Poly(ADP-ribose) Polymerase 1 Is Inhibited by a Histone H2A Variant, MacroH2A, and Contributes to Silencing of the Inactive X Chromosome*

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Poly(ADP-ribose) polymerase 1 (PARP-1) is a nuclear enzyme that is involved in modulating chromatin structure, regulation of gene expression, and sensing DNA damage. Here, we report that PARP-1 enzymatic activity is inhibited by macroH2A, a vertebrate histone H2A variant that is enriched on facultative heterochromatin. MacroH2A family members have a large C-terminal non-histone domain (NHD) and H2A-like histone domain. MacroH2A1.2 and PARP-1 interact in vivo and in vitro via the NHD. The NHD of each macroH2A family member was sufficient to inhibit PARP-1 enzymatic activity in vitro. The NHD of macroH2A1.2 was a mixed inhibitor of PARP-1 catalytic activity, with effects on both catalytic activity and the substrate binding affinity of PARP-1. Depletion of PARP-1 by RNA interference caused reactivation of a reporter gene on the inactive X chromosome, demonstrating that PARP-1 participates in the maintenance of silencing. These results suggest that one function of macroH2A in gene silencing is to inhibit PARP-1 enzymatic activity, and this may affect PARP-1 association with chromatin.

In eukaryotic cells DNA is packaged into chromatin, and this packaging impacts all DNA-templated processes, including transcription. Regulated changes in chromatin structure are crucial to establish and maintain the diverse expression profiles that characterize the hundreds of cell types in multicellular organisms (1). The nucleosome is the structural unit of chromatin and comprises 147 bp of DNA wrapped around a histone octamer, which is composed of two copies each of the four core histones H2A, H2B, H3, and H4.

Chromatin structure can be affected through the action of ATP-dependent chromatin remodeling enzymes or by the covalent modification of histone proteins, creating binding sites for additional regulatory proteins (1, 2). In addition, chromatin structure can be modulated through the binding of effector proteins to nucleosomes. Poly(ADP-ribose) polymerase 1 (PARP-1)5 is an example of a nucleosome binding protein that can affect chromatin structure (3, 4). PARP-1 is the prototypical member of a family of PARP proteins, which catalyze the transfer of ADP-ribose units from donor nicotinamide adenine dinucleotide (NAD+) molecules to target proteins (5). PARP-1 functions as a structural component of chromatin, modulator of chromatin structure, and a sensor of DNA damage through its intrinsic enzymatic activity (4, 6, 7). In the absence of NAD+, PARP-1 binds to nucleosomes, compacts chromatin, and inhibits its transcription in vitro (3). Furthermore, catalytically inactive PARP-1 maintains silencing of heterochromosomal retrotransposons in Drosophila (8). However, at physiological concentrations of NAD+, PARP-1 is enzymatically active and does not bind nucleosomes (3). Despite this, PARP-1 binds chromatin in vivo and is implicated in transcriptional silencing, suggesting that modulation of PARP-1 activity in vivo may be a mechanism that is employed to direct changes in chromatin structure.

Chromatin structure can also be regulated by replacement of core histones with histone variants. MacroH2A1 and macroH2A2 are vertebrate-specific variants that replace H2A in an estimated three percent of nucleosomes (9). MacroH2A family members consist of a histone domain that is highly similar to H2A and a large C-terminal non-histone domain (NHD). The NHD domain is related to a family of proteins that includes a class of ADP-ribose processing enzymes and NAD+ metabolite binding proteins (10–12). MacroH2A1 consists of two isoforms, macroH2A1.1 and macroH2A1.2, that are produced by

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5 The abbreviations used are: PARP-1, poly(ADP-ribose) polymerase 1; NHD, non-histone domain; GFP, green fluorescent protein; mH2A, macroH2A; BRCT, BRCA1 C-terminal region; BAL, B-aggressive lymphoma; PBS, phosphate-buffered saline; Xi, inactive X chromosome; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HA, hemagglutinin; RNAi, RNA interference; 5-Aza-dC, 5-aza-2′-deoxycytidine; TSA, trichostatin A; shRNA, short hairpin RNA.
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alternative splicing and differ by only 30 amino acids in the NHD. All three forms of macroH2A are enriched in regions of silent chromatin, such as the inactive X chromosome (Xi) and senescence-associated heterochromatin (13–17). Depletion of macroH2A1 in female cells causes reactivation of genes on the Xi, demonstrating a role in the maintenance of silent chromatin (18). MacroH2A1 is also required for silencing of the IL-8 gene in a cell-type specific fashion, indicating that it regulates gene expression at individual loci as well as larger domains (19).

