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Nitish Gulve

University of Wuerzburg

Zhong Deng

The Wistar Institute

Samantha Soldan

The Wistar Institute

Olga Vladimirova

Gene Expression and Regulation Program, The Wistar Institute

Jayamanna Wickramasinghe

The Wistar Institute

Hongwu Zheng

Weill Cornell Medicine

Andrew Kossenkov

Wistar Institute

Paul Lieberman (✉ lieberman@wistar.org)

The Wistar Institute

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DAXX-ATR-X-H3.3 Regulation of p53 Chromatin Binding and DNA Damage Response

Nitish Gulve¹, Zhong Deng¹, Samantha S. Soldan¹, Olga Vladimirova¹, Jayamanna Wickramasinghe¹, Hongwu Zheng², Andrew V. Kossnekov¹, and Paul M Lieberman^{1*}

¹ The Wistar Institute, Philadelphia, PA 19104

² Weill School of Medicine, Cornell. University, New York, NY

*Corresponding Author

lieberman@wistar.org

Abstract

DAXX and ATRX are tumor suppressor proteins that form a histone H3.3 chaperone complex and are frequently mutated in cancers with the alternative lengthening of telomeres (ALT), such as pediatric glioblastoma. Rapid loss of function of either DAXX or ATRX are not by themselves sufficient to induce the ALT phenotype. However, cells lacking DAXX or ATRX can be readily selected for ALT-like features. Here, we show that DAXX and ATRX null glioblastoma cells with ALT-like features have defects in p53 chromatin binding and DNA damage response regulation. RNA-seq analysis of DAXX or ATRX null U87 glioblastoma cells with ALT-like features revealed that p53 pathway is among perturbed. ALT-selected DAXX and ATRX-null cells had aberrant response to DNA damaging agent etoposide. Both DAXX and ATRX-null ALT cells showed a loss of p53 binding at a subset of response elements. Complementation of DAXX null cells with a wild-type DAXX transgene rescued p53 binding and transcription, while the tumor associated mutation L130R that disrupts ATRX binding was incapable of rescuing p53 chromatin binding. We show that histone H3.3 binding is reduced in DAXX-null cells especially at subtelomeric p53 binding sites and telomere repeats. These findings indicate that DAXX and ATRX function to enable p53 chromatin binding through modulation of histone H3.3 binding, especially at sub-telomeric sites.

Introduction

DAXX and ATRX have been implicated as tumor suppressor proteins that restrict the alternative lengthening of telomeres (ALT). ALT is a recombination-based mechanisms that arises in 10-15% of cancer through poorly understood genetic and epigenetic changes^{5,6,7}. Whole genome sequencing studies have identified loss of function mutations in ATRX, DAXX, and histone H3.3 to be significantly associated with ALT induction in different classes of gliomas⁸⁻¹⁰, sarcomas, and pancreatic neuroendocrine tumors^{1,2}. Moreover, analysis of 22 immortalized ALT cell lines have also underlined the involvement of ATRX/DAXX complex in ALT activation¹¹. ALT cells modulate chromatin and DNA repair pathways that facilitate telomeres elongation through homologous DNA recombination¹². Telomere recombination is known to be inhibited by telomere repeat binding factors and telomeric heterochromatin^{3,4}. Both DAXX or ATRX are known to contribute to telomeric heterochromatin and to limit homologous recombination^{5,6,7,8}. However, loss of DAXX or ATRX is not sufficient to establish telomere recombination and cell proliferation associated with ALT^{9,10}.

DAXX and ATRX are thought to suppress ALT, in part, through their ability to load histone H3.3 into chromatin^{5,9,11}. ALT cells have altered telomeric heterochromatin and accumulate high levels of single-strand DNA and DNA damage marked by association with γ H2AX and 53BP1 at telomeres⁹. This DNA damage signaling reflects both double strand breaks and homologous recombination repair. The extent and tolerance of DNA damage increases further upon mutation of TP53 (p53)¹³. TCGA pan cancer atlas study of ALT related genes show *TP53* to be one of the top mutated genes¹⁴. p53 is a DNA binding protein and master transcriptional regulator of genome integrity controlling transcription of gene networks for cellular senescence and apoptosis^{15,16}. p53 binds to several loci within the cellular genome including promoters, enhancers, transcriptionally inactive regions as well as retrotransposon-like

elements in many human subtelomeres^{17,18}. The inter-relationship between DAXX-ATRX, and p53 DNA-damage response function in ALT is not completely understood.

