Ataxin 1, a *SCA1* neurodegenerative disorder protein, is functionally linked to the silencing mediator of retinoid and thyroid hormone receptors

Chih-Cheng Tsai*†‡§, Hung-Ying Kao*†¶, Akifumi Mitzutani‡, Ester Banayo*†, Harini Rajan‡, Michael McKeown‖, and Ronald M. Evans*†§**

*Howard Hughes Medical Institute, †Gene Expression Laboratory, and ‖Molecular Biology and Virology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA 92037; and ‡Department of Physiology and Biophysics, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, 683 Hoes Lane, Piscataway, NJ 08854

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Ataxin 1 (Atx1) is a foci-forming polyglutamine protein of unknown function, whose mutant form causes type 1 spinocerebellar ataxia in humans and exerts neurotoxicity in transgenic mouse and fly expressing mutant Atx1. In this study, we demonstrate that Atx1 interacts with the transcriptional corepressor SMRT (silencing mediator of retinoid and thyroid hormone receptors) and with histone deacetylase 3. Atx1 binds chromosomes and mediates transcriptional repression when tethered to DNA. Interaction with SMRT-related factors is a conserved feature of Atx1, because Atx1 also binds SMRTER, a *Drosophila* cognate of SMRT. Significantly, mutant Atx1 forms aggregates in *Drosophila*, and such mutant Atx1-mediated aggregates sequester SMRTER. Consistently, the neurodegenerative eye phenotype caused by mutant Atx1 is enhanced by a *Smrter* mutation and, conversely, is suppressed by a chromosomal duplication that contains the wild type *Smrter* gene. Together, our results suggest that Atx1 is a transcriptional factor whose mutant form exerts its deleterious effects in part by perturbing corepressor-dependent transcriptional pathways.

Spinocerebellar ataxia 1 (SCA1) is a progressive neurodegenerative disease caused by glutamine repeat expansion in ataxin 1 (Atx1) (1, 2). Other than its involvement in *SCA1*, the exact function of Atx1 is currently unknown. SCA1 pathology is characterized by ataxia, progressive motor deterioration, and loss of Purkinje cells in the cerebellum (3). Neurodegeneration has also been observed in transgenic *SCA1* mouse and in transformed *SCA1* fly when human mutant (glutamine repeat-expanded) Atx1 is ectopically expressed in mouse Purkinje cells and in *Drosophila* eyes, respectively (4, 5). These results suggest that specific conserved pathways are perturbed by mutant Atx1.

A recent genetic screen in *Drosophila* (5) that sought to identify modulators of the Atx1-mediated eye phenotype has identified components involved in protein folding, protein clearance, RNA processing, and transcriptional repression as potential targets for Atx1. Although the identification of heat shock response protein/chaperone (protein folding) and of ubiquitin/ubiquitin conjugase (protein clearance) in this screen has verified previous findings that these proteins are linked to polyglutamine diseases (6–10), the association that was revealed between Atx1 and several transcriptional corepressors, including Sin3 and Rpd3 (the *Drosophila* histone deacetylase 1), remains unexplained.

We were drawn to the results from this genetic screen in part because our earlier results indicated that silencing mediator for retinoid and thyroid hormone receptors (SMRT)-related ecdysone receptor-interacting factor (SMRTER) interacts with dSin3A (11), and because a similar interacting profile has also been observed for their vertebrate counterparts, such as SMRT and vertebrate Sin3A (12). Additionally, SMRT forms nuclear foci (13, 14) that resemble those formed by Atx1 (15, 16). These observations led us to hypothesize that SMRT and SMRTER may interact with Atx1. In this study, using cellular, molecular, and biochemical assays, we confirm our hypothesis by demonstrating the interactions between Atx1 and SMRT in mammalian cells and between Atx1 and SMRTER in *Drosophila*. Our results establish that Atx1 is involved in gene transcriptional regulation and that SMRT and its related factors represent conserved components targeted by Atx1 across phyla.

Materials and Methods

Constructs. ***Atx1 variants.*** Wild-type *Atx1* (30 CAG-repeat) was first isolated from a human cerebellum polyA RNA library (Clontech) by PCR by using two primers corresponding to the 5′ and 3′ ends of the human *Atx1* gene. Mutant *Atx1* (82 CAG-repeat) was obtained from Harry Orr (University of Minnesota, Minneapolis) (17). CAG-repeat-deleted *Atx1* (0 CAG-repeat) was generated by three-way ligation involving two PCR fragments corresponding to Atx1 amino acids 1–196 and 227–816.

Mammalian expression constructs. DNA fragments encoding amino acids 4–816 of human Atx1 [wild type (30Q), mutant (82Q), or (0Q)] were generated by PCR. EcoR1/*Nhe*I-digested PCR DNA fragments were then subcloned into a cytomegalovirus-based expressing vector/cyan fluorescent protein (CFP) vector to generate CFP-Atx1(30Q), CFP-Atx1(82Q), and CFP-Atx1(0Q), respectively. A similar approach was used to generate FLAG-Atx1 and Gal4-Atx1 variants with the three different glutamine repeat lengths. Yellow fluorescent protein (YFP)-SMRTER (2094–3040) was generated by subcloning a PCR fragment corresponding to the (2094–3040) region of SMRTER to CMX-YFP vector. FLAG-histone deacetylase (HDAC) 1 was obtained from M. Downes (The Salk Institute for Biological Studies) (14). Gal4-E52, which contains a nonrepressive domain from SMRTER, was described previously (11).

