Introduction

Architecture of chromatin regulates numerous cellular processes, including gene transcription, DNA replication/repair and export of RNA. Heterochromatin is the key element in this architecture and is involved in regulation of genome transcription, chromosome segregation and genome stability. Heterochromatic regions are predominantly present in the centromeric (CEN), pericentromeric (periCEN) and telomeric regions of chromosomes and consists of tandem repeats (TRs) comprising up to 10% of genomes in majority of higher organisms. The CEN regions of human chromosomes contain the largest TR family in the human genome called α-satellite DNA. It was proposed that transcription from this region is necessary for proper formation and function of centromeres. The periCEN region in humans consists of simple sequence satellites arrays; one of examples of these arrays is human satellite DNA 3 (HS3). HS3 arrays of different size are located at periCEN regions of most human chromosomes. Several chromosome-specific HS3 subfamilies have been reported in reference 6; among them, HS3 of chromosome 9 (HS3-9) was found to transcribe upon external stress application, including heat shock (HS); yet, functional significance of transcription and/or transcripts and chromatin remodeling processes associated with the HS-induced periCEN transcription remains incompletely characterized. The key factor that mediates stress-induced transcription is the heat shock factor 1 (HSF1). Upon stress, HSF1 forms nuclear stress bodies (nSBs) at actively transcribed periCEN regions. Daxx is a multi-functional nuclear protein that is mainly associated with nuclear structure ND10/PML NBs in normal conditions. PML NBs are linked to several processes, including the maintenance of intranuclear homeostasis by sequestering proteins into “nuclear depot” and releasing associated proteins into nucleoplasm upon stress application. Thus, according to proposed “nuclear depot” model, PML NBs may not represent site of Daxx activity but predominantly are sites of sequestration...
and segregation whereas Daxx is outside of this structure. In agreement with this model, besides the major accumulation at PML NBs, Daxx is also found associated with a subset of interphase centromeres in human cells;\(^{13}\) in mouse cells, Daxx is enriched at condensed heterochromatin (MaSat) at the end of S/ beginning of G2 phase,\(^ {9}\) suggesting CEN/periCEN heterochromatin as potentially "active" location of Daxx.

The functional significance of Daxx association with CEN/periCEN was dim until recent findings that identified Daxx-containing complex as a novel chaperone for histone H3.3.\(^ {18-20}\) Incorporation of this H3 variant into chromatin is replication-independent and is associated with elevated gene transcription.\(^ {15}\) Previously identified histone chaperone HIRA mediates loading of H3.3 into gene-rich areas of genome, specifically into gene regulatory elements.\(^ {11}\) Several recent studies have shown enrichment of H3.3 in repetitive regions of genome including telomeric and pericentromeric heterochromatin;\(^ {16}\) moreover, Daxx-mediated incorporation of H3.3 into MaSat in mouse cells correlates with transcription elevation from this region of genome.\(^ {16}\) It was also suggested that integration of H3.3 is important for the transcription-associated maintenance of heterochromatin structure at these locations.\(^ {17}\)

Given the major difference between human periCEN and CEN as well as in organization between mouse and human heterochromatin, it is very appealing to investigate function of Daxx in the incorporation of H3.3 into both CEN/periCEN regions of human chromatin and to analyze the consequences of this incorporation on transcription of these regions; in both normal and stress-induced conditions. Pursuing this direction we found that depletion of Daxx in human cells diminishes recruitment of histone H3.3 into both CEN and periCEN and reduces transcription from these regions of genome. Rather unexpectedly we observed that accumulation of Daxx at CEN/periCEN upon HS prevents changes in epigenetic modifications at these locations. Our findings suggest that Daxx-containing complex secures chromatin integrity by H3.3 incorporation in normal conditions and protects CEN/periCEN epigenetic steady-state upon stress application.

