Histone chaperone Anp32e removes H2A.Z from DNA double-strand breaks and promotes nucleosome reorganization and DNA repair

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The repair of DNA double-strand breaks (DSBs) requires open, flexible chromatin domains. The NuA4–Tip60 complex creates these flexible chromatin structures by exchanging histone H2A.Z onto nucleosomes and promoting acetylation of histone H4. Here, we demonstrate that the accumulation of H2A.Z on nucleosomes at DSBs is transient, and that rapid eviction of H2A.Z is required for DNA repair. Anp32e, an H2A.Z chaperone that interacts with the C-terminal docking domain of H2A.Z, is rapidly recruited to DSBs. Anp32e functions to remove H2A.Z from nucleosomes, so that H2A.Z levels return to basal within 10 min of DNA damage. Further, H2A.Z removal by Anp32e disrupts inhibitory interactions between the histone H4 tail and the nucleosome surface, facilitating increased acetylation of histone H4 following DNA damage. When H2A.Z removal by Anp32e is blocked, nucleosomes at DSBs retain elevated levels of H2A.Z, and assume a more stable, hypoacetylated configuration. Further, loss of Anp32e leads to increased Ctp1-dependent end resection, accumulation of single-stranded DNA, and an increase in repair by the alternative non-homologous end joining pathway. Exchange of H2A.Z onto the chromatin and subsequent rapid removal by Anp32e are therefore critical for creating open, acetylated nucleosome structures and for controlling end resection by Ctp1. Dynamic modulation of H2A.Z exchange and removal by Anp32e reveals the importance of the nucleosome surface and nucleosome dynamics in processing the damaged chromatin template during DSB repair.

NHEJ | DSB repair | H2A.Z | Anp32e | genome instability

The repair of DNA double-strand breaks (DSBs), which cleave the DNA backbone, requires remodeling of the local chromatin architecture. This reorganization of the chromatin is important for promoting access to the site of damage, for creating a template for the repair machinery, and for re-packaging the chromatin and resetting the epigenetic landscape following repair. Chromatin remodeling at DSBs is linked to changes in post-translational modification of histones. DSBs activate the ataxia-telangiectasia mutated (ATM) and DNA–PKcs kinases, which phosphorylate multiple DNA repair proteins, including histone H2AX. Phosphorylated H2AX (γH2AX) provides a binding site for mdc1, which promotes spreading of γH2AX for hundreds of kilobases either side of the break (1–3). DSBs also promote complex patterns of chromatin ubiquitination, including ubiquitination of H2A/H2AX by the RNF8/RNF168 ubiquitin ligases, which, in turn, creates binding sites for repair proteins such as 53BP1 and brca1 (4–7). DSBs also lead to increased methylation of histone H3 on lysine 9 (8, 9) and methylation of H3 on lysine 9 (10), which drives activation of the Tip60 acetyltransferase and the ATM kinase (11, 12). Further, the NuA4–Tip60 complex (5, 13–15) promotes acetylation of histone H4 at DSBs and drives the formation of open, flexible chromatin domains at DSBs (5, 13, 14). The repair of DSBs is therefore fundamentally a chromatin-driven process requiring dynamic changes in histone modification and chromatin reorganization, which directly promote recruitment of DSB repair proteins (16).

The NuA4–Tip60 remodeling complex plays a central role in nucleosome reorganization at DSBs (16), NuA4–Tip60 is a 16 subunit complex containing 2 key subunits—the p400 SWI/SNF ATPase and the Tip60 acetyltransferase. The p400 ATPase promotes exchange of H2A for the histone variant H2A.Z (13, 17). This increase in H2A.Z at DSBs then promotes acetylation of histone H4 by the Tip60, creating open, flexible chromatin at sites of DNA damage (5, 11, 14). Inactivation of NuA4–Tip60 blocks both H2A.Z exchange and H4 acetylation, leading to a reduction in chromatin mobility at DSBs. Consequently, loss of H2A.Z exchange leads to defective DSB repair, increased sensitivity to DNA damage, and genomic instability (13, 18, 19).