Both DAXX and ATRX are multifunctional tumor suppressor proteins^{5, 12}. ATRX and DAXX form a complex together with the histone variant H3.3 which is responsible for depositing H3.3 at heterochromatin regions and telomeric regions of the genome²²⁻²⁴. In addition to their function in loading H3.3 onto telomeric chromatin, DAXX and ATRX bind many other proteins, including p53 and MDM2, and can localize with PML-containing nuclear bodies (PML-NBs) that associate with ALT telomeres. ATRX/DAXX/H3.3 and p53 mutations frequently co-occur in ALT cancers⁹, suggesting that these pathways may be additive in cancer cell progression. In a previous study, we demonstrated that loss of ATRX or DAXX is not sufficient for ALT, but that additional genetic and/or epigenetic events can lead to the emergence of clones with ALT-like features⁹. Here, we investigate the relationship between DAXX and ATRX loss of function and p53 DNA damage sensing pathway using sets of syngenic glioblastoma cell lines with CRISPR/Cas9 knock-out of DAXX or ATRX that have acquired ALT-like features relative to parental controls. Our findings suggest that the DAXX-ATRX-H3.3 axis is required for p53 chromatin binding and DNA damage response, and that disruption of these pathways are critical for ALT cell survival.

RESULTS

Deregulation of p53 pathway in DAXX and ATRX knock-out cells with ALT-like features.

We have previously reported the generation of ATRX and DAXX deficient U87-T glioblastoma cell lines that have acquired ALT-like features⁹. In that study, we found that transient depletion of DAXX or ATRX led to an increase in senescent cells, but that continuous passage enabled the isolation of clonal survivors with hallmarks of ALT. To gain further insight into the common

features of these ALT-like DAXX_KO and ATRX_KO cells, we performed RNA-seq and transcriptomic analysis. We found overlap of 2724 genes significantly (FDR<5%) affected by both DAXX_KO and ATRX_KO relative to parental U87-T control cells (**Fig. 1a**). Gene enrichment analysis of the overlap revealed a number of significantly affected regulators (FDR<10⁻⁵, Z>2) with p53 reaching the most significance and predicted to be more active in the KO cell lines (**Fig. 1b**). Seven known p53 direct targets among 28 top upregulated genes (FDR<5% in both, >5 fold up regulation) were identified, including TMTC1, PTGES, CSF1, PLAGL1, MRAS, RTN1, and CD70 (**Fig. 1c**). Several of these gene have been implicated in glioblastoma tumorigenesis^{13, 14, 15, 16, 17}.

We re-validated that the DAXX_KO cell line had ALT-like features (**Fig. S1**). Southern blot analysis shows that DAXX knock-out (DAXX_KO) cell lines have more heterogeneous telomere length distribution compared to parental controls (Ctrl) (**Fig. S1b**). Consistent with our previous publication¹⁸, the bulk of telomere DNA signals reduced in both Ctrl and DAXX_KO cells treated with topoisomerase inhibitor etoposide. Telomere circles (t-circles) were observed in DAXX_KO cells by 2D agarose gel analysis, and their frequency was reduced by re-introduction of a wild-type (WT) DAXX transgene (**Fig. S1c**). ALT-associated PML-nuclear bodies (APBs) with enlarged PML-NBs and colocalization with telomere DNA, as measured by IF and FISH respectively, were observed at high-frequency in DAXX_KO relative to WT cells, and this could be suppressed in cells with DAXX WT transgene but not by DAXX L130R mutant transgene (**Fig. S1d and e**). DAXX L130R is a tumor derived mutation of DAXX with biochemical defect in ATRX binding and histone H3.3 chaperone function. Furthermore, DAXX_KO cells had elevated telomere C-circles as measured by rolling circle assay, and this was suppressed in cells expressing WT DAXX but not DAXX L130R transgene (**Fig. S1f**). These findings are consistent with our previous study showing ALT-like features in these and related cell lines with ATRX and DAXX knock-outs⁹.

Attenuated DNA damage response in ALT-like cells lacking ATRX or DAXX.