Yeast constructs. DNA fragments corresponding to amino acids 4–703 of Atx1 (0Q), (30Q), or (82Q) were subcloned into pGAD424 vector to generate GAD-Atx1(0Q), GAD-Atx1(30Q), and GAD-Atx1(82Q), respectively. GBT-SMRT constructs were described previously (13). GBT-SMRTER(2094–3040) was generated by subcloning a PCR fragment corresponding to the 2094–3040 region of SMRTER to GBT9 vector.

GST fusion constructs. DNA encoding Atx1 amino acids 477–575 was subcloned into pGEX-4T1 based vector to generate a

Abbreviations: Atx1, ataxin 1; CFP, cyan fluorescent protein; CtBP, C-terminal binding protein; GAD, Gal4 activation domain construct; GBT, Gal4 DNA binding domain construct; HDAC, histone deacetylase; SCA1, spinocerebellar ataxia 1; SMRT, silencing mediator of retinoid and thyroid hormone receptors; SMRTER, SMRT-related ecdysone receptor interacting factor; YFP, yellow fluorescent protein.

§To whom correspondence may be addressed. E-mail: tsaich@umdnj.edu or evans@salk.edu.

¶Present address: Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, OH 44106.

**Present address: Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI 02912.

GST-Atx1 fusion protein. This fusion protein was then used to generate anti-Atx1 antibody in guinea pig.

Antibodies. ***Antibodies for mammalian cultured cells.*** Antibodies used were anti-SMRT (PA1-843, Affinity BioReagents, Neshanic Station, NJ), anti-HDAC3 (H3034, Sigma), and anti-HDAC1 (06-720, Upstate Biotechnology, Lake Placid, NY).
Antibodies for Western blot analysis. Antibodies used were anti-FLAG (M2, Upstate Biotechnology), anti-SMRT (PA1-843, Affinity BioReagents), anti-Sin3A (AK-11, Santa Cruz Biotechnology), anti-C-terminal binding protein (CtBP) (H-440, Santa Cruz Biotechnology), anti-HDAC1 (06-720, Upstate Biotechnology), anti-HDAC3 (2632, Cell Signaling Technology, Beverly, MA), and anti-HDAC8 (H145, Santa Cruz Biotechnology).
Antibodies for* Drosophila *tissues and polytene chromosomes. Antibodies used were anti-Atx1 (477–575) and anti-SMRTER. (11).

The secondary antibodies, Texas red-conjugated or FITC-conjugated anti-mouse, anti-rabbit, or anti-guinea pig antibodies, were purchased from Jackson ImmunoResearch.

Immunofluorescence and Microscopic Analysis. Routine immunofluorescent staining procedures were applied to cultured cells, *Drosophila* tissues, and *Drosophila* polytene chromosomes. After the last washing step, the samples were mounted with VECTASHIELD medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories). The images were examined and captured by using a Nikon E1000 microscope and were analyzed by using IMAGEPRO software.

***Drosophila* Stocks and Salivary Gland Isolation.** Flies were raised and crossed at 25°C. *P{Hsp70-Gal4}* (BL 1799), *P{GMR-Gal4}* (BL 1104), *P{Smrter^{BG01648}}/FM7a* (BL 13116), and *Dp(1;Y)BSC5* (BL 5796) were obtained from the Bloomington Stock Center. *P{UAS-Atx1(82Q)}* was a gift from M. Feany (Brigham and Women's Hospital, Harvard Medical School, Boston) (18). *P{UAS-dHdj1}* was a gift from P. Kazemi-Esfarjani (State University of New York, Buffalo) (19). The wild-type control line was *w^{1118}*.

Results

The Formation of Atx1 Nuclear Foci Is Independent of Glutamine Repeat Length. Previous studies have indicated that, in transfected cells, both wild-type (30Q) and mutant (82Q) Atx1 localize to small dense nuclear bodies (15, 16). This observation raises the question of whether the glutamine repeat is required for Atx1-nuclear foci formation, and why Atx1(82Q) does not form protein aggregates, because long polyglutamine repeats alone are capable of forming a single large protein aggregate (20, 21). To investigate this question, we generated and tested vectors encoding CFP, a variant of GFP, fused to wild-type Atx1(30Q), mutant Atx1(82Q), and Atx1(0Q) (Fig. 1 *A* and *B*). In human embryonic kidney cells (HEK-293), both wild-type and mutant CFP-Atx1s display the previously reported stereotypical focal nuclear pattern (Fig. 1 *C* and *D*) (15, 16). To our surprise, a similar nuclear pattern was also observed for CFP-Atx1(0Q) (Fig. 1*E*). Because Atx1(0Q) does not include the glutamine repeat tract, this result indicates that Atx1 nuclear foci are formed by a mechanism that is distinctly different from nuclear inclusions *in vivo* and from the polyglutamine-mediated protein aggregates found in cultured cells.