Here, we demonstrate that H2A.Z exchange at DSBs is dynamic, with H2A.Z accumulating at DSBs within minutes of damage, followed by rapid H2A.Z eviction. Further, we show that Anp32e, an H2A.Z-specific histone chaperone, binds specifically to the docking domain of H2A.Z and is required to remove H2A.Z from the damaged chromatin template. Failure to remove H2A.Z leads to defects in DSB repair, including a loss of H4 acetylation, defects in non-homologous end joining (NHEJ), and increased end resection of DSBs.

Results

Here, we examined the role of the histone chaperone Anp32e (20) in regulating H2A.Z exchange at DSBs. DNA damage created by laser microirradiation (“laser stripping”) led to an increase in γH2AX and accumulation of Anp32e (Fig. 1). No focal accumulation of Anp32e was seen in untreated cells (Fig. S1A). However, Anp32e accumulation was delayed relative to γH2AX formation, so that

Significance

DNA is wrapped around nucleosomes, so that repairing double-strand breaks (DSBs) requires significant nucleosome reorganization. Nucleosome reorganization requires exchange of histone H2A.Z. This work reveals that H2A.Z only transiently accumulates at breaks and demonstrates that Anp32e, a histone chaperone, is recruited to DNA breaks and removes H2A.Z from the nucleosomes. Further, removal of H2A.Z from the intact nucleosome by Anp32 promotes acetylation of histone H4, remodels the local chromatin, and facilitates DNA repair. The dynamic exchange and removal of H2A.Z by Anp32e is therefore critical for chromatin reorganization at DSBs. Further, modulation of the nucleosome surface by Anp32e directs histone modification, recruitment of repair proteins, and the processing and repair of DSBs.

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Anp32e is rapidly recruited to DSBs. (A) U2OS cells expressing Flag-Anp32e were exposed to laser microirradiation and allowed to recover for the indicated times. γH2AX and Flag-Anp32e were detected by immunofluorescence. Percent of γH2AX stripes colocalizing with Anp32e is shown. (B and C) The 293T cells were incubated with nonspecific (siCon) or Anp32e-specific siRNAs (siAnp32e or siAnp32f) for 48 h, followed by transfection with vector (+) or the p84-ZFN (+) to create DSBs. Cells were processed for ChIP 18 h later using Anp32e antibody (B) or H4Ac antibody (C), followed by real-time PCR (RT-qPCR) with primers located 500 bp to the right of the DSB. Results are ±SE (n = 3). (D) U2OS cells were incubated with nonspecific (siControl) or Anp32e-specific (siAnp32e) siRNA for 48 h, followed by laser stunning. Cells were either fixed immediately (0 min) or allowed to recover for the indicated times, followed by immunofluorescent staining with γH2AX and H2A.Z antibodies.

Anp32e is rapidly recruited to DSBs. (Fig. 1D). Surprisingly, H2A.Z was only transiently retained on the damaged chromatin, with essentially all of the H2A.Z removed within 10 min of damage (Fig. 1D). Further, depletion of Anp32e did not block accumulation of H2A.Z (Fig. 1D); rather, loss of Anp32e blocked removal of H2A.Z, leading to prolonged retention of H2A.Z on the chromatin at DSBs (Fig. 1D). The H2A.Z chaperone Anp32e therefore rapidly removes H2A.Z from sites of DNA damage. Further, because loss of Anp32e blocks H4Ac at DSBs (Fig. 1C), this implies that removal of H2A.Z by Anp32e may trigger H4Ac at DSBs.

The crystal structure of Anp32e bound to H2A.Z indicates that Anp32e makes multiple contacts with H2A.Z, including T103/I104 in the C-terminal docking domain (20, 21) (Fig. 2A). Although the Anp32e interacting residues are conserved in H2A, the extra glycine residue in H2A (blue circle; Fig. 2A) disrupts Anp32e–H2A interaction (20, 21), so that Anp32e only binds H2A.Z. We therefore replaced the Anp32e binding region of H2A.Z with the equivalent region from H2A, including the extra glycine, creating the chimeric H2A.Z protein H2A.ZNG (Fig. 2A). We then examined whether Anp32e–H2A.Z interaction was required for H2A.Z removal.

