Nuclear accumulation of HDAC4 in ATM deficiency promotes neurodegeneration in ataxia telangiectasia

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Ataxia telangiectasia is a neurodegenerative disease caused by mutation of the Atm gene. Here we report that ataxia telangiectasia mutated (ATM) deficiency causes nuclear accumulation of histone deacetylase 4 (HDAC4) in neurons and promotes neurodegeneration. Nuclear HDAC4 binds to chromatin, as well as to myocyte enhancer factor 2A (MEF2A) and cAMP-responsive element binding protein (CREB), leading to histone deacetylation and altered neuronal gene expression. Blocking either HDAC4 activity or its nuclear accumulation blunts these neurodegenerative changes and rescues several behavioral abnormalities of ATM-deficient mice. Full rescue of the neurodegeneration, however, also requires the presence of HDAC4 in the cytoplasm, suggesting that the ataxia telangiectasia phenotype results both from a loss of cytoplasmic HDAC4 as well as its nuclear accumulation. To remain cytoplasmic, HDAC4 must be phosphorylated. The activity of the HDAC4 phosphatase, protein phosphatase 2A (PP2A), is downregulated by ATM-mediated phosphorylation. In ATM deficiency, enhanced PP2A activity leads to HDAC4 dephosphorylation and the nuclear accumulation of HDAC4. Our results define a crucial role of the cellular localization of HDAC4 in the events leading to ataxia telangiectasia neurodegeneration.

Ataxia telangiectasia is a hereditary multisystemic disease resulting from mutations in the ATM gene, which encodes a 370-kD member of the phosphatidylinositol 3 (PI3)-kinase family of kinases. The symptoms of ataxia telangiectasia include non-neurological phenotypes such as immune-system defects, germ-cell defects, hypersensitivity to ionizing radiation and increased susceptibility to cancer. However, it is the loss of neuronal cells, the most direct cause of the devastating ataxia, that is arguably the least understood phenotype in this disease. We report a newly discovered role for HDAC4 in this process.

Class I and class IIa histone deacetylases (HDACs) have key roles in brain development and neuron survival. HDAC4 (class IIa) is abundant in neurons, where it is predominantly cytoplasmic. Relevant to the phenotype of ataxia telangiectasia, HDAC4 deficiency in mice is marked by a postnatal atrophy of the cerebellum, with surviving Purkinje cells having a notable reduction in dendritic complexity. HDAC4 is normally phosphorylated by calcium/calmodulin-dependent kinases (CaMks), enabling its binding to the 14-3-3 family of protein chaperones, HDAC4, like other class IIa HDACs, associates with the prosurvival transcription factors MEF2A and CREB, repressing their transcriptional activity. Though well studied in other cell types, the relationship between HDAC4 and MEF2A- or CREB-dependent gene expression in neurons is largely unexplored.

RESULTS
Nuclear HDAC4 reduces MEF2A/CREB-dependent transcription

The similar phenotypes of neuron death and dendritic atrophy in the cerebella of ATM- or HDAC4-deficient mice prompted us to examine HDAC4 in human ataxia telangiectasia cerebella. Normally, HDAC4 immunoreactivity is found in Purkinje cell cytoplasm (Fig. 1a). However, the Purkinje cell nuclei in the ataxia telangiectasia samples had strong HDAC4 staining (Fig. 1a,b). The nuclear accumulation of HDAC4 was specific: despite structural and functional similarities to HDAC5 and HDAC9 showed little nuclear accumulation in ataxia telangiectasia (Supplementary Fig. 1a). We replicated these observations in mice. HDAC4, but not HDAC5 or HDAC9 (Supplementary Fig. 1b), showed significant nuclear accumulation in Atm−/− but not wild-type mouse Purkinje cells (Fig. 1a,c). We found similar shifts in HDAC4 localization in other mouse brain regions, including the neocortex and brain stem (Supplementary Fig. 1c). The shift was also seen with exogenous protein; GFP-HDAC4 transfected into cultured Atm−/− neurons also localized to the nucleus (Fig. 1d). Further, after exposure of cultured cortical neurons to siRNA against Atm, HDAC9 remained in the cytoplasm, whereas endogenous HDAC4 shifted to the nucleus (Supplementary Fig. 1d).

