Deubiquitinase USP7 contributes to the pathogenicity of spinal and bulbar muscular atrophy

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Supplemental Information

Gene	Encoded protein	Fold enrichment with AR112Q				
		anti-AR Ab		3B5H10 Ab		
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 3
Ahi1	Jouberin	**2.12**	**1.51**	ND	ND	1.24
Alg2	Alpha-1,3-mannosyltransferase	**4.35**	**1.75**	1.26	**2.75**	ND
Anp32b	Acidic leucine-rich nuclear phosphoprotein 32 family member B	**1.54**	**1.62**	ND	ND	ND
Capn2	Calpain-2 catalytic subunit	**2.2**	-1.18	**1.66**	1.34	**1.92**
Chaf1a	Chromatin assembly factor 1 subunit A	ND	1.01	**1.56**	**1.67**	ND
Dars	Aspartate-tRNA ligase	**1.78**	**1.59**	ND	1.37	**2.09**
Fmr1	Fragile X mental retardation protein 1 homolog	**1.65**	**1.77**	ND	ND	1.26
Gpc1	Glypican 1	ND	**1.58**	**6.63**	**1.52**	**4.2**
Hmbox1	Homeobox containing 1	**2.06**	1.23	**1.51**	**1.53**	1.17
Hspa1a	Heat shock 70 kDa protein 1A/1B	1.14	-1.29	1.22	**2.94**	**1.88**
Ipo5	Importin 5	**1.82**	**2.56**	**2.01**	ND	1.44
Ldha	L-lactate dehydrogenase A chain	**1.75**	**3.93**	ND	ND	**1.58**
Lgals3bp	Galectin-3-binding protein	**2.76**	1.41	**1.82**	**2.7**	1.13
Man2c1	Alpha-mannosidase 2C1	-1.15	1.29	**3.12**	**3.15**	**2.66**
Myo5a	Unconventional myosin-Va	ND	**-1.7**	**1.9**	-1.41	**1.68**
Nckap1	Nck-associated protein 1	**1.71**	-1.14	**1.73**	-1.01	**1.53**
Nisch	Nischarin	**1.69**	1.16	1.31	**1.52**	**1.53**
Pdcd6	Programmed cell death protein 6	**2.32**	1.4	1.18	**1.85**	**1.67**
Plat	Tissue-type plasminogen activator	**2.35**	**2.7**	**1.75**	ND	**2.81**
Rpl3	60S ribosomal protein L3	1.33	1.04	1.14	**1.7**	**1.53**
Rpl5	40S ribosomal protein L5	1.45	-1.21	1.19	**1.76**	**1.56**
Rpl27a	60S ribosomal protein L27a	1.25	-1.07	-1.01	**1.55**	**1.55**
Rpl36	60S ribosomal protein L36	1.31	1.07	1.46	**2.09**	**1.52**
Rplp1	60S acidic ribosomal protein P1	1.25	-1.05	1.11	**1.55**	**1.9**
Rps5	40S ribosomal protein S5	1.38	1.18	1.23	**2.06**	**1.57**
Rps17	40S ribosomal protein S17	1.45	1.04	1.06	**1.85**	**1.56**
Sart3	Squamous cell carcinoma antigen recognized by T-cells 3	**1.69**	**1.77**	ND	ND	-1.42
Slc3a2	4F2 cell-surface antigen heavy chain	1.05	**2.11**	ND	**2.5**	**2.4**
Slc7a5	Large neutral amino acids transporter small subunit 1	-1.06	**1.91**	ND	**1.62**	**2.23**
Srebf1	Sterol regulatory element-binding protein 1	ND	**3.77**	**2.01**	**3.9**	ND
Srebf2	Sterol regulatory element-binding protein 2	ND	**2.58**	**1.62**	**3.51**	ND
Tufm	Elongation factor Tu, mitochondrial	ND	**1.51**	**2.55**	**1.55**	-1.16
Usp7	Ubiquitin carboxyl-terminal hydrolase 7	**3.36**	**1.96**	**2.56**	**3.08**	**2.25**
Zfhx3	Zinc finger homeobox protein 3	**5.1**	**1.59**	**2.48**	**1.94**	-1.44

Table S1 related to Figure 1. Proteins enriched with AR112Q by 1.5 fold or more (shown in bold) in at least two pull-down experiments with anti-AR or 3B5H10 antibodies. The level of enrichment is shown for each protein. ND, protein was not detected. Negative number indicates protein enrichment with AR10Q.

