miR-19, miR-101, and miR-130 co-regulate ATXN1 levels to potentially modulate SCA1 pathogenesis.

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Supplementary Methods

Immunofluorescence and confocal microscopy

Animals were anesthetized with avertin and transcardially perfused with 1X phosphate buffered saline (PBS) for 2 minutes followed by 10% formalin (Sigma) for 10 minutes, and immunostaining was performed as described previously [1]. Brains were removed and fixed in 10% formalin overnight at 4 °C, and then transferred to 30% sucrose at 4 °C. Once brains had equilibrated in sucrose, they were frozen in O.C.T. and stored at -80 °C until sectioning. For all experiments, 45 micrometer sagittal brain sections from mutant and wild type animals were prepared side-by-side and placed directly into 1X PBS. Sections were blocked for 1 hour at 4 °C in PBS/0.3% Triton-X 100/2% normal goat serum. They were then incubated with primary antibodies for 2-3 days at 4 °C followed by secondary antibody incubation for 2-3 days, also at 4 °C. Sections were mounted onto slides using Prolong anti-fade Gold Mounting Media (Molecular Probes), according to the manufacturer's instructions. Image analysis was carried out using a Zeiss LSM 510 confocal microscope. Mouse anti-calbindin (Sigma) was used at 1:1000; and Goat anti-mouse Alexa488 was used at 1:500. Incubation with Toto3 (Molecular Probes) (1:10, 000) was carried out for 20 minutes prior to mounting. Immunofluorescence experiments were carried out using 2 *SCA1*[82Q] Tg/Tg, 2 *SCA1*[82Q] Tg/+ and 2 FVB age and sex matched wild type mice at 11 weeks in 2

independent experiments. Two-three sections per animal were imaged, and representative images are shown. For each experiment, equivalent sections were incubated with secondary antibody alone (no primary control) and did not yield a signal above background.

Accelerating rotating rod test

This experiment was carried out as described previously [2]. Age matched *SCA1*[82Q] Tg/+ (n=11) and *SCA1*[82Q] Tg/Tg (n=7) animals were tested to measure the ability of mice to improve motor skill performance with training.

Transfection with miRNA duplexes and inhibitors

MCF7, NIH3T3, HEK293T or HeLa cells were plated the day before transfection at 2 x 10^5 cells per well in 6-well plates. The following day, 120 pmoles of siRNA or miRNA duplexes or 200 pmoles of 2′-O-methyl inhibitors were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and cells were cultured in DMEM medium with 10% fetal bovine serum. After 48 hrs or 72 hrs incubation of the cells transfected with 2′-O-methyl inhibitors or duplexes, cells were harvested and lysed with lysis buffer (0.5% NP-40, 20mM Tris-HCl [pH 8.0], 150~180mM NaCl, 1mM EDTA, and complete protease inhibitor cocktail (Roche)) for 15min on ice. After centrifugation, 10~20 μg of cell lysate was subject to 4~12% NuPAGE (Invitrogen) and western blot analysis for ATXN1 and GAPDH. The ATXN1 primary antibody 11750 was used as described previously [3]. All of the siRNAs, miRNA duplexes and miRNA inhibitors were purchased from Dharmacon Co.. The target sequences of sihATXN1 and simATXN1 are 5′-GGGAAUAGGUUUACACAAA-3′ and 5′-GAUCUAACGUGGGCAAGUA-3′, respectively.

Subcloning of the hATXN1 3′UTR

A full length or partial 3′UTR of human *ATXN1* mRNA was amplified by PCR using HeLa cDNA. For the full length 3′UTR (1~7015) amplification, 5′-TCTAGAGGCAGCGTGGGGGAAAGGAAACG-3′ (forward primer) and 5′-GCTAGCTGTATCCTACAAATAGACACACCACG-3′ (reverse primer) were used. For a partial 5′ end of 3′UTR (1~1600) amplification, 5′-TCTAGAGGCAGCGTGGGGGAAAGGAAACG-3′ (forward primer) and 5′-GCTAGCAACCTTTCCCACAATGTTATTGG-3′ (reverse primer) were used. For a partial 3′ end of 3′UTR (4200~7015) amplification, 5′-TCTAGACCTGCATCTTGTGGATTACTCCTTAG-3′ (forward primer) and 5′-GCTAGCTGTATCCTACAAATAGACACACCACG-3′ (reverse primer) were used. Primers were designed to have XbaI and NheI restriction enzyme sites at 5′ and 3′ ends of PCR products, respectively. All of the PCR products were subcloned into pCR4 Blunt-TOPO vectors (Invitrogen) followed by sequencing. The 3′UTRs were then subcloned into pGL3-control (Promega) or FLAG-hATXN1[86Q] expression vector (pcDNA3-F86Q) using the XbaI site at 3′end of firefly luciferase or hATXN1[86Q] genes.

Mutagenesis of miRNA target sites

Mutagenesis of each putative miRNA target site was carried out using QuickChange XL Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. For mutagenesis of the first miR-19 target site, 5′-GATGAACTTTTAAAGAACCTTGCGATTTGCTGATATTGAGTTTATAACTTGTG-3′ (upper primer) and 5′-

