Death domain-associated protein (Daxx) is a multifunctional protein that modulates both apoptosis and transcription. Within the nucleus, Daxx is a component of the promyelocytic leukemia protein (PML) nuclear bodies (NBs) and interacts with a number of transcription factors, yet its precise role in transcription remains elusive. To further define the function of Daxx, we have isolated its interacting proteins in the nucleus using epitope-tagged affinity purification and identified X-linked mental retardation and α-thalassaemia syndrome protein (ATRX), a putative member of the SNF2 family of ATP-dependent chromatin remodeling proteins that is mutated in several X-linked mental retardation disorders. We show that substantial amounts of endogenous Daxx and ATRX exist in a nuclear complex. Daxx binds to ATRX through its paired amphipathic alpha helices domains. ATRX has ATPase activity that is stimulated by mononucleosomes, and patient mutations in the ATPase domain attenuate this activity. ATRX strongly represses transcription when tethered to a promoter. Daxx does not affect the ATPase activity of ATRX, however, it alleviates its transcription repression activity. In addition, ATRX is found in the PML-NBs, and this localization is mediated by Daxx. These results show that the ATRX-Daxx complex is a novel ATP-dependent chromatin-remodeling complex, with ATRX being the core ATPase subunit and Daxx being the targeting subunit. Moreover, the localization of ATRX to the PML-NBs supports the notion that these structures may play an important role in transcription regulation.

Daxx\textsuperscript{1} was initially identified as a pro-apoptotic molecule that binds to the intracellular tail of the death receptor CD95 (Fas/Apo-1) and enhances apoptosis through the induction of the c-Jun NH\textsubscript{2}-terminal kinase (JNK) pathway (1). Daxx associates with apoptosis signal-regulating kinase 1, an apical kinase of the JNK pathway, causing it to become active (2). Subsequent studies have revealed a role for Daxx in apoptosis induced by other stimuli, including transforming growth factor-β and interferons (3, 4). Despite these cytoplasmic interactions, Daxx is mainly found in the nucleus, and its cytosolic translocation appears to be regulated. For example, the translocation of Daxx to the cytoplasm can be induced by CD95 engagement in a heat shock protein 27-inhibitable manner (5). In addition, ASK1 can enhance the cytosolic localization of Daxx (6). On the contrary, accumulation of Daxx in the nucleus is observed during the activation of splenocytes (7).

In the nucleus, Daxx is found in the promyelocytic leukemia protein nuclear bodies (PML-NBs) (8–10), macromolecular complexes that regulate a wide range of cellular processes (11, 12). The PML-NBs are dispersed in over 95% of cases of acute promyelocytic leukemia (APL) by the PML-retinoic acid receptor fusion protein generated by a t(15,17) translocation. Treatment of APL patients with all-trans-retinoic acid or arsenic oxide induces degradation of the fusion protein, restoration of normal PML-NB appearance, and concomitant remission of the disease, implicating a critical function of the PML-NBs in protecting against APL pathogenesis (11–13). In addition, the PML-NBs are disrupted by various viral proteins and increase in both size and number in response to anti-viral cytokine interferons that up-regulate PML-NB components, suggesting a role for the PML-NBs in anti-viral responses (14). Furthermore, cells deficient in PML are resistant to a wide range of apoptotic stimuli, including UV radiation and engagement of CD95 (15), and Daxx appears to be able to regulate apoptosis from the PML-NBs (7, 8). How the PML-NBs function in tumor suppression, anti-viral responses, and apoptosis is not well defined. Several cellular proteins have been found in the PML-NBs in addition to PML and Daxx, including p53, pRb, Sp100, SUMO (small ubiquitin-like modifier), CBP, HAUSP, and BLM, and a role for the PML-NBs in transcription has been proposed (16). However, a better understanding of PML-NB function requires the identification of additional key components that associate with these structures.

