Paeoniflorin eliminates a mutant AR via NF-YA-dependent proteolysis in spinal and bulbar muscular atrophy

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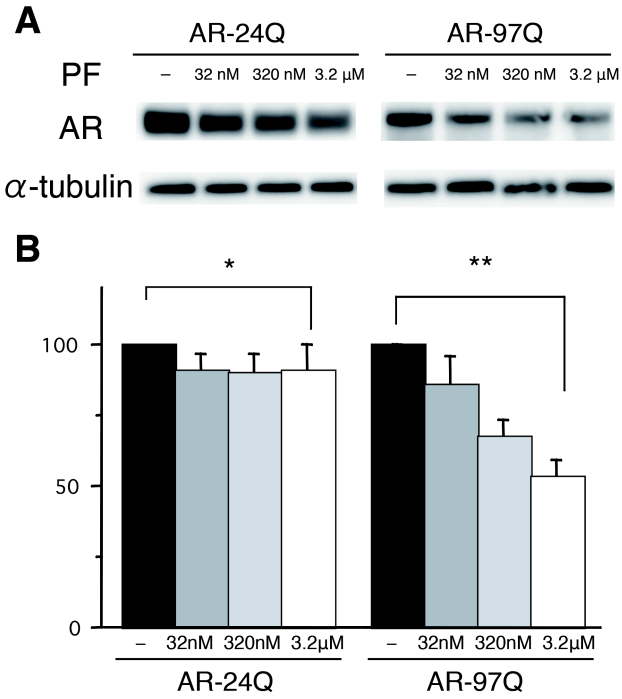
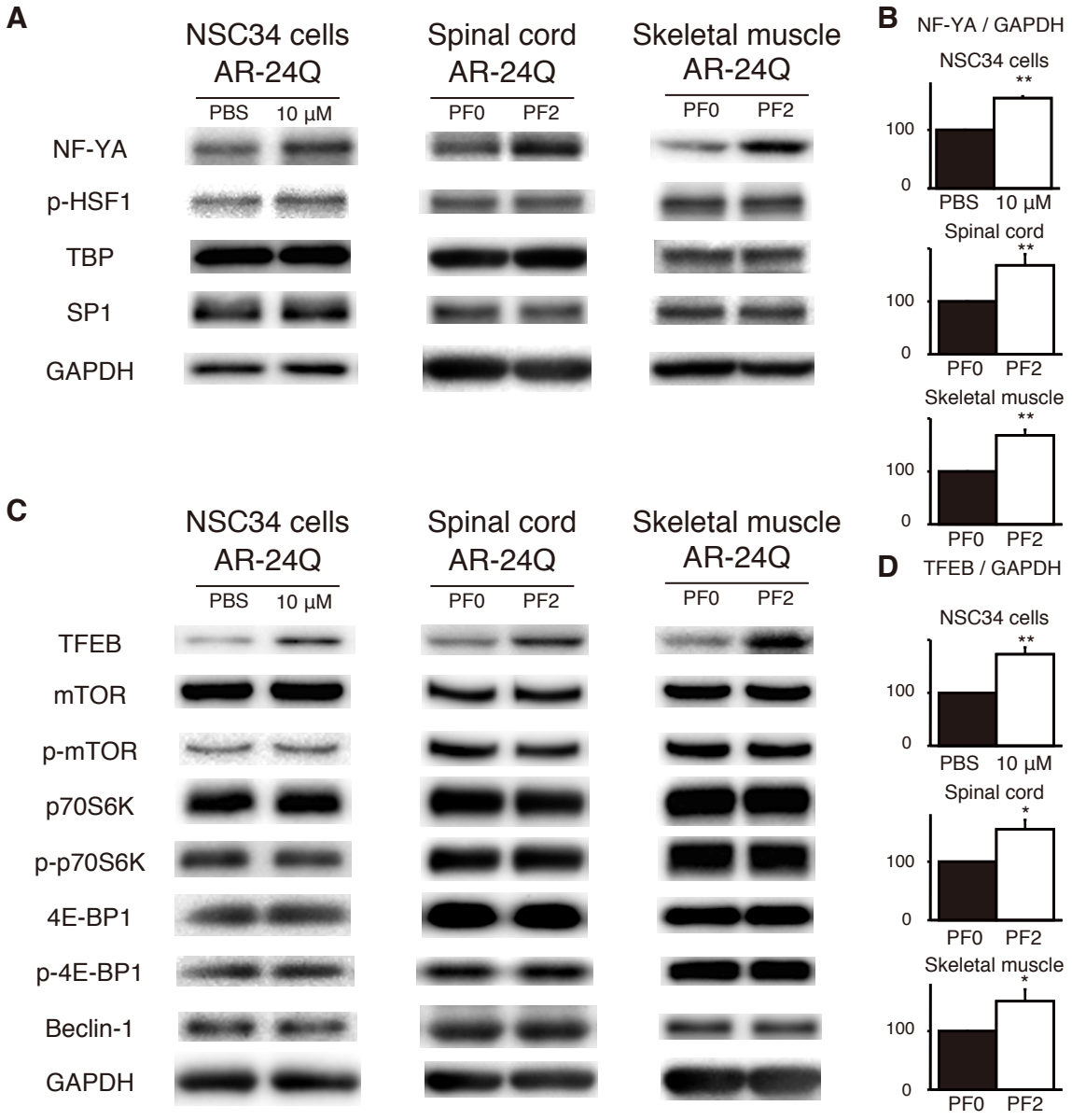
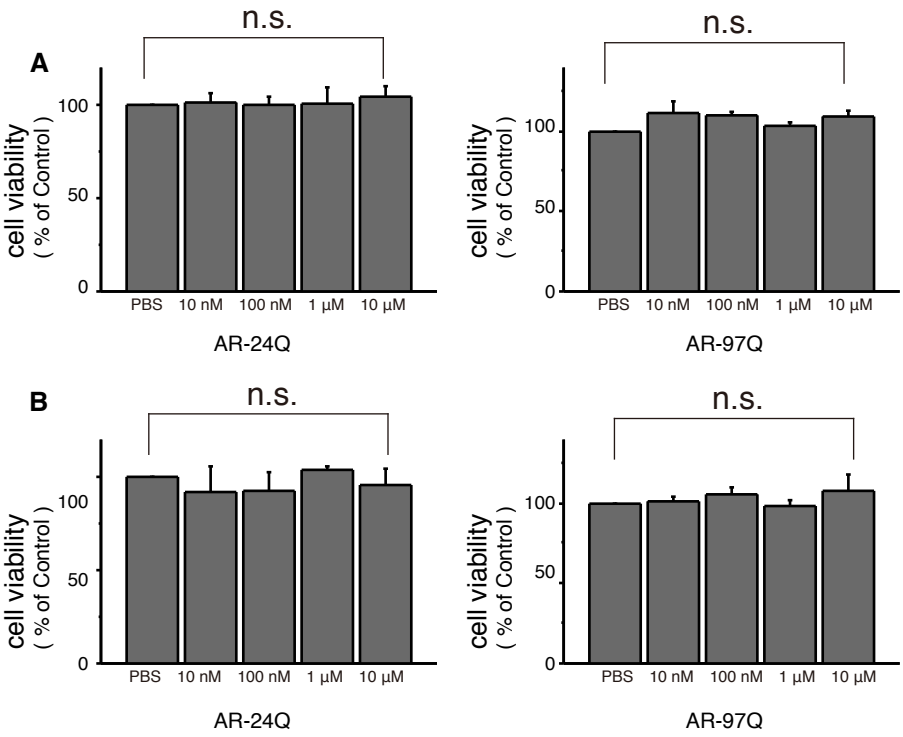