Endogenous SMRT Colocalizes with Atx1s in Cultured Cells. In recent studies, we have reported the partitioning of the nuclear corepressor SMRT into nuclear dots or compartments, termed matrix-associated histone deacetylase nuclear bodies (13, 14). The similarity between the appearance of matrix-associated histone deacetylase bodies of SMRT and of the nuclear foci of Atx1 led us to explore the potential overlap between the Atx1 foci and SMRT dots. Accordingly, the cells transfected with CFP-Atx1(30Q), CFP-Atx1(82Q), or CFP-Atx1(0Q) were subjected to immunofluorescent staining by using SMRT-specific antibody. In untransfected cells, such as in HEK-293, endogenous SMRT shows a typical diffuse background with occasional foci formation (data not shown). The dotted nuclear pattern of SMRT intensifies in cells transfected with either of the three CFP-Atx1 variants, revealing an apparent colocalization of SMRT and Atx1s (Fig. 1 *C–E* and *C′–E′*).

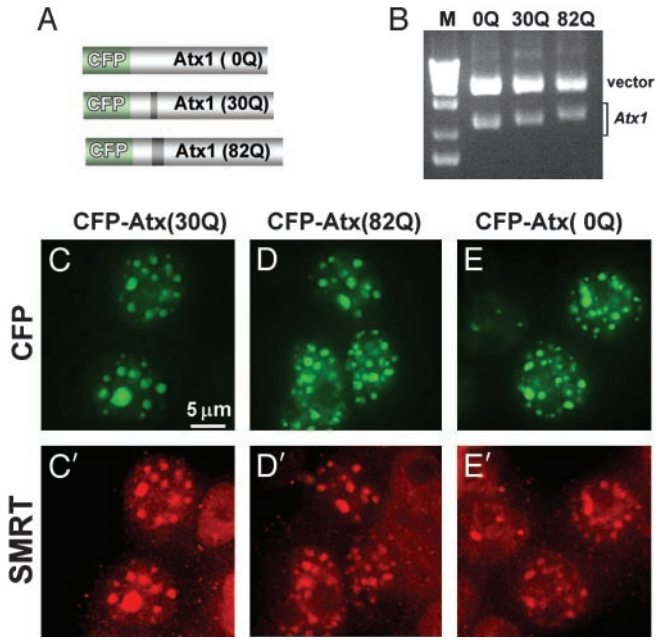

Fig. 1. Atx1 forms nuclear foci and associates with SMRT independent of its glutamine repeat length. (*A*) Diagram of cytomegalovirus promoter-driven CFP-tagged Atx1 variant constructs used in the transfection experiments. Atx1(30Q), Atx1(82Q), and Atx1(0Q) represent wild-type Atx1, mutant (expanded) Atx1, and glutamine repeat-deleted Atx1, respectively; the polyglutamine tract is indicated by a dark gray box. (*B*) Restriction enzyme digestion patterns for CFP-Atx1 constructs. (*C–E*) Nuclear focal patterns for different CFP-Atx1s and their effects on SMRT expression. HEK-293 cells were transfected with CFP-Atx1(30Q) (*C*), CFP-Atx1(82Q) (*D*), or CFP-Atx1(0Q) (*E*), respectively. CFP signal was captured in black and white and then rendered in green by using PHOTOSHOP (Adobe Systems, Mountain View, CA). The endogenous SMRT protein was detected by an indirect immunostaining method using an anti-SMRT antibody and Texas red-conjugated secondary antibody (*C′–E′*).

Atx1 Interacts with SMRT and SMRTER in Yeast. To provide evidence for a direct association between Atx1 and SMRT, a yeast two-hybrid assay was used (22). A Gal4 activation domain-Atx1 fusion [GAD-Atx1(30Q)] containing amino acids 4–703 of Atx1 was constructed and tested against a series of Gal4 DNA binding domain–SMRT constructs (GBT-SMRTs), including GBT-SMRT (2–316), GBT-SMRT(159–683), GBT-SMRT(699–1035), GBT-SMRT(1060–1831), and GBT-SMRT(1755–2518) (Fig. 2*A*). Among the tested GBT-SMRT constructs, the C-terminal (1755–2518) region of SMRT interacts strongly with Atx1, as revealed by the strong β-galactosidase enzymatic activity (Fig. 2*B*, lane 2, blue dots).

A similar interaction profile is also observed between Atx1s and SMRTER (Fig. 2B, lane 3), a counterpart of SMRT in *Drosophila* (11). This interaction is also mediated by means of the C-terminal region of SMRTER(2094–3040), indicating that interaction with nuclear corepressors is a conserved feature of Atx1 and that the Atx1-interacting motif(s) reside at the C-terminal regions of SMRT and SMRTER. As shown in Fig. 2*C*,