Daxx has been shown to be a transcription regulatory protein and to associate with several sequence-specific transcription factors, such as Pax3, Pax5, ETS1, and the glucocorticoid receptor.
To further define the function of Daxx, we have devised a convenient affinity purification strategy to isolate Daxx-associated proteins. Here, we describe the identification and characterization of ATRX as a Daxx-interacting protein. ATRX is a large protein of ~280 kDa that contains a putative ATPase/helicase domain homologous to that found in members of the SNF2 family of chromatin-remodeling proteins. In addition, it also contains a plant homeodomain (PHD) zinc finger domain, which is most similar to the DNA methyltransferase 3 family of proteins. Mutations in the X-linked ATRX gene are most commonly located within the putative ATPase/helicase domain and the PHD motif causing a severe mental retardation syndrome that is associated with urogenital abnormalities, facial dysmorphism, and frequently α-thalassemia (the ATR-X syndrome) (23–25). Despite its similarity to chromatin-remodeling proteins, the precise function of ATRX remains largely unknown. We show that endogenous Daxx and ATRX are present in a nuclear complex. Daxx interacts with ATRX through its NH2-terminal paired amphipathic alpha helices (PAH) domains. In addition, we demonstrate that ATRX possesses mononucleosome-stimulated ATPase activity, and patient mutations attenuate this activity. Furthermore, we demonstrate that ATRX is localized to the PML-NBs. Daxx recruits ATRX to the PML-NBs and inhibits ATRX-mediated transcription regulation. These results suggest that the ATRX-Daxx complex is a novel chromatin remodeling complex and that Daxx may regulate ATRX activity by altering its cellular localization. The localization of ATRX to the PML-NBs also supports the notion that the PML-NBs regulate diverse cellular processes by modulating transcription.

Experimental Procedures

Reagents—The following reagents were obtained from the indicated sources: monoclonal antibodies against FLAG (M2) and HA (HA-7) and their agarose affinity gels and corresponding peptides (Sigma); anti-Daxx (M-122), anti-PML (PG-M3), and anti-ATRX (H-300) antibodies (Santa Cruz Biotechnology); protein A- and G-Sepharose beads, [γ-32P]ATP (3000 Ci/nmol), and DNase I (10 units/µl, fast protein liquid chromatography-pure) (Amersham Pharmacia); protease inhibitors (CompleteMini, EDTA-free) and human interferon-γ (Roche Applied Science). The affinity-purified sheep anti-ATRX (Fxnp5) was previously described (26).

Expression Plasmids—For expression in mammalian cells, full-length Daxx protein and the deletion mutants of Daxx and ATRX were fused to an NH2-terminal FLAG tag in pRK5 (27). pGal, expressing the DNA binding domain of Gal4 (amino acids 1–148), and pGal-ATRX, expressing a fusion of theGal4 DNA-binding domain of ATRX, were also made in pRK5. The ATRX-GFP fusion plasmids were made in either pEGFP-C2 (for the full-length protein or pEGFP-C1 (for deletion mutants) (Clontech), whereas RFP-PML was made in pdsRed-N1 (Clontech). For expression in insect cells, human ATRX and ISWI proteins SNF2H and SNF2L were cloned into baculovirus (Clontech) with either a COOH-terminal HA tag (for ATRX) or an NH2-terminal FLAG tag (for SNF2H and SNF2L). The ATRX point mutations were made using the XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instruction and verified by DNA sequencing. The following mutations were made: PHD, the common R246C mutation located in the PHD zinc finger domain; PED3 and PED23, the Y2084H and D2035V mutations in the putative ATPase/helicase domain which have been previously described (23); and K1600R mutation in the conserved ATP binding site.

Biochemical Identification of Daxx-binding Proteins—To prepare nuclear extracts, human HeLa S3 cells from 10-liter suspension culture (obtained from the National Cell Culture Center) were lysed in five cell volumes of buffer A (20 mM HEPES-NaOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Cells were further disrupted by Dounce homogenization, and the nuclei were collected by centrifugation at 1000 × g for 10 min. The nuclei were suspended in buffer B (20 mM HEPES, pH 8.0, 25% glycerol, 1.5 mM MgCl2, 400 mM NaCl, 0.2 mM EDTA), and the soluble fraction was diluted 1:1 with a low salt buffer (10 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA) to yield nuclear extracts. To generate recombinant FLAG-Daxx protein in mammalian cells, FLAG-Daxx/pRK5 or the control vector FLAG/pRK5 was transfected into HeLa cells seeded in thirteen 10-cm plates with 5 µg of DNA/plate by the calcium phosphate precipitation method. Twenty-four hours after transfection, cells were washed with ice-cold saline-buffered saline and lysed with 1 ml of lysis buffer A (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The cell lysates were incubated with protein G-Sepharose beads for 4 h. After extensive washing, the beads were incubated overnight with 10 mg of HeLa nuclear extracts. The beads were washed four times with IP-lysis buffer and three times with buffer C (10 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM EDTA). The bound proteins were eluted with 100 µl of buffer C containing 100 µg/ml FLAG peptide for 1 h. The eluted proteins were stored at −80 °C. An aliquot of eluted proteins was resolved on SDS-PAGE gel, and Daxx-associated proteins were analyzed by mass spectrometry.