CACAAGTTATAAACTCAATATCAGCAAATCGCAAGGTTCTTTAAAAGTTCATC-3′ (lower primer) were used. For mutagenesis of the second miR-19 target site, 5′-GTAATCATAAAATCCACTCTTTGCTGAGTTTGATCTTTACTGAAATATGTTGC-3′ (upper primer) and 5′-GCAACATATTTCAGTAAAGATCAAACTCAGCAAAGAGTGGATTTTATGATTAC-3′ (lower primer) were used. For mutagenesis of the third miR-19 target site, 5′-GGAAACTCCAAACAACTTGCTGACCTCTACTCCGGAGCTG-3′ (upper primer) and 5′-CAGCTCCGGAGTAGAGGTCAGCAAGTTGTTTGGAGTTTCC-3′ (lower primer) were used. For mutagenesis of the fourth miR-19 target site, 5′-CTAGGGAAGGAATGATGTTTTGCTGCTTATTGAAAAGAAAATTTTAAGTGC-3′ (upper primer) and 5′-GCACTTAAAATTTTCTTTTCAATAAGCAGCAAAACATCATTCCTTCCCTAG-3′ (lower primer) were used. For mutagenesis of both the first and the second miR-101 target sites, 5′-CCTTATCATTTGTATCCAGATTACTCTACTCTAGGCTAAAATAACACAG-3′ (upper primer) and 5′-CTGTGTTATTTTAGCCTAGAGTAGAGTAATCTGGATACAAATGATAAGG-3′ (lower primer) were used. For mutagenesis of the second miR-101 target site, 5′-CATTTGTATCCAGATTACTGTACGTAAGGCTAAAATAACACAG-3′ (upper primer) and 5′-CTGTGTTATTTTAGCCTTACGTACAGTAATCTGGATACAAATG-3′ (lower primer) were used. For mutagenesis of the third miR-101 target site, 5′-GTCTTTGAACTCTAGTACGTATTATAGTTCATGACTATGGACAAC-3′ (upper primer) and 5′-GTTGTCCATAGTCATGAACTATAATACGTACTAGAGTTCAAAGAC-3′ (lower primer) were used. For mutagenesis of the fourth miR-101 target site, 5′-

GGTCTCTCATTCAACAAGTACGTAATCTCACTTTAAACTCTTTGG-3′ (upper primer) and 5′-CCAAAGAGTTTAAAGTGAGATTACGTACTTGTTGAATGAGAGACC-3′ (lower primer) were used. For mutagenesis of the first miR-130 target site, 5′-GTTGCCTTCTGAAACCCTTTGCAGATCAATTTGCACCAGGTG-3′ (upper primer) and 5′-CACCTGGTGCAAATTGATCTGCAAAGGGTTTCAGAAGGCAAC-3′ (lower primer) were used. For mutagenesis of the second miR-130 target site, 5′-CCAGTGTAAAATATTGTACTTGCAGAAGCTTTTTTAAACAAATATTAAAAAATGGAAG-3′ (upper primer) and 5′-CTTCCATTTTTTAATATTTGTTTAAAAAAGCTTCTGCAAGTACAATATTTTACACTGG-3′ (lower primer) were used.

Dual luciferase assay

HeLa cells were plated the day before transfection at 5 x 10^4 cells per well in 24-well plates. The following day, 30 pmoles of miRNA duplexes, 50 ng of firefly luciferase expression vectors (pGL3), and 12.5 ng of renilla luciferase expression vectors (pRL-TK) were co-transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Thirty hrs later, luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocols. The experiments were carried out in triplicate.

RT-PCR

HeLa total RNA was prepared using Trizol (Invitrogen) and 5 μg of total RNA was subject to cDNA synthesis using Superscript II (Invitrogen) and Oligo-dT primers (Invitrogen) according

to the manufacturer's protocols. For detection of human *ATXN1* mRNA levels, 5′-GACCTCGGTGGAGCTTGGTTTAC-3′ (forward primer) and 5′-TGCTGCTCAGCCTTGTGTCC-3′ (reverse primer) were used. For detection of GAPDH mRNA levels, 5′-CGTATTGGGCGCCTGGTCACCAG-3′ (forward primer) and 5′-GTTGTCATGGATGACCTTGGCCAG-3′ (reverse primer) were used.

Northern blot analysis for microRNAs

Twenty-four week-old C57/B6 WT male mice were used to prepare total RNA from whole brain, cerebral cortex, or cerebellum. Fifty μg of RNA was loaded in each lane. Northern blot analysis was carried out as described previously [4]. The probe sequences are 5′-TCAGTTTTGCATGGATTTGCACA-3′ (anti-miR-19b), 5′-CTTCAGTTATCACAGTACTGTA-3′ (anti-miR-101a), 5′-ATGCCCTTTTAACATTGCACTG-3′ (anti-miR-130a), and 5′-ATGCCCTTTCATCATTGCACTG-3′ (anti-miR-130b), respectively.

***In situ* hybridization (ISH) for miRNAs**

Tissue preparation and automated ISH for miRNA were performed as previously described [5,6,7] and as described online at http://www.genepaint.org/RNA.htm with the modifications as described here. Briefly, brains of C57/B6 male adult mice (P56) were embedded in O.C.T. and fresh frozen in a custom made freezing chamber that allows precise alignment of the specimen. Serial sections at 25 μm thickness were cut with a cryostat. After paraformaldehyde fixation, acetylation and dehydration, the slides were assembled into flow-through hybridization chambers and placed into a Tecan (Mannedorf, Switzerland) Genesis 200 liquid-handling robot, which

executes a script that performs non-radioactive ISH using DIG labeled miRNA antisense LNA probes (Exiqon) in less than 24 hrs. The DIG labeled LNA probes were used at a concentration of 30nM. A "scrambled" sequence with no homology to known miRNAs was used as a negative control. Hybridized LNA probes were detected by catalyzed reporter deposition (CARD) using biotinylated tyramide followed by colorimetric detection of biotin with avidin coupled to alkaline phosphatase [7,9]. This results in a dark blue precipitate in cells containing the transcript of interest. The amount of precipitate is proportional to the number of detected transcripts [8].