Both the H2A-like domain and the NHD of macroH2A are implicated in regulation of gene expression. When the macroH2A1 H2A-like domain is incorporated into nucleosomes, it interferes with SW1/SNF nucleosome remodeling in vitro (20). The NHD associates with histone deacetylases in vivo, interferes with the binding of NF-κB in vitro, and also inhibits the initiation of transcription (20–23), suggesting that macroH2A regulates transcription at multiple levels. Here, we provide evidence for a mechanism by which macroH2A can regulate chromatin structure and gene expression. We find that macroH2A associates with PARP-1 through the NHD in vivo and in vitro and that macroH2A blocks PARP-1 enzymatic activity in vitro. We also demonstrate that the NHD of macroH2A can promote recruitment of PARP-1 to the Xi. Finally, knockdown of PARP-1 results in reactivation of a gene on the Xi, consistent with a role for PARP-1 in maintenance of heterochromatic silencing. Together, these data suggest that transcriptional repression by macroH2A may be mediated through recruitment of PARP-1 and inhibition of its enzymatic activity.

EXPERIMENTAL PROCEDURES

HEK293 Cell Culture and Immunopurification—5 × 10^8 293 cells or 293-histone-green fluorescent protein (GFP) cells were washed in phosphate-buffered saline (PBS) and resuspended in lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 mM sodium butyrate, 1 mM phenylmethylsulfonyl fluoride, 1× phosphatase inhibitor mixtures I and II (Sigma), 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.7 μg/ml pepstatin). The resulting lysate was sonicated and centrifuged to remove debris, and the extract was adjusted to 1.8 mg ml⁻¹. GFP-tagged histones were affinity purified from the supernatant from 293-histone-GFP cells using 2 μg of rabbit polyclonal anti-GFP antibody (Abcam) per mg of cell extract. Endogenous macroH2A1 was purified from the supernatant of 293 cells using 20 μg of rabbit polyclonal antibodies that recognize macroH2A1.1 and macroH2A1.2 (24). Immunoprecipitations were performed at 4 °C for 2 h and captured on protein-A coated magnetic beads (Dynal), and the beads were washed three times in room temperature lysis buffer. Construction of H2A-GFP and macroH2A1.2-HD-GFP was performed by PCR of sequences from cDNAs generated from HEK293 cells and cloning into a backbone originating from pBOS-H2B-GFP (Clontech), generating a construct that expresses either H2A or macroH2A-HD tagged with GFP on the C terminus. After sequencing to ensure integrity, plasmids were transfected into HEK293 cells using FuGENE 6 (Roche Diagnostics) and stable lines were selected for using Blasticidin S (Invitrogen). Western blotting was performed according to standard procedures. Antibodies used were mouse C-2–10 anti-PARP (Trevigen) 1:1000, rabbit anti-PARP-1 antibody directed against the DNA-binding region 1:2000 (3), rabbit anti-macroH2A1 antibody 1:1000 (24), rabbit anti-macroH2A1 antibody 1:2000 (Upstate), rabbit anti-EZH2 1:400 (25), mouse anti-HA.11 (Covance) 1:1000, and mouse JL-8 anti-GFP (Clonetech) 1:2000.

Mass Spectrometry—In gel digestion and mass spectrometry (ProtTech, Inc.) identified 12 peptides, comprising 17.85% coverage of PARP-1.

Chromosome Spreads—Cells were exposed to 1 μg/ml colchicine (Sigma) for 4 h at 37 °C, and mitotic cells were harvested by washing off plate. Cells were then washed twice in PBS, swollen in 0.075 M KCl for 30 min at 37 °C. Approximately 10,000 cells were spun down onto ethanol washed slides at 1300 rpm for 10 min in a Shandon Cytospin. Cells were then incubated in KCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and 0.1% Triton X-100) for 8 min, fixed in 2% paraformaldehyde diluted in 1x PBS for 10 min, and washed in PBS + 0.2% Tween 20, then processed for immunofluorescence as described (26). Anti-PARP-1 catalytic domain rabbit antibody was used at 1:200 (3) and was detected with 1:200 Texas Red anti-rabbit secondary (Jackson ImmunoResearch Laboratories). All microscopy was performed on an Olympus BX-60 Fluorescence microscope. Images were captured using a Hamamatsu ORCA-ER CCD camera and Openlab Digital Darkroom software (Improvision).