The association of MEF2A and CREB with HDAC4 was markedly higher in Atm−/− cerebellar extracts than in wild-type extracts (Fig. 1e,f and Supplementary Fig. 2a). That interaction with HDAC4 served to inactivate MEF2A and CREB because we observed reduced promoter occupancy of these two proteins at known target genes in Atm−/− mice. We performed chromatin immunoprecipitation (ChIP) and assayed by quantitative PCR (qPCR) the extent to which various promoter regions were occupied: Egr3, Fos and Mef2a for MEF2A and Af, Nrxn1, Nrxn3 and Egr1 for CREB. In each region, we found lower

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Nuclear HDAC4 suppresses neuronal gene expression

Enhanced HDAC4 in Atm−/− neuronal nuclei suggests a reduction of histone acetylation in these cells. Immunostaining for total histone 3 (H3) and H4 in Atm−/− cerebella was similar to that in wild-type cerebella (Fig. 2a). By contrast, we found less immunostaining for acetylated histone 3 (AcH3) and AcH4 in Atm−/− mice compared to wild-type mice. We found the same result in the neocortex and hippocampus (Supplementary Fig. 2d). We confirmed these findings using western blots (Fig. 2b–d). We then examined the association of AcH3 and AcH4 with specific gene promoters. We performed ChIP followed by qPCR assays with extracts from wild-type and Atm−/− mouse cerebella (Fig. 2e–g). The ChIP of H3 revealed no consistent difference between wild-type and Atm−/− samples in chromatin association at the promoters we examined (Fig. 2e). By contrast, the associations of AcH3 and AcH4 with multiple neuronal growth genes were consistently lower in Atm−/− samples compared to wild-type samples. The loss of histone acetylation in a promoter region suggests a closed chromatin configuration and reduced transcription. Accordingly, in Atm−/− compared to wild-type samples, we observed less transcription of multiple neuronal genes at which there was lower promoter occupancy by acetylated histones, for example, Bdnf, Grin2a (also known as Nr2a2), Nrnx1 and Nrnx3 (Fig. 2f–g). The occupancy of these promoters by H3 (Fig. 2e) and H4 (data not shown) in Atm−/− mice was equivalent to that in wild-type mice.

We next performed ChIP with HDAC4 and found that it directly associates with chromatin (Supplementary Fig. 3a,b). We analyzed the HDAC4-precipitated DNA with ChIP sequencing (ChIP-seq) using a SOLiD DNA fragment library platform. The aligned ChIP-seq tags revealed patterns of HDAC4-chromatin binding that differed substantially between wild-type and Atm−/− mice (Fig. 2h and Supplementary Fig. 3c). To validate the ChIP-seq results, we performed ChIP-qPCR of selected genes using primers designed to be within the ChIP-seq–identified Atm−/− peak, as well as primers in two sites flanking the peak by approximately 900 bp (Supplementary Fig. 3d–g). All four qPCR assays showed...
increased HDAC4 binding to the region of the peak predicted by sequencing and peak finding in the Atm−/− samples.

**Inhibiting HDAC4 prevents neuronal dysfunction in Atm−/−**

Our data indicate that shifting the location of HDAC4 from the cytoplasm to the nucleus is neurotoxic. Notably, HDAC4 knockout mice have smaller brains than wild-type mice, suggesting that genetic deletion of HDAC4 is also neurotoxic. To explore this potential discrepancy, we treated Atm−/− and wild-type mice with trichostatin A (TSA), a classic HDAC inhibitor. We used treatment with sodium butyrate, a class I HDAC inhibitor, as a control. After 7 d of TSA treatment, the concentrations of markers of degeneration, such as cleaved caspase-3 and the cell cycle markers proliferating cell nuclear antigen (PCNA) and cyclin D1, were substantially lower than those normally found in Atm−/− cerebellum (Fig. 3a,b); by contrast, the concentrations of neuronal survival proteins were higher (Fig. 3c,d). Treatment with sodium butyrate had little effect on the levels of these markers (Supplementary Fig. 4a,b).