Cell death assay

HEK293T cells were plated the day before transfection at 2~4 x 10^4 cells per well in 24-well plates. The following day, 2′-O-methyl inhibitors or FLAG-hATXN1[86Q] expressing vectors were transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Fifty pmoles of 2′-O-methyl inhibitors (Control or a mixture of inhibitors specific for each miRNA (12.5 pmole each)) and 0.25 μg of pcDNA3-F86Q-3UTR WT were transfected into the cells in each well for the experiment in Figure 3d. In case of the experiment in Figure 3g, the amount of plasmids used for each transfection is as follows; 0.5 μg of pcDNA3, 0.5 μg of pcDNA3-F86Q-3UTR WT, 0.5 μg of pcDNA3-F86Q Mut, or 0.25 μg each of pcDNA3-F86Q and pcDNA3. One well was treated with a mixture of Lipofactamine 2000 and Opti-MEM (Invitrogen) to get the number of 'untransfected cells' that is used for calculation of cell survival rates after cell counting. The cells were cultured in 0.5ml of DMEM medium with 10% fetal bovine serum. After 48h or 72h, cells were detached by pipetting and subjected to direct cell counting using a hemacytometer. The experiments were carried out in triplicate.

Statistical analysis

For statistical analysis, all experiments were carried out more than 3 times independently. The western blots were quantified using ImageJ software package. Statistical significance between control values and experimental values was determined using Student's *t* test. All data are reported as means ± standard deviations (s.d.) and statistical significance is presented with asterisks.

References

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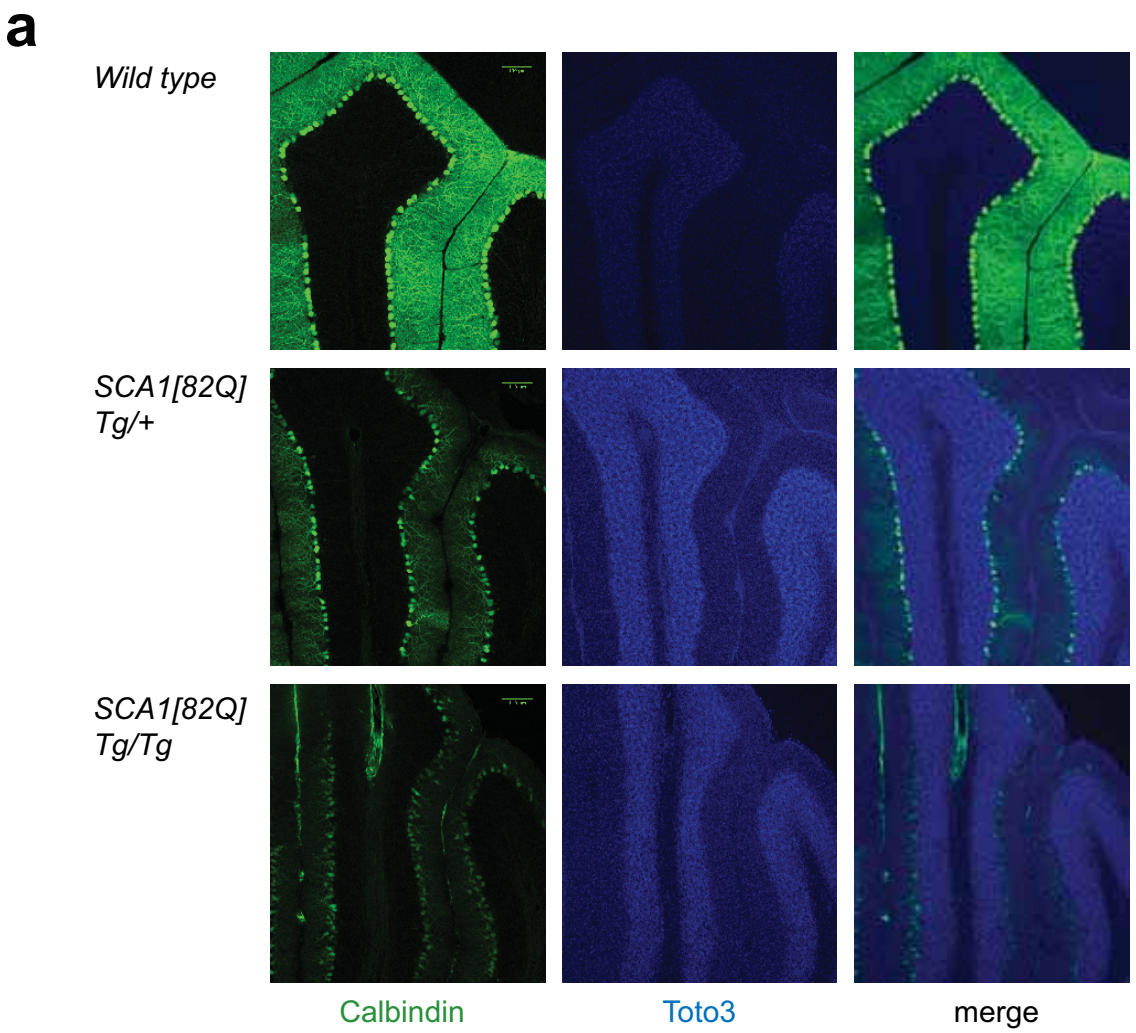


Figure S1. Increasing dosage of the *SCA1*[82Q] transgene is associated with more severe SCA1 neuropathology. (a) Immunofluorescence for calbindin, a marker of Purkinje cell dendritic arborization, and Toto3, a nuclear dye, on sagittal cerebellar sections from wild type, *SCA1*[82Q] Tg/+ and *SCA1*[82Q] Tg/Tg reveals a decrease in calbindin staining intensity in *SCA1*[82Q] mice relative to wild type at the mid-symptomatic disease stage (11-12 weeks). This decrease is more marked in Tg/Tg cerebellum compared to Tg/+. The scalebar represents 100 μm. (b) Performance of *SCA1*[82Q] Tg/+ and *SCA1*[82Q] Tg/Tg mice on an accelerating rotating rod apparatus. Six week old animals were tested for four trials per day for 4 consecutive days on an accelerating RotaRod. *SCA1*[82Q] Tg/Tg mice show impaired performance improvement as compared with age-matched *SCA1*[82Q] Tg/+ mice. * P<0.03 and ** P<0.01.