Supplementary Figures and Table



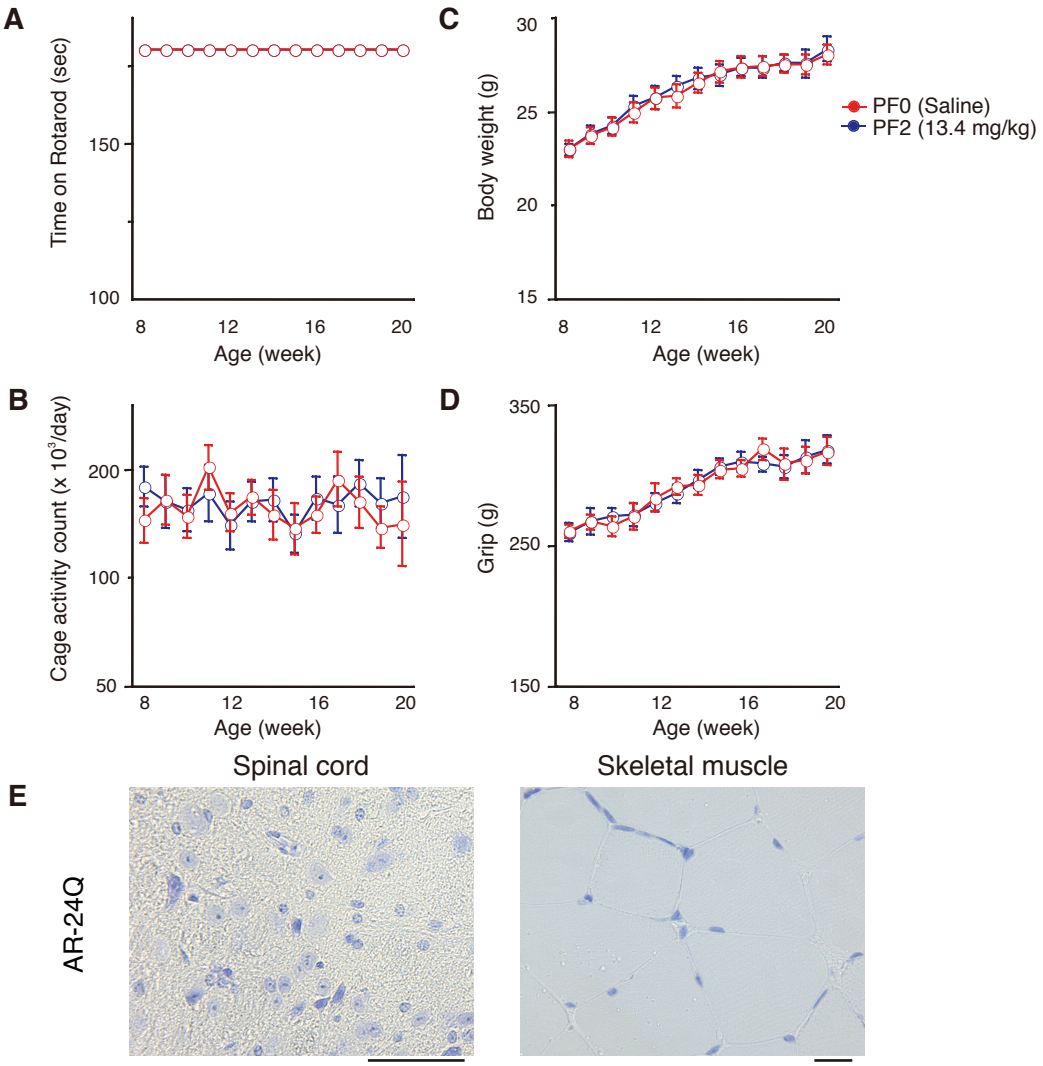
Supplementary Figure 1. Effects of paeoniflorin (PF) on the expression levels of wild-type (24Q) and mutant (97Q) AR in Neuro2A cells. (A and B) Western blots and densitometric analyses of ARs from Neuro2A cells transiently transfected with AR-24Q and AR-97Q and treated with the indicated dose of PF. The decrease in AR-97Q was significantly greater than AR-24Q. (* $P < 0.05$; ** $P < 0.01$, $n = 5$, One-way ANOVA with Tukey-Kramer *post-hoc* test). Error bars (B), s.e.m.