**Results**

HS increases accumulation of Daxx at CEN/periCEN. Daxx is mainly associated with ND10/PML NBs in normal conditions (Fig. 1A).\(^ {11,15}\) but in small sub-population of cells, Daxx formed additional foci associated with CEN (visualized by CREST antibody; Fig. 1E, inset 2). According to proposed "nuclear depot" model,\(^ {15}\) PML NBs response to extracellular stress by releasing associated proteins into nucleoplasm; thus, we tested localization of Daxx upon physiological hyperthermia (heat shock, HS) application. After 1 h of HS at 42°C, number of PML NBs increased 2- to 5-fold in agreement with previously published data (Fig. 1B).\(^ {15,16}\) most likely due to balance changes in post-translational modification of PML itself and/or other components of PML NBs; Daxx remained nuclear as in other stresses conditions;\(^ {15}\) still co-localizing with majority of PML NBs (Fig. 1B). In addition to PML NBs localization, Daxx also appeared in multiple (20–40) PML-negative domains in the majority of cells (Fig. 1B). Number and shape of these domains was proportional to CEN. Indeed, we observed that Daxx co-localized with CEN in most cells after 1 h of HS (Fig. 1F). Daxx remained associated with CEN at 1 h after HS recovery (Fig. 1G and inset 1) and also accumulated juxtaposed to CEN (Fig. 1G and inset 2). At 2 h after HS recovery, number of PML NBs, Daxx-PML and Daxx-CEN association mostly returned to the pre-stress conditions (Fig. 1D and H). Thus, Daxx, which in normal condition mainly resided at PML NBs and had minor association with CEN, became predominantly associated with CEN in response of HS application, although it was still observed at PML NBs. Statistical analysis is presented in Figure 1M; stability of Daxx did not change upon stress and recovery (Fig. S1G). Similar HS-induced dynamics of Daxx was observed in HeLa and MCF7 cancer cell lines and MCF10A, untransformed breast epithelial cell line (not shown).

Upon HS recovery, in addition to CEN, Daxx accumulates adjacent to CEN (Fig. 1G and inset 2), potentially at periCEN. Upon HS, transcription factor HSF1 activates transcription from periCEN (satellite HS3–9),\(^ {19}\) accumulating at these TRs in so-called stress bodies (SBs).\(^ {19}\) Thus, we used HSF-1 as a surrogate marker to monitor Daxx localization relative to the transcriptionally active periCEN. HSF-1, that was nuclear homogeneous in normal conditions (Fig. 1I), formed SBs upon HS application (Fig. 1J); HSF-1-stained SBs were juxtaposed to some of Daxx accumulations (presumably at CEN), reflecting the expected adjacent localization of CEN and periCEN in interphase chromosome territories (Fig. 1F and J, insets). Daxx co-localized with SBs at 1 h after HS recovery (Fig. 1K and compare inserts 2/3 with inset 2 at Fig. 1G). At 2 h after HS recovery, Daxx was associated with subpopulation of SBs (Fig. 1L). Thus, in addition to CEN, Daxx was also associated with SBs (that should mark periCEN) upon stress recovery.

To analyze Daxx association with CEN/periCEN TRs more directly, we developed CEN and periCEN probes for fluorescent in situ hybridization (FISH) to combine with immunostaining for Daxx. Specificity of CEN (pan-CEN) and periCEN (HS3–9) probes was tested in mitotic spreads for B (human fibroblasts) and HEP2 cells. As expected, pan-CEN produced signal at all chromosomes, while HS3–9 hybridized with two chromosomes in B and three chromosomes in HEP2 cells, that are tri-sonic for chromosome 9 (Fig. S1); CEN and periCEN probes had adjacent/partly overlapping localization in mitotic spreads and also in interphase cells (Figs. S1 and S2), reflecting neighboring positions of CEN/periCEN at chromosomes. In agreement with immunofluorescence results (Fig. 1E), Daxx had minor association with both CEN and periCEN probes in normal condition (Fig. S2A). Daxx co-localized with CEN signal after HS and 1 h of recovery (Fig. S2B and C), it was associated with periCEN mostly at 1 h of stress recovery (Fig. S2C). Thus, DNA FISH/immunofluorescence data proved increase of stress-induced association of Daxx with CEN/periCEN.