**Hypophosphorylation of HDAC4 induces its nuclear accumulation**

To determine whether HDAC4 has a role in the neuronal DNA damage response, we exposed cultures of wild-type and Atm−/− neurons to low doses of etoposide, a topoisomerase inhibitor known to induce DNA double-strand breaks. This exposure enhanced the activation of caspase-3, but this effect was tenfold greater in Atm−/− neurons than in wild-type neurons (Fig. 4a,b). When we used shRNA against HDAC4 in addition to and at the same time as the etoposide exposure, however, the cell death induced by etoposide increased in the wild-type neurons, but the shRNA had little effect on cell death in the Atm−/− neurons (Fig. 4a,b). Thus, loss of cytoplasmic HDAC4...
Figure 3 Inhibition of HDAC4 and blocking the nuclear accumulation of HDAC4 partially reverses the ataxia telangiectasia phenotype. (a) TSA injection reverses neuronal degeneration markers in the Atm−/− cerebellum. Fluorescent images of Atm−/− brain sections immunostained for cleaved caspase-3, as well as for PCNA and cyclin D1. The white arrows indicate labeled Purkinje cells. (b) Quantification of the degeneration markers for the experiment shown in a. Each bar represents the average of three independent experiments. Error bars, s.e.m. (c) Immunoblot assays of neuronal and cell-cycle proteins in cerebellar lysates prepared from DMSO- or TSA-injected wild-type and Atm−/− mice. (d) Quantification of the western blot bands shown in c. Error bars, s.d. (e) Effects of TSA on the motor function of Atm−/− and wild-type mice. Motor performance was measured as the average latency before falling from a rotarod. Each treatment group consisted of 4–6 mice. *P < 0.05 by analysis of variance (ANOVA). (f,g) Effects of TSA on the spontaneous locomotor activities (f) and the exploratory activities (g) in Atm−/− mice, as observed by open-field test. Data are means ± s.e.m.

makes neurons more sensitive to DNA damage. However, HDAC4 itself does not respond to DNA damage. We exposed wild-type and Atm−/− mice to 5 Gy of whole-body irradiation, which activated ATM in wild-type mice but not in Atm−/− mutant mice (Fig. 4c). The cytoplasmic location of HDAC4 nonetheless was unchanged after irradiation in mice of both genotypes (Fig. 4c).

We prepared cytoplasmic and nuclear fractions from wild-type and Atm−/− mouse cerebella and probed them with an antibody against phosphorylated Ser632 (phospho-Ser632) of HDAC4 or total HDAC4. In the wild-type cerebellum, most of the HDAC4 was cytoplasmic and phosphorylated (Fig. 4d,e). In the Atm−/− cerebellum, however, a large portion of the HDAC4 was nuclear and was almost entirely unphosphorylated at that location. The total HDAC4 was the same in wild-type and Atm−/− mice (Fig. 4d,e). Homogenates of human ataxia telangiectasia cerebellar samples behaved similarly to the Atm−/− mouse samples (Fig. 4f), and the use of a second HDAC4 phosphospecific antibody (to phospho-Ser246) confirmed the phospho-Ser632 results (Fig. 4f). The lower amount of HDAC4 phosphorylation in Atm−/− mice compared to wild-type mice led to a lower association of HDAC4 with the 14-3-3 protein, as assayed by communoprecipitation from human ataxia telangiectasia cerebellum (Fig. 4g,h) and Atm−/− mouse brain (Supplementary Fig. 5a,b).