GeI Filtration Chromatography and Co-immunoprecipitation—Ten milligrams of HeLa S3 nuclear extract were separated on a Superose 6 column driven by an Amersham Biosciences ÄKTA fast protein liquid chromatography system. Fractions were analyzed with trichloroacetic acid and by SDS-PAGE and immunoblotting. To detect the interaction between endogenous ATRX and Daxx, about 2 × 106 HEK293T cells were lysed in IP-lysis buffer. Cell extracts were incubated with the indicated antibodies, followed by incubation with protein A beads. The immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotting analyses. To map the interaction domain on Daxx, FLAG-tagged wild-type Daxx and various mutant Daxx proteins were expressed in 293T cells. The cell extracts were immunoprecipitated with M2 beads and endogenous ATRX protein bound to the beads was detected by immunoblotting. Reciprocally, various FLAG-tagged ATRX constructs were expressed in 293T cells, and their ability to associate with endogenous Daxx was examined by immunoprecipitation assays.

Expression and Purification of Recombinant ATRX, SNF2H, and SNF2L—The baculovirus insect cells were co-transfected with baculovirus DNA into insect Sf21 cells (Clontech) to generate recombinant baculoviruses according to the manufacturer's instruction. Following several rounds of amplification, the recombinant virus was used to infect insect Sf21 cells. Forty-eight hours post-infection, the cells were harvested and recombinant proteins were purified as previously described with modifications (28). The cell pellet was suspended in lysis buffer 20 M HEPES, pH 7.5, 0.5 M NaCl, 10 mM KCl, 2 mM MgCl2, 5% glycerol, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.1% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitors (CompleteMini, EDTA-free) and human interferon-γ (Roche Applied Science). The affinity-purified sheep anti-ATRX (Fxnp5) was previously described (26).

IPAse Assay—The standard assay of recombinant human ATRX, SNF2H, and SNF2L were resolved as previously described with modifications (29). Purified recombinant proteins (5–200 ng) were incubated with 25.25 µCi of [γ-32P]ATP (3000 Ci/mmol), plus 2.94 nM pUC19 plasmid DNA (100 ng) or 0.83 pmol (250 ng) of mononucleosomes where indicated, in a total volume of 20 µl of ATPase assay buffer (25 mM HEPES-KCl, pH 7.5, 5% glycerol, 20 mM KCl, 5 mM MgCl2, 25 mM bicine, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% NP-40, 0.005% Nonidet P-40). The mixture was stopped after incubation at 27 °C for 20 min by adding 2 µl of 0.5 mM EDTA (pH 8.0). One microliter of the reaction mixture was separated by thin layer chromatography with a polyethylenimine-cellulose plate (J.T. Baker) in 0.5 mM LiCl and 1 M acetic acid. The ratio of free phosphate to ATP was measured by liquid scintillation counting.
Daxx Associates with the ATR-X Syndrome Protein

Identification of ATRX as a Novel Daxx-interacting Protein—To identify Daxx-associated proteins, we expressed a FLAG-tagged Daxx protein (FLAG-Daxx) in HeLa cells and affinity-purified the protein using anti-FLAG monoclonal antibody M2 beads. The immobilized FLAG-Daxx was then incubated with a large quantity of HeLa nuclear extract to identify its interacting proteins. This approach has two major advantages. First, the bait protein is made in mammalian cells and is therefore likely to have the correct post-translational modifications. Second, it avoids the lengthy (and sometimes unachievable) process of establishing stable transfectants. As shown in Fig. 1A, a protein around 300 kDa in size in the HeLa nuclear extract specifically associated with the FLAG-Daxx beads but not the control beads. This protein was analyzed by mass spectrometry, and thirteen peptide sequences were obtained, all of which matched the sequence of ATRX (Fig. 1A).