Protein Purification—All macroH2A non-histone domain GST/His₆ constructs were generated by PCR cloning and insertion into pGEX-4T bacterial expression construct, followed by sequencing to ensure integrity of plasmids. BL21-DE3pLysS (Stratagene) bacteria transfected with constructs were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside after growth to an absorbance of A₆₀₀ 0.6 for 3 h and harvested. Cells were lysed in EQ buffer (50 mM sodium Phosphate, pH 7.0, 300 mM NaCl, 1 mM PMSF, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin, and 0.1% Nonidet P-40). Lysates were clarified by centrifugation, loaded onto Talon Metal affinity beads, washed with 20 column volumes of EQ buffer, then 5 column volumes of high salt EQ buffer (50 mM sodium phosphate, pH 7.0, 1 M NaCl, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1% Nonidet P-40) to ensure removal of contaminating DNA, washed in 10 column volumes of EQ buffer with 20% glycerol, and then eluted with buffer EQoff (50 mM sodium phosphate, pH 7.0, 1 M NaCl, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1% Nonidet P-40) to ensure removal of contaminating DNA, washed in 10 column volumes of EQ buffer with 20% glycerol, and then eluted with buffer EQoff (50 mM sodium phosphate, pH 7.0, 1 M NaCl, 1 mM PMSF, 150 mM imidazol, 20% glycerol, and 0.1% Nonidet P-40). Fractions containing eluted proteins were then bound to glutathione resin in EQ buffer with 2 M DTT added, washed in GST wash buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 M DTT). Proteins were eluted in GST wash buffer plus 10 mM glutathione, then dialyzed against GST dialysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 M DTT, 20% glycerol). FLAG-tagged human PARP-1 was expressed and purified from baculovirus infected SF9 insect cells as described previously (3).

GST IP Protocol of Recombinant and in Vitro Transcribed Proteins—PARP-1 in vitro transcription templates were generated using PCR primers that allowed for the addition of a T7
promoter to the 5’ end and introduction of a HA epitope to the C terminus of the PARP-1 fragment. Templates were added to a TNT T7 Quick for PCR DNA (Promega) in vitro transcription and translation kit to generate HA-tagged proteins. 500 nM GST bait constructs were mixed with 75 nM FLAG-hPARP-1 or 16% of in vitro translated and transcribed PARP-1 pieces in GST IP buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 0.02% Nonidet P-40, 10% glycerol, 1 mM PMSF) and incubated with glutathione beads (Amersham Biosciences) for 1.5 h at 4 °C, then washed five times with 1 ml of GST IP buffer, then boiled in SDS-loading buffer, and proteins were separated by SDS-PAGE. Recombinant PARP-1 was detected using a 1:1000 dilution of anti-PARP-1 antibody directed against the DNA-binding region (3), and HA was detected using mouse anti-HA.11 (Covance) 1:1000.

PARP Enzyme Assays—PARP-1 activity assays to determine an IC₅₀ with macroH2A family members were performed under the following conditions (1 μl of high specific activity human PARP-1 (Trevigen), 50 mM Tris-HCl, pH 8.0, 25 mM MgCl₂, 10 mM β-mercaptoethanol, 400 μM NAD⁺, and [³²P]NAD⁺ to a final specific activity of 0.5 μCi/nmol NAD⁺) in a final volume of 100 μl. Inhibition by Gst-macroH2A1.2-NHD-His deletion constructs was performed in 100-μl assays with 65 mM purified FLAG-hPARP-1, 50 mM Tris-HCl, pH 8.0, 25 mM MgCl₂, 1 mM DTT, and 400 μM NAD⁺, and [³²P]NAD⁺ to a final specific activity of 0.5 μCi/nmol NAD⁺. Kinetic analysis of GST-macroH2A1.2-NHD-His of PARP-1 was performed as above, except trichloroacetic acid-precipitable counts were normalized for the specific activity of NAD⁺ for each reaction (μCi/nmol NAD⁺). Each reaction was incubated with either 10 μl of dilution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol) or the appropriate purified GST construct in dilution buffer. PARP-1 and GST constructs were pre-incubated for 30 min at 30 °C, then NAD⁺ was added for 10 min at 30 °C, and reactions were stopped with the addition an ice-cold solution of 5% trichloroacetic acid/1% inorganic phosphate. Reactions were precipitated on ice for 10 min, spun at 14,000 rpm in a microcentrifuge for 10 min, and washed twice more with ice cold 5% trichloroacetic acid/1% inorganic phosphate. Precipitable counts were measured using a Beckman scintillation counter. Trichloroacetic acid-precipitable PARP activity was in the linear range for reaction conditions (data not shown).