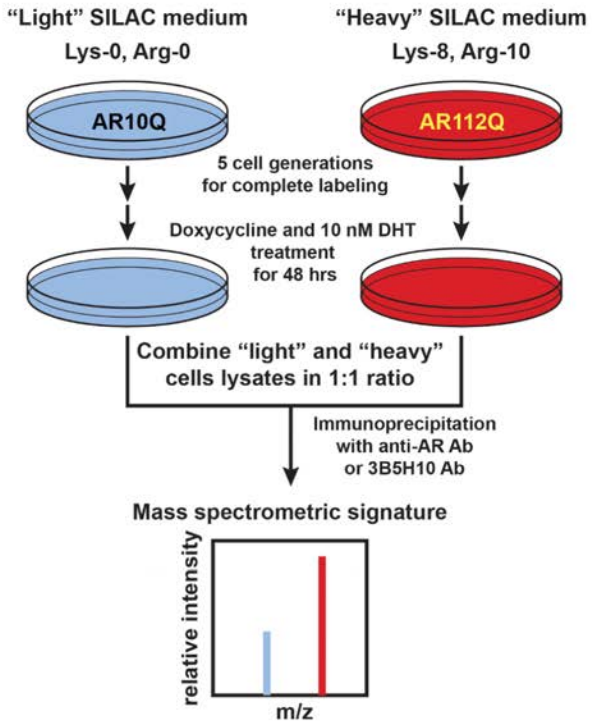
Gene	Encoded protein	Fold enrichment with AR10Q anti-AR Ab	
		Exp. 1	Exp. 2
Banf1	Barrier-to-autointegration factor	**1.86**	**2.08**
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	**1.7**	**2.24**
Hspb1	Heat shock protein beta 1	**3.47**	**3.82**
Hsph1	Heat shock protein 105 kDa	**2.05**	**2.54**
Luc7l2	Putative RNA-binding protein Luc7-like 2	**1.51**	**2.35**
Sri	Sorcin	**3.62**	**2.49**
Tgm2	Tissue transglutaminase	**4.05**	**4.74**
Tpi1	Triosephosphate isomerase	**2.47**	**3.09**
Usp26	Ubiquitin carboxyl-terminal hydrolase 26	**5.66**	**4.65**

Table S2 related to Figure 1. Proteins enriched with AR10Q by at least 1.5 fold in two pull-down experiments with anti-AR antibody

Peptide sequence	Position of ubiquitinated lysine	Fold change miR *Usp7* #1 H/ miR control L	Fold change miR *Usp7* #1 L/ miR control H	Fold change miR control H/ miR control L
VYPRPPSKTYR	K17	-2.8	-1.69	-1.08
EASGAPTSSKDNYLGGTSTISDNAK	K220	-2.45	-1.57	1.01
QLVHVVKWAK	K717	-2.31	-1.78	-1.02
NQKFFDELR	K825	-3.64	-1.73	-1.16
ALLLFSIIPVDGLKNQKFFDELR	K825	-3.79	ND	-1.21
ALLLFSIIPVDGLKNQK	K825	-1.84	-1.93	-1.01
MNYIKELDR	K836	-2.63	-1.68	-1.22
RFYQLTKLLDSVQPIAR	K861	-2.88	-1.82	-1.31
FYQLTKLLDSVQPIAR	K861	-2.56	-2.02	-1.13
ILSGKVKPIYFHTQ	K910, 912	-2.61	-1.67	-1.21
VKPIYFHTQ	K912	-2.19	-1.64	-1.15

Table S3 related to Figure 11. Knockdown of USP7 results in changes in AR112Q ubiquitination state. PC12 cells inducibly expressing AR112Q and constitutively expressing *Usp7*-directed miRNA (miR *Usp7* #1) or non-targeting miRNA (miR control) grown in SILAC heavy (H) or light (L) medium, were treated with 10 nM DHT for 48 hrs and with 10 μM MG132 for the last 6 hrs prior to harvest. The whole ubiquitinome analysis was performed on cell lysates (mixed in 1:1 ratio as indicated) (see Experimental Procedures). Identified AR peptides (with diGly remnants) are shown. The ubiquitinated sites are shown in red. For all listed ubiquitinated sites the localization probability was 1. ND, peptide was not detected.

A



B

Experiment	Antibody used for IP	AR112Q/AR10Q ratio
1	anti-AR	0.74
	3B5H10	28.7
2	anti-AR	1.2
	3B5H10	93.4
3	3B5H10	30.5

Figure S1, related to Figure 1. Quantitative interaction screen for wild-type and polyQ-expanded AR. (**A**) Schematic of the experimental design. PC12 cells expressing AR10Q or AR112Q were grown in light or heavy SILAC medium for 5 generations (see Experimental Procedures). Cells were lysed and the lysates combined in a 1:1 ratio. IPs were carried out with an anti-AR antibody (G122-434, BD Biosciences), which immunoprecipitates both AR10Q and AR112Q with similar efficiency (as indicated by the AR112Q/AR10Q ratio in (**B**)), or with 3B5H10 antibody which preferentially immunoprecipitates AR112Q. Immunoprecipitated proteins were analyzed by mass spectrometry. (**B**) Ratio of immunoprecipitated AR112Q to AR10Q by either anti-AR or 3B5H10 antibody from three experiments described above based on heavy/light ratio for AR peptides obtained by mass spectrometry analysis. The fold enrichment of the AR interacting proteins was corrected for the differences in recovery of AR using the anti-AR antibody (see **Table S1, S2**).