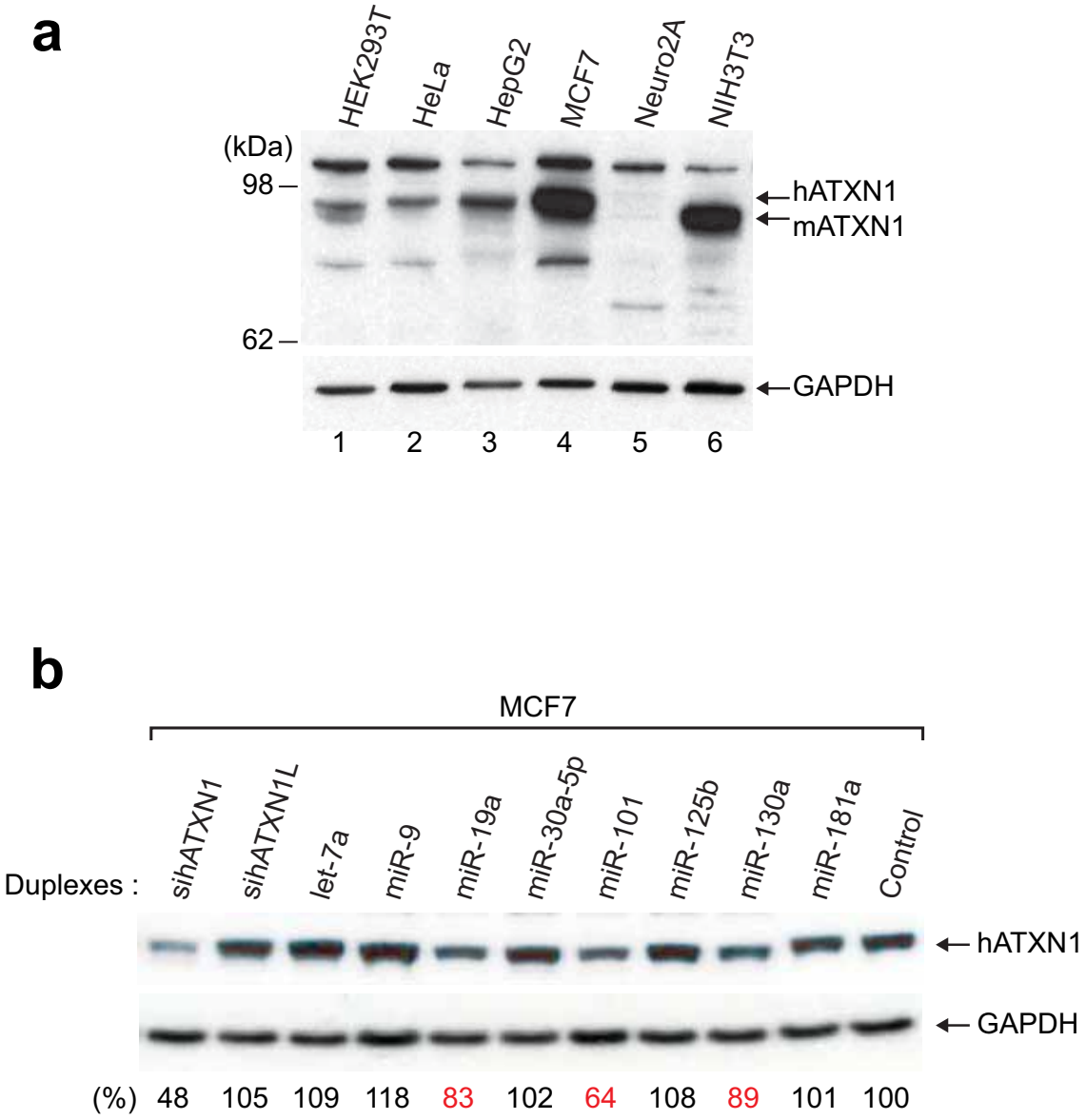


Figure S2. Validation of the selected miRNAs in MCF7 cells. a) Comparison of ATXN1 levels among various human and mouse cell lines and b) validation of the selected miRNAs by Western blot analysis for ATXN1 using the 11750 antibody. Protein extracts were prepared from cells 72hrs after transfection with each miRNA duplex. Relative levels of ATXN1 (control = 100%) are presented under the blot image.

