Supplementary materials and methods

Northern blot analysis

Northern or dot blot analysis was carried out using Human Brain MTN Blot II or Tissue Expression Array (Clonetech 7755-1 and 7776-1, respectively). RNA blots were hybridized with ^{32}P-labeled human *Boat*, *ataxin-1*, *Smrt*, or *β-Actin* cDNA respectively.

Yeast two hybrid assays

Yeast two hybrid assays were performed by transforming Y190 yeast cells with both pGBT9- and pGAD424-based constructs according to manufacturer's instructions (Clontech). Transformants were selected on SD -Leu/-Trp plates for 2 days. For X-gal dot blot assays, cells grown in SD -Leu/-Trp liquid media overnight were transferred into fresh YEPD media and grown until mid-log phase (OD_{600}=0.6-0.8) at 30˚C (~4 hrs). 0.2 OD_{600} units of cells were applied to each well of a dot-blot manifold (Bio-Rad) that sandwiches two Whattman filters. Yeast cells retained on the top layer of filter after vacuum application were lysed by freeze and thaw before adding X-gal reaction solution.

Cell Culture and transfection

HEK-293 or MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) at 37˚C in 5% CO_2. The media were supplemented with 10% calf bovine serum, 50 U/ml penicillin G, and 50μg/ml streptomycin sulfate. Transfection of cells was carried out at least 12 hours after plating. For immunofluorescent staining, co-immunoprecipitation, and Luciferase reporter assays, cells were plated in Lab-Tek Chamber Slides (4.2 cm^2/ well) (Nalge Nunc), in tissue-culture dishes (10cm) (Becton Dickinson), and in 24-well cell culture clusters (Costar) respectively. For transient transfection, plasmids were mixed with appropriate amounts of Effecten transfection reagent (Qiagen) or Lipofectamine 2000 (Invitrogen) and Opti-MEM1 medium (Gibco BRL) according to the manufacturers' instructions. For using Effecten, four hours after transfection, the Opti-MEM1 medium was replaced with DMEM and incubated for another 15-20 hours before further analysis.

Immunoprecipitation

Transfected cells from a 100mm plate were washed twice with ice-cold PBS, and lysed in 1ml of lysis buffer (50mM Tris-Hcl pH8.0, 150mM NaCl, 10% glycerol, 0.5% NP-40, Complete protease inhibitors (Roche)) for 15min at 4˚C with rocking. Cell lysates were transferred into new tubes

and were sheared by passing through a 19-gauge syringe ten times, followed by a 22-gauge five times. The lyates were cleared by centrifugation at 15,000 rpm for 15min at 4˚C. After pre-blocking with 60μl of Protein A/G agarose beads (50% slurry, Santa Cruz), 1 ml of lysate was incubated with 120μl (50% slurry) of Anti-FLAG-M2 agarose affinity gel (Sigma A-2220) for 1.5 hr to overnight at 4˚C, followed by four times wash with ice-cold washing buffer (50mM Tris-Hcl pH8.0, 150mM NaCl, 0.5% NP-40, Complete protease inhibitors). The bound proteins were eluted in 60μl of 2xSDS loading buffer and subjected to western blot analysis.

For immunoprecipitation experiments with Hela nuclear extracts (a gift from Dr. Joseph Fondell), 2mg of total proteins were used. The nuclear extracts, after pre-blocking with 120μl (25% slurry) protein A/G agarose beads, were incubated with 240μl (25% slurry) antibody (SMRT, ataxin-1, or IgG)-bound protein A/G agarose beads at 4˚C for overnight. The remaining Co-IP experimental procedures are similar to the methods described above.

GST pull-down assay

25μl of GST or GST-ataxin-1(20-197) or GST–Boat (20-197) fusion protein bound agarose beads were incubated with in vitro translated ^{35}S-methionine labeled SMRT fragments (TNT-T7 Coupled Reticulocyte Lysate System, L4610, Promega) in 1ml of ice-cold binding buffer (50mM Tris-Hcl pH8.0, 150mM NaCl, 10% glycerol, 0.5% NP-40, Complete protease inhibitors (Roche)). The binding and washing conditions were similar to those used in immunoprecipitation experiments. Binding proteins were eluted in 20μl of 2x SDS sample buffer, and subjected to SDS-PAGE.

Preparing frozen sections of mouse brains and Drosophila eyes

For brain cryosectioning, three-week-old or eight-week-old *wild-type FVB/N* mice or *SCA1 B05* mice were anesthetized and transcardially perfused with ice-cold PBS followed by ice-cold PBS-buffered 4% paraformaldehyde (PH7.4) fixation. After perfusion, brains were dissected, post-fixed for 3 hours at 4°C, and washed in PBS at 4˚C overnight. Fixed brains were then cryoprotected with PBS-buffered 30% sucrose and embedded in O.C.T. medium (Tissue-Tek). Serial 12-μm-thick sections of the brain were cut using a Leica freezing microtome. For immunostaining experiments, prior to adding antibodies, all brain sections were treated with 10mM citric acid monohydrate (PH 6.0) at 65^{0}C for 1.5 hours to expose the antigens. Anatomic identification of regions in mouse brains is based on The Mouse Brain (Academic Press).