Short Hairpin RNA Interference (shRNAi) and X Chromosome Reactivation Experiments—Plasmid for RNAi constructs carrying short hairpin RNA sequences expressed under the control of the U6 or the H1 promoter were made as previously described (27, 28). The Parp-1 RNAi was obtained from G. M. Shah and subcloned into pSuper-RetroPuromycin (Oligo-Engine) (29). Mel-18 and macroH2A1 RNAi plasmid have been described previously (18). Reactivation of GFP in SV40 T antigen-transformed mouse embryonic fibroblasts with the X-inactivated GFP transgene was performed as described before (18). Briefly, Phoenix cells were transfected and used to generate retroviral stocks, and mouse embryonic fibroblasts cultures were transduced with the viral supernatants in the presence of polybrene (4 μg/ml). Puromycin selection was added to the cultures 24 h after viral transduction (4 μg/ml). After selection, the cells were exposed to 5-aza-2’-deoxycytidine (5-Aza-dC) (4 days at 300 nM) and to 500 nM trichostatin A (TSA) for the last 24 h. The cells were subjected to fluorescence-activated cell sorter analysis counting a minimum of 100,000 cells per sample. Experiments were performed in duplicate at least three times.

RESULTS

MacroH2A1.2 Associates with PARP-1 via the NHD—MacroH2A1.2 contains a large C-terminal NHD of unknown function. To investigate function of the NHD, we identified proteins that associated with full-length macroH2A1.2 (mH2A1.2) but not canonical H2A or the histone domain of macroH2A1 (mH2A1-HD). HEK293 cell lines expressing mH2A1.2, H2A, or the mH2A1-HD fused to GFP were generated. All three GFP-tagged histones were detected in the nucleus and on mitotic chromosomes, demonstrating they were incorporated into chromatin (data not shown). Whole cell extracts from all three histone-GFP lines and HEK293 cells were immunoprecipitated with GFP antibodies. SYPRO Red staining of immunoprecipitated material showed co-precipitation of the core histones with the GFP-tagged histones (Fig. 1A), confirming that all three fusion proteins were incorporated into chromatin. A prominent band of ~110 kDa present only in the mH2A1.2-GFP immunoprecipitates was identified as PARP-1 by mass spectrometry, a protein implicated in regulation of chromatin structure. Western blotting of GFP immunoprecipitates confirmed that mH2A1.2-GFP exhibited significant PARP-1 binding, while H2A-GFP and mH2A1-HD-GFP did not (Fig. 1B). In addition, we detected co-precipitation of endogenous PARP-1 with macroH2A1 from HEK293 cells, using polyclonal antiserum that recognizes both macroH2A1 splice variants (mH2A1.1 and mH2A1.2 (24); Fig. 1C). Together, these data show that mH2A1.2 interacts physically with PARP-1 and that the interaction requires the NHD.

MacroH2A1.2 is enriched on the Xi relative to the active X chromosome and autosomes, and sequences sufficient for enrichment on the Xi lie within the mH2A1-HD (21, 30). We examined whether PARP-1 was also enriched on the Xi by immunostaining for PARP-1 in mitotic chromosome spreads from the macroH2A1.2-GFP, mH2A1-HD-GFP, H2A-GFP, and parental HEK293 cell lines. In mitotic spreads from HEK293 cells, PARP-1 was not appreciably detectable (data not shown). While the H2A-GFP line showed an overall increase in the amount of PARP-1 staining of all chromosomes, it did not show enrichment of PARP-1 on a particular chromosome. Both mH2A1.2-GFP and mH2A1.2-HD-GFP are enriched on the Xi (24), which allows for easy identification of the Xi in spreads. In the mH2A1.2-GFP line, PARP-1 was enriched on the Xi in 79.3 ± 5.1% of spreads (Fig. 1D), while in the mH2A1-HD-GFP line PARP-1 was enriched on the Xi in 1 ± 0.8% of spreads (n = 400). These data indicate that enrichment of macroH2A1.2-GFP on the Xi promotes PARP-1 association in vivo, consistent with a role for the NHD in recruiting PARP-1.