Given the transcriptomic alteration of p53 response pathway in ATRX_KO and DAXX_KO cells, we assessed their ability to respond to DNA damage relative to parental control U87-T cell line. We found that the topoisomerase inhibitor etoposide induced a potent p53-response and corresponding growth inhibition in parental U87-T cells (**Fig. 2**). Western blot analysis indicated that p53 and phospho-S15 p53 levels were induced in U87-T parental, and to a lesser extent in DAXX_KO (**Fig. 2a**) and ATRX_KO (**Fig 2b**) cell lines. In contrast, γ H2AX levels were modestly increased in KO cells relative to parental control. These findings suggest that KO cell lines have attenuated p53 response and accumulate higher levels of γ H2AX DNA damage in response to etoposide treatment. Cell cycle distribution of each cell type with or without etoposide treatment was assayed by flow cytometry (**Fig. 2c and d**). We found that DAXX_KO and ATRX_KO cells were enriched in G1 and depleted in S/G2 relative to control under normal growth conditions, while the percent in S phase increased in KO cells relative to control after etoposide treatment. A clonogenicity assay also revealed that DAXX_KO and ATRX_KO had defective growth inhibition relative to parental control in response to etoposide treatment (**Fig 2e and f**). Taken together, these findings indicate that DAXX and ATRX KO U87-T cells have changes in cell cycle distribution and cell cycle arrest defects in response to DNA damage.

Altered p53 response transcriptome in U87 ALT-like cells lacking ATRX or DAXX.

We next assayed the RNA-seq (Quantseq) transcriptomic response to DNA damaging agent etoposide in DAXX_KO and ATRX_KO U87 ALT-like cells relative to parental control U87-T cells (**Fig. 3a**). 933 genes in DAXX_KO and 1562 genes ATRX_KO were found to be less responsive to etoposide (upregulated in WT at FDR<5%, but significantly less upregulated in KO condition at $p<0.05$). There was an overlap of 512 genes between the two cell lines, a 6.4 fold more than expected by chance alone indicating very similar functional effect between the

two KO cell lines. Enrichment analysis showed several the most affected regulators ($FDR < 10^{-3}$, $Z > 2$) including p53 to be significantly inhibited in response to etoposide in DAXX_KO or ATRX_KO cells (**Fig. 3b**). There was a strong correlation of the magnitude of the attenuation effect between DAXX_KO and ATRX_KO (Spearman $r = 0.34$, **Fig. 3c**). Among the genes with the most reduced response (at least 4 fold, **Fig. 3c**) there were many well-characterized p53 response genes, including GADD45A, TNFRSF10A (TRAIL), MAFB, TP53INP1, USP2, and TIMP3. RT-qPCR demonstrated that some p53 response genes, such as GADD45A, CYP4F3, and PAR6G were affected by DAXX-KO and ATRX_KO, while other genes, such as CDKN1A, were not significantly affected in DAXX_KO (**Fig. 3d**).

Altered p53 chromatin binding in DAXX_KO and ATRX_KO U87 ALT cells.

Since many p53 response genes were affected in both DAXX_KO and ATRX_KO cells, we assayed p53 binding by ChIP-seq in DAXX_KO and control cells with or without etoposide treatment (**Fig. 4**). After etoposide treatment we observe significant loss of p53 peak signal throughout the genome in DAXX KO cells (**Fig. 4a**). We identified a subset of p53 binding sites that were more significantly affected as compared to other regions where there was only modest reduction. We highlight sites at the CDKN1A, GADD45A, and miR-34 genes that showed significant disruption in DAXX_KO cells compared to controls. A consensus sequence analysis using JASPAR and HOMER indicated that two subtypes of p53 consensus binding sites were most affected in DAXX_KO cells (**Fig. 4b, upper panel**). Most p53 binding sites have a consensus sequence, variations of these sequences such as quarter vs half-sites¹⁸, high-binding cooperativity vs low-binding cooperativity²⁶ or non-canonical binding sequences have different outcomes in terms of regulating gene expression. We also identified several other consensus binding sites, such as ATF and Forkhead transcription factors Foxf1, FoxL2, and FoxP1 that had weak, but significant enrichments with p53 sites and differential gene expression (**Fig. 4b, lower panel**). We validated these p53 binding by ChIP-qPCR for several

p53 binding sites, including those associated with well-known response genes p21 and GADD45, as well as at sub-telomeric p53 binding sites at chromosomes 18q and 13q (**Fig. 4c**). Negative control genomic regions like TMCC1 and CCDC170 demonstrate the specificity of the p53 ChIP assay. Western blot analyses indicate that p53 levels are nearly identical in DAXX_KO and ATRX_KO after etoposide treatment (**Fig. 2a and b**), so it is unlikely that total levels of p53 account for these substantial differences in chromatin binding. Further, we assayed another ALT cell line, the osteosarcoma derived U2OS, that is known to have an ATRX null background^{19, 20}. Although p53 was stabilized and phosphorylated upon etoposide treatment as measured by Western blot (**Supplemental Fig. S2a**), we observe weak (less than 2-fold) stabilization of p53 at the response elements in the promoters of CDKN1A and GADD45A and no stabilization at the subtelomeric response elements (**Supplemental Fig. S2b**). These findings suggest that DAXX or ATRX are necessary for efficient chromatin binding in ALT cells.