We could find no canonical Ser/Thr-Gln ATM target site on HDAC4 itself or on the kinases responsible for its phosphorylation, CaMKII and CaMKIV7,17,29. Therefore, we asked whether the HDAC4 protein phosphatase, PP2A30, was involved in the hypophosphorylation of HDAC4 in Atm−/− mice. PP2A is a heterotrimERIC protein31 that must associate with its enzymatic target for full phosphatase activity. It consists of a structural A subunit (PP2A-A, also known as PR65), a tissue-specificity–regulatory B subunit (PP2A-B, also known as PR55) and a catalytic C subunit (PP2A-C, also known as Pp2ca). Immunoprecipitations of HDAC4 from human control lysates revealed little interaction of HDAC4 with either the PP2A-A or PP2A-C subunit. By contrast, in the patients with ataxia telangiectasia, we observed a robust association of HDAC4 with both the A and C subunits of PP2A (Fig. 4i). We also found this greater HDAC4-PP2A association in the Atm−/− mouse brain (Supplementary Fig. 5c). Thus, the lower phosphorylation of HDAC4 in ATM-deficiency is likely caused by a greater association of HDAC4 with its phosphatase, PP2A.

PP2A mediates ATM-dependent HDAC4 nuclear translocation Ser401 of PP2A-A is a highly probable site of ATM phosphorylation32. We therefore performed immunoprecipitation with antibodies to PP2A-A, PP2A-C or HDAC4 and probed the resulting western blots with an antibody to phosphorylated serine or threonine preceding a glutamine residue (phospho-Ser/Thr-Gln), the canonical ATM phosphorylation site. The resulting bands were phosphorylated on Ser401 by ATM in wild-type and Atm−/− mouse cerebella (Supplementary Fig. 5a).

BDNF is known to be essential for proper neuronal development33. We therefore asked whether the delayed development of Purkinje neurons in Atm−/− mice was associated with a reduction in BDNF expression. We measured CREB phosphorylation and BDNF expression by immunoblotting and found that CREB phosphorylation was decreased in Atm−/− mice compared to wild-type mice (Fig. 4j). In addition, the BDNF expression in cerebellum was decreased by 25% in Atm−/− mice compared to wild-type mice (Fig. 4j). Therefore, the reduced BDNF expression in Atm−/− mice likely contributes to the ataxia telangiectasia phenotype, as BDNF has been shown to be required for Purkinje cell survival34.

PP2A mediates ATM-dependent HDAC4 nuclear translocation Ser401 of PP2A-A is a highly probable site of ATM phosphorylation32. We therefore performed immunoprecipitation with antibodies to PP2A-A, PP2A-C or HDAC4 and probed the resulting western blots with an antibody to phosphorylated serine or threonine preceding a glutamine residue (phospho-Ser/Thr-Gln), the canonical ATM and ataxia telangiectasia and Rad3 related (ATR) target binding site.
We found a strong phospho-Ser/Thr-Gln signal on the PP2A-A band from wild-type but not Atm−/− mice on the western blot (Fig. 5a). We found no phospho-Ser/Thr-Gln signal for either wild-type or Atm−/− mice with the PP2A-C or HDAC4 immunoprecipitates (Fig. 5a). As further proof that HDAC4 itself is not an ATM substrate, we also found no phospho-Ser/Thr-Gln signal in N2a cells overexpressing Flag-tagged HDAC4 (Flag-HDAC4) (Supplementary Fig. 6a). We verified that Ser401 is the predominant ATM phosphorylation site on HDAC4 using in vitro kinase assays (Fig. 5b); a form of PP2A-A carrying the S401A mutation could not be phosphorylated by ATM. We overexpressed Flag-HDAC4 with GFP-tagged isoforms of PP2A-A and analyzed the Flag-HDAC4 immunoprecipitates for the presence of PP2A-A. We found a strong HDAC4-PP2A association in the non-phosphorylatable (S401A) PP2A-A isoform (S401A) (Fig. 5c) but not in wild-type or in the phosphomimetic isoform (S401D) (Fig. 5c). We confirmed this result by probing PP2A-A immunoprecipitates for Flag-HDAC4 (Fig. 5c).