For quantitative analysis of Daxx association with CEN/periCEN, we next performed G4IP assay using HEP2 cell line modified for stable expression of FLAG-HA-Daxx; microscopy characterization of this cell line confirmed that HS-induced
The intranuclear dynamics of FLAG-HA-Daxx recapitulates endogenous Daxx protein. Daxx association with CEN (α-satellite, Fig. 1N) was minor at 37°C, was elevated ~10-fold upon HS (p < 0.001), was still high, but reduced, upon 1 h of recovery and returned to pre-stress levels at 2 h and 3 h of recovery. Daxx association with periCEN (HS3-9) was minimal at 37°C, elevated upon HS (p < 0.001) and 1 h of recovery, and reduced to almost pre-stress levels by 3 h of recovery. Thus, alternative method of
Figure 1 (See opposite page). Daxx accumulation at CEN/periCEN. HEp2 cells were fixed at 37°C or exposed to 42°C for 1 h and either fixed immediately or recovered at 37°C for 1–12 h. Daxx is mostly associated with PML NBs (visualized by anti-PML antibody) in control conditions (CEN) but forms additional domains after HS and at 1 h recovery (B and C) and is mostly returned to pre-stress localization after 2 h recovery (D). Daxx co-localizes with centromeres (visualized by CEN-TRFs), human autoimmune sera (H) in some cells at 37°C (G, inset 2); this co-localization is obvious in most cells after HS and 1 h recovery (F and G, inset 1); and is decreased after 2 h recovery (H). In addition, Daxx accumulates juxtaposed to centromeres upon recovery (G, inset 2). HS is nuclear homogenous in control conditions (I) and forms stress bodies (SBs) upon HS. Daxx is adjacent to SBs after HS (I, inset 1) and co-localizes with majority of SBs (inset 2) at 1 h recovery (K, insets 1 and 2). At 2 h recovery, Daxx is associated with some SBs (L, inset 1 high association, inset 2 low association). (M) Colocalization analysis (Pearson’s correlation coefficient upon HS and recovery) has been used to quantify the degree of association for Daxx/PML and Daxx/centromeres (CEN). Data are the means of three experiments, and the standard deviation is shown. (N) ChIP analysis of Daxx association with CEN/periCEN in HEp2 cells stably expressing FLAG-HA-Daxx. Daxx association with CEN (α-satellite) was minimal at 37°C, was elevated (~10-fold upon HS (p < 0.001), was still high, but reduced, at 1 h of recovery and returned to pre-stress levels at 2 h and 3 h. Daxx association with periCEN (HS 3–9) was minimal at 37°C, was elevated upon HS (p < 0.001) and 1 h of recovery, and reduced to almost pre-stress levels at 3 h of recovery.

Since HS-induced transcription of periCEN TRs is mediated by HSFI and it was suggested that Daxx participates in HSFI activation upon stress, we tested the ability of Daxx to affect transcription of another HSFI target, HSP70 ([HS3A1]). We found that depletion of Daxx in HEp2 cells did not reduce expression of HSP70, but rather slightly increased it (Fig. S3C); thus, Daxx may affect CEN/periCEN transcription not by previously demonstrated HSFI activation, but via an alternative mechanism.

Daxx is required for H3.3 association with CEN/periCEN. Recently, several groups have identified Daxx as a chaperone of histone H3.3.46–48 Considering that deposition of this variant of histone H3 is usually associated with elevated transcription, we reasoned that Daxx-dependent expression of CEN/periCEN could be explained by differential incorporation of H3.3. To test this idea, control- and Daxx-depleted HEp2 cells were modified for stable expression of FLAG-HA-tagged H3.3 or H3.1 by transduction with pOZ vector (Fig. S4) for cell lines characterization, note that both H3.1 and H3.3 have mostly homogenous nuclear localization). Next, we used a ChIP assay to analyze association of H3.3 and H3.1 with CEN and periCEN in control- and Daxx-depleted cells (Fig. 3). In normal conditions, incorporation of H3.3 into CEN (α-satellite) and periCEN (HS3–9) repeats was reduced by Daxx depletion (p < 0.001), while deposition of H3.1 was not affected. Despite robust accumulation of Daxx at CEN/periCEN loci upon HS application, we did not observe major changes in H3.3 incorporation into both TRs in control-depleted (and also in Daxx-depleted) cells upon 1 h of recovery. We concluded that Daxx is required for deposition of H3.3 at CEN and periCEN at 37°C, but not during HS application/recovery.

Daxx protects epigenetic signature of heterochromatin. We did not observe major changes in H3.3 loading at CEN/periCEN at 1 h of HS recovery (Fig. 3), the time-point of (or immediately after) maximal Daxx association with these TRs (Figs. 1 and S2), indicating a potential alternative role of Daxx at heterochromatin during stress/recovery. In order to examine this novel Daxx function, we analyzed epigenetic modifications at CEN/periCEN by ChIP assay. It was previously shown that periCEN is enriched with repressive chromatin marker H3K9Me3, while active chromatin marker H3K4Me2 is specifically associated with CEN,24 thus, we focused our analysis on these epigenetic markers of chromatin before, during and after HS application.
At 37°C, Daxx depletion resulted in reduction of H3K9Me3 levels at both CEN and periCEN (p < 0.01) that was not further affected by HS (Fig. 4 and right). HS slightly reduced H3K0Me3 at periCEN in control-depleted cells; this data may explain recent observation that loading of HP1 (that is H3K9Me3-dependent) is diminished in response to HS.17