The PARP-1 Catalytic Domain Interacts with the MacroH2A1.2 NHD—To test whether the macroH2A1.2 NHD and PARP-1 interact directly, in the absence of additional proteins, as well as to map the domains of each protein required for association, we performed in vitro binding assays. The NHD
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A.

B.

C.

D. PARP-1  GFP  DAPI

mH2A1.2-GFP  mH2A1-HD-GFP
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consists of a basic region, a leucine zipper-like region, and an ADP-ribose phosphoesterase-like region (Fig. 2A). We expressed and purified GST and His₆-tagged mH2A1.2-NHD, basic region (Basic), leucine zipper-like region (LZ), ADP-ribose phosphoesterase-like region (ADP), and the NHD without the leucine zipper-like region (NHDΔLZ). These were employed as bait in pulldown experiments, using recombinant, FLAG-tagged PARP-1 as the prey. PARP-1 associated strongly with the NHD, the leucine zipper-like region, the ADP-ribose phosphoesterase-like region, and the NHD without the leucine zipper-like region (Fig. 2B, lanes 3, 4, 6, and 7) but not with the basic region or GST alone (Fig. 2B, lanes 2 and 5).

Thus, both the leucine zipper-like region and the ADP-ribose phosphoesterase-like region of the mH2A1.2-NHD can each independently interact with PARP-1 in vitro.

Next, we examined which regions of PARP-1 associated with the macroH2A1.2 NHD. PARP-1 consists of a zinc finger (ZnF) DNA binding region, a BRCA1 C-terminal repeat region (BRCT) automodification domain, and a catalytic (CAT) domain (Fig. 2C). HA-tagged versions of these three regions of PARP-1 were transcribed and translated in vitro and used as prey in the presence of GST-His₆ tagged mH2A1.2-NHD, which served as bait. Compared with GST-alone, the GST-mH2A1.2-NHD interacted with the zinc finger region (Fig. 2D, lane 8) and interacted even more strongly with the

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**FIGURE 1.** MacroH2A1.2 interacts with PARP-1 through its NHD. A, A SYPRO-stained 4–20% SDS-PAGE gel of input cellular extracts and anti-GFP immunoprecipitations from the parental HEK293 cell line (lanes 1 and 5) or stable lines expressing GFP-tagged H2A (lanes 2 and 6), the histone domain of macroH2A1 (mH2A1.2-HD) (lanes 3 and 7), or full-length macroH2A1.2 (mH2A1.2) (lanes 4 and 8). The gel shows 25 µg of input cellular extract (left) and the entire immunoprecipitation (right) for each sample. Labeled on the left are the identities of the major bands. Histones and GFP-histones were identified by size. The mono-ubiquinated form of macroH2A1.2-GFP (mH2A1.2-GFP-ubi) (24) and PARP-1 were identified by mass spectrometry. The asterisk denotes the location of heavy chain from the GFP antibody. For each immunoprecipitation, 1.8 mg of total extract was incubated with 2 µg of antibody. B, Western blot confirming the specific enrichment of PARP-1 with the macroH2A1.2 NHD. Immunoprecipitations were performed from 1.8 mg of total cellular extracts from HEK293 cells (lanes 1 and 5) and stable lines expressing GFP-tagged H2A (lanes 3 and 7), mH2A1.2-HD (lanes 4 and 8), or mH2A1.2 (lanes 2 and 6). On the right are PARP-1 (upper) and GFP (lower) Western blots. The asterisk shows the location of heavy chain. Input corresponds to 25 µg of total cellular extract. For each immunoprecipitation, 1.8 mg of total extract was incubated with 2 µg of antibody. C, Western blots depict PARP-1 co-immunoprecipitates with endogenous macroH2A1 but not with IgG. Immunoprecipitations were performed from 1.8 mg of total cellular extracts from HEK293 cells using either 20 µg of rabbit IgG as a control or 20 µg of a rabbit polyclonal antibody to macroH2A1. Immunoprecipitated material was blotted for the presence of either macroH2A1 (lower panel) or PARP-1 (upper panel). D, in metaphase chromosome spreads, immunofluorescence reveals that PARP-1 is enriched on the macroH2A1.2-GFP-labeled Xi. Chromosome spreads from cells expressing either mH2A1.2-GFP (upper) or mH2A1-HD-GFP (lower) were immunostained for PARP-1, and the DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI).
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catalytic domain of PARP-1 (Fig. 2D, lane 12). The mH2A1.2-NHD did not interact with the BRCT automodification domain (Fig. 2D, lane 10). These data show that multiple domains of each protein facilitate direct interaction of macroH2A1.2 with PARP-1.

