted from dynamic nucleosomes. The lower exchange of H2A.Z might suggest that following transient eviction, H2A.Z is recycled, while H3 is exchanged.

**Misregulation of H2A.Z expression promotes exchange**

A biochemical approach to studying nucleosome dynamics is the use of competition assays to test if transiently evicted histone proteins can be captured by ectopic DNA. As an alternative approach that is applicable to our in vivo assays, we designed a

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**Figure 7.** Steady state abundance of H2A.Z at different genomic loci. Four loci were selected based on previous data (Albert et al., 2007; Kobor et al., 2004) that have high (PTC6), high/medium (RSA4), medium (ABP1), and low (SPA2) H2A.Z abundance in log phase. A) Abundance of H2A.Z-V5 was determined with respect to abundance of H3-V5 (each IP/input). B) Abundance of H2A.Z-HA-6xHis with respect to H3-HA-6xHis. Average of three biological replicates ± SEM.
Figure 8. Global exchange of H3 and H2A.Z reflects exchange in chromatin. Exchange of H2A.Z and H3 (new/old, HAH/V5) was determined by ChIP-qPCR for three regions in the genome: A) PTC6; B) RSA4; C) ABP1. Average of three biological replicates ± SEM. Strains NKI8050 and NKI8053.
competition assay by ectopic overexpression of H2A.Z, whereby incorporation into nucleosomes and replacement of existing H2A.Z in non-replicating cells indicates that resident H2A.Z is not stably incorporated into nucleosomes (Cheng and Gartenberg, 2000). To that extent, we employed the RITE assay, in which the tagged histone genes are under control of their endogenous promoters, in combination with an extra copy of new H2A.Z or H3 under control of the galactose-inducible GAL1 promoter integrated at the HO locus (RITE+, Fig. 9). For analysis of histone exchange by RITE+, cells were grown, arrested by starvation, and switched in media containing raffinose as a neutral carbon source. Cells were released in galactose media to induce the GAL1 promoter. Under these conditions, new histones are synthesized from the endogenous locus as well as the rapidly induced ectopic copy. Since yeast cells grow slower in galactose than glucose media (Fig. 10), the kinetics of release and exchange are not directly comparable. Therefore, regular RITE strains lacking the ectopic new copy were grown under identical conditions and used for comparison. Ectopic

**Figure 9.** Outline of RITE+ assay to introduce ectopic copies of new RITE-tagged proteins. RITE is targeted to the endogenous locus of the gene of interest and thereby under control of the endogenous promoter. In addition, a copy of the gene tagged with the new tag is introduced at the HO locus and under control of the galactose-inducible GAL1 promoter ($P_{GAL1}$). In the presence of glucose or raffinose, and before the switch of the endogenous RITE-tagged gene, the extra copy is not induced. After inducing a switch of the endogenous copy, the cells are released in galactose-containing media, which leads to expression of the endogenous copy as well as early and ectopic expression of the $P_{GAL1}$-driven copy.
Figure 10. Ectopic expression of H2A.Z or H3 does not alter cell cycle progression during a switch-release assay. Analysis DNA content by flow cytometry of RITE and RITE+ strains (NKI8050; NKI8053; NKI2359; NKI2364) as described in Figs. 11-12.
expression led to increased new H2A.Z levels in the cell (Fig. 11). In addition, the bulk level of old H2A.Z decreased. Applying RITE+ to H3 led to a modest increase in new histone protein (Fig. 11). The magnitude of the effect was much smaller than that of H2A.Z, possibly due to the higher levels of histone H3 already present in the cell compared to H2A.Z, which is present in only a fraction of the genome. ChIP analyses confirmed that ectopic H2A.Z expression led to exchange of H2A.Z in chromatin and that histone H3 exchange was less affected (Fig. 12). Together, the in vivo competition assay suggests that despite the low level of histone exchange observed under physiological conditions, H2A.Z is not stably bound to nucleosomes.

**DISCUSSION**

H2A.Z is a multi-functional variant of H2A that has been implicated in various key cellular processes. Many studies point to a role for H2A.Z in influencing gene regulation by nucleosome dynamics. Indeed, it has become evident that histone dynamics can be used by the cell as a mechanism to alter chromatin structure and function. However, the mechanisms by which H2A.Z acts in vivo in this process are still poorly understood. Here we used a combination of in vivo assays to track the dynamic properties of H2A.Z itself. Unexpectedly, H2A.Z exchange is high in replicating cells but limited in non-replicating cells in G1, even in nucleosomes that undergo exchange of histone H3.

Since disassembly of nucleosomes occurs by first evicting H2A-H2B (or H2A.Z-H2B) dimers and then removing of H3-H4 tetramers, these observations suggest that evicted old H2A.Z molecules are reassembled while evicted histone H3 proteins are replaced by new ones. Misregulated overexpression of H2A.Z supports such a recycling model of H2A.Z. The extra H2A.Z proteins were readily incorporated into chromatin in non-replicating cells and replaced part of the resident H2A.Z, indicating that H2A.Z-containing nucleosomes are dynamic structures. Our findings suggest that in a dynamic nucleosome, the different histones can have different fates when evicted. In nucleosomes where H3 is evicted and replaced by new H3 protein, evicted H2A.Z can be recycled (Fig. 13).