Rescue of p53 binding by DAXX requires H3.3 chaperone function.

To more rigorously determine whether the loss of p53 binding is a specific phenotype of DAXX KO and not an off-target effect of CRISPR gene editing, we generated a rescue DAXX KO cell line by expressing full length wild-type YFP-tagged hDAXX (hDAXX) (**Fig. 5a**). We noticed that DAXX overexpression led to an increase in p53 protein levels in response to etoposide treatment. This suggests p53 protein expression levels may be partly dependent on DAXX. To determine if an interaction between DAXX and p53 may be altered, we assayed this potential interaction by coIP (**Supplementary Fig. S3**). However, we were unable to demonstrate a stable interaction between p53 and DAXX, although MDM2 was identified in p53 coIP, and ATRX was identified in DAXX co-IP (**Supplementary Fig. S3**). ChIP-qPCR studies showed that the p53 binding at various p53 response elements was defective in DAXX_KO cells, but was restored in the cells expressing hDAXX (**Fig. 5b**). This was confirmed at the p21 promoter,

GADD45A gene, and subtelomeres 18q and 13q. We next tested whether the DAXX mutant deficient for ATRX binding (L130R) and H3.3 binding histone chaperone function could rescue p53 binding (**Fig. 5c-d**). In contrast to wild-type hDAXX, L130R mutant failed to rescue p53 binding at each of the response elements tested (**Fig. 5d**). This suggests that ATRX binding and H3.3 loading are important for p53 chromatin binding. To test this more directly, we assayed H3.3 binding by ChIP-qPCR. We found that DAXX_KO cells were depleted for histone H3.3 at several p53 binding sites, including those at subtelomeres 13q and 18q (**Fig. 5e**). We further assayed H3.3 loading at telomere repeats by dot blot analysis, and that DAXX_KO cells had reduced H3.3 at telomere repeats relative to controls, and relative to actively transcribing GAPDH gene (**Fig. 5h**). Taken together, these results suggest that p53 chromatin binding depends on H3.3 loading by DAXX-ATRX in these ALT-like cells.

DISCUSSION

DAXX and ATRX function as tumor suppressors that are frequently mutated in ALT cancers, yet their precise role in regulating ALT is not completely clear. Here, we show that loss of DAXX or ATRX in a U87 derived glioblastoma cell line gives rise to ALT-like phenotype, with corresponding aberrations in the p53-dependent DNA damage response. We found that U87-T cells lacking DAXX or ATRX had increase p53 pathway activation. Paradoxically, these same cells failed to mount a robust p53-response to exogenous DNA damage from etoposide. Our findings suggest that loss of DAXX or ATRX result in a chronic DNA damage tolerance that limits p53 response to further DNA damage. Our data further suggests that this DNA damage tolerance is linked to the failure to load histone H3.3 at p53 response elements and telomere repeat DNA (**Fig. 6**).

Both DAXX and ATRX have been implicated in regulation of p53 and DNA damage response pathways. DAXX has been shown to co-purify with p53 in several cell types. A recent study has found that DAXX functions as a protein foldase and its interaction with p53 regulates interaction with MDM2 and p53 functional activity²¹. In a mouse genetic study, heterozygous deletions of DAXX led to an increase sensitivity to low dose radiation and higher incidence of carcinomas, but no overt defect in p53 function or pathway was reported²². In a different mouse study, DAXX loss led to the de-repression of endogenous retroviral elements (ERVs) that could trigger DNA damage signaling²³. In HEK cells, DAXX knock-out had little detectable effect on p53 target genes in response to DNA damage²⁴. On the other hand, DAXX knock-out in PanNET cells led to the derepression of oncogenic drivers, including STC2, which was found be regulated by DAXX/H3.3/H3K9me3 pathway²⁵. The ability to reverse ALT phenotype by the re-introduction of ATRX and DAXX is also context and cell-type dependent^{31,32}. These findings suggest that DAXX may interact with p53 pathway through various mechanisms that depend on cell or cancer type.