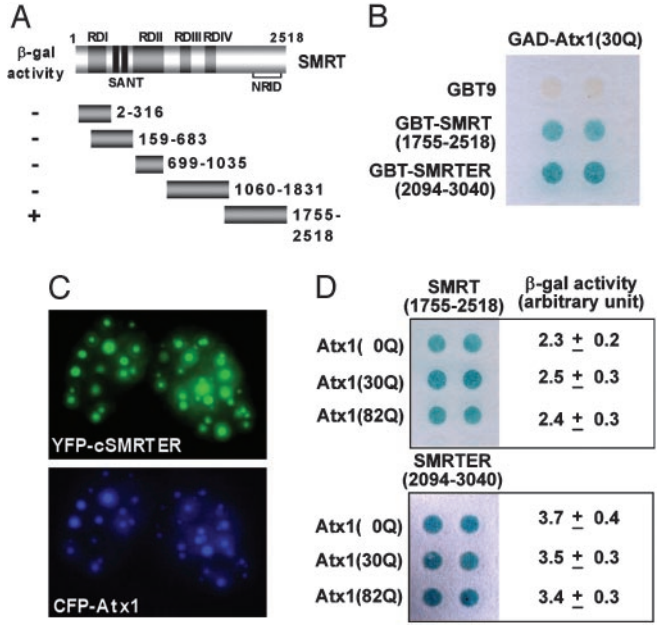

Fig. 2. Atx1 interacts with SMRT and SMRTER in yeast. (*A*) Summary of yeast two-hybrid assays for Atx1 and SMRT. The diagram shows the regions of SMRT used in the assays and also the known functional domains of SMRT, including SWI3/Ada 2/N-CoR/TFIIIB′ domains, repression domains (RD I, RD II, RD III, RD IV), and nuclear receptor-interacting domains (NRID). Yeast 190 cells were transformed with GAD-Atx1 (4–703) along with each of the GBT-SMRT variants. The Atx1-interacting domain was mapped to amino acids 1755–2518. Positive β-galactosidase activities were based on color reaction from the lifting assays. (*B*) Yeast two-hybrid assays for Atx1 and SMRT or SMRTER. Yeast 190 cells were transformed with GAD-Atx1 (4–703)(30Q), along with an empty GBT9 vector, with GBT-SMRT(1755–2518), or with GBT-SMRTER(2094–3040). Positive interactions revealed by 5-bromo-4-chloro-3-indolyl-β-D-galactoside reaction are shown as blue dots. (*C*) Expression patterns of CFP-Atx1 and YFP-SMRTER in the transfected cells. HEK-293 cells were transfected with plasmids expressing both YFP-SMRTER(2094–3040) and CFP-Atx1(30Q). (*D*) Yeast two-hybrid assays for Atx1 variants and SMRT or SMRTER. Yeast 190 cells were transformed with GBT-SMRT(1755–2518) or GBT-SMRTER(2094–3040), along with GAD-Atx1(0Q), GAD-Atx1(30Q), or GAD-Atx1(82Q). The β-galactocidase activities from the liquid assays are shown on the right.

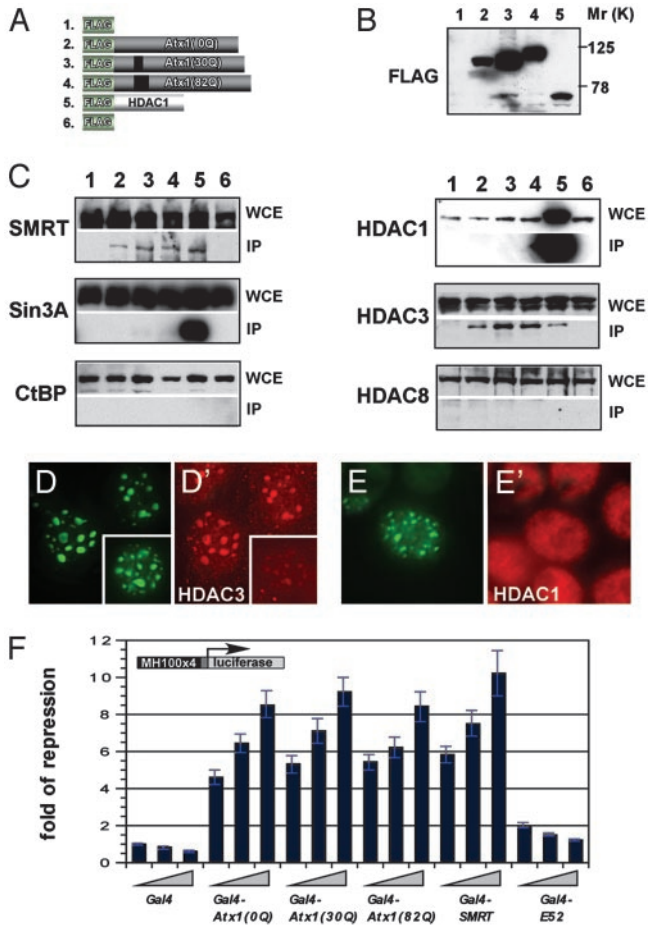

Fig. 3. Atx1 interacts selectively with HDAC3 *in vitro* and *in vivo*. (*A*) Diagram of FLAG-tagged Atx1 and HDAC1 constructs used in the coimmunoprecipitation experiments. Lanes 1 and 6, FLAG alone; lane 2, FLAG-Atx1(0Q); lane 3, FLAG-Atx1(30Q); lane 4, FLAG-Atx1(82Q); lane 5, FLAG-HDAC1. The glutamine repeat tract is indicated by a black box. (*B*) Western blot analysis for FLAG-Atx1s and FLAG-HDAC1 expression. Whole-cell extracts (WCE) prepared from the transfected cells with plasmids corresponding to *A* were subjected to Western blot analysis by using the anti-FLAG M2 antibody. (*C*) Coimmunoprecipitation experiments to identify Atx1-associating proteins. WCE and immunoprecipitated complex (IP) prepared from the FLAG-Atx1 transfected cells were subjected to Western blot analysis by using anti-SMRT, anti-Sin3A, anti-CtBP, anti-HDAC1, anti-HDAC3, or anti-HDAC8 antibodies, respectively. (*D* and *E*) HDAC3 and HDAC1 staining patterns in cells transfected with CFP-Atx1. HEK-293 cells (*D Inset* and *E*) or MCF-7 cells (*D*) were transfected with CFP-Atx1(82Q) and were immunostained by using anti-HDAC3 antibody (*D′*) or anti-HDAC1 antibody (*E′*). (*F*) The Gal4 reporter assays for Gal4-Atx1 fusions. HEK-293 cells were cotransfected with a Gal4 responsive luciferase reporter (MH100x4), along with three different concentrations of plasmids corresponding to empty Gal4, Gal4-Atx1(0Q), Gal4-Atx1(30Q), Gal4-Atx1(82Q), Gal4-SMRT, or Gal4-E52. Whereas Gal4-SMRT is used here as a positive control, both Gal4-DBD and Gal4-E52 are used as negative control. Luciferase reporter activity was normalized with a β-galactosidase expressing a CMX-lacZ control construct.