At 37°C, depletion of Daxx resulted in reduction of H3K4Me2 levels at CEN [α-satellite, Fig. 4 left top, p < 0.01]. The difference disappeared after HS application: H3K4Me2 level in control cells decreased, while it was not affected in Daxx-depleted cells. Level of active chromatin marker H3K4Me2 correlated with the dynamics of CEN transcription (compare Fig. 4 to Fig. 2): at 37°C, relative amount of transcripts was reduced in Daxx-depleted cells; after HS, however, level of H3K4Me2 in control cells decreased, while it was not affected in Daxx-depleted cells. Our data are in agreement with the observation that the level of centromeric transcription is reduced in the absence of H3K4Me2.2 Depletion of Daxx did not affect H3K4Me2 at periCEN (H3S-9) in normal conditions (Fig. 4 and bottom); however, this modification accumulated during stress recovery, at 2- to 3-fold at 1 h (p < 0.01) and almost 10-fold at 12 h (p < 0.001, Fig. 4). Thus, depletion of Daxx resulted in post-stress enrichment of periCEN with active chromatin marker H3K4Me2. We concluded that depletion of Daxx leads to epigenetic changes of heterochromatin in response to stress, specifically elevating presence of active chromatin marker H3K4Me2 at periCEN. This observation suggests a function for Daxx-containing complex in “epigenetic signature” protection at CEN/periCEN heterochromatin upon stress application.

Discussion

Daxx is a multi-functional ND10/PML NBs associated protein9,10 that was identified recently as a histone chaperone incorporating histone H3.3 into pericentromeric MaSat in mouse fibroblasts.37 Here we present evidence that Daxx possesses a similar histone chaperone activity incorporating H3.3 in human periCEN, suggesting a conservative chaperone function of Daxx, despite the major differences in pericentromeric heterochromatin structures between mouse and human species.30 In addition, we show that Daxx possesses the same H3.3 loading activity toward CEN chromatin in human cells. Thus, in combination with published data in reference 16 and 17, we conclude that Daxx incorporates H3.3 into both centromeric and pericentromeric regions in human and mouse cells. This implicates involvement of Daxx in the establishment of a “H3 barcode” at heterochromatin regions,31 confirming previously suggested model.32 In agreement with the reported role of H3.3 in transcription activation, Daxx-dependent incorporation of H3.3 may mechanistically explain transcription reduction from both TRs upon Daxx depletion (Fig. 2).

In normal conditions, Daxx is associated with ND10/PML NBs, in agreement with previously published observation,14 we also found minor accumulation at CEN/periCEN in a subpopulation of cells, suggesting potential cell cycle dependence of this transient association. Daxx was previously found at pericentromeric heterochromatin (MaSat) at the end of S/beginning of G2 phases in mouse cells,15 suggesting that Daxx-containing chaperone complex may incorporate H3.3 into human CEN/periCEN upon transient association with these heterochromatic regions, likely at the end of S/G phase.

We found dramatic elevation of Daxx association with CEN/periCEN in response to HS (Fig. 1). HS-induced Daxx accumulation does not significantly affect H3.3 incorporation to CEN/periCEN, suggesting an alternative function of Daxx-containing complex at this region of heterochromatin during stress/recovery. In our search for this function, we turned to the epigenetic modifications of heterochromatin. Based on the currently accepted model,30 CEN/periCEN regions are associated with a specific set

![Figure 2. Daxx-dependent expression of CEN/periCEN upon HS and recovery. RNA was purified from control (CTL) and Daxx-depleted HEp2 cells at 37°C, immediately after HS at 42°C for 1 h or after recovery at 37°C for 1–12 h. qPCR analysis of α-satellite repeats transcripts (CEN, left) and H3S-9 transcripts (periCEN, right). Transcript levels were normalized to GAPDH expression levels. Bars represent the mean between replicates (mean ± SD).](image-url)
can be explained by reduced transcription from corresponding TRs and may potentially reduce heterochromatin formation. What happens with “epigenetic signature” of heterochromatin in response to stress, including HS, is not well characterized, though burst of periCEN transcription may suggest some adaptive changes in heterochromatin epigenetics. In this regard, sequential waves of epigenetic modifications were documented at heterochromatin after HS; it was proposed that transcription from periCEN is necessary for the post-stress reconstitution of heterochromatin structure. In agreement with this model, depletion of Daxx induces robust changes in the epigenetic modifications at periCEN regions upon stress recovery. Specifically, we found elevation of H3K4Me2 that, in turn, may perturb the epigenetic balance important for centromere/kinetochore structure and function. As H3K4Me2 at CEN is necessary to maintain CENP-A balance, depletion of Daxx may also result in post-stress reduction in CENP-A loading.