Mutations in ATRX and DAXX can occur in combination with missense mutations in TP53 in many glioblastomas^{26, 27}. These missense mutations may have partial and altered activities, and in combination with DAXX or ATRX, result in a further loss of p53 tumor suppressor function. Many TP53 mutations confer gain-of-function activity that affects chromatin accessibility²⁸. G-quadruplex DNA was identified as a feature that was differentially recognized by some p53 gain of function mutants. Some gain of function mutations in p53 were found to have differential binding by ChIP-seq and this binding was found dependent on the chromatin modifying complex MLL²⁹. ATRX is important for suppression of G4 quartet formation³⁰ and G4 quartets can inhibit p53 binding³¹. So, it is possible that ATRX deficiency prevent p53 binding due to formation of G4 quartet structures at p53 response elements.

Our study finds that DAXX and ATRX KO cells with ALT-like phenotype have aberrant p53 DNA damage response pathways. These cells have an increase in the constitutive

activation of p53 response genes, but a failure of p53 to respond to exogenous damage. We note that p73 and p63 are elevated under these conditions and may partly account for the basal activation of the p53 pathway in the absence of p53 functionality due to ATRX or DAXX loss. The loss of p53 functionality correlates with reduced binding to consensus sequences throughout the genome with more drastic effects at some subtelomeric sites. We find that the DAXX-dependent defect in p53 correlates with its interaction with ATRX and the assembly of H3.3 on chromatin, including telomeres. We conclude that the DAXX-ATRX-H3.3 axis plays an important role in regulating p53 access to its binding sites and regulation of the p53 DNA damage response. We propose that this aberrant DNA damage response is a key contributor to the acquisition of the ALT phenotype.

METHODS

Cell lines

U87, U2OS and HEK293T cells used in this study were obtained from American type culture collection (ATCC). U87, U87-T, U87 derived cell lines were described previously⁹. U87-T cells were generated by transducing U87 cells with pBabe-Lox-TERT-Lox. ALT positive single cell clones of DAXX_KO and ATRX_KO U87 cells were previously described and characterized¹⁹. DAXX_KO cells is previously referred to as U87-T 4-17⁹. ATRX_KO is previously referred to as U87-T A16L⁹. U87-T sgCon (referred to as Control) has empty Cas9 lentivirus vector with TERT transgene. U87-T derived and HEK293T cells were cultured in DMEM (life technologies) supplemented with 10% FBS and penicillin/streptomycin. U2OS cells were cultured in McCoy's 5A medium (life technologies) supplemented with 10% FBS and penicillin/streptomycin.

Antibodies and Chemicals

The following antibodies were used: p53 DO-1 (sc-126X for ChIP), p53 DO-1 (Millipore OP-43L for Western blots), γ H2AX JBX-301 (Millipore 05-636), phospho-p53 (Ser-15) (Cell Signaling technology 9284), ATRX (Bethyl Laboratories A301-045A), DAXX (Sigma 07-471) and MDM2 (Cell Signaling technology 86934). Etoposide Sigma (E1383) was dissolved as 1 mM stock solution in DMSO and used at concentrations and times indicated.

Analysis of cell cycle kinetics

U87-T WT shControl, Daxx_KO, and ATRX_KO cells were seeded at 1×10^5 cells/well in 6-well plates and exposed to etoposide (10 μ M) or DMSO control in biological triplicates per each condition. After 24h, cells were permeabilized with cold, 70% ethanol and resuspended in PBS containing PI (10 mg/mL) and RNase A solution (100 μ g/mL). Flow cytometry was performed on a BD-LSR II (BD Biosciences; Bedford, MA) and FloJo software (Ashland, OR) was used for cell cycle analysis.

RNA-seq

Total RNA was extracted using Trizol following manufacturer's instructions. RNA was treated with Turbo DNA-free kit (life technologies) and quality checked with TapeStation (Agilent). Libraries were prepared using Quant-seq 3'-mRNA kit (Illumina) and sequencing was performed on Illumina Nextseq500 on high-output mode in a 75 bp single end run. RNA-seq data was aligned using STAR³² algorithm against hg19 human genome and RSEM v1.2.12 software³³ was used to estimate read counts using gene information from Ensemble transcriptome version GRCh37.p12. Raw counts were used to estimate significance of differential expression difference between any two experimental groups using DESeq2³⁴. Overall gene expression changes were considered significant if passed FDR<5 threshold. Gene set enrichment analysis was done using QIAGEN's Ingenuity Pathway Analysis software (IPA, QIAGEN Redwood

City, www.qiagen.com/ingenuity) using “Upstream Regulators” options. Most significant regulators ($p < 0.001$, unless stated otherwise) that had a significantly predicted activation state ($|Z| > 2$) were reported.