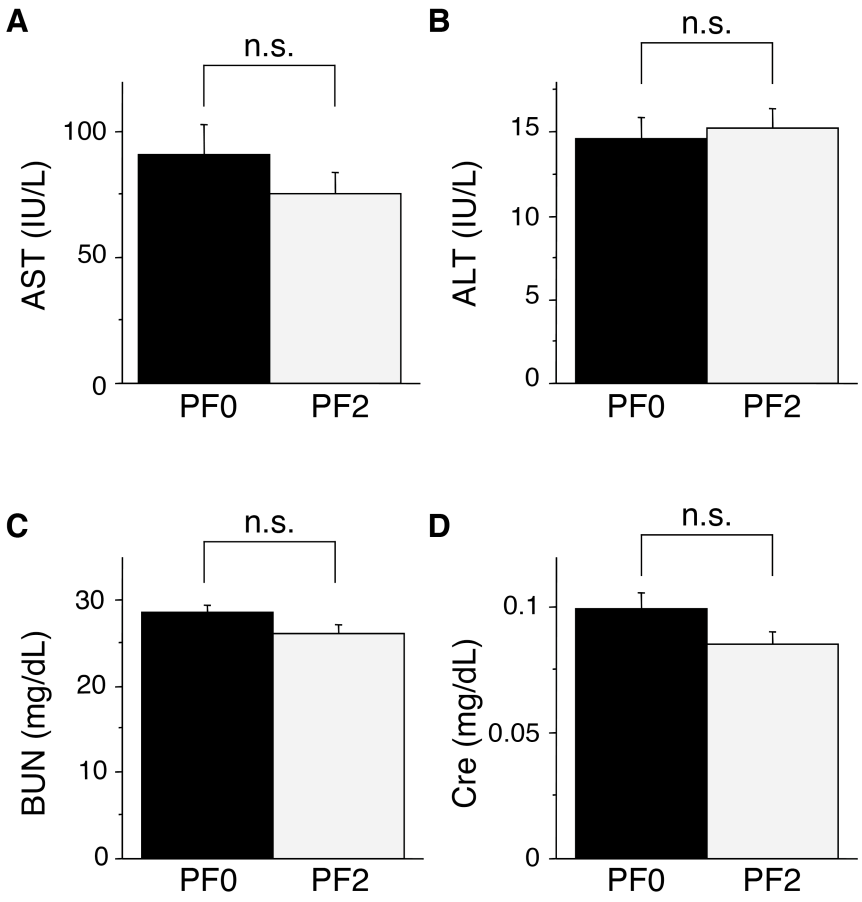
Supplementary Figure 2. Effects of paeoniflorin (PF) on the molecular chaperone-UPS and the autophagy system in cultured cell and mouse models. (A and B) Western blots and densitometric analyses of the expression levels of molecules related to the molecular chaperone-UPS in NSC34 cells stably expressing wild-type AR (24Q) and 16-week-old AR-24Q mice administered 13.4 mg/kg (PF2) PF. (** $P < 0.01$, $n = 4$, unpaired t-test). (C and D) Western blots and densitometric analyses of the expression levels of molecules related to autophagy in NSC34 cells stably expressing wild-type AR (24Q) and 16-week-old AR-24Q mice administered 13.4 mg/kg (PF2) PF. (* $P < 0.05$; ** $P < 0.01$, $n = 4$, unpaired t-test). Error bars (B and D), s.e.m.