ATM-dependent phosphorylation of PP2A-A also alters the localization of the PP2A holoenzyme itself. Endogenous PP2A-A was predominantly cytoplasmic in wild-type neurons but was predominantly nuclear in Atm−/− neurons (Fig. 5d). In wild-type primary neurons, when we coexpressed mutant or wild-type GFP–PP2A-A with mCherry-tagged PP2A-C (mCherry–PP2A-C), both subunits were located primarily in the cytoplasm (Fig. 5e). In Atm−/− neurons, however, unmodified PP2A-A and PP2A-C translocated to the nucleus, whereas the S401D PP2A-A mutant was located predominantly in the cytoplasm, even in ATM-deficient neurons. The S401A PP2A-A mutant was nuclear in both wild-type and Atm−/− neurons (Fig. 5e). In contrast, the locations of the HDAC4 kinases CaMKII and CaMKIV were unaffected by ATM deficiency (data not shown).

We inhibited ATM activity with 2 mM caffeine or 10 mM KU-55933. Both inhibitors caused HDAC4 to move from cytoplasm to nucleus within 3 h of administration (Fig. 5f and Supplementary Fig. 6b). This translocation of HDAC4 could be blocked by simultaneously knocking down PP2A (Fig. 5g and Supplementary Fig. 6c) or by pretreating the cells with the PP2A inhibitor endothall (Fig. 5h and Supplementary Fig. 6d). The genotype-dependent nuclear translocation of endogenous HDAC4 could also be blocked by infecting Atm−/− mice with Pr65 (also known as Ppp2r1a) or Pp2ca shRNAs (Fig. 5h).

Cytoplasmic HDAC4 improves the phenotype of Atm−/−

To verify that cytoplasmic HDAC4 prevents cell cycle reentry and other degenerative changes in Atm−/− mice, we co-injected lentiviral particles encoding human wild-type HDAC4, a nuclear localization HDAC4 mutant (4A, which has the R269A, R280A, K280A and R281A mutations), a nuclear export mutant (L1062A) or a non-phosphorylatable mutant (3SA, which has the S246A, S467A and R281A mutations), into wild-type and Atm−/− mouse cerebella. Heat shock protein 90 (Hsp90) and HDAC1 were used as cytoplasmic and nuclear markers, respectively. (a) Quantification of the bands shown in d. *P < 0.05 by Student’s t test. (f) Immunoblot assays of HDAC4 and phospho-HDAC4 in protein extracts prepared from frozen cerebellar samples from four human controls without ataxia telangiectasia and four individuals with ataxia telangiectasia. (g,h) Coimmunoprecipitations showing the interaction between HDAC4 and 14-3-3 protein in lysates of cerebellar tissue from human control and ataxia telangiectasia brains. WB, western blot. (i) Coimmunoprecipitations showing the association of HDAC4 with the PP2A subunits in lysates of cerebellar tissue from human control and ataxia telangiectasia brain. IB, immunoblot.