ChIP-seq

ChIP-seq was performed as previously described¹⁸ with certain modifications. Etoposide (10 μ M) treated cells were harvested after 24 hours of treatment, fixed with formaldehyde for 10 min followed by quenching with glycine. 25 million cells per sample were sonicated using Covaris sonicator to obtain fragments of size between 100-500 bp. Chromatin was incubated with 10 mg of p53 antibody (Santa Cruz sc-126X) overnight followed by pull-down using protein- G Dynabeads (life technologies). Beads were washed, eluted and reverse cross-linked overnight. IP DNA was purified using Sigma PCR purification kit. Quality control was performed on TapeStation (Agilent). Libraries were prepared using Illumina protocol and sequencing 75bp single end on Illumina Nextseq500 on high-output mode. The data was aligned using bowtie³⁵ against hg19 version of the human genome and HOMER³⁶ was used to generate bigwig files and call significant peaks vs input using `-style factor` option. Peaks that passed FDR<5% threshold were considered significant. Normalized signals for significant peaks were derived from bigwig files using `bigWigAverageOverBed` tool from UCSC toolbox³⁷ with `mean0` option. Fold differences between samples were then calculated and p53 peaks that reduced signal at least 2-fold were considered as affected by DAXX KO. Genes were associated with peaks based on 3kb from any transcript TSS threshold.

ChIP-qPCR

ChIP-qPCR was performed similar to ChIP-seq with few changes. 1 million cells per sample were used and sonication was performed using Diagenode water bath sonicator at high range

30s on 30s off for a total of ~20 min with intermittent incubation on ice to obtain fragment size between 100-500 bp. qPCR primers were designed using NCBI primer blast and the run was performed on ABI QuantStudio 7. Primers used are listed in Supplemental Table 1.

qRT-PCR

Total RNA was extracted using Trizol following manufacturer's instructions. RNA was treated with Turbo DNA-free kit (life technologies). Primers were designed using NCBI primer blast and the run was performed on ABI QuantStudio 7. Primers used are listed in Supplemental Table 1.

Telomere Restriction Fragment (TRF) Analysis of Telomeric DNA Length

TRF analysis of telomere DNA length was performed as described previously⁹. Briefly, genomic DNA was prepared using Wizard genomic DNA purification kit (Promega), digested with *AluI*+*MboI* restriction endonucleases, fractionated in a 0.7% agarose gel, denatured, and transferred onto a GeneScreen Plus hybridization membrane (PerkinElmer). The membrane was cross-linked, hybridized at 42°C with 5'-end-labeled ³²P-(TTAGGG)₄ probe in Church buffer, and washed twice for 5 min with 0.2 M wash buffer (0.2 M Na₂HPO₄ pH 7.2, 1 mM EDTA, and 2% SDS) at room temperature and once with 0.1 M wash buffer at 42°C. The images were analyzed by Phosphor-imager, visualized by Typhoon 9410 Imager (GE Healthcare).

Two-dimensional (2D) gel electrophoresis

Two-dimensional (2D) gel electrophoresis was performed as previously described⁹. Briefly, equal amounts of *AluI*+*MboI* digested DNA (15 µg) was subjected to electrophoresis in a 0.4% agarose gel (first dimension) at room temperature and 30 V for 12-14 hr, and then in a 1.2% agarose gel (second dimension) containing 0.3 µg/ml ethidium bromide at 4°C and 150V for 6 hr. The gel was processed as described above for the Southern blot analysis.

C-circle assay

C-circle assay was performed as described previously⁹. Briefly, sample DNA digested with *A*/ul and *M*bol (30 ng/10 µl) was combined with 10 µl reaction mix [0.2 mg/ml BSA, 0.1% Tween, 0.2 mM each dATP, dGTP, dTTP, 2 × φ29 Buffer, and 7.5 U φDNA polymerase (NEB)]. The reactions were incubated at 30°C for 8 hrs, and then at 65°C for 20 min. The reaction products were diluted to 400 µl with 2 × SSC, dot-blotted onto a GeneScreen Plus membrane, and hybridized with a ³²P-labeled (CCCTAA)₄ probe at 37°C for overnight to detect C-circle amplification products. The blots were washed four times at 37°C in 0.5 × SSC/0.1% SDS buffer, exposed to Phosphor-imager screens, visualized by Typhoon 9410 Imager (GE Healthcare Life Sciences), and quantified with ImageQuant 5.2 software (Molecular Dynamics).