To prepare cryosections of Drosophila eye/head, adult flies were first anesthetized with CO_2. The proboscis was first removed before tearing the head from the body by forceps. The dissected heads were then treated with ice-cold PBS-buffered 4% paraformaldehyde (PH7.4) for 2 hours. After rinsing with several rounds of PBS, the fly heads were cryoprotected with PBS-buffered 30% sucrose for overnight. The heads were then embedded in O.C.T. medium for sectioning. For our immunostaining experiments, we used 14-μm-thick cross-sections of the fly head.

Immunofluorescence and microscopic analysis

Similar immunofluorescent staining procedures were applied to mouse brain, human cultured cells, and Drosophila tissues: samples were first incubated with the blocking solution PBTS for 30min, followed by incubation with selective primary antibody(ies) (diluted in PBTS) for 2 hours at room temperature or overnight at 4˚C. The samples were washed 3x with PBTS (5min/wash) and then incubated with secondary antibody(ies) (diluted in PBTS) for 1 hr. After 3x wash with PBTS (5min/wash), the samples were mounted with Vectashield medium with 4', 6-diamidino-2-phenylindole (DAPI) (Vecter). The images were captured using Nikon E1000 microscope, DXM 1200 digital camera, and ACT-1 software.

Antibodies

Immunostaining for mammalian cultured cells: anti-SMRT (Affinity Bioreagent PA1-843, 1:300); anti-HDAC3 (BD Biosciences 612635, 1:200); anti-HDAC1 (Upstate 06-720, 1:200); anti-ataxin-1(477-575) (1:500); anti-Boat (284-412) (1:500). *For western blot analysis:* anti-FLAG (Sigma F-7425, 1:2000); anti-ataxin-1(477-575) (1:1000), and anti-Boat (284-412) (1:1000). *For Drosophila salivary glands and eyes:* anti-ataxin-1(477-575) (1:500); anti-ataxin-1(4-197) (1:500); anti-Boat (284-412) (1:500); anti-SMRTER (1:200) (Tsai et al., 2004); anti-USP (AB11, 1:100) (Christianson et al., 1992); anti-HA(Upstate 06-831, 1:400); anti-FLAG (Sigma F-7425, 1:400); and anti-22C10 (Developmental Studies Hybridoma Bank, 1:150) (Zipursky et al., 1984). *For mouse brain tissues:* anti-ataxin-1(477-575) (1:400); anti-Boat (284-412) (1:400); anti-SMRT(972-1151) (1:400); anti-NeuN (Chemicon, MAB377, 1:500); anti-Calbindin D-28K (Chemicon, AB1778, 1:500). *The secondary antibodies:* Texas-Red conjugated or FITC conjugated anti-mouse, anti-rabbit, or anti-guinea pig antibodies (1:400) were purchased from Jackson ImmunoResearch.

In situ hybridization for mouse brains

Frozen mouse brains were post-fixed with PBS-buffered 3.7% formaldehyde for 20 min. The fixed tissues were washed twice with PBS, followed by proteinase K (10 μg/ml in PBS) treatment for 10 min. After washing once with PBS, the tissues were post-fixed with PBS-buffered 3.7% formaldehyde for 15 min again. The tissues were rinsed with distilled water twice before treatment with 0.1 M triethanolamine-HCl pH8.0 with 0.25% acetic anhydride solution. The tissues were washed with PBS for 5 min and then covered with 500μl of prehybridization solution (50% formamide, 5X SSC, 5mM EDTA, 1X Denhardt's, 0.1 mg/ml heparin, 0.1% Tween 20, 0.3 mg/ml yeast RNA). The prehybridization step was carried out at 65°C for 2 hours, followed by the hybridization step (500μl of hybridization solution plus 5μl of Dig-labeled sense or anti-sense RNA probes) at 65°C for 12-16 hours.

After the hybridization step, the slides were rinsed with 2X SSC at 67°C twice and room temperature three more times (5 min/each wash). The tissues were treated with 2 μg/ml RNase A (in 2 X SSC) at 37°C for 30 min, followed by two washing steps (0.2 X SSC at 65°C for 30 min/wash). The tissues were rinsed with PBT twice for 10-20 min each time and blocked with a blocking solution (PBT+10% goat serum) for 30 min to an hour. The tissues were incubated with Dig-antibody (Roche, 1333089) (diluted 1:2000 in PBT+10 % goat serum) for 1-2 hours at room temperature.

After the antibody treatment, the tissues were first washed with PBT three times (15 min/wash), followed by a wash with alkaline-phosphatase buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20, and 5mM levamisole) for 5 min. Color reaction was carried out by incubating the tissues with 1ml of alkaline-phophatase buffer with 20 μl of NBT /BCIP stock solution (Roche, 1681451). After the color reaction and step-wise washing steps with PBS buffered 25%, 50%, 75%, 100% ethanol, the tissues were mounted in Permount medium.

The digoxigenin-labled RNA probes were generated using the Dig-RNA labeling kit (Roche, 1177025). The template (Bluescript as the backbone) contains a DNA fragment corresponding to the 132-448 region of Boat. The anti-sense probes were generated by T3 RNA polymerase; the sense probes were generated by T7 RNA polymerase.

This is a modified version of a protocol described in (Gray et al., 2004).

References

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