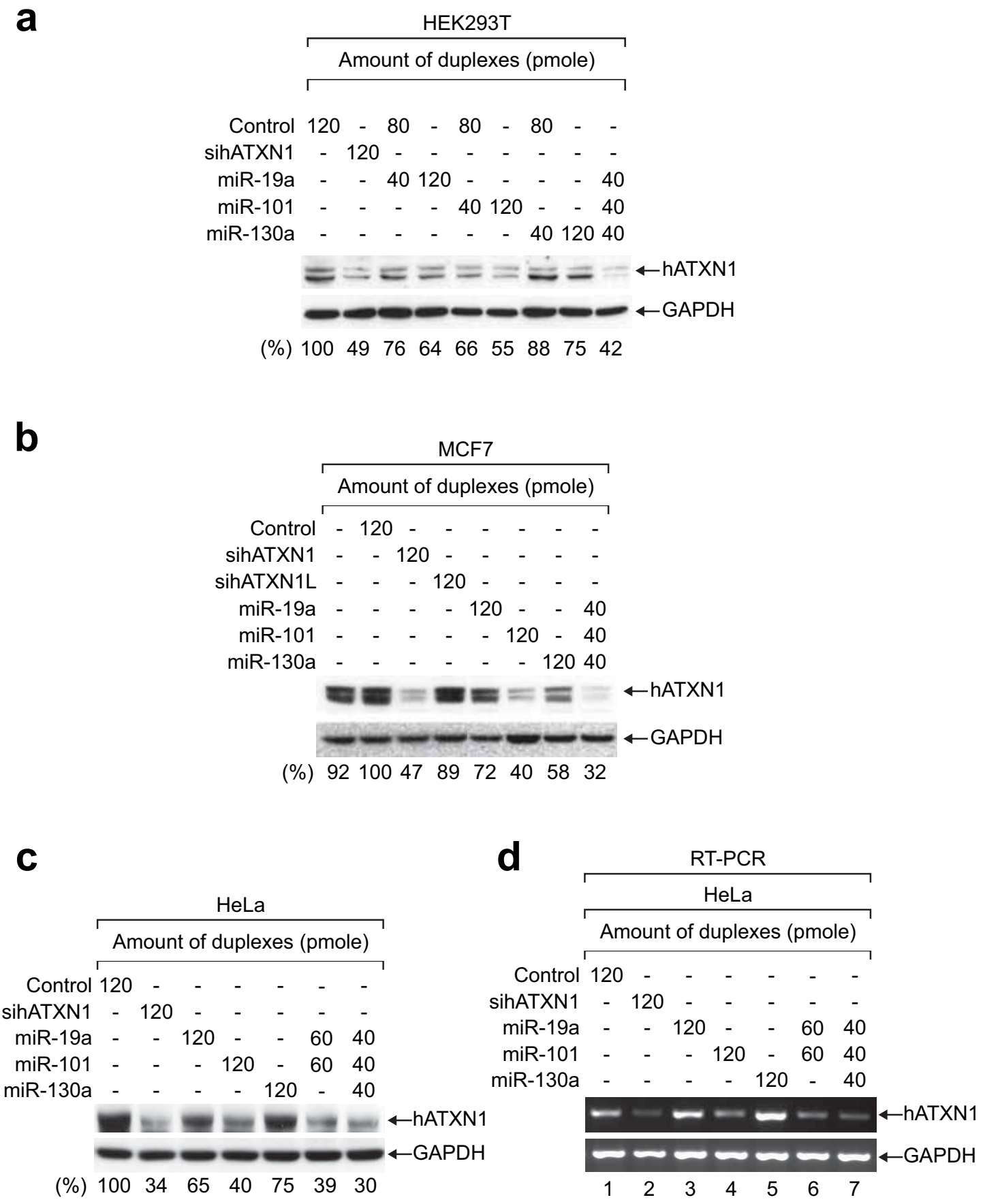


Figure S3. miR-19a, 101, and 130a regulate the level of ATXN1 in various human cells.
Western blot analysis for ATXN1 proteins using cell lysates of HEK293T (a), MCF7 (b) and HeLa (c). Relative levels of ATXN1 (control = 100%) are presented under the blot image. (d) RT-PCR for *ATXN1* mRNA using HeLa total RNA. Protein extracts and total RNA were prepared from cells 72hrs after transfection with each miRNA or siRNA duplex. Total amount of duplexes used for each transfection is identical.

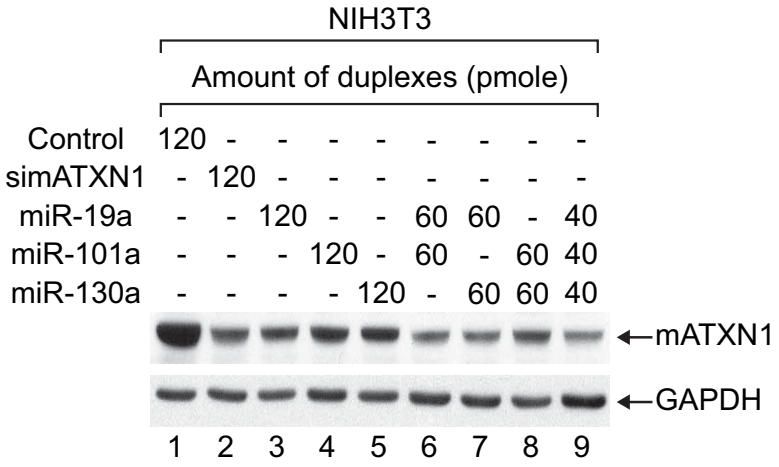


Figure S4. miR-19a, 101a, and 130a regulate the level of ATXN1 in NIH3T3 cells.
Western blot analysis for mouse ATXN1 proteins. Protein extracts were prepared from cells 72hrs after transfection with each miRNA or siRNA duplex. Total amount of duplexes used for each transfection is identical.