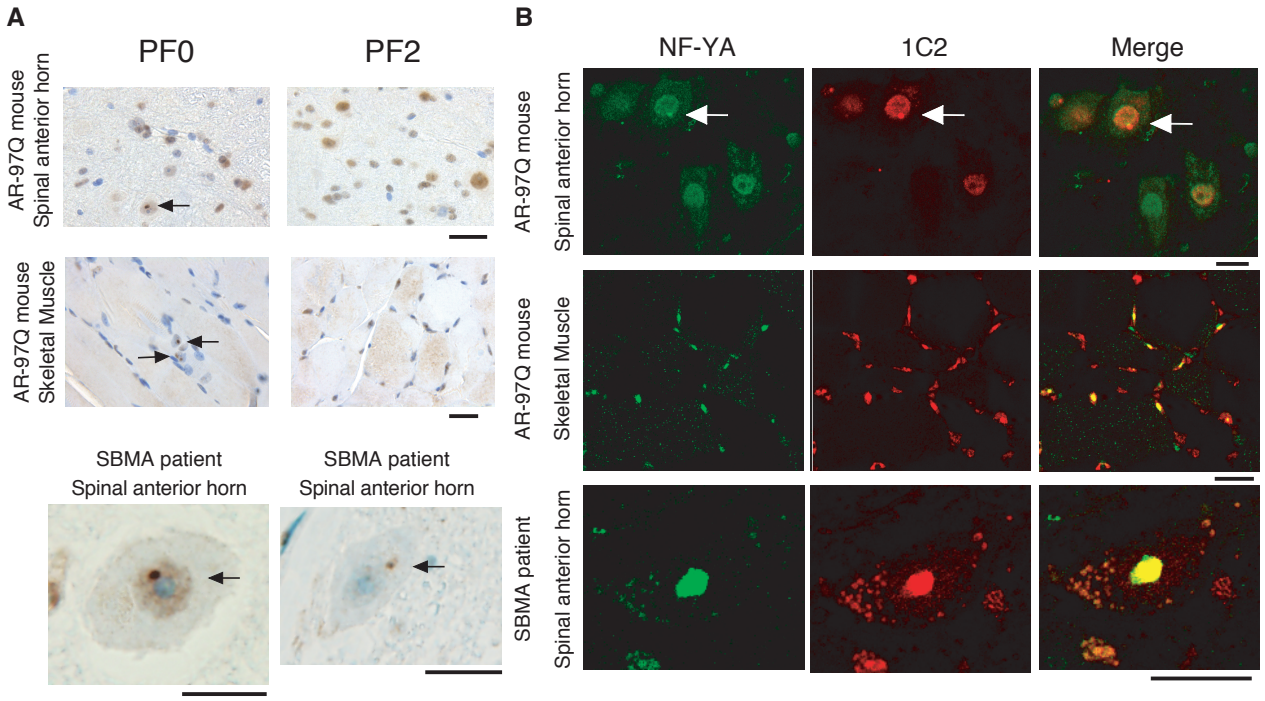
Supplementary Figure 3. Effects of paeoniflorin (PF) on cell viability in NSC34 cells stably expressing mutant AR. Cell viability was measured using the WST-1 (A) and MTS assay (B). The data are expressed by the mean of percent cell viability compared to control (n = 4).



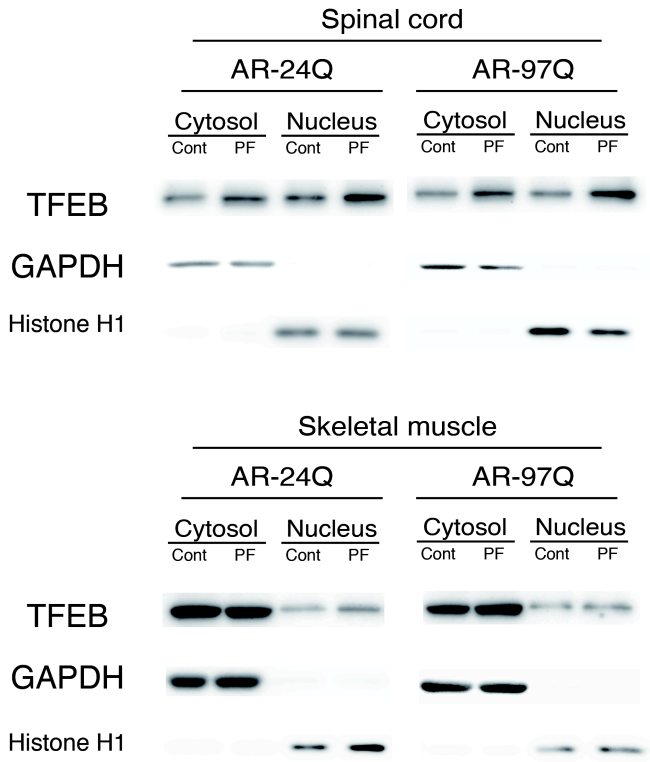
Supplementary Figure 4. Effects of paeoniflorin on the phenotype of male AR-24Q mice. The rotarod task (A), cage activity (B), body weight (C) and grip strength (D) of AR-24Q mice treated with saline (PF0) or paeoniflorin (PF2) at a dose of 13.4 mg/kg. (*n* = 12, One-way ANOVA with Tukey-Kramer *post-hoc* test). (E) Immunohistochemistry of the AR-24Q mice using the 1C2 antibody (16 weeks old). Error bars (A-D), s.e.m. Scale bar, 50 μm.