a YFP fusion to SMRTER(2094–3040) colocalizes with CFP-Atx1(30Q). These data suggest that Atx1 and SMRTER interact in HEK-293 cells in a manner dependent on the SMRTER C-terminal domain.

We next studied the effect of differing Atx1 glutamine repeats on Atx1 interaction with SMRT or SMRTER in yeast. In addition to GAD-Atx1(30Q), we generated and tested two other GAD-Atx1 variants, encoding either the glutamine repeat expanded form (82Q) or the deleted form (0Q) of Atx1, against GBT-SMRT(1755–2518) and GBT-SMRTER(2094–3040) in yeast (Fig. 2*D*). The β-galactosidase activity (from both lifting and liquid assays) found in yeast transformed with GAD-Atx1(0Q), GAD-Atx1(30Q), or Atx1(82Q) were all comparable for both SMRT and SMRTER, indicating that altering the glutamine repeat length in Atx1 does not impair Atx1-SMRT or -SMRTER interactions.

Atx1 Forms Complexes with HDAC3 and Functions as a Transcriptional Repressor. We also studied the interaction between Atx1 and factors known to associate with SMRT, such as HDACs, that mediate the repressive activity of SMRT (13, 23–27). A panel of potential associating proteins was examined in Atx1-immunoprecipitated complexes by Western blot. In addition to three type 1 HDACs (HDAC1, HDAC3, and HDAC8), we also tested Sin3A and CtBP in light of their genetic interactions in the Atx1-mediated eye phenotype in *Drosophila* (5). HEK-293 cells were transfected with plasmids expressing FLAG, FLAG-tagged Atx1(0Q), Atx1(30Q), Atx1(82Q), or HDAC1, respectively (Fig. 3*A*) and were first monitored for their protein expression (Fig. 3*B*). In these experiments, FLAG-HDAC1 was used as a positive control, because HDAC1 has been found to associate with SMRT and several of the examined protein factors.

Consistent with our earlier data, SMRT is present in the

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immunoprecipitated complexes of all three forms of Atx1 (Fig. 3*C*). The level of immunoprecipitated SMRT correlates directly to Atx1 expression (Fig. 3*B*) and is not affected by variation in the glutamine repeat length. SMRT was also detected in the complex associated with FLAG-HDAC1, in agreement with previous observations (12). Surprisingly, and in contrast to the genetic evidence, we were unable to detect HDAC1, Sin3A, or CtBP in the Atx1 coimmunoprecipitated complexes (Fig. 3*C*). These interactions were not observed despite the robust detection of Sin3A in the coimmunoprecipitated complex for FLAG-HDAC1 in the parallel experiments. These results indicate that the interactions between Atx1 and HDAC1, Sin3A, or CtBP in HEK-293 cells may be too unstable or transient to be detected in these assays.

We did, however, identify HDAC3 as a preferential target for Atx1 (Fig. 3*C*). This selective interaction between Atx1 and HDAC3 is noteworthy, because HDAC3 is so far the only HDAC that has been identified in purified SMRT and nuclear receptor corepressor complexes (23–25, 27). The association between Atx1 and HDAC3 is also observed in cell cultures. Immunofluorescent staining in HEK-293 or MCF-7 cells transfected with CFP-Atx1(82Q) revealed that HDAC3, but not HDAC1, shifts from a dispersed pattern to a speckled nuclear pattern after the formation of Atx1 nuclear foci (Fig. 3 *D*, *D′*, *E*, and *E′*). As in the case of SMRT, the association between Atx1 and HDAC3 appears independent of the glutamine repeat in Atx1 (data not shown).

Given the known roles of SMRT and HDAC3 in transcriptional repression, we examined the ability of Atx1 to repress transcription in HEK-293 cells. To examine the effect of Atx1 on transcriptional repression, Atx1 was directed to a characterized artificial promoter by fusion to a Gal4 DNA binding domain. In this assay, Gal4-Atx1(0Q), Gal4-Atx1(30Q), and Gal4-Atx1(82Q) all functioned as transcriptional repressors in HEK-293 cells (Fig. 3*F*), similar to the repressive effect exercised by Gal4-SMRT. The repressive effects of Atx1s appear to be independent of glutamine repeat as well. On the contrary, transcriptional repressive effects were not observed for Gal4 and for a control Gal4-E52 fusion, which does not encode a transcriptional repressive domain (11).