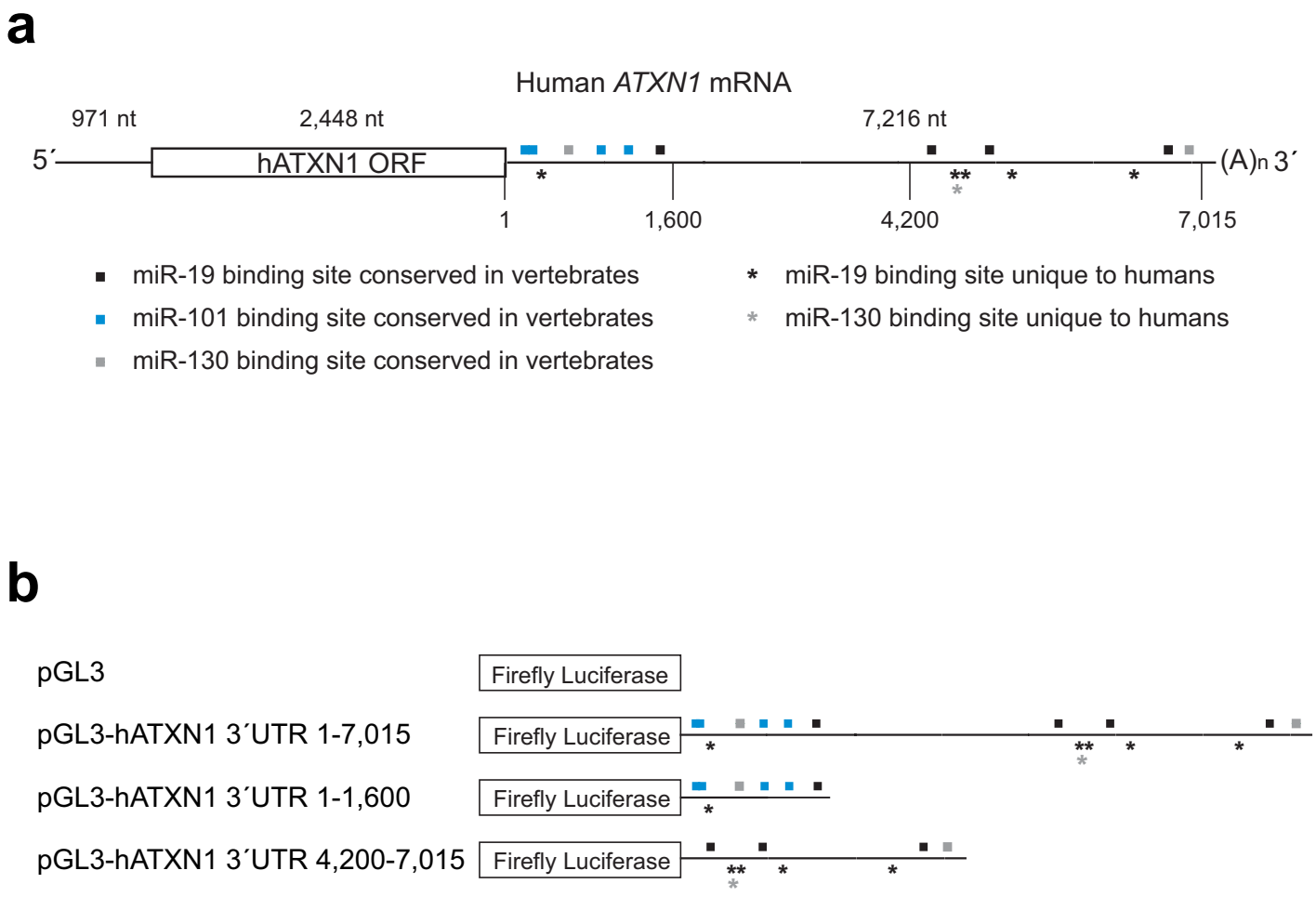
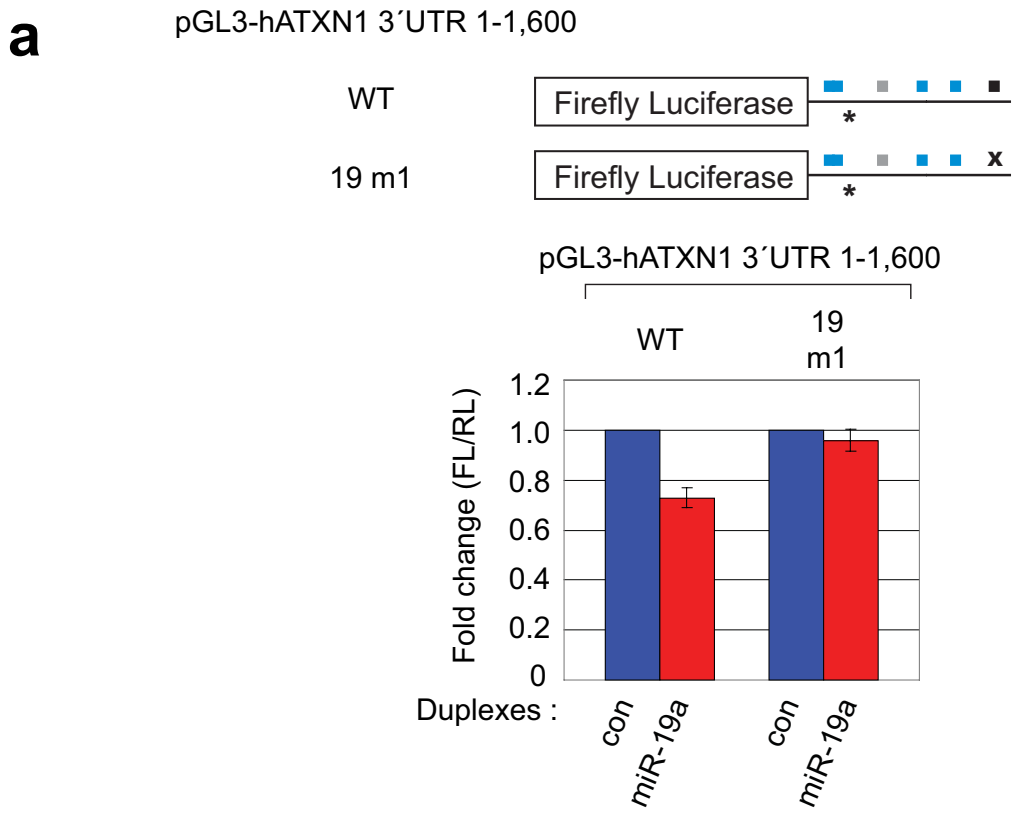


Figure S5. Schematic diagrams of human *ATXN1* mRNA and firefly luciferase reporters containing the human *ATXN1* 3′UTR. (a) Schematic diagram of human *ATXN1* mRNA and putative target sites of three different miRNAs. (b) The luciferase constructs containing the partial or full length human *ATXN1* 3′UTR used for the luciferase assays in Figure 1e.