To confirm the Daxx-ATRX interaction in another cell line as well as the interaction between the endogenous proteins, we performed immunoprecipitation assays using extracts of human 293T cells. Endogenous Daxx was immunoprecipitated, and ATRX was readily detected by Western blot analysis in the anti-Daxx immunoprecipitates but not in control immunoprecipitates with an unrelated antibody (Fig. 1B, lane 2 versus 1). Reciprocally, Daxx was present in the anti-ATRX immunoprecipitates (lane 3). These assays also revealed that a significant portion of endogenous Daxx and ATRX are in the same complex (data not shown). To further characterize the Daxx/ATRX complex, we fractionated HeLa cell nuclear extracts using a gel filtration column and analyzed fractions from the column by Western blot analysis with anti-ATRX and anti-Daxx antibodies. The Daxx and ATRX proteins were co-eluted in a complex of ~350 kDa, suggesting a stoichiometric ratio of Daxx and ATRX in the complex. Recently, ATRX was shown to be present in a larger (1 MDa) complex that contained six polypeptides, including Daxx (31). The differences we observed are likely attributable to the sensitivity of the complex to differing salt concentrations used for purification.

Daxx Associates with ATRX via its NH₂-terminal PAH Domains—To delineate the region of Daxx that mediates its interaction with ATRX, we constructed various deletion mutants of Daxx, each tagged with an NH₂-terminal FLAG epitope (Fig. 2A). The FLAG-Daxx proteins were transiently expressed in 293T cells and precipitated with anti-FLAG M2 beads, and the presence of endogenous ATRX in the immunoprecipitates was examined by Western blotting. As shown in Fig. 2B, all Daxx mutants that contained the NH₂-terminal 160 amino acids, including the first paired amphipathic helix (PAH), were able to associate with ATRX (lanes 2–6), albeit the interaction was stronger if the second PAH domain was also present (amino acids 1–260, lanes 2–5 versus lane 6). In contrast, all of the mutations that were missing the first PAH domain failed to bind to ATRX (lanes 7–9). Together, these results demonstrate that the NH₂-terminal PAH region of Daxx mediates its interaction with ATRX. Notably, this is the first indication of a function for the PAH domains, as all of the Daxx-interacting

with 1% paraformaldehyde for 15 min at room temperature and treated with 0.2% Triton X-100 for 15 min on ice. Cells were then incubated for 1 h with anti-Daxx (M-112), anti-PML (PG-M3), or anti-ATRX (H-300), as indicated, followed by incubation with fluorescein isothiocyanate- or Texas Red-conjugated secondary antibodies. Fluorescence signals were analyzed on a Bio-Rad 1024-ES confocal laser-scanning microscope unless otherwise indicated.

Reporter Gene Assays—HEK293T cells were seeded in 24-well plates and transfected on the second day by the calcium phosphate precipitation method, with 0.4 μg of 5xGal-TK-luciferase reporter gene plasmid (30), 50 ng of pCMV-β-galactosidase, and various amounts of pGal, pGal-ATRX, and Daxx. The total amount of DNA was made constant by adding the pRK5 vector. Twenty-four hours after transfection, cells were harvested and assayed for luciferase activity with a firefly luciferase assay kit (Promega), according to the manufacturer’s instructions. Luciferase activities were normalized on the basis of the activities of the co-transfected β-galactosidase. Data shown are representative of three independent experiments done in duplicate.

RESULTS

Identification of ATRX as a Novel Daxx-interacting Protein—To identify Daxx-associated proteins, we expressed a FLAG-tagged Daxx protein (FLAG-Daxx) in HeLa cells and affinity-purified the protein using anti-FLAG monoclonal antibody M2 beads. The immobilized FLAG-Daxx was then incubated with a large quantity of HeLa nuclear extract to identify its interacting proteins. This approach has two major advantages. First, the bait protein is made in mammalian cells and is therefore likely to have the correct post-translational modification. Second, it avoids the lengthy (and sometimes unachievable) process of establishing stable transfectants. As shown in Fig. 1A, a protein around 300 kDa in size in the HeLa nuclear extract specifically associated with the FLAG-Daxx beads but not the control beads. This protein was analyzed by mass spectrometry, and thirteen peptide sequences were obtained, all of which matched the sequence of ATRX (Fig. 1A).