Immuno-FISH assay. Indirect immunofluorescence (IF) combined with fluorescence *in situ* hybridization (FISH) analysis was performed as described previously⁹. Primary antibody to PML (ab96051, Abcam) was prepared in 1:500 dilution in blocking solution. After IF, cells were fixed in 4% paraformaldehyde in 1x PBS for 10 min, washed in 1x PBS, dehydrated in ethanol series (70%, 95%, 100%), and air-dried. Coverslips were denatured for 5 min at 80-85°C in hybridization mix [70% formamide, 10mM Tris-HCl, pH 7.2, and 0.5% blocking solution (Roche)] containing telomeric PNA-Tamra-(CCCTAA)₃ probe, and hybridization was continued for 2 hrs at room temperature in the dark moisturized chambers. Coverslips were washed, counterstained with 0.1 µg/ml DAPI in blocking solution, and mounted with VectorShield (Vector Laboratories). Images were captured with a 63x lens on a Zeiss LSM780 confocal laser scanning microscope (Carl Zeiss). Cells with >5 PML foci colocalizing with telomere DNA foci were scored as APB positive. The quantification was generated from at least three independent Immuno-FISH experiments.

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Competing Interests

PML declares an ownership interest and advisor to Vironika, LLC. No other authors declare competing interests.

Author Contributions

NG, SSS and ZD contributed experimental data, conceptualization and writing. **AVK** contributed data analysis, methodology and writing. **OV** contributed experimental data. **JW** contributed data analysis. **HZ** provided cell line reagents. **PML** contributed conceptualization, resources, and writing.

Figure Legends

Figure 1. Transcriptomic profile of DAXX_KO and ATRX_KO U87 cells. **a.** Venn diagram showing overlap of gene transcripts affected by DAXX_KO or ATRX_KO relative to control U87-T cells based on RNAseq (Quantseq). **b.** IPA for enriched regulators activated (red) or inhibited (blue) by DAXX_KO and ATRX_KO. **c.** Heat map analysis showing top 20 differentially regulated mRNA for DAXX_KO and ATRX_KO relative to WT control. Ratio of KO to WT (K/W) and False Discovery Rate (FDR) are shown. Binding site for p53 within 10kb of transcription start sites is indicated (purple).

Figure 2. Aberrant DNA damage response in DAXX_KO and ATRX_KO U87 cells. **a.** Western blot for Control or DAXX_KO cells treated with DMSO (D) or 1 μ M etoposide (E) for 24 h probed with antibody to DAXX, p53, pS15p53, or Actin. **b.** Western blot for ATRX_KO cells treated same as in panel **a.** **c.** Cell cycle analysis of U87T Control, Daxx_KO, and ATRX_KO cells treated with DMSO or Etoposide (10 μ M) for 24 hours (<G1=black, G1=red, S= green, G2= blue). **d.** Percent increase in S phase populations in Etoposide (10 μ M) treated cells compared to DMSO treated controls. (ANOVA $p=0.0079$; Tukey's multiple comparisons test * $p < 0.05$, ** $p < 0.01$). **e.** Clonogenicity assay with U87-T parental control, DAXX_KO, or ATRX_KO cells treated with DMSO or etoposide followed by replating and staining with crystal violet. **f.** Quantification of clonogenicity assay shown in panel d. Error bars are sdm and *** p -value $< .001$ by student t-test.

Figure 3. Transcriptomic analysis of DNA damaging response in DAXX_KO and ATRX_KO U87 cells. **a.** Venn diagram showing overlap of genes differentially regulating in DAXX_KO and ATRX_KO relative to WT control cells in response to treatment with etoposide. **b.** IPA regulators most affected by etoposide treatment common for both DAXX_KO and

ATRX_KO cells. **c.** Heat map analysis of top 20 differentially regulated genes showing differences in DAXX_KO and ATRX_KO relative to WT control. P53 binding sites within 10kb of gene is indicated in purple. **d.** RT-qPCR for DAXX_KO (top row) or ATRX_KO (lower row) relative to Ctrl after DMSO (grey) or etoposide (red) treatment. Genes analyzed are indicated, CDKN1A, GADD45A, CYP4F3, and PARG6.