H2A.ZNG and H2A.Z were efficiently expressed (Fig. S2A) and exchanged onto the chromatin at the p84-ZFN DSB (Fig. 2B). HA-H2A.Z accumulated at sites of laser DNA damage, and was removed by 30 min postirradiation, so that less than 5% of cells retained HA-H2A.Z (Fig. 2D). However, HA-H2A.ZNG was exchanged onto the damaged chromatin but was not removed (Fig. 2D), being retained for at least 30 min. This result is consistent with the ChIP data (Fig. 2B), where higher levels of H2A.ZNG accumulated at DSBs. Further, Anp32e was recruited to DSBs in the absence of H2A.Z (Fig. S2B), indicating that retention of H2A.ZNG was not due to failure to recruit Anp32e to the DSB. Binding of Anp32e to H2A.Z’s docking domain is therefore required to remove H2A.Z from DSBs, but is not for the initial exchange of H2A.Z onto the damaged chromat. Further, removal of H2A.ZNG at DSBs blocked H4Ac after DNA damage (Fig. 2C).

Thus, retention of H2A.Z on damaged chromat via loss of Anp32e may trigger H4Ac at DSBs. However, HA-H2A.Z was removed within 10 min of damage (Fig. 1D), so that less than 5% of cells retained HA-H2A.Z (Fig. 2D). Further, accumulation of H2A.ZNG at DSBs blocked H4Ac after DNA damage (Fig. 2C).

16% of γH2AX stripes contained Anp32e immediately after DNA damage (Fig. 1A). Despite this short delay, Anp32e was eventually loaded onto all of the γH2AX “stripes.” To further confirm that Anp32e is recruited to DSBs, we used the well-characterized p84-zinc finger nuclease (p84-ZFN) (13) to create a targeted DSB on chromosome 19 (Fig. S1C). ChIP demonstrated significant enrichment for Anp32e at the p84-ZFN DSB (Fig. 1B). Further, two siRNAs to Anp32e (which reduced Anp32e but not the cutting efficiency of p84-ZFN; Fig. S1 C and D) blocked Anp32e accumulation at the DSB (Fig. 1B). Anp32e is therefore rapidly loaded onto the chromatin at DSBs. Because Anp32e is an H2A.Z chaperone (20), we examined how Anp32e impacted the ability of the NuA4 complex to promote H2A.Z exchange (by the p400 ATPase) (5, 13) and H4 acetylation (H4Ac; by the Tip60 acetyltransferase) at DSBs. Loss of Anp32e reduced H4Ac at DSBs (Fig. 1C), indicating that Anp32e is required for H4Ac after DNA damage. We next examined if Anp32e contributed to H2A.Z exchange. H2A.Z rapidly accumulated at sites of laser damage (Fig. 1D). Further, depletion of Anp32e did not block accumulation of H2A.Z (Fig. 1D); rather, loss of Anp32e blocked removal of H2A.Z, leading to prolonged retention of H2A.Z on the chromatin at DSBs (Fig. 1D). The H2A.Z chaperone Anp32e therefore rapidly removes H2A.Z from sites of DNA damage. Further, because loss of Anp32e blocks H4Ac at DSBs (Fig. 1C), this implies that removal of H2A.Z by Anp32e may trigger H4Ac at DSBs.