The MacroH2A NHD Inhibits PARP-1 Enzymatic Activity—
The interaction between the macroH2A1.2 NHD and the catalytic domain of PARP-1 prompted us to investigate whether the NHD had any affect on PARP-1 catalytic activity. Human PARP-1 and [32P]NAD+ were incubated with GST-mH2A1.2-NHD-His6, GST, or buffer alone and the products of the PARP-1 enzymatic reaction visualized by SDS-PAGE and autoradiography. The high molecular weight smear observed when PARP-1 and [32P]NAD+ were incubated with buffer or GST alone is indicative of the addition of poly(ADP-ribose) oligomers to PARP-1 or PARP-1 automodification (Fig. 3A, lanes 2–4). GST-mH2A1.2-NHD-His6 significantly inhibited PARP-1 automodification relative to buffer or GST alone (Fig. 3A, lanes 2–4). Quantitation of PARP-1 activity showed that GST-mH2A1.2-NHD-His6 inhibited PARP-1 enzymatic activity by ~10-fold (Fig. 3B). To examine whether inhibition of PARP-1 was a common feature of the macroH2A family, we purified and tested the NHDs of other macroH2A variants: macroH2A1.1 and macroH2A2 (Fig. 2B, lanes 3 and 4). We found that GST fusions of these NHDs also inhibited PARP-1 activity ~10-fold (Fig. 3B). In titration experiments, the NHDs of all three macroH2A isoforms exhibited a half-maximal inhibitory concentration (IC50) of ~100 ± 20 nM (Fig. 3C), indicating that the ability to inhibit PARP-1 enzymatic activity is conserved among all members of the macroH2A family. When GST-mH2A1.2-NHD-His6 was added to either free poly(ADP-ribose) chains or poly(ADP-ribosylated) PARP-1, there was no reduction in the amount of free poly(ADP-ribose) chains or poly(ADP-ribose) chains on PARP-1, suggesting that the mH2A1.2-NHD inhibits PARP-1 catalysis directly rather than degrading the products of the PARP-1 enzymatic reaction (supplemental Fig. 1).

To gain insight into the mechanism of inhibition of PARP-1 enzymatic activity, we analyzed PARP-1 enzyme kinetics under different concentrations of GST-mH2A1.2-NHD-His6 and NAD+. GST-mH2A1.2-NHD-His6 acted as a non-competitive inhibitor of PARP-1 enzymatic activity, with increasing concentrations of the mH2A1.2-NHD lowering the kcat of the reaction, with a calculated Ks of 80 ± 13 nM (Fig. 3, D and E). GST-mH2A1.2-NHD-His6 also lowered the Kcat from 429 ± 35 to 111 ± 11 μM (Fig. 3D). There was no difference in the overall length of the poly(ADP-ribose) chains produced in reactions containing GST-mH2A1.2 NHD-His6, indicating that the macroH2A1.2 NHD does not regulate chain length (supplemental Fig. 2). Together, these data show that macroH2A1.2 NHD acts as a mixed inhibitor of PARP-1 enzymatic activity, with effects on both the kcat and the Km, and does not affect overall polymerase processivity.