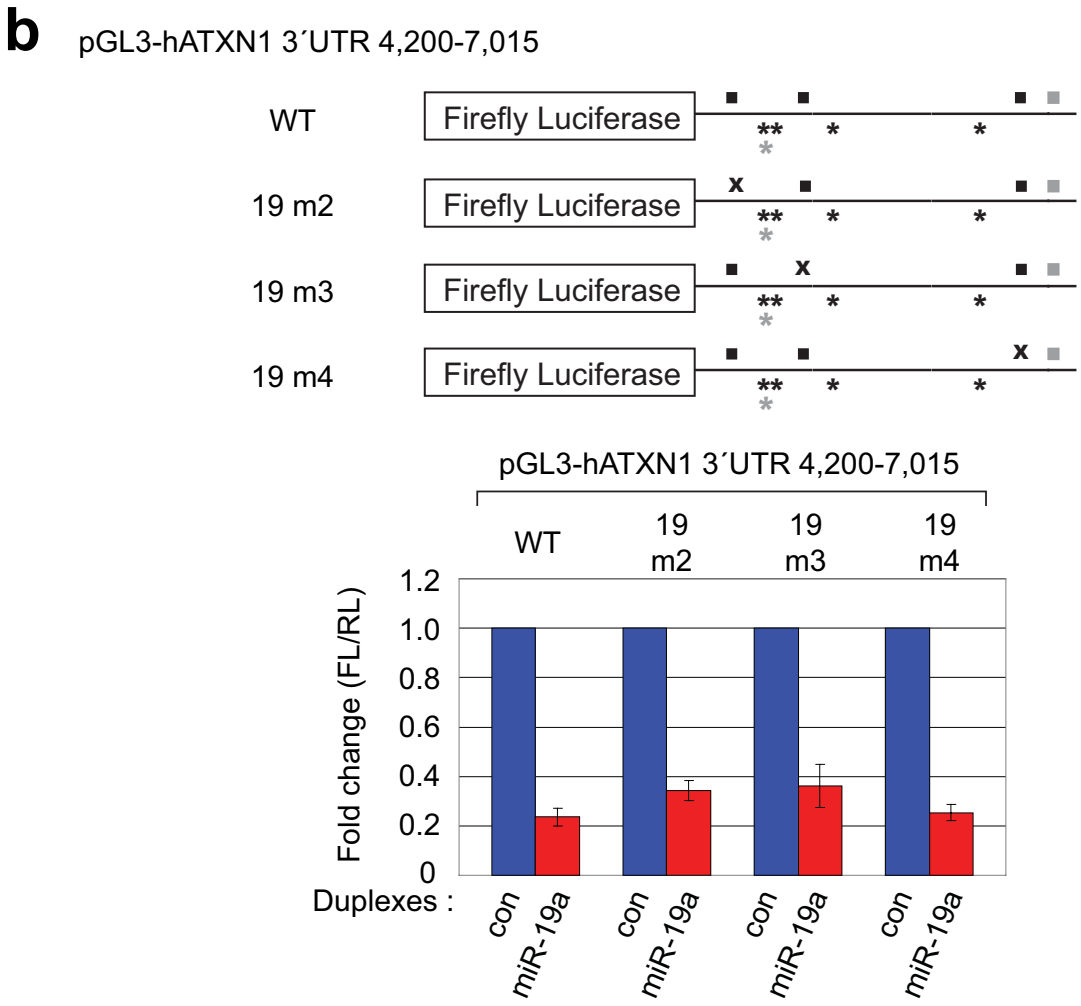


Figure S6. Validation of putative miR-19 target sites. Luciferase assays using (a) pGL3-hATXN1 3´UTR 1-1,600 and (b) pGL3-hATXN1 3´UTR 4,200-7,015. The upper panel shows schematic diagrams of reporters containing a mutated miR-19 target site, which is depicted as 'x'. Mean relative levels of luciferase activity (negative control =1) and standard deviation are presented in the lower panel. This experiment was carried out in triplicate.