Chromatin compaction involves binding of the H4 tail of one nucleosome to an acidic patch on the surface of an adjacent nucleosome. Acetylation of the H4 tails blocks this interaction and promotes unpacking of chromatin fibers (22–26). The acidic patch on the nucleosome surface is formed from a short stretch of acidic amino acids (the acidic domain) in the docking domain of H2A and H2A.Z (Fig. 2A). This acidic domain is adjacent to (and partially overlaps with) the Anp32e interaction domain on H2A.Z (20, 27–29) (Fig. 2A). Anp32e binding may therefore promote H4Ac by either displacing the H4 tail from the adjacent acidic domain or by removing H2A.Z and eliminating the entire H4 binding surface. To test this hypothesis, we used the LANA protein of Kaposi's sarcoma herpes virus (27). LANA protein binds tightly to the acidic patch (30, 31), using the same acidic amino acids to which the H4 tail binds. The 23 amino acid domain of LANA, which binds to the acidic patch, and a control, in which an essential arginine was mutated to glycine (30), were fused to GFP and expressed in cells (Fig. S3A). GFP–LANA and GFP–Con had minimal impact on damage-induced H4Ac in control cells (Fig. 3A). However, GFP–LANA (but not GFP–Con)
that retention of H2A.Z at DSBs tends to stabilize the nucleosomes. Because expression of H2A.ZNG also inhibits H4Ac (Fig. 3 A and B), we used the LANA peptide to restore H4Ac in H2A.ZNG cells. However, even though the LANA peptide can restore H4Ac (Fig. 3B), it only partially restored the increase in NaCl solubility of histone H3 caused by DNA damage in H2A.ZNG cells (Fig. 3C). Increased H4Ac, on its own, is therefore insufficient to decrease nucleosome stability. This result suggests that removal of H2A.Z, in combination with increased H4Ac, are required to decrease nucleosome stability at DSBs.

Previous studies implicated H2A.Z exchange in regulation of end processing of DSBs by CtIP (13). In fact, cells expressing H2A.ZNG or lacking Anp32e (Fig. 4 A and B) exhibited a significant loss of Ku70 recruitment to laser stripes. In addition, ChIP analysis demonstrated an increase in the ssDNA binding protein RPA32 at DSBs in H2A.ZNG cells (Fig. 4B). This finding suggests that retention of H2A.Z at the DSB (by expressing H2A.ZNG or depleting Anp32e) directs processing of the DNA ends toward ssDNA production, favoring RPA binding over Ku70/80 binding. Further, GFP-LANA, which binds to the nucleosomal surface and partially rescues H4Ac and formation of open chromatin in H2A.ZNG cells (Fig. 3), substantially restored Ku70/80 loading in the absence of ssDNA production, favoring RPA binding over Ku70/80 binding. Importantly, depletion of CtIP (Fig. S3B), which is essential for end resection, also restored Ku70 loading in the absence of Anp32e (Fig. 4E). Loss of Ku70 binding and increased ssDNA in Anp32e-depleted cells is therefore mediated through increased end resection of the break by CtIP. Because depletion of Anp32e blocks H2A.Z removal (Fig. 3) and H4Ac (Fig. 4), this result implies that dynamic H2A.Z exchange (by NuA4–Tip60) and removal by Anp32e, coupled with H4Ac, creates a chromatin conformation that functions to restrain CtIP-mediated end resection.

Fig. 2. Anp32e uses H2A.Z’s docking domain to remove H2A.Z. (A) Sequence alignment of human H2A and H2A.Z, with conserved amino acids in yellow. The acidic patch in H2A (blue circle) is indicated. Red lines delineate acidic domain. Sequence of chimeric H2A.ZNG is indicated. (B) The 293T cells expressing vector, HA-H2A.Z, or HA-H2A.ZNG were transfected with vector (−) or p84-ZFN (+) to create DSBs. Cells were processed for ChIP 18 h later using HA antibody and primers were located 500 bp to the right of the DSB. Results are ±1SE (n = 3). (C) The 293T cells expressing HA-H2A.Z or HA-H2A.ZNG were transfected with vector (−) or p84-ZFN (+) to create DSBs. Cells were processed for ChIP 18 h later using H4Ac antibody and primers were located 500 bp to the right of the DSB. Results are ±1SE (n = 3). (D) U2OS cells expressing HA-H2A.Z or HA-H2A.ZNG were exposed to laser striping and either fixed immediately (0 min) or recovered for 30 min. H2A.Z is detected with HA antibody. Percent of γH2AX stripes colocalizing with H2A.Z is shown.