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proteins previously reported in the literature associate with the COOH region of Daxx (1, 3, 17–21).

Mapping the Daxx-interacting Region of ATRX—To determine the Daxx-interacting domain of ATRX, we generated various FLAG-tagged ATRX deletion mutants (Fig. 3A). Our initial analysis demonstrated that both halves of the ATRX protein can interact with Daxx, indicating that ATRX may contain two distinct Daxx-interacting domains (Fig. 3B, lanes 2 and 7). Further analysis revealed that all of the ATRX fragments containing amino acids 1189–1326 interacted well with Daxx, whereas the COOH fragments of ATRX that were missing these amino acids failed to do so (lanes 11–14). Thus, amino acids 1189–1326 constitute a Daxx-interacting domain. In addition, the region between amino acids 321 and 865 may contain a second Daxx-interacting domain (lanes 4 and 9). However, the first domain bound to Daxx more strongly than the second one and therefore may be the domain mainly responsible for the ATRX-Daxx interaction (lanes 11, 12, and 14 versus lane 9). In contrast, two previously identified domains of ATRX, the NH2-terminal PHD domain and the COOH-terminal ATPase/helicase domains, are not required for the interaction with Daxx (Fig. 3B).

ATRX Possesses ATPase Activity That Is Stimulated by Nucleosomes—The ATPase/helicase domain of ATRX shares significant homology with those found in members of the SNF2 family of chromatin-remodeling proteins. We therefore sought to determine whether the ATRX protein had ATPase activity. We expressed recombinant ATRX and, for comparison, recombinant mammalian ISWI proteins-SNF2H and SNF2L-in insect cells using baculovirus expression plasmids. The recombinant ATRX protein was purified by an anti-HA affinity column and analyzed by SDS-PAGE followed by silver staining and immunoblotting with either anti-ATRX or anti-FLAG antibody as indicated. FL, the full-length protein. Asterisk, anti-FLAG M2 heavy chain.

![FIG. 2. The PAH domains of Daxx mediate its interaction with ATRX. A, schematic representation of the domains that comprise Daxx and of the Daxx fragments used in this study. PAH, paired amphipathic alpha helices domain. AD, acid-rich region; SPT, Ser/Pro/Thr-rich domain. The amino acids present in each fragment are indicated on the left. B, the PAH domains of Daxx mediate its interaction with ATRX. FLAG-tagged Daxx proteins were expressed in 293T cells and immunoprecipitated with M2 beads. The precipitating proteins were analyzed by Western blotting using either anti-ATRX or anti-FLAG antibody as indicated. FL, the full-length protein. Asterisk, anti-FLAG M2 heavy chain.](http://www.jbc.org/)

![FIG. 3. Mapping the Daxx-interacting domain of ATRX. A, schematic diagram of the full-length ATRX protein and the deletion mutants used in this study. The plant homeomain (PHD), ATPase/ Helicase domains, and first Daxx-interacting domain (DID) are indicated for the full-length ATRX protein. The deletion mutants were designated as fragments (F) 1 to 14, with the amino acids present in each fragment shown in parentheses. The FLAG tag and green fluorescent protein (GFP) were fused to the NH2 termini of the fragments. B, the domains of ATRX that mediate its interaction with Daxx. FLAG-tagged ATRX proteins were expressed in 293T cells and immunoprecipitated with M2 beads. The precipitating proteins (left panels and top right panel) or cell extracts (bottom right panel) were analyzed by Western blotting using anti-Daxx, anti-FLAG, or anti-ATRX (when the anti-FLAG Western blotting signals were too weak) antibody as indicated. *, anti-FLAG M2 heavy chain. **, a nonspecific band (in lanes 8, 10–14).](http://www.jbc.org/)
ATP in a dose-dependent manner (lanes 10–13 versus 1). To determine the relative specific activities of the different proteins, we plotted the dose-dependent ATPase activities of the purified proteins from three independent preparations and found that the ability of ATRX to hydrolyze \[\gamma^{33}\text{P}]\text{ATP}, based on the molar concentration of protein, was only slightly less than that of SNF2H but significantly higher than the ATPase activity of SNF2L (Fig. 4D). It is clear that the observed ATPase activity originated from the ATRX protein as opposed to one or more contaminant proteins in the preparation, because the activity was either diminished or completely impaired when ATRX mutations were introduced (see below).