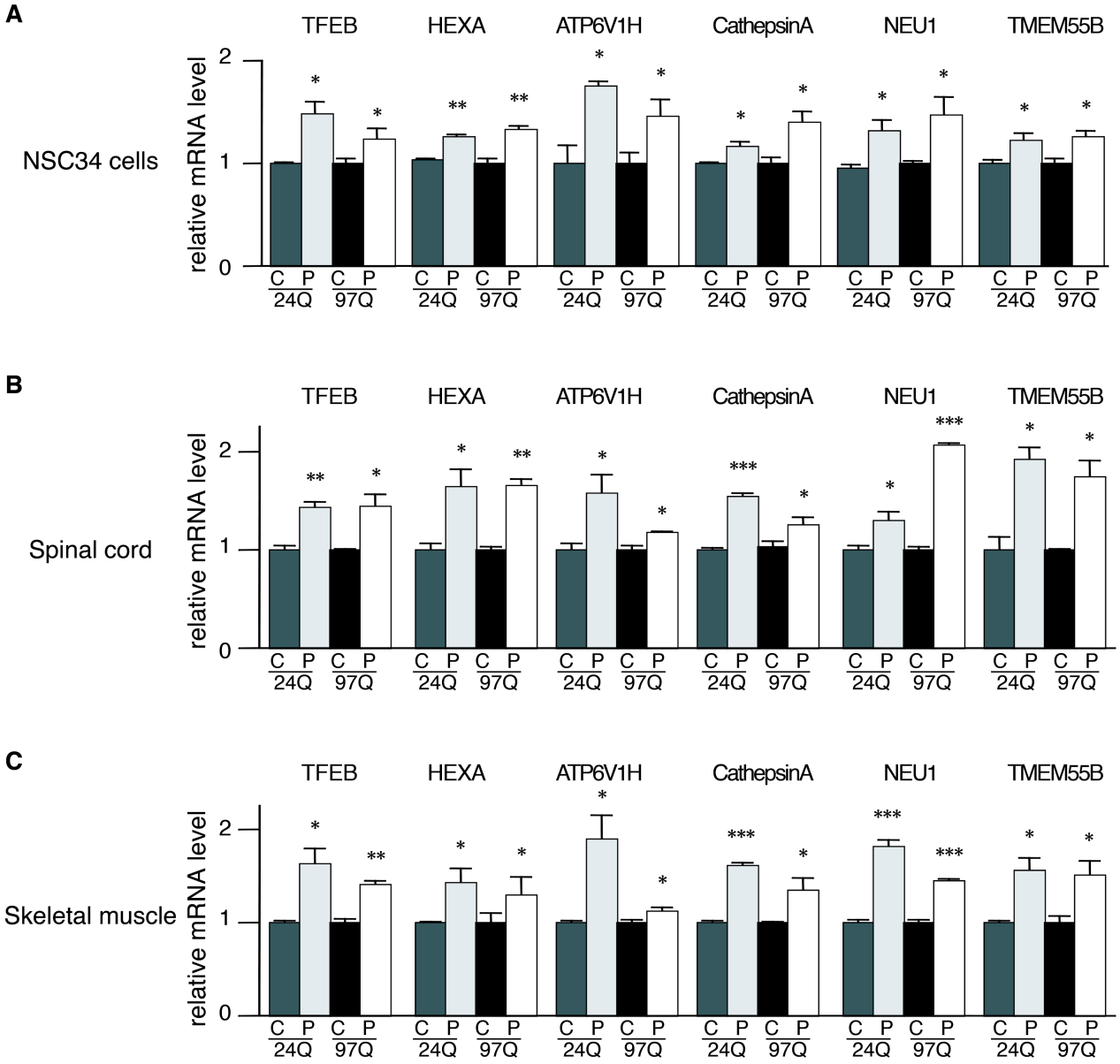
Supplementary Figure 5. The hematological examination of male AR-97Q mice treated with PF. Levels of (A) aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), (C) blood urea nitrogen (BUN) and (D) serum creatinine (Cre) were assayed in untreated AR-97Q mice (cont; 16 weeks of age) and in AR-97Q mice treated with 13.4 mg/kg PF (PF2; 16 weeks of age). The laboratory results showed no significant differences in the levels of any of the above compounds in the PF2 mice compared with the control mice, demonstrating the lack of toxicity due to PF treatment. Values are expressed as the mean ± SE (n=20, unpaired t-test).