Mutant Atx1 Forms Aggregates and Sequesters SMRTER in *Drosophila*. Because human Atx1 interacts with *Drosophila* SMRTER *in vitro*, we next examined whether such an Atx1-SMRTER interaction can be recapitulated in *Drosophila* tissues. We ectopically expressed Atx1(82Q) in *Drosophila* using the binary Gal4/UAS system (28). *UAS-Atx1(82Q)* flies, in which *Atx1* (82Q) is placed under the control of multimerized Gal4 responsive elements (18), were crossed to *Hsp70-Gal4* flies, which carry a heat-shock inducible Gal4 driver. Although *Hsp70-Gal4* was initially chosen for its heat-inducible feature, further analysis revealed that *Hsp70-Gal4* is selectively expressed in the salivary gland without heat shock treatment (Fig. 4).

Expression of Atx1(82Q) in the salivary gland is detrimental to salivary gland development, because salivary glands from the *Hsp70-Gal4*;*UAS-Atx1(82Q)* late third-instar larvae displayed clear deformations, including a significant reduction in cell volume and a notable disarray in tissue organization (Fig. 4*A*). In contrast, no obvious defects were observed in the salivary glands of the control *UAS-Atx1(82Q)* larvae (Fig. 4*B*). This result indicates that the deleterious effects caused by overexpression of Atx1(82Q) are not restricted to neuronal cells in *Drosophila*. We next examined whether Atx1(82Q) forms aggregates in *Drosophila* salivary gland and, if it does, whether SMRTER expression is affected accordingly. Simultaneous immunostaining of *Hsp70-Gal4*;*UAS-Atx1(82Q)* salivary glands with both Atx1- and SMRTER-specific antibodies revealed that Atx1(82Q) forms protein aggregates within the nuclei of salivary gland cells (Fig. 4 *C* and *D*). Significantly, these Atx1(82Q)-mediated aggregates sequester endogenous SMRTER (Fig. 4*D′*). As a result of this sequestering effect by Atx1(82Q)-aggregates, SMRTER is depleted from other parts of the nucleus. On the contrary, in the control *UAS-Atx1(82Q)* larvae, a granular SMRTER pattern is detected in the nucleus of salivary gland cells (Fig. 4*E*).

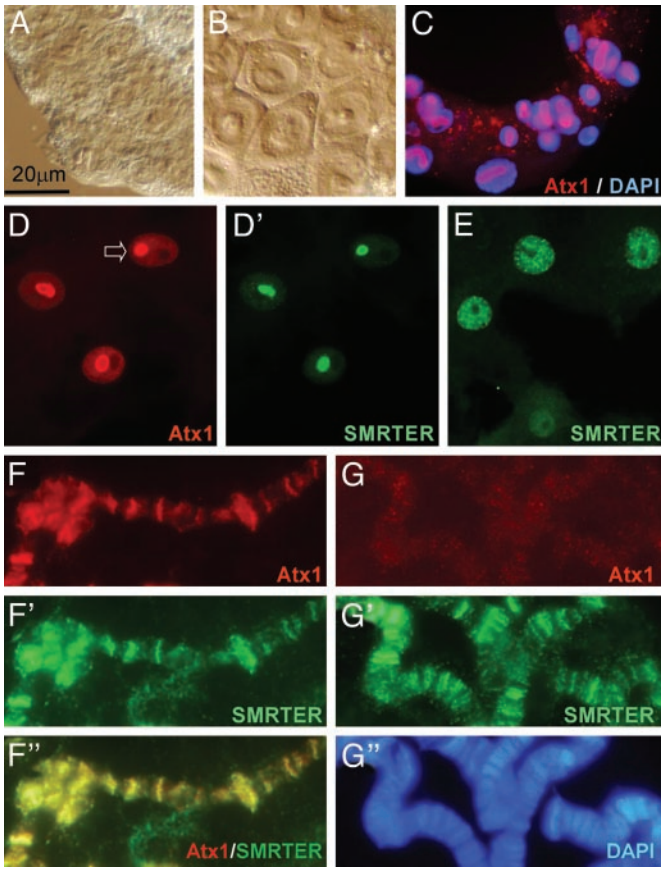

Fig. 4. Atx1(82Q) binds chromosomes and sequesters SMRTER into its aggregates. (*A* and *B*) Mutant Atx1 causes salivary gland deformity. Shown are Nomarski images corresponding to salivary gland cells isolated from the *Hsp70-Gal4*;*UAS-Atx1(82Q)* larvae (*A*) and from the control *UAS-Atx1(82Q)* (*B*). (*C*) Atx1(82Q) forms aggregates in salivary gland. Nonsquashed salivary gland isolated from *Hsp70-Gal4*;*UAS-Atx1(82Q)* larvae were immunostained with anti-Atx1 (Texas red) antibody. The salivary gland is also counterstained with 4′,6-diamidino-2-phenylindole. (*D* and *E*) Atx1(82Q)-mediated aggregates sequester SMRTER. Squashed salivary gland cells isolated from *Hsp70-Gal4*;*UAS-Atx1(82Q)* larvae (*D*) or from the control *UAS-Atx1(82Q)* larvae (*E*) were immunostained with anti-Atx1 (Texas red) (*D*) and with anti-SMRTER (FITC) antibodies (*D′* and *E*). An arrow indicates the region where Atx1 aggregates form. (*F* and *G*) Atx1 and SMRTER localize to overlapping chromosomal loci. The polytene chromosomes isolated from *Hsp70-Gal4*;*UAS-Atx1(82Q)* salivary glands (*F*) or from the control *UAS-Atx1(82Q)* salivary glands (*G*) were subjected to indirect immunostaining by using anti-Atx1 (Texas red) (*F* and *G*) and anti-SMRTER (FITC) (*F′* and *G′′*). *F′′* is a merged image. G′′ shows the chromosomes counterstained with 4′,6-diamidino-2-phenylindole.