Fig. 3. Binding of LANA to nucleosomal surface rescues H4Ac in Anp32e-deficient cells. (A) The 293T cells expressing HA-H2A.Z, HA-H2A.ZNG, GFP-Con, or GFP–LANA were transfected with vector (−) or p84-ZFN (+). ChiP was performed 18 h later using H4Ac antibody and primers were located 500 bp to the right of the DSB. Results are ±1SE (n = 3). (B) The 293T cells expressing GFP-Con or GFP–LANA were transiently transfected with siRNA to Anp32e (siAnp32e). Cells were transfected 48 h later with vector (−) or p84-ZFN (+) and performed for ChIP using H4Ac antibody. (C) Cells expressing HA-H2A.Z or HA-H2A.ZNG were untreated or exposed to bleomycin (7.5 μM) as indicated. Nuclei were extracted in 1.0 M NaCl, salt soluble proteins separated by SDS/PAGE, and H3 was detected by Western blot. Ponceau S staining indicates loading.
resection, retaining intact DSB ends and therefore favoring Ku70 binding.

Finally, we examined how Anp32e contributes to DSB repair. Loss of Anp32e (Fig. 5A) or expression of H2A.ZNG (Fig. 5B) increased radiosensitivity, consistent with a key role for Anp32e and H2A.Z removal in DSB repair. Neither depletion of Anp32e or expression of H2A.ZNG impaired cell kinetics (Fig. S3C). However, loss of Anp32e reduced NHEJ activity in cells (Fig. 5C), consistent with the loss of Ku70 at DSBs in the absence of Anp32e (Fig. 4E). Alternative-NHEJ (alt-NHEJ) is a backup pathway used when components of NHEJ, such as Ku70, are inactivated (32, 33). Indeed, cells lacking Anp32e showed increased repair through the alt-NHEJ pathway (Fig. 5D), consistent with the increase in CtIP-dependent ssDNA and loss of Ku70 binding (Fig. 4). Finally, depletion of Anp32e led to a small but significant increase in repair by homologous recombination (HR) (Fig. 5E), suggesting that the increase in ssDNA following Anp32e depletion may also increase repair by the HR pathway.

Discussion

We have demonstrated that the Anp32e histone chaperone is a DNA damage response protein which directs removal of H2A.Z from nucleosomes at DSBs. Previous work indicated that NuA4–Tip60 promotes both H2A.Z exchange and H4Ac on nucleosomes at DSBs, creating open chromatin domains that are required for DSB repair (Fig. 6) (11, 13, 14). Our results now extend this work to demonstrate that H2A.Z is only retained transiently at DSBs, and that rapid removal of H2A.Z by Anp32e is required to promote H4 acetylation and nucleosome reorganization at DSBs. The NuA4–Tip60 complex and Anp32e therefore function together to coordinate dynamic accumulation and removal of H2A.Z from nucleosomes during the repair of DSBs.

We propose a model (Fig. 6) in which the initial exchange of H2A.Z by NuA4–Tip60 stabilizes nucleosomes at the break, limiting chromatin mobility and maintaining chromatin structure. Anp32e then removes the entire H2A.Z–H2B dimer, eliminating both H2A.Z and the acidic patch, and freeing the H4 tail for acetylation by Tip60 and processing of the DSB. How H2A.Z alters nucleosome function is complex and may depend on associated histone modifications and histone variants (29). For example, H2A.Z is associated with open chromatin (34–37),

![Fig. 4. Removal of H2A.Z by Anp32e is required for retention of Ku70 at DSBs.](image-url)

![Fig. 5. Loss of Anp32e promotes repair by the alt-NHEJ pathway.](image-url)
Control of nucleosome dynamics by Anp32e-directed removal of H2A.Z

Fig. 6. Control of nucleosome dynamics by Anp32e-directed removal of H2A.Z at DSBs. Unacetylated H4 tails bind to the acidic patch on adjacent nucleosomes. Exchange of H2A.Z by the p400 subunit of NuA4 creates a binding site for Anp32e on the nucleosome surface. Anp32e then removes the entire H2A.Z–H2B dimer, resulting in loss of the acidic patch. Loss of the acidic patch releases the H4 tail, allowing for acetylation by Tip60 and a shift to a more flexible chromatin structure. The resulting partial nucleosome may then be reconstituted by the addition of new H2A–H2B dimers or may contribute to the nucleosome-depleted region identified at DSBs.