In summary, we found that Daxx is important for loading of histone H3.3 on human CEN/periCEN TRs by transient association of epigenetic modifications: centromeric CENP-A-containing nucleosomes (centromere-specific variant of histone H3) are interspersed with transcriptionally-prompted H3K4Me2 nucleosomes that participate in recruitment of CENP-A chaperone HJURP to centromere and maintenance of CENP-A balance. Flanking heterochromatic TRs (periCEN) are mostly lacking H3K4Me2, but are enriched in repressive H3K9Me3 modification. This “epigenetic signature” is necessary for genome integrity and proper CEN/periCEN function; forced changes in these modifications lead to the loss of centromere structure/function, as shown recently for the human artificial chromosome. We found that depletion of Daxx results in decrease of both modifications at CEN/periCEN in normal conditions; whether these changes are associated with reduced incorporation of histone H3.3 can be an appealing model that is awaiting further confirmation. TRs transcription participates in RNAi-mediated attraction ofClr4/Su(var)3–9 to CEN/periCEN, that in turn methylates H3K9 thus recruiting HP1 for heterochromatin establishing. Thus, decrease of H3K9Me3 at CEN/periCEN upon Daxx depletion can be explained by reduced transcription from corresponding TRs and may potentially reduce heterochromatin formation.

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with these genomic regions in normal conditions. H3.3 loading is associated with elevated transcription at both TRs: at CEN, in normal conditions, and at periCEN, in stress-induced conditions. After stress, “epigenetic signature” is protected via Daxx-containing complex assembly at CEN/periCEN. Based on this model, repression or mutations of Daxx suppose to: (1) reduce incorporation of H3.3 on TRs; (2) reduce CEN/periCEN transcription; (3) change “epigenetic signature” of CEN/periCEN chromatin. Combination of these changes may compromise essential heterochromatin functions, therefore potentially increasing genomic instability and initiating malignant transformation of cells. In this context, the recent finding that mutations in Daxx and its H3.3 chaperone partner ATRX are elevated in pancreatic cancer and glioblastoma may suggest function of Daxx/ATRX H3.3 chaperone in tumorigenesis.

Several questions associated with this model remained unresolved: (1) What is the mechanism of Daxx association with CEN/periCEN in normal conditions and upon stress application? (2) How can Daxx protect “epigenetic signature” at CEN/periCEN after stress? (3) Finally, what is the function of CEN and periCEN transcription and/or transcripts in normal conditions, upon stress application and during cell transformation, given elevated TR expression recently described in epithelial cancers. Future studies are needed to elucidate mechanism of propagation and maintenance of heterochromatin, which may clarify functions of this part of the genome in normal and pathological conditions.

Materials and Methods

Cell lines and stress conditions. HEp2 cell lines stably expressing Daxx and control shRNA were additionally transfected with either Daxx siRNA or Non-Targeting siRNA correspondingly (Daxx siGENOME SMARTpool M004420-01-0005; siGENOME Non-Targeting siRNA Pools D-001206-13, Dharmacon, Lafayette, CO) 48 h before experiments to achieve maximal Daxx depletion. HEp2 cells stably expressing Flag tagged H3.3 and H3.1 were created on the background of Daxx and control shRNA cells using pOZ plasmid (Fig. S4). All cells were grown at 37°C in a humidified 5% CO2 atmosphere. For HS, cells were
placed at 42°C in a humidified 5% CO₂ atmosphere for 1 h; for recovery, cells were returned to 37°C in a humidified 5% CO₂ atmosphere for indicated periods of time.

Quantitative RT-PCR. Total RNA was prepared using the Tri Reagent according to the manufacturer instructions (Sigma, St. Louis, MO). RNA was treated with the Turbo DNA-free kit (Applied Biosystems, Carlsbad, CA) to remove the potential traces of DNA. cDNA was produced with SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA) using 1 μg of isolated RNA. Quantitative PCR was performed on the StepOne RT-PCR machine (Applied Biosystems, Foster City, CA) with either Fast SYBR Green Master Mix (HS3-9, HSPPA) or Power SYBR Green Master Mix (to-satellite) (Applied Biosystems, Foster City, CA); see Table S1 for primers and conditions. Expression was normalized to the GAPDH using the formulas: ΔC(T) = C(T(HS3-9 or to-satellite) - C(T(GAPDH)); ΔΔC(T) = C(T(input)) - C(T(37°C)). RQ = 2^{-ΔΔC(T)}.