Figure 4. Attenuation of p53 chromatin binding in DAXX_KO and ATRX KO U87 cells. **a.** ChIP-seq tracks for p53 in control (blue) or DAXX_KO (red) at gene loci for CDKN1A (top), GADD45A (middle), or miR-34 (lower). **b.** Consensus motifs for p53 ChIP-seq sites with most differential binding between DAXX_KO and WT (top) and for overlapping sites with known factors other than p53. **c.** ChIP-qPCR for p53 in DAXX_KO and WT control cells treated with DMSO or etoposide (red) for binding sites at the p21 promoter, GADD45A, 18q subtelomere, 13q subtelomere, TMCC1 or CCDC170 gene loci. ChIP-qPCR with IgG control is shown in lower panels. **d.** Same as in panel c, except for ATRX_KO cells.

Figure 5. DAXX and H3.3 dependency for p53 function in DAXX_KO U87-T cells. **a.** Western blot of Control (Ctrl), DAXX_KO, or DAXX_KO+hDAXX cells treated with DMSO (D) or 50 μ M etoposide (E) and probed with antibody to DAXX, p53, or Actin. **b.** p53 ChIP-qPCR for cells treated as in panel **a.** **c.** Western blot of Ctrl or DAXX_KO+L130R cells treated with DMSO (D) or etoposide (E) and probed with antibody to DAXX, p53, or Actin. **d.** p53 ChIP-qPCR for cells treated as in panel **c.** **e.** H3.3 ChIP-qPCR in Ctrl or DAXX_KO cells treated with DMSO or etoposide at p21 promoter, GADD45A, 18q subtelomere, 13q subtelomere, or CCDC170 gene loci. **f.** ChIP assay for H3.3 or H3 probed for telomere DNA or control GAPDH DNA in Ctrl or DAXX_KO cells.

Figure 6. Schematic model of p53 chromatin binding dependence on ATRX-DAXX-H3.3 chromatin assembly. H3.3 loading onto p53 response elements at DNA damage response genes and subtelomeres depends on the histone chaperone activity of ATRX and DAXX. Loss of ATRX or DAXX restricts the binding of p53 to chromatin resulting in a failure to activate p53 response genes, such as GADD45 and p21 and aberrant DNA damage response (DDR). Failure to load H3.3. at telomeres and subtelomeres leads loss of telomere T-loops, generation of chronic telomere DNA damage signaling and homologous recombination-based alternative lengthening of telomeres.

Supplemental

Figure S1. ALT-like phenotype in DAXX_KO U87 cells. **a.** Western blot of U87-T control (Ctrl) or DAXX_KO cells with antibody to DAXX (upper) or Actin (lower). **b.** Southern blot analysis of telomere DNA from Ctrl or DAXX_KO cells treated without (-) or with (+) 1 μ M etoposide for 24 hr was detected with TelC probe. **c.** 2D agarose gel analysis of DNA from DAXX_KO cells or DAXX_KO cells reconstituted with hDAXX probed with TelC or stained with ethidium bromide (EtBr). **d.** Immunofluorescence analysis of Ctrl, DAXX_KO, DAXX_KO+hDAXX, or DAXX_KO+L130R DAXX, imaged for Telomere (TelG, red) + DAPI (blue), PML (green) or merged. **e.** Quantification of APBs in Ctrl or DAXX_KO alone, or with hDAXX or L130R DAXX cells. ** indicates p value <.01, student t-test. **f.** C-circle assay showing representative data (insert) and quantification of Ctrl or DAXX_KO alone, or with hDAXX or L130R DAXX cells. ** indicates p value <.01, student t-test.

Figure S2. P53 functionality in U2OS ALT cells. **a.** Western blot of U2OS cells treated with DMSO or 50 mM etoposide for 24 hr, assayed with antibody to p53, pS15-p53, or Actin. **b.** ChIP assay for U2OS cells treated as in panel **a** with either DMSO (grey) or etoposide (red) using antibody to p53 (top row) or igG control (lower row), and assayed by qPCR with primers

for p53 binding sites at the p21 promoter, GADD45A, 18q subtelomere, 13q subtelomere, or TMCC1.

Figure S3. CoIP of p53 with MDM2 but not DAXX or ATRX in U87 cells. U87T control cells were subject to IP with p53, DAXX, or IgG control, and then assayed by Western blot for DAXX, ATRX, p53 or MDM2. Input (5%) is show for each antibody.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6.

Supplementary Data

Fig S1.

Fig S2.

Fig S3.

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