Previous studies have shown that the ATPase activity of both the SNF/SWI and the ISWI subfamilies of chromatin remodeling molecules is stimulated by DNA. However, the responses of these molecules to naked and mononucleosomal DNA are different. SNF/SWI proteins are stimulated equally well by both types of DNA, whereas ISWI proteins are stimulated only by nucleosomes (32). To characterize the effects of these DNA substrates on the ATPase activity of ATRX, we examined whether naked DNA or mononucleosomes could stimulate the ATPase activity of ATRX. The ATPase activity of ATRX approximately doubled in response to mononucleosomes but was not stimulated by plasmid DNA (Fig. 5A, lanes 10, 11 versus 9). The effect of the mononucleosomes, albeit moderate, was very reproducible. In addition, the response of ATRX alone was slightly different than the activity of the ATRX-Daxx complex, which could be stimulated equally well by both naked DNA and chromatin (31). As a control, we examined the ATPase activity of the two recombinant mammalian ISWI proteins in the presence of either DNA or mononucleosomes and observed that it was consistent with previous studies, with the ATPase activity of SNF2H being stimulated by both and the ATPase activity of SNF2L being only enhanced by mononucleosomes (Fig. 5A, lanes 1–7) (32).

**ATR-X Syndrome-associated Mutations Impair the ATRX ATPase Activity**—ATR-X syndrome is associated with a variety of mutations in the ATRX gene, suggesting that the prevailing disease mechanism is the loss of ATRX protein function. However, the absence of mutations in highly conserved residues within the ATPase domain implies that such changes to this domain would be lethal. Thus, patient mutations may only attenuate activity without abolishing it altogether. To distinguish whether known mutations abolish or attenuate ATPase activity, we generated recombinant ATRX proteins harboring known mutations within the ATPase domain (Ped3-Y2084H and Ped23-D2035V), an ATPase-dead mutant (Mu-K1600R), and as a control, the common R246C mutation located within the PHD domain (PHD). All mutant proteins were expressed in insect cells, and three different preparations were purified to homogeneity before examining ATPase activity. Although the point mutation in the PHD domain had no effect on ATPase activity, mutations in the ATPase domain severely impaired activity (Fig. 5B, lanes 3–5). As expected, the Lys-to-Arg mutation in the catalytic site completely abolished the ATPase activity (lane 6). These results demonstrate that patient muta-
Daxx Associates with the ATR-X Syndrome Protein

FIG. 5. Effects of DNA substrates, Daxx, and patient-associated mutations on the ATPase activity of ATRX. A, the effects of plasmid and mononucleosomal DNA on the ATPase activity of ATRX, SNF2H, and SNF2L. ATPase assays for the indicated recombinant proteins (SNF2H, 10 ng; SNF2L, 50 ng; and ATRX, 5–10 ng) were performed in the presence of plasmid DNA (0.1 μg of pUC19, D) or the mononucleosome (0.25 μg of both histone and DNA, N), as indicated. C, no protein or DNA was added. B, patient-associated mutations affect the ATPase activity of ATRX. Middle: recombinant ATRX proteins were affinity-purified with anti-ATRX antibody Fxnp5 and visualized by silver staining after being resolved on SDS-PAGE. C, sample from uninfected cell lysate. Top: the ATPase activity was assessed by TLC as in Fig. 4C. Bottom: relative ATPase activity according to liquid scintillation counts. Daxx does not alter the ATPase activity of ATRX. Left, FLAG-tagged Daxx and DaxxC (amino acids 349–740) expressed in HeLa cells, affinity-purified, and resolved by SDS-PAGE followed by Coomassie staining. Right, the ATPase assay was performed with recombinant ATRX in the absence (−) or presence (+) of the indicated Daxx proteins.

The ATPase Activity of ATRX Is Not Affected by Daxx—Having shown that ATRX has ATPase activity that is defective in certain ATR-X patients, we examined whether Daxx regulates the ATPase activity of ATRX. FLAG-tagged Daxx and DaxxC, the latter missing the ATRX interacting domain, were expressed in mammalian cells and affinity-purified to homogeneity (Fig. 5C, left panel). When these Daxx proteins were added to the ATPase assay reaction, no effect on ATP hydrolysis was observed (Fig. 5C, lanes 3 and 4 versus 1). Thus, Daxx does not appear to modulate the ATPase activity of ATRX.