**Figure 4** HDAC4 cytoplasmic localization requires phosphorylation of HDAC4 and is independent of DNA damage. (a) The effect of Hdac4 shRNA on caspase-3 activation in Atm−/− neurons. Activation of caspase-3 (red) was used as an index of impending neurodegeneration; microtubule-associated protein 2 (Map2) (green) was used as a neuronal marker. Eto, etoposide; NT, no treatment; shGapdh, Gapdh shRNA. (b) Cell death quantified by counting the number of activated caspase-3-immunostained cells and expressing these numbers as a percentage of the total Map2-stained neurons. Data are mean ± s.e.m. (c) Mice treated with or without 5 Gy whole-body irradiation (IR). Cryostat sections of Atm+/+ and Atm−/− cerebella immunostained for HDAC4 (green) and H2A histone family member X (γ-H2AX) or phospho-Ser15 of p53 (both in red). At least three pairs of age-matched mice were used for each experiment. White boxes indicate areas magnified in the two single-channel insets. (d) Immunoblot assays of HDAC4 and phospho-S632 of HDAC4 in nuclear (Nuc) or cytoplasmic (Cyt) extracts prepared from Atm+/+ and Atm−/− mouse cerebella. Heat shock protein 90 (Hsp90) and HDAC1 were used as cytoplasmic and nuclear markers, respectively. (e) A quantification of the bands shown in d. *P < 0.05 by Student’s t test. (f) Immunoblot assays of HDAC4 and phospho-HDAC4 in protein extracts prepared from frozen cerebellar samples from four human controls without ataxia telangiectasia and four individuals with ataxia telangiectasia. (g,h) Coimmunoprecipitations showing the interaction between HDAC4 and 14-3-3 protein in lysates of cerebellar tissue from human control and ataxia telangiectasia brains. WB, western blot. (i) Coimmunoprecipitations showing the association of HDAC4 with the PP2A subunits in lysates of cerebellar tissue from human control and ataxia telangiectasia brain. IB, immunoblot.
S632A mutations) together with Hdac4 shRNA (which is specific for the mouse Hdac4 message) in the cerebella of wild-type and Atm−/− mice. One week after injection of the two lentiviruses, we monitored gene transfer by immunohistochemistry using a human-specific antibody to HDAC4 (Supplementary Fig. 7c,d). In Atm−/− cerebellar neurons infected with the two lentiviruses, cytoplasmic HDAC4 (4A) prevented cell cycle reentry and caspase-3 activation (Fig. 6a,b). By contrast, nuclear HDAC4 (L1062A and 3SA) resulted in cell cycle reentry and caspase-3 activation in both Atm−/− and wild-type lentivirus-infected mice (Fig. 6a,b). Reduced histone acetylation accompanied the higher concentration of nuclear HDAC4 in Atm−/− compared to wild-type mice, as confirmed by immunocytochemistry of Ach3 (Supplementary Fig. 7e). Overexpression of unmodified human HDAC4 had little effect on either wild-type or Atm−/− neurons.

In primary neurons, overexpression of HDAC4 showed that neuronal cell cycle activity was tightly correlated with the presence of HDAC4 nuclear localization (Supplementary Fig. 6e,f). Cytoplasmic HDAC4 (4A) was protective and prevented Atm−/− neuronal cell cycle reentry. This offered us the opportunity to separately test the functions of nuclear and cytoplasmic HDAC4 and relate each to the presence or absence of behavioral abnormalities in Atm−/− mice. We co-injected lentiviruses encoding Hdac4 shRNA (against the Hdac4 mouse message) together with viruses encoding exclusively cytoplasmic HDAC4 (H4) into mouse cerebellum at postnatal day (P) 18. At this age, the area infected by the virus should be larger than that infected after a comparable injection into an adult mouse. At P39, when the transgene expression is maximal, we subjected the mice to the same training and trials described above. Atm−/− mice injected with cytoplasmic 4A were able to stay on the rotarod significantly longer (P < 0.05) than Atm−/− sham-injected mice (Fig. 6c), and the results of the spontaneous locomotor activity observed in the open-field test were consistent with this difference (Fig. 6d). The exploratory activity (Fig. 6e) of 4A-injected Atm−/− mice was significantly greater (P < 0.05) than the activity of mice injected with nuclear L1062A (Fig. 6d,e). L1062A-injected wild-type mice performed substantially worse than sham-injected wild-type mice (Fig. 6c,e). To test the involvement of PP2A in these results, we injected a lentivirus carrying wild-type PP2A- A or the PP2A-A S401A or S401D isofrom into both wild-type and Atm−−/− cerebella. The PP2A-A S401D mutant blocked HDAC4 nuclear translocation and inhibited cell-cycle and caspase-3 activation. However, wild-type PP2A-A and the PP2A-A S401A mutant had little effect on these activities (Supplementary Fig. 8a,b). The effect of the PP2A-A S401D mutant on the behavioral abnormalities in the Atm−/− mice, however, was modest (Fig. 6c,e). This might be a result of the broad range of substrates whose phosphorylation would...
be changed by PP2A activity as well as of the indirect nature of the effect of PP2A on the ataxia telangiectasia phenotype.