Atx1 and SMRTER Localize to Overlapping Chromosomal Regions. The molecular, biochemical, and genetic linkage between Atx1 and components involved in transcriptional repression suggests that Atx1 may associate with chromosomes. Simultaneous treatment of *Hsp70-Gal4*;*UAS-Atx1(82Q)* chromosomes with both anti-Atx1 and SMRTER antibodies revealed that Atx1 indeed binds to chromosomes (Fig. 4*F*) and, remarkably, that it localizes to the same chromosomal regions where SMRTER is found (Fig. 4

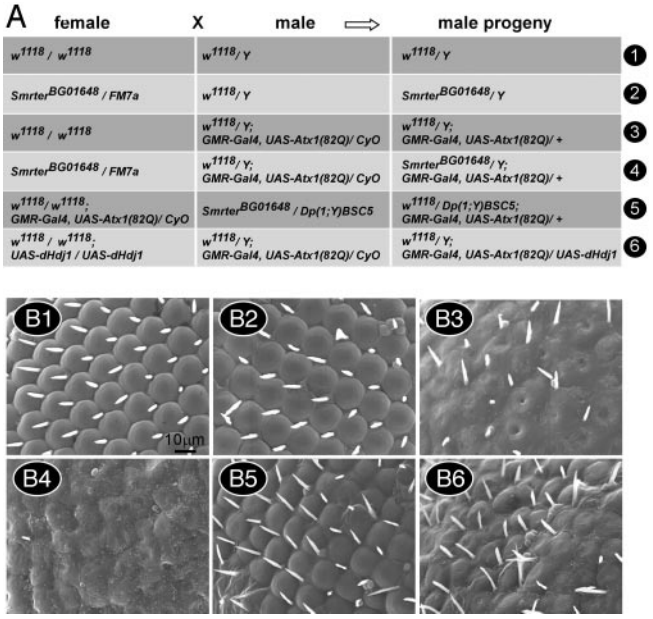

Fig. 5. *Smrter* mutation modulates the Atx1(82Q)-mediated eye phenotype. (*A*) Scheme of genetic crosses using indicated females and males to produce male progeny with corresponding genetic background. The numbered rows (1–6) correspond to the images shown in *B*. (*B1–B6*) *Smrter* and *Atx1* interact genetically. Shown are scanning electron microscopy images of retinas corresponding to adult wild-type (w^{1118}/Y) (*B1*), $Smrter^{BG1648}/Y$ (*B2*), w^{1118}/Y;*GMR-Gal4,UAS-Atx1(82Q)*/+ (*B3*), $Smrter^{BG1648}/Y$;*GMR-Gal4,UAS-Atx1(82Q)*/+ (*B4*), w^{1118}/*Dp(1;Y)BSC5*; *GMR-Gal4,UAS-Atx1(82Q)*/+ (*B5*), and w^{1118}/*Y;GMR-Gal4,UAS-Atx1(82Q)/UAS-dHdj1* (*B6*).

F–F″). In comparison, only background Atx1 staining can be detected for the polytene chromosomes isolated from the control *UAS-Atx1(82Q)* salivary glands (Fig. 4*G*). This result not only demonstrates that Atx1 is a chromosomal binding factor but also verifies that Atx1 and SMRTER interact *in vivo*. Interestingly, both Atx1 and SMRTER are absent from the banding regions (where 4′,6-diamidino-2-phenylindole staining is strong) and from the centromere area (not shown), indicating that Atx1 and SMRTER are excluded from heterochromatic regions on chromosomes.

***Smrter* Mutation Enhances the Eye Phenotype Caused by Mutant Atx1.** In light of the above observation, we examined whether the reported eye phenotype of Atx1(82Q) flies could be enhanced or suppressed by *Smrter* mutations. The semilethal $Smrter^{BG01648}$ allele, which displays no significant morphological eye phenotype, was used to study the genetic interaction between *Smrter* and *Atx1*. $Smrter^{BG01648}/FM7a$ or control wild-type (w^{1118}) females were crossed with the recombined *GMR-Gal4,UAS-Atx1(82Q)/CyO* males, and male offspring were scored for eye morphology (the scheme for various genetic crosses is shown in Fig. 5*A*). Although $Smrter^{BG01648}/Y$ flies are viable and display a normal eye structure similar to that of w^{1118}/Y (Fig. 5 *B1* and *B2*), a rough eye phenotype was observed for w^{1118}/Y,*GMR-Gal4,UAS-Atx1(82Q)*/+ flies (Fig. 5*B3*). This phenotype is similar to the phenotype reported previously (5, 18). However, the eye phenotype is enhanced in $Smrter^{BG01648}/Y$,*GMR-Gal4,UAS-Atx1(82Q)*/+ flies, resulting in a glazed eye surface, severely disorganized ommatidia, and, most prominently, loss of bristles (Fig. 5*B4*).