ATRX Represses Transcription When Tethered to DNA and This Transcriptional Repression Is Alleviated by Daxx—We next analyzed the effect of Daxx on ATRX-mediated transcriptional regulation. Because ATRX is a chromatin-remodeling molecule, we speculated that tethering ATRX to a promoter would affect transcription. We fused ATRX to the Gal4 DNA-binding domain (Gal-ATRX) and introduced this fusion into 293T cells together with a luciferase reporter plasmid containing five Gal4 DNA-binding sites upstream of the thymidine kinase (TK) promoter. Gal-ATRX strongly inhibited expression of the TK promoter-driven luciferase (Fig. 6A, columns 3–6 versus 1 and 2). This transcription repression was dependent on the targeting of ATRX to the promoter, because FLAG-tagged ATRX failed to inhibit luciferase expression (column 7). Thus, ATRX can function as a transcription repressor. This is consistent with previous findings that ATP-dependent chromatin-remodeling molecules can repress the expression of some genes while activating the expression of others (33). When Daxx was included in this reporter assay, transcription repression by ATRX was reduced in a Daxx dose-dependent manner (Fig. 6B, columns 2 and 3 versus 1). Therefore, Daxx inhibits the function of ATRX in transcription regulation.

Localization of ATRX to the PML-NBs—Because Daxx had no effect on the ATPase activity of ATRX but still inhibited ATRX-mediated transcriptional activity, we investigated the possibility that Daxx alters the cellular localization of ATRX. Given that Daxx is found in the PML-NBs, we tested whether ATRX is also localized to these nuclear bodies and, if so, whether this localization is dependent on Daxx. Endogenous ATRX in HeLa cells showed a diffuse staining pattern with several prominent nuclear speckles that nicely co-stained with an anti-PML antibody (Fig. 7A, panels a–c). In addition, consistent with previous studies, the size of the PML-NBs was enhanced upon interferon treatment, and concomitantly, more ATRX was found to be present in the PML-NBs (Fig. 7A, panels d–f). These results confirm that ATRX is a PML-NB component.

Daxx Targets ATRX to the PML-NBs—To investigate the role of Daxx in the targeting of ATRX to the PML-NBs, we first tested whether Daxx enhances the PML-NB localization of ATRX. Expression of a GFP fusion of ATRX in 293T cells showed a pattern of nuclear dots against a diffuse nuclear distribution that was similar to the endogenous protein. The GFP-ATRX dots were co-localized with Daxx in the PML-NBs (Fig. 7B, panels a–c). Notably, this localization was enhanced...
Daxx Associates with the ATR-X Syndrome Protein

when Daxx was co-transfected with GFP-ATRX (panels d–f). To facilitate PML-NB targeting of Daxx, we included PML in the experiments as previous studies have shown that PML recruits Daxx to the nuclear bodies (7, 9, 18). We next analyzed the structural determinants of ATRX that mediate its PML-NB localization. Various ATRX fragments used for the interaction assays (Fig. 3A) were fused to GFP. When these GFP-ATRX fragment fusions were expressed in mammalian cells, most of them, including those consisting of the first Daxx-interacting domain, showed a diffuse distribution in the nucleus and/or cytosol. However, two ATRX fragments (F1 and F5) appeared in nuclear speckled structures (Fig. 8A). Nevertheless, these two fragments, which were missing the first Daxx-interacting domain (see Fig. 3A), did not localize to the PML-NBs even when they were co-expressed with PML and Daxx (Fig. 8B, panels d–i; Fig. 8C, panels a–c; and data not shown). Interestingly, the ATRX fragments containing the first Daxx-interacting region (F6, F11, F12, and F14) localized to the PML-NBs when they were co-expressed with Daxx and PML (Fig. 8C, panels d–o). In contrast, the fragments missing the first Daxx-interacting domain were not localized to the nuclear bodies in the presence of Daxx and PML (Fig. 8C, panels d–c and data not shown). The correlation between the ability to bind to Daxx and the PML-NB localization of the ATRX fragments indicates that Daxx targets ATRX to the nuclear bodies. Finally, we co-expressed GFP-ATRX(F11) with either full-length Daxx or with a Daxx deletion mutant lacking either the NH₂-terminal ATRX-interacting domain or the COOH-terminal PML-interacting domain. The expression of full-length Daxx, but not of the deletion mutants, was correlated with GFP-ATRX accumulation in the PML-NBs. Taken together, these results show that Daxx recruits ATRX to the PML-NBs most likely through its ability to bind to both PML and ATRX.