Supplementary Figure 6. NF-YA is recruited into the nuclear and cytoplasmic inclusions of mutant AR. (A) Immunohistochemical staining of the untreated AR-97Q mice (PF0) and AR-97Q mice treated with 13.4 mg/kg PF (PF2) with NF-YA-specific antibody (16 weeks old). The PF2 mice show increased expression levels of nuclear NF-YA in the spinal cord and skeletal muscle of AR-97Q mice. NF-YA is localized to the nuclear and cytoplasmic inclusions (arrow) in the spinal anterior horn and skeletal muscle of 16-week-old AR-97Q mice and SBMA patients. (B) Double-immunofluorescence staining for NF-YA (green), expanded-polyQ (red) and the overlay of the two signals (yellow) reveal that NF-YA and mutant AR co-localize in NIs (shown in yellow, arrow) in 16-week-old AR-97Q mice and SBMA patients. Scale bars, 20 μm.



Supplementary Figure 7. Paeoniflorin (PF) regulates TFEB nuclear translocation. To verify that PF induces TFEB activation, the nuclear translocation of TFEB was investigated using western blot analysis. PF increased the amount of TFEB in the nucleus at a dose of 13.4 mg/kg in 16-week-old AR-24Q and AR-97Q mice.



Supplementary Figure 8. Paeoniflorin (PF) induces the expression of wide-ranged lysosomal genes. Real-time RT-PCR of lysosomal genes normalized to β2 microglobulin levels in NSC34 cells stably expressing AR-24Q or AR-97Q (A), the spinal cord (B) and skeletal muscle (C) of 16-week-old AR-24Q and AR-97Q mice. C, control; P, 10 μM PF in cells, 13.4 mg/kg PF in mice. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, $n = 3$, unpaired t-test). Error bars (A-C), s.e.m.

Supplementary Table 1. List of primer pairs for quantitative real-time reverse transcription-PCR.

Gene	Forward primer	Backward primer
AR	GGCTATGAATGTCAGCCCAT	TTGAGGCTAGAGAGCAAGGC
NF-YA	CGAAGAAGCCATGACACAGA	CCCACTGGAAGTCAGTCCAT
TFEB	AACAGTGCTCCCAACAGTCC	TCTCAGGGTTGATGAGCCC
HEXA	GACGCTACCGTAACCTGCTC	GACGGAGACCACCAGAATGT
ATPaseV1H	CTGCTCACGATGTTGGAGAA	GGCCAGAAGAGCATTGTAGC
Cathepsin A	CACTGCTCTTGTTGCTGCTC	GTCCGATGCTCTGAGGTAGC
Neu1	TCTCCTCAGTGATGACCACG	AGCCATCTGGAAGCTCGTAG
TMEM55	CGTCTGTCAGTCTCCGATCA	AGACAATTACAGGGGCATCG
β2 microgloblin	CTGACCGGCCTGTATGCTAT	CCGTTCTTCAGCATTTGGAT