There is some evidence that such recycling mechanisms are more generally applied by the cell. The HIR complex in yeast has been suggested to reassemble H3 in cis (Kim et al., 2007), and the histone chaperone Asf1 has been shown to bind to H3-H4 dimers harboring chromatin-associated PTMs during DNA replication stress (Groth et al., 2007). Two chaperones have been identified for H2A.Z: Nap1 which is implicated in the nuclear import of H2A and H2A.Z, and Chz1 which is a nuclear chaperone exclusive for H2A.Z (Andrews et al., 2010; Billon and Côté, 2012; Liu and Churchill, 2012; Luk et al., 2007; Mosammaparast et al., 2002; Straube et al., 2010). However, deletion of these chaperones, alone or in combination, did not alter global exchange of bulk H2A.Z (data not shown). This result suggests
Figure 11. Ectopic expression increases the dynamics of H2A.Z. A) Representative anti-LoxP immunoblot of exchange of H2A.Z in the absence (-) or presence (+) of an ectopic galactose-induced copy (strains NKI8053 and NKI2364). H2B was used as loading control. Experimental set up is the same as for Fig. 3A except for the use of different carbon sources. B) Representative anti-LoxP immunoblot of H3 exchange with and without ectopic H3 expression. (strains NKI8050 and NKI2359). C-F) Quantification of anti-LoxP immunoblots of H2A.Z and H3 for RITE or RITE+. Average of three biological replicates ± SEM.
that there are redundant mechanisms that deliver H2A.Z to the SWR1 complex. More subtle or localized effects of Nap1 or Chz1 may be detected by chromatin association assays or ChIP experiments. For example, it has recently been shown that there is a dynamic relocalization of H2A.Z during the cell cycle of mouse trophoblast cells (Nekrasov et al., 2012).

H2A.Z is found mostly in promoter regions, where H3 is known to have a high exchange (Dion et al., 2007; Jamai et al., 2007; Verzijlbergen et al., 2010). Also...
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H2A.Z localization negatively correlates with localization of H3K56acetylation, which is a mark for new H3 and is correlated positively with H3 exchange (Kaplan et al., 2008; Rufiange et al., 2007). A recent study showed that H3K56ac stimulates the SWR1 complex to replace H2A.Z in the nucleosome by H2A, whereas H3K56 that is not acetylated stimulates the SWR1 complex to replace H2A by H2A.Z (Watanabe et al., 2013). Watanabe et al. hypothesize that this is a mechanism of the cell to open up a promoter region. First, the SWR1 complex replaces H2A in the nucleosome by H2A.Z, which makes the nucleosome unstable and easier to remove. Next, H3 is exchanged and the new H3 is acetylated on K56, which stimulates the SWR1 complex to replace the H2A.Z in the nucleosome by H2A. This model would suggest that H2A.Z is very dynamic. Nevertheless, we did not detect an H2A.Z exchange level higher than that of H3. Therefore, the role of SWR1 in the dynamic behavior of H2A.Z in vivo and its role in assembly and recycling needs to be further examined. Other candidates for recycling H2A.Z are INO80, which can exchange H2A.Z for H2A (Papamichos-Chronakis et al., 2011), and the FACT complex, which has previously been implicated in both eviction and reassembly of nucleosomes (Formosa, 2011).

The stability of H2A.Z in nucleosomes undergoing H3 replacement indicates that in nucleosomes where H3 is evicted and replaced by new H3 protein, transiently evicted H2A.Z can be recycled. This process uncouples destabilization of nucleosomes from replacement of modified histones by new naïve ones and may thereby help to preserve epigenetic signals present on H2A.Z. Although histone variants have different modes of regulation than canonical histones (see Chapter 1) and are generally believed to be constitutively expressed and incorporated in a replication-independent manner (Skene and Henikoff, 2013), our studies on H2A.Z show that new H2A.Z incorporation is highest in replicating cells. This observation is in line with the peak of H2A.Z mRNA expression in late S-phase (Fig. 14). Furthermore, ectopic expression by the GAL1 promoter, which leads to overexpression of H2A.Z already in G1, enables new H2A.Z deposition. Therefore, our results show that the decision for recycling or replacement is determined by histone timing and expression level. Interestingly, H2A.Z is overexpressed in several types of cancer (Dryhurst et al., 2012; Hua et al., 2008; Svolotis et al., 2009, 2010; Valdés-Mora et al., 2012). It will be interesting to determine whether this overexpression influences the recycling of H2A.Z and maintenance of epigenetic signals.

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Figure 13. Model for fate of H2A.Z and H3 in a dynamic nucleosome. Upon destabilization and disassembly of a nucleosome, H3 can be evicted and replaced by newly synthesized H3, leading to erasure and resetting of epigenetic information. H2A.Z is transiently evicted and recycled, providing access for regulatory factors to the nucleosomal DNA and yet preserving epigenetic information on H2A.Z proteins.

Figure 14. Cell cycle regulation of H2A.Z expression. Analysis of expression of H2A.Z using SCEPTRANS (Kudlicki et al., 2007; Pramila et al., 2006; Spellman et al., 1998) shows that H2A.Z mRNA levels are periodic and show maximum expression in late S-phase, somewhat later than histone H3 and the other canonical histones.
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