DISCUSSION

Daxx was first identified as a regulator of apoptosis through JNK activation. Subsequent studies have shown that it is also a key component of the PML nuclear bodies and that it regulates transcription through its interactions with various DNA-binding proteins. In the present study, we demonstrate that Daxx interacts with ATRX, providing new insights as to the role of Daxx in transcriptional regulation.

Sequence homology has shown that ATRX is a member of the SNF2 family of ATP-dependent chromatin remodeling proteins (23, 24). Here we demonstrate that ATRX has nucleosome-stimulated ATPase activity that, in the case of purified recombinant ATRX, is equivalent to other recognized chromatin remodeling proteins. In addition, Xue et al. (31) recently showed that an ATRX-Daxx complex appeared to have ATP-dependent remodeling activity unique from that of other SWI/SNF-like protein complexes in that it may function at the DNA entry/exit sites of nucleosomes.

Although the ATPase domain of ATRX is a common site of patient mutations (25%), the defects in this domain lie outside of the most highly conserved residues, thereby suggesting that mutations in conserved amino acids may be lethal (23). Consistent with this idea, we demonstrated that two patient mutations (Ped3 and Ped23) introduced into recombinant ATRX attenuated ATPase function, whereas a mutation in the highly conserved catalytic site (K1600R; Mu) totally abolished it. In contrast, a mutation in the PHD domain had normal ATPase activity, suggesting that mutations in this domain have no direct effect on ATPase activity but rather alter ATRX function.
by another mechanism. Previous studies have indicated that chromatin-remodeling molecules can activate the expression of some genes and repress the expression of others. Interestingly, we found that ATRX, when tethered to DNA, strongly repressed transcription driven by the promoter of the thymidine kinase (Fig. 6). Consistent with this finding is the localization of ATRX to pericentromeric heterochromatin and its interaction with HP-1, both further substantiating a repressive role for ATRX. Moreover, with regard to the present study we expect that the ATPase-dependent chromatin remodeling activity of ATRX is required for transcription repression.

Daxx does not affect the ATPase activity of ATRX. Rather, it appears to regulate the cellular localization of ATRX. We demonstrate that Daxx can target ATRX to the PML-NBs, and, therefore, it may modulate ATRX activity by sequestering ATRX away from its target genes (Fig. 9). It remains to be determined whether Daxx also targets ATRX to sequence-specific transcription factors. This scenario is plausible because Daxx interacts with ATRX through its NH₂-terminal PAH domains but with sequence-specific transcription factors, such as Pax3, Pax5, ETS1, and the glucocorticoid receptor, through its COOH terminus (17–21). Daxx may thus function as a molecular bridge between DNA-binding proteins and chromatin remodeling complexes under certain circumstances and facilitate the assembly of transcription regulatory complexes.

The PML-NBs regulate a wide range of cellular processes, including tumor and growth suppression, apoptosis, anti-viral responses, and cellular senescence (11, 12). The underlying mechanisms of the PML-NBs, however, are not well defined. Nevertheless, accumulating evidence suggests a role for the PML-NBs in transcriptional regulation (16). Several transcription regulatory proteins have been found in the PML-NBs, including transcription factors such as p53 and pRB, as well as CBP, a member of the histone acetyltransferase protein family. The identification of ATRX, a chromatin-remodeling protein, within the PML-NBs strengthens the notion that these nuclear structures may be critical compartments for the regulation of transcription. It is conceivable that the PML-NBs may regulate transcriptional activity by restricting the access of regulatory proteins to their target genes. Alternatively, it may facilitate the assembly of transcriptional regulatory complexes by bringing the complex components within close proximity of each other.

The function of an ATRX-Daxx complex can only be speculated at this point. However, we know that both proteins are required for normal development. Mutations in ATRX lead to a severe mental retardation disorder, whereas mice deficient in Daxx are embryonic lethal. It is thus possible that the ATRX-Daxx complex regulates the transcription of developmentally important genes. Confirmation of such a function will require the identification of specific target genes.
Daxx Associates with the ATR-X Syndrome Protein

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