Next we assayed whether any region of the mH2A1.2-NHD was sufficient to inhibit PARP-1 enzymatic activity. PARP-1 and [32P]NAD+ were incubated with buffer, GST, or GST-His6 tagged regions of the macroH2A1.2-NHD, and PARP-1 enzymatic activity was assayed. The ADP-ribose phosphoesterase-like region and the NHD lacking the leucine zipper-like region of mH2A1.2 both inhibited PARP-1 to levels comparable with the full NHD (Fig. 3F). The leucine zipper-like region inhibited PARP-1 enzymatic activity to a lesser degree, while the basic region did not inhibit PARP-1 enzymatic activity (Fig. 3F). Thus, the ADP-ribose phosphoesterase-like and the leucine zipper-like regions, both of which were found to interact with PARP-1, were each individually sufficient to inhibit PARP-1 enzymatic activity.

PARP-1 Maintains Silencing on the Xi—MacroH2A1.2 is one of many factors that contribute to stable silencing of genes on the Xi. We investigated whether mouse Parp-1 played a role in the maintaining silencing on the Xi by depleting Parp-1 from female mouse embryo fibroblast cells with a GFP transgene integrated on the Xi. This approach has been used previously to look at the contribution of multiple mechanisms to the maintenance of gene silencing on the Xi, such as DNA methylation, histone deacetylation, and incorporation of macroH2A1 (18, 31). When these cells were exposed to 5-Aza-dC and TSA, loss of DNA methylation and histone acetylation induced expression of the normally silent GFP transgene in 2.7 ± 0.4% of cells (Fig. 4A). Depletion of macroH2A1 by RNA interference using a short hairpin RNA (shRNA) combined with 5-Aza-dC and TSA treatment caused further reactivation, with 5.6 ± 0.6% of cells showing expression of the Xi-linked GFP transgene (Fig. 4, A and B), as shown previously (18). shRNA-mediated depletion of Parp-1 in drug-treated cells resulted in a similar increase in GFP-positive cells (6.7 ± 0.3%) (Fig. 4, A and B), indicating that, like macroH2A1, Parp-1 plays a role maintenance of silencing on the Xi.