The genetic interaction between Atx1 and SMRTER is further confirmed by the suppression of the Atx1-mutant eye phenotype by a Y chromosome (*Dp(1;Y)BSC5*), which contains a duplication of a small chromosomal interval (11B14-C2), in which the *Smrter* gene resides. A restoration of wild-type-like eye structure is seen in w^{1118}/*Dp(1;Y)BSC5*;*GMR-Gal4,UAS-Atx1(82Q)*/+ flies (Fig. 5*B5*). This suppressing effect caused by *Dp(1;Y)BSC5* resembles that caused by *Drosophila* heat-shock 40 protein (dHDJ1) (Fig. 5*B6*), a molecular chaperone known to suppress polyglutamine-induced neurotoxicity in *Drosophila* (5, 8, 19, 29). Together, these results establish genetic and physiological evidence for a functional interaction between Atx1 and SMRTER.

Discussion

Atx1 is a polyglutamine disease protein, whose mutant (glutamine repeat-expanded) form is involved in SCA1 (1, 2). Although Atx1 was identified a decade ago, its exact function, other than its involvement in SCA1, remains unclear. Over the past few years, several Atx1-interacting factors have been identified, including GAPDH (30), leucine-rich acidic nuclear protein/pp32 (16), A1Up (a ubiquitin-like nuclear protein) (31), polyglutamine-tract binding protein 1 (32), and 14-3-3 (33). The known properties of these Atx1-interacting proteins, however, reveal little about the exact nature of Atx1. In this study, spurred by the resemblances between matrix-associated histone deacetylase-bodies of SMRT (14) and the nuclear focal pattern of Atx1 (15, 16), we established a functional link between Atx1 and SMRT. We first showed the coincidence of Atx1 foci with SMRT matrix-associated histone deacetylase bodies *in vivo* (Fig. 1 *C–E*) and then demonstrated a direct link between Atx1 and SMRT by using yeast two-hybrid assays and coimmunoprecipitation studies (Figs. 2*B* and 3*C*). We also showed that Atx1 forms complexes with HDAC3 (Fig. 3 *C* and *D*), a histone deacetylase copurified with the SMRT complex (23, 24). The linkage between Atx1 and SMRT and HDAC3, two well characterized components involved in transcriptional repression (34–36), thus establishes that Atx1 is involved in gene transcriptional regulation.

In addition to SMRT, Atx1 also interacts with nuclear receptor corepressor, chick SMRT (data not shown), and *Drosophila* SMRTER (Fig. 2*B*), a SMRT- and nuclear receptor corepressor-related factor in the fly (11). Evidently, interacting with SMRT-related factors is a conserved feature of Atx1. The interaction between Atx1 and SMRTER enabled us to further investigate the role of SMRTER in Atx1-mediated phenotypes in *Drosophila*. We were able to establish that mutant Atx1 forms protein aggregates in *Drosophila* salivary gland cells and that such Atx1(82Q)-mediated aggregates sequester endogenous SMRTER (Fig. 4 *D* and *D′*). We also demonstrated that Atx1 and SMRTER colocalize with each other on *Drosophila* polytene chromosomes (Fig. 4 *F–F″*). This *in vivo* observation confirms our hypothesis that Atx1 is a chromatin binding factor and is likely involved in gene transcriptional regulation. Moreover, our genetic analysis establishes that the Atx1(82Q)-mediated eye phenotype in *Drosophila* is enhanced by a *Smrter* mutation and, conversely, is suppressed by a chromosomal duplication that carries the *Smrter* gene locus (Fig. 5*B*, rows 2–5). In the latter case, however, we should caution that we cannot entirely exclude the possibility that other translocated genes, from a small X-chromosome interval to Y chromosome, may contribute to the suppressing effect.

In parallel with this study, several other polyglutamine disease proteins have also been found to associate with transcriptional corepressors. For example, huntingtin (Htt) interacts with nuclear receptor corepressor and Sin3A (37, 38); atrophin-1 recruits Sin3A and HDAC2 in transfected cells (39); and androgen receptor, a nuclear receptor itself, was recently found to interact with SMRT as well (40, 41). It is thus becoming evident that certain aspects of polyglutamine-protein functions are mediated through transcriptional corepressors. Intriguingly, several recent results also indicate that a transcriptional coactivator, cAMP-response element-binding protein (CBP), is also targeted by polyglutamine-disease proteins, including Htt, androgen recep-

tor, and atrophin 1 (38, 42, 43). Because CBP is sequestered into polyglutamine-mediated nuclear inclusions (NIs), it has been hypothesized that compromised functioning of CBP may contribute to the cellular toxicity of these diseases (44–46). The link between CBP and NIs has also raised the possibility of using HDAC inhibitors (HDACi) as a therapeutic agent to treat polyglutamine diseases (47–49). Our finding that the normal function of Atx1 is linked to SMRT, along with other evidence that Htt, atrophin 1, and androgen receptor are associated with components involved in the transcriptional repression apparatus, indicates that caution will be needed before using HDACi to treat all polyglutamine diseases. HDACi might interfere not only with the pathological properties but also with the transcriptional regulatory properties of various polyglutamine disease proteins. Therefore, our discovery that Atx1 interacts with SMRT and SMRTER provides a model for assessing the potential benefits or hazards of HDACi to treat SCA1.

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