Table S1

Table S1

| Gene          | Accession Number |
|---------------|------------------|
| HS3-9         | HS3-9            |
| to-satellite  | to-satellite     |

Isolation Buffer (15 mM Tris pH 7.5, 15 mM NaCl, 60 mM KCl, 0.34 M sucrose) supplemented with 1 mM CaCl₂. Nuclei were isolated by centrifugation at 1,000x g for 5 min and resuspended in Chromatin TE buffer and treated with proteinase K for 2 h. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Chromatin was eluted with a buffer containing 10 mM Tris pH 8.0, 1% SDS, 5 mM EDTA at 65°C for 30 min. Cross-link was reversed by addition of 5 M NaCl and incubation at 65°C for 4 h. The samples were diluted with equal volume of TE buffer and treated with proteinase K for 2 h. DNA was purified by Phenol/Chloroform extraction and ethanol precipitation. Enrichment for Flag IP was calculated as follows: ΔC(T) = C(T bound fraction - CT input fraction). ΔΔC(T) = ΔC(T (pOZ-Flag-H3.1 or pOZ-Flag-H3.3) - ΔC(T (pOZ-Flag, 37°C)). RQ = 2^{-ΔΔC(T)}.

To analyze association of Daxx with CEN and periCEN, HEp2 cells were modified for stable expression of Flag tagged Daxx using pOZ plasmid. Formaldehyde was added directly to cells media to final concentration 1%. The reaction was quenched after 10 min by adding Glycine to final concentration 0.125 M for 5 min. Cells were washed twice with PBS, resuspended in Lysis buffer (50 mM TRIS-HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton) and incubated for 10 min with rotation. Cells were centrifuged at 1,000 g for 5 min, supernatant was discarded and pellet was resuspended in IP buffer (50 mM Tris-Hcl, pH 7.4; 400 mM NaCl, 1 mM EDTA, 1% Triton) supplemented with 0.04% SDS, transferred into 5 ml Falcon round-bottom tube and sonicated using Misonix sonicator (3000 (6 cycles 10 sec on/50 sec off, power 2.5). Insoluble material was removed by centrifugation. Soluble chromatin fraction was diluted with equal volume of IP buffer to reduce SDS concentration to 0.02%. Chromatin was immunoprecipitated with anti-Flag M2 Magnetic Beads (Sigma) for 2 h with rotation. Beads were washed once with IP buffer supplemented with 0.02% SDS then three times with IP buffer and finally with TE buffer. Chromatin was eluted with a buffer containing 10 mM Tris pH 8.0, 1% SDS, 5 mM EDTA at 65°C for 30 min. Cross-link was reversed by addition of 5 M NaCl and incubation at 65°C for 4 h. The samples were diluted with equal volume of TE buffer and treated with proteinase K for 2 h. DNA was purified by Phenol/Chloroform extraction and ethanol precipitation. Enrichment for Flag IP was calculated as follows: ΔC(T) = C(T bound fraction - CT input fraction). ΔΔC(T) = ΔC(T (pOZ-Flag-Daxx) - ΔC(T (pOZ-Flag)). RQ = 2^{-ΔΔC(T)}.

Immunofluorescence. Immunofluorescence analysis was completed on cells grown overnight on coverslips in 24 well plates (Corning Inc.) as described previously in reference 15. The following primary antibodies were used: Daxx 5.14 monoclonal,27 PML 5.16 monodo- nal,30 PML 14 rabbit,31 HSFI rat polyclonal (Abcam), CREST human autoimmune antibodies (gift of Dr Gerd Maul, Wistar Institute). Cells were stained with Hoechst 33342 (Sigma) for DNA visualization and mounted on slides with Fluoromount G (Southern Biotech). Images were analyzed using Leica TCS SP5 confocal microscope. Colocalization analysis (Pearson’s correlation coefficient)35 on confocal images has been used to quantify the degree of association for Daxx/PML (for PML, NBCs) and Daxx/CREST (for centromeres). For calculation of the Pearson’s correlation coefficient, images were processed and analyzed using MetaMorph software, version 6.0. At least 50 cells were analyzed for each time point; experiments were repeated three times.
No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
landebioscience.com/journals/article/2018/01
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