DISCUSSION

In the absence of its substrate, NAD+, PARP-1 binds nucleosomes, causing chromatin compaction and transcriptional repression in vitro (3). PARP-1 is chromatin associated in vivo, at NAD+ concentrations that promote PARP-1 automodification and release from chromatin (3). In this study, we present evidence that macroH2A inhibits PARP-1 enzymatic activity and may therefore promote PARP-1 retention on chromatin under conditions where its automodification and release from chromatin would otherwise be stimulated. We find that macroH2A1.2 interacts with PARP-1 in vitro and in vivo, through the NHD. The macroH2A1.2 promotes PARP-1 localization to chromatin via the NHD, when expressed as a GFP fusion protein. Furthermore, the macroH2A1.2 NHD inhibits PARP-1 NAD+-dependent poly(ADP-ribose) polymerization in vitro. Together, these data suggest that PARP-1 retention on chromatin may be a mechanism by which macroH2A isoforms promote transcriptional silencing. In this model, PARP-1 might function to compact chromatin in regions of the genome packaged with macroH2A-containing nucleosomes, contributing to heterochromatin formation and transcriptional repression. While this work was under review, Ouarrarhni et al. (32) reported that macroH2A1.1 associates with and inhibits Parp-1 and that transcriptional repression of a heat shock gene is mediated by macroH2A1.1 and PARP-1, consistent with a role for the macroH2A-PARP-1 interaction in transcriptional silencing.
FIGURE 3. All macroH2A NHDs inhibit PARP-1 enzymatic activity in vitro. A, the macroH2A1.2-NHD inhibits PARP-1 automodification in vitro. An SDS-PAGE gel of a PARP-1 automodification reaction shows that 1 μM GST-macroH2A1.2-NHD-His6 inhibits PARP-1 compared with 1 μM GST. B, quantitation of macroH2A1.1, macroH2A1.2, and macroH2A2 NHD inhibition of PARP-1 generated trichloroacetic acid-precipitable poly(ADP-ribose). GST-His6-tagged NHDs from each of the macroH2A family members (1 μM) (Fig. 2B) are inhibitory relative to GST (2.5 μM) in PARP-1 activity assays described under “Experimental Procedures.” PARP-1 activity is normalized to the buffer alone control and set to one. Values are mean and S.D. of two independent experiments. C, dose-dependent inhibition of PARP-1 activity by the NHDs of the macroH2A family. PARP-1 activity assays (as described under “Experimental Procedures”) carried out with 0, 10.24, 25.6, 64, 400, 1000, or 2500 nM GST-mH2A1.2-NHD-His and GST-mH2A1.1-NHD-His or 0, 4.096, 10.24, 25.6, 64, 400, 1000, or 2500 nM GST-mH2A2-NHD-His. An IC50 of 101 ± 18 nM, 127 ± 22 nM, and 100 ± 18 nM was calculated for GST-mH2A1.2-NHD-His6, GST-mH2A1.1-NHD-His6, and GST-mH2A2-NHD-His6, respectively. PARP-1 activity is normalized to the buffer alone and set to one. Values are mean and S.D. of two independent experiments. D, non-competitive inhibition of PARP-1 by macroH2A. PARP-1 activity was measured over NAD concentration ranging from 10 to 800 μM as described under “Experimental Procedures,” with the addition of either 0 μM (red), 0.1 μM (blue), 0.5 μM (green), or 2.5 μM GST-mH2A1.2-NHD-His6 (black). The different Vmax values at varied [GST-macroH2A1.2-NHD-His6] clearly indicate non-competitive inhibition. Each value is the mean and S.D. from three independent experiments. E, GST-mH2A1.2-NHD-His6 inhibits PARP-1 by directly suppressing catalysis. The effect of GST-mH2A1.2-NHD-His6 on the PARP-1 kcat is best described by the equation $k_{cat} = (k_{cat,int}/(1 + [GST-mH2A1.2-NHD-His6]/K_i)) + k_{cat,sat}$, where $k_{cat,int}$ is the intrinsic PARP-1 $k_{cat}$ and $k_{cat,sat}$ is the PARP-1 $k_{cat}$ at saturating levels of GST-mH2A1.2-NHD-His6. This analysis yields values for the $K_i = 80 ± 14 nM$, $k_{cat,int} = 16.1 ± 0.7 s^{-1}$ and $k_{cat,sat} = 0.5 ± 0.5 s^{-1}$. However, the fit of the data is similar if the $k_{cat,sat}$ term is included. F, domain analysis of macroH2A1.2 inhibition. 1 μM concentration each of GST-His6-tagged macroH2A1.2 NHD fragments (Fig. 2B) was tested in PARP-1 activity assays as described under “Experimental Procedures.” Each value is the mean and S.D. from three independent experiments.
PARP-1 Is Inhibited by MacroH2A

Like macroH2A1, PARP-1 was necessary for stable gene silencing on the Xi, as depletion of Parp-1 led to reactivation of a silent GFP transgene on the Xi in mouse cells. This is the first report of Parp-1 involvement in maintenance of silencing on the Xi. Homozygous deletion of Parp-2 combined with heterozygous deletion of Parp-1 results in female-specific lethality that is associated with X chromosome instability (33). The involvement of Parp-1 in maintenance of gene silencing on the Xi suggests that loss of proper dosage compensation in these mutant mice may contribute to the chromosome instability phenotype, due selection against the reactivated Xi.

The macroH2A1.2 NHD acted mainly as a non-competitive inhibitor of PARP-1 and association with the macroH2A1.2 NHD does not occlude access of NAD$^+$ to the PARP-1 active site. This suggests that macroH2A is binding PARP-1 in a location other than the active site and that macroH2A NHD mediates its effect through an allosteric interaction with PARP-1.

Previous investigation into the nucleotide binding properties of PARP-1 revealed its effect through an allosteric interaction with PARP-1. This suggests that macroH2A is binding PARP-1 in a location other than the active site and that macroH2A NHD mediates its effect through an allosteric interaction with PARP-1.

The function of the macro family of proteins has been under intense investigation, and they have been shown to be involved in diverse functions centered on NAD$^+$ metabolism, ranging from resolving products of tRNA splicing to associating with a

![FIGURE 4. Depletion of Parp-1 leads to reactivation of a gene on the Xi.](image-url)
product of histone deacetylation reactions (10–12). Inhibition of PARP-1 catalytic activity provides a new function for macroH2A. We also show that PARP-1 plays a role in maintaining silencing at the Xi, a region of the genome where macroH2A is enriched. Our data provide a link between macroH2A and PARP-1, which can collaborate to modulate chromatin structure and repress transcription.

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