but is present in heterochromatin and can stabilize nucleosomes (38, 39). In addition, the acidic domain of H2A.Z is longer than that of H2A (Fig. 2A) and favors the formation of more compact chromatin (29, 39), presumably by increasing interaction between the H4 tail and the acidic patch. Further, we showed H2A.Z retention at DSBs blocked the shift to open chromatin, indicating that the initial exchange of H2A.Z stabilizes chromatin immediately after DNA damage. Other repressive factors, including HP1 and NuRD (10, 40–42), are also rapidly, but transiently recruited to DSBs. Transient loading of H2A.Z and other repressors may therefore temporarily “heterochromatinize” nucleosomes at breaks, stabilizing the damaged chromatin, and allowing the cell time to process the chromatin for DNA repair (Fig. 6).

H2A.Z contains a specific binding site for Anp32e (20, 21), which partially overlaps with the acidic domain to which the H4 tail binds (24–27). Anp32e binding to H2A.Z may displace the H4 tail, promoting H4 acetylation. The observation that the LANA protein, which binds the acidic domain (30), can rescue H4 acetylation in the absence of Anp32e supports this finding. However, Anp32e, in common with many histone exchange reactions, actually removes the entire H2A.Z–H2B dimer, allowing the cell time to process the chromatin for DNA repair (Fig. 6).
Chromatin Immunoprecipitation. Chip assays used the SimpleChIP Chromatin IP Kit (Cell Signaling Technology). Cells were crosslinked with formaldehyde (0.2% final concentration). Cells were permeabilized in ChIP buffer (Cell Signaling Technology) and sonicated (Fisher BioRuptor U200). Part of the supernatant was digested with proteinase K (65 °C for 2 h), the DNA isolated by spin columns, and input DNA quantitated by real time PCR. Equivalent amounts of chromatin were incubated with primary antibody (overnight at 4 °C) followed by protein-G agarose beads precoated with sperm DNA. Immune complexes were washed in low and high salt ChIP buffers (Cell Signaling Technology), eluted and incubated in NaCl (65 °C for 2 h), and then digested with proteinase K. Purified DNA was quantitated by RT-qPCR using the Step One Plus real time PCR system (Applied Biosystems). Primer sequences, Chip gradients, and PCR conditions are in SI Materials and Methods.

Laser Microirradiation and Immunofluorescence. Laser damage was produced using a 30-watt 405-nm diode laser focused through the 40×-plan Apochromat (N.A. 1.2) objective. Laser irradiation was defined by the laser spot size, laser power density, and laser exposure time. Laser power density was equally distributed across the laser spot size. Laser spot size for all experiments was defined by the objective focal length and objective numerical aperture. The laser output was 40–60% power to limit DNA damage to the laser path without noticeable cytotoxicity. Cells were preincubated with Hoechst 33258 (1 μg/ml for 5 min at 37 °C) before exposure to laser light. Cells were fixed with PBS/parafomaldehyde (4% [vol/vol]). Time between laser exposure and fixation was 5 min. Cells were permeabilized in methanol, washed in PBS, and incubated in Triton X-100 (0.2% for 5 min). Cells were washed twice in PBS and blocked with PBS (10% for 30 min). For Ku70, cells were preextracted in buffer D (10 mM Pipes pH 7.0; 100 mM NaCl; 300 mM sucrose; 3 mM MgCl2; 0.5% Triton X-100) before fixation. Slides were incubated with primary and secondary antibody with washing between each step, mounted with Fluoromount-G (Southern Biotech), and imaged with a Zeiss Axiolager Z1 microscope equipped with an AxioCam MRC Rev.3 color digital camera and ImageJ (version 1.45s). Acquisition software and image processing used the Zeiss Axiosvision software package (Zeiss Imaging).

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