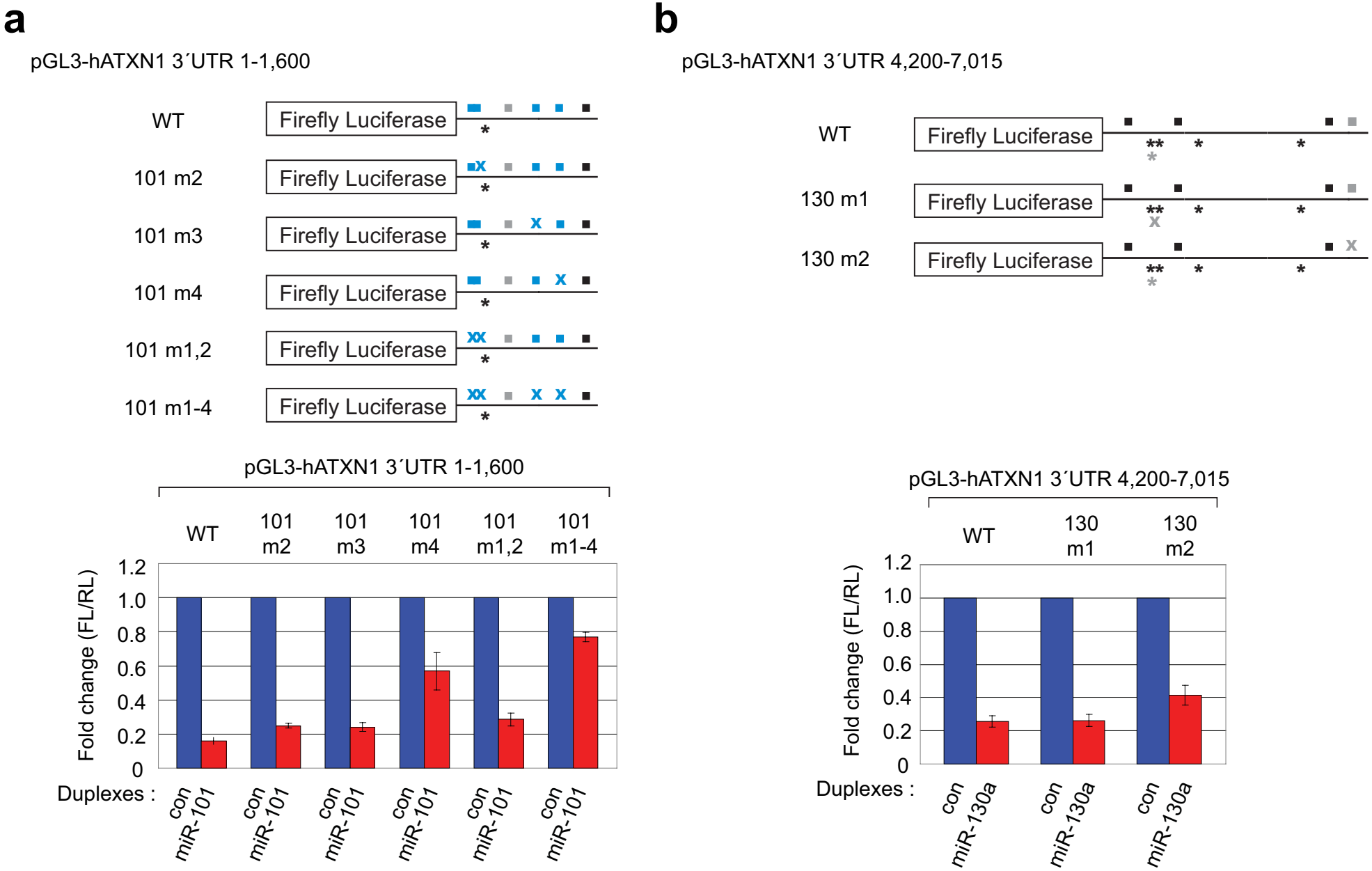


Figure S7. Validation of putative miR-101 and miR-130 target sites. The same experiment as described in Figure S6, except for using the reporters containing mutated (a) miR-101 or (b) miR-130 target sites.

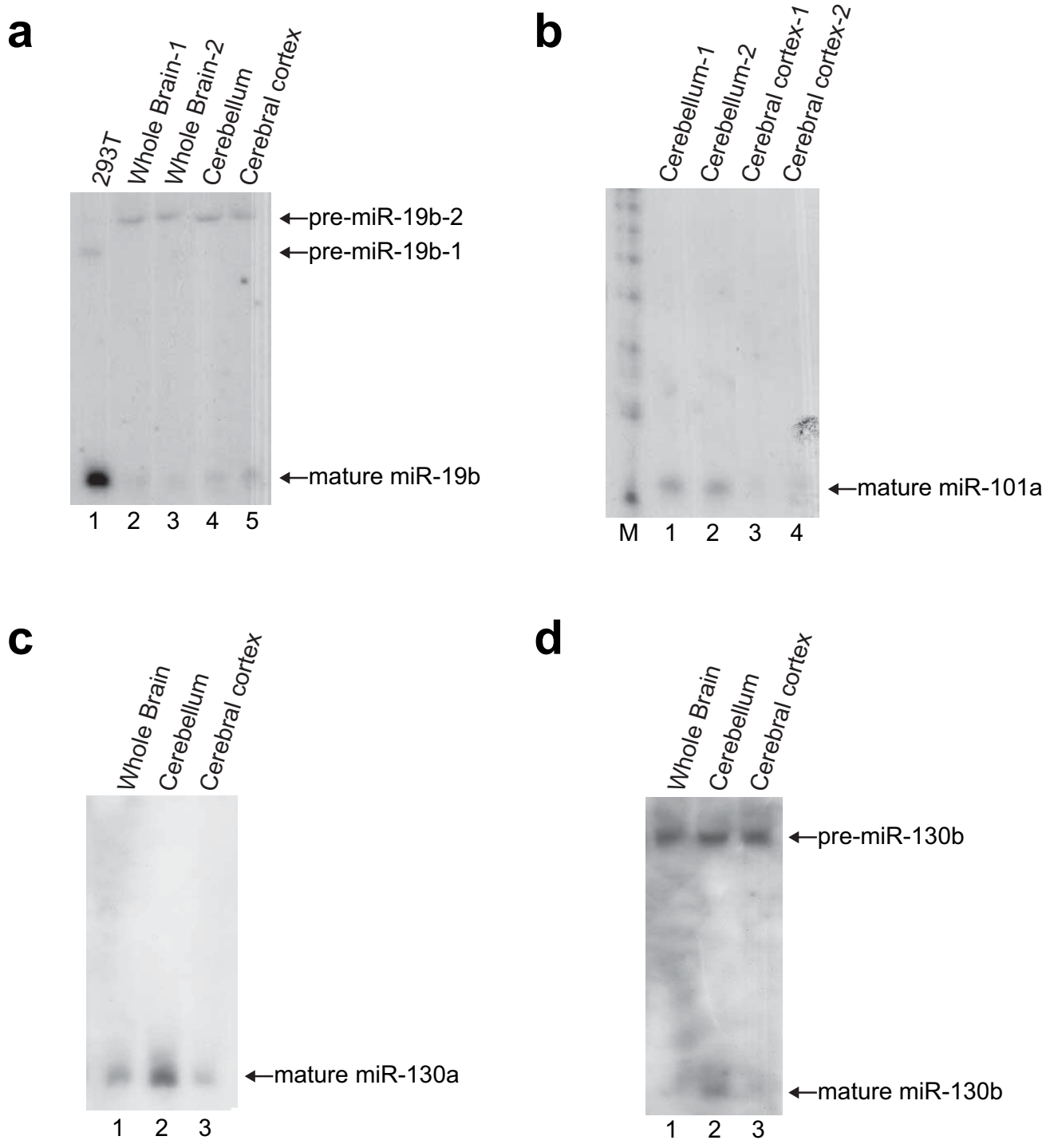


Figure S8. Expression profiles of miR-19, miR-101, and miR-130 in the mouse cerebellum. Northern blot analysis for (a) miR-19b, (b) miR-101a, (c) miR-130a, and (d) miR-130b. Total RNA was prepared from whole brain, cerebral cortex, or cerebellum of 24 week-old C57/B6 WT male mice. Fifty μg of RNA was loaded in each lane.