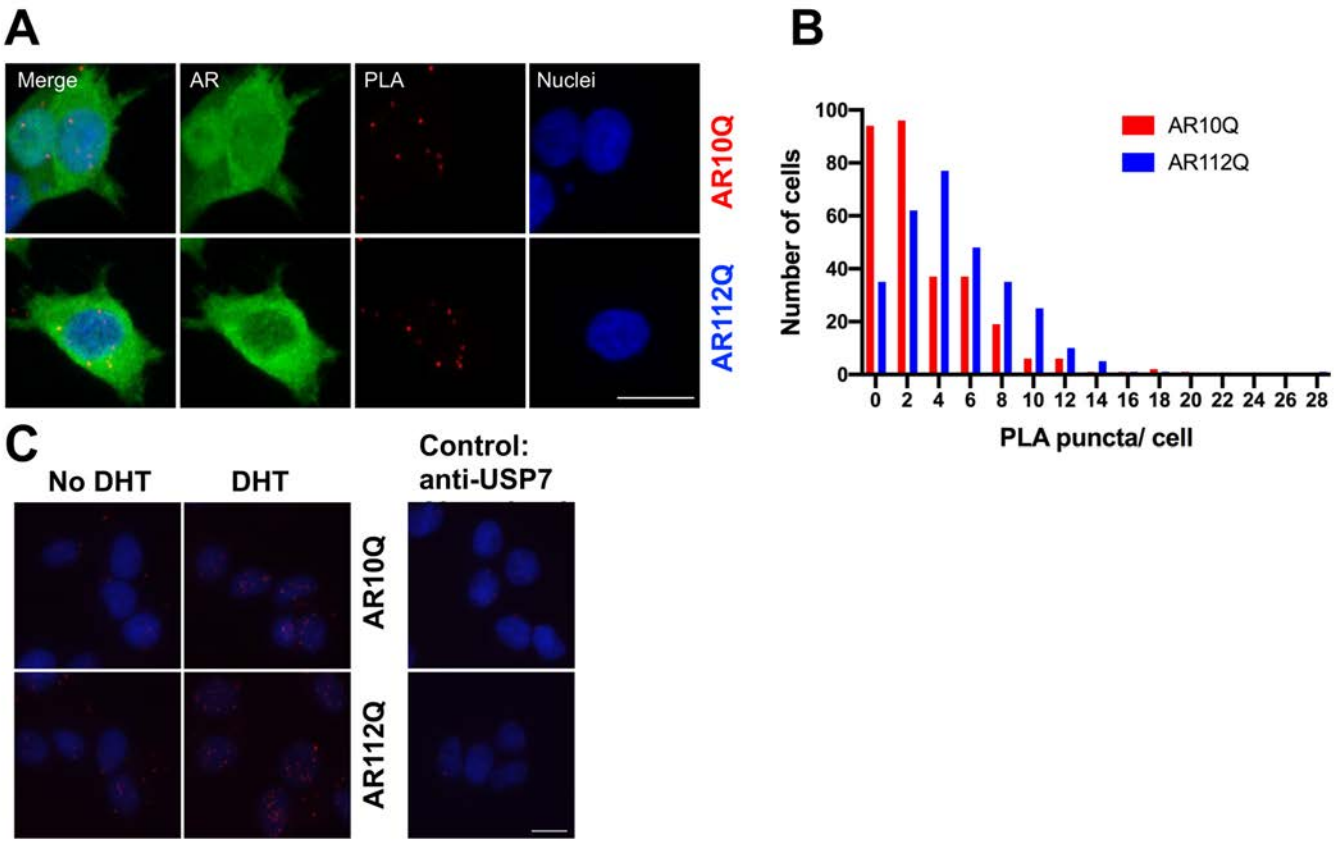


Figure S2, related to Figure 2. USP7 interaction with AR in PC12 cells under conditions in which AR is predominantly cytoplasmic (i.e. in the absence of DHT) was evaluated by proximity ligation assay. (**A**) Red immunofluorescent puncta represent physical proximity between antigen-associated anti-USP7 and anti-AR antibodies. Subsequent immunostaining (green) with anti-AR antibody established the mostly cytoplasmic distribution of total AR. Scale bar represents 10 μm. (**B**) Quantification of the interaction in cells expressing AR10Q and AR112Q, respectively. One hundred cells were evaluated per condition and were done in triplicate. $p<0.0001$ determined by the Kolmogorov-Smirnov test. (**C**) Typical immunofluorescent images used to evaluate the number of puncta per cell under each experimental condition. These images were taken prior to immunostaining for total AR with controls shown on the right.