**DISCUSSION**

Our data provide insights into the functions of both ATM and HDAC4 in the health and survival of central nervous system neurons. HDAC4 is normally maintained in the neuronal cytoplasm through its interaction with the 14-3-3 protein. This interaction requires CaMKII- or CaMKIV-mediated phosphorylation of the 14-3-3 protein. This interaction stabilizes HDAC4 in the cytoplasm and prevents its nuclear translocation.

This study uncovers several major features of the epigenetic landscape of the *Atm*−/− neuronal genome. First, our ChIP analysis showed genome-wide changes in the location of AcH3 and AcH4 in *Atm*−/− mice compared to wild-type mice, which is in contrast to the results seen with unmodified histone H3 or H4. Second, by performing ChIP-seq with antibodies to HDAC4, we provide data in support of the idea that HDAC4 is itself associated with specific genomic locations and that these locations differ depending on the *Atm* genotype of the neuron. Most models of HDAC4 action are based on its binding to and inhibiting proteins, such as MEF2A, CREB and CREB but show high amounts of association with HDAC4 itself. Third, in the promoter region of each genetic locus we examined, the changes in gene transcription predicted by a more open conformation in the presence of acetylated histone proteins were borne out by the results from both the RT-PCR and western blot analyses. This supports the hypothesis that changes in the pattern of histone acetylation and transcription factor inhibition that occur as a result of the presence of nuclear HDAC4.

This concept suggests several new targets for pharmacological intervention in the treatment of ataxia telangiectasia. For example, inhibiting HDAC4 activity and blocking its nuclear accumulation both reverse the altered histone acetylation pattern and rescue various aspects of the neurodegenerative phenotype of mouse ATM-deficient neurons. Notably, the phenotypic rescue of both the neurodegenerative changes of *Atm*−/− cerebellar neurons in vivo as well as the improvements in motor behavior when HDAC4 nuclear activity is inhibited or when cytoplasmic HDAC4 is overexpressed. The lentivirus injection studies illustrate this concept on a cell-by-cell basis; the TSA injections make the same point on the level of the whole brain. TSA is admittedly a very broad spectrum HDAC inhibitor, however, the reversal of cell cycle protein expression as a result of TSA treatment was noteworthy. In addition, the failure of sodium butyrate to elicit a comparable effect suggests no
involvement of class I HDAC activities. This picture of a two-edged sword—neuroprotection of HDAC4 in the cytoplasm but neurotoxicity of HDAC4 in the nucleus—suggests that it may be more strategic to focus on HDAC4 location rather than HDAC4 activity as the most useful ataxia telangiectasia therapeutic target.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

J.L and K.H. designed the experiments, analyzed data and wrote the manuscript. C.L.R. and R.P.H. developed, carried out and analyzed data for the ChiP-seq analyses. J.L and J.C. carried out the immunocytchemistry experiments. J.C. performed all of the qPCR experiments. J.L. and A.K. carried out the mouse cerebellar lentiviral injections. M.S.S., J.L. and A.K. carried out behavioral tests.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

ChIP-seq analysis. ChIP-seq libraries were constructed from chromatin immunoprecipitated with antibodies to HDAC4 using the SOLiD DNA fragment library kit (Invitrogen) following the standard ChIP-seq protocol. Libraries containing Atm+/+ or Atm−/− samples were bar coded, applied to beads with emulsion PCR, enriched and sequenced using the SOLiD System v3.5 at the Waksman Genomics Laboratory of Rutgers University. A sample prepared without immunoprecipitation from the Atm+/+ samples was used as the input control. The resulting sequence files and quality scores are available from the NIH Sequence Read Archive under the accession number SRA023500. Sequenced reads and quality strings were aligned to the mouse genome (mm9) using Bowtie 0.12.5 (ref. 35) to identify the single, best-quality match location. Results were converted to BAM format using SAMtools96. Peaks were selected, comparing Atm+/+ or Atm−/− against the input control, using Find Peaks 4.0 (ref. 37). Peak tracks were created and visualized using the UCSC Genome Browser, where subsets of peaks were chosen for qPCR validation.

Administration of TSA. TSA and sodium butyrate were from Sigma. The inhibitors were administered by a single intraperitoneal (i.p.) injection to 2-month-old wild-type and Atm−/− mice (a random mix of both sexes) twice a week at doses of 10 mg/kg (TSA) or 50 mg/kg (sodium butyrate). Seven days after the injection, the mice were killed and samples were collected. For the behavioral experiments, TSA and sodium butyrate were administered i.p. for 3 weeks, beginning at 4 weeks of age.

Lentivirus production and cerebellar injections. The hairpin sequences for MISSION shRNA Lentiviral Transduction Particles used in cerebellum infection, including shRNA to Hdac4-3 and Hdac4-4 (Supplementary Table 3), were from Sigma. Human HDAC4 and PR65 lentiviral constructs were from GeneCopoeia. Site-directed mutation of lentiviral PR65 to S401A and S401D and lentiviral HDAC4 to 4A (R269A, R280A, K280A and R281A), L1062A and 3SA (S246A, S467A and S632A) were performed using a QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). The lentiviral particles were prepared with Lenti-Pac Expression Packaging Kits (GeneCopoeia) in HEK293T cells and were purified using Ultra-Pure Lentivirus purification Kits (Applied Biological Material Inc. Vancouver). Stereotaxic intracerebellar infusions were delivered to wild-type and Atm−/− mice (8 weeks of age)38,39 under isoflurane anesthesia. Mice were positioned in a Kopf stereotaxic apparatus, burr holes were drilled into the skulls, and a 5-µl Hamilton syringe fitted with a 33-gauge needle was lowered into the cerebellum for vector delivery. The coordinates of the burr hole and ventral location for infusion were −7.2 mm from bregma, 1.0 mm lateral from the midline and 3 mm ventral. For each cerebellum, slow infusion over 15 min of 3–5 µl lentiviral particles (1–5 × 107 IU/ml) was performed, with the needle being withdrawn 5 min after completing the infusion. For knocking down endogenous HDAC4 and overexpressing human HDAC4, we delivered shRNA to mouse HDAC4 and human HDAC4 lentiviruses in a 1:1 ratio. After surgery, mice were injected subcutaneously with 0.3 ml pre-warmed saline to avoid dehydration and were allowed 7 d recovery before tissue collection. For the behavioral experiments, lentiviruses were injected into mice cerebella at P18. Three weeks after injection, mice were trained for behavioral tests.

Rotarod test. For the rotarod test, age-matched mice were given two training trials (with an intertrial interval of 2 h) with the rotarod adjusted to accelerate from 6 rpm to 40 rpm over a 5 min period each day. Latency to fall off was measured. After 1 week, mice were tested using the rotarod adjusted to maintain a constant speed for the entire 5 min test period. Each mouse was tested with the rotarod set at 10 rpm and 16 rpm.

Open-field test. The open-field test was conducted as previously described40. Briefly, a floor open field (63 cm × 57 cm × 28 cm) was used to assess general motor activity. The floor of the arena was divided into a grid of 30 squares, with each square measuring −11 cm on a side. After placing a mouse at the center of the arena, locomotor activity, measured by the number of crossed grids, and exploratory activity, measured by the number of rearings on the hind feet, were recorded during a 5 min period. The total distance traveled was measured from the recorded videotapes using video-tracking software (Spontaneous Motor Activity Recording and Tracking (SMART), San Diego instruments, San Diego, CA).

Statistical analyses. All data are presented as the means ± s.e.m. of a minimum of three replicates. For all of the analyses reported here, we evaluated statistical differences either by Student’s t test or, for the behavioral tests, by ANOVA. For all analyses, we considered P < 0.05 to be statistically significant.

Additional methods. Detailed methodology is described in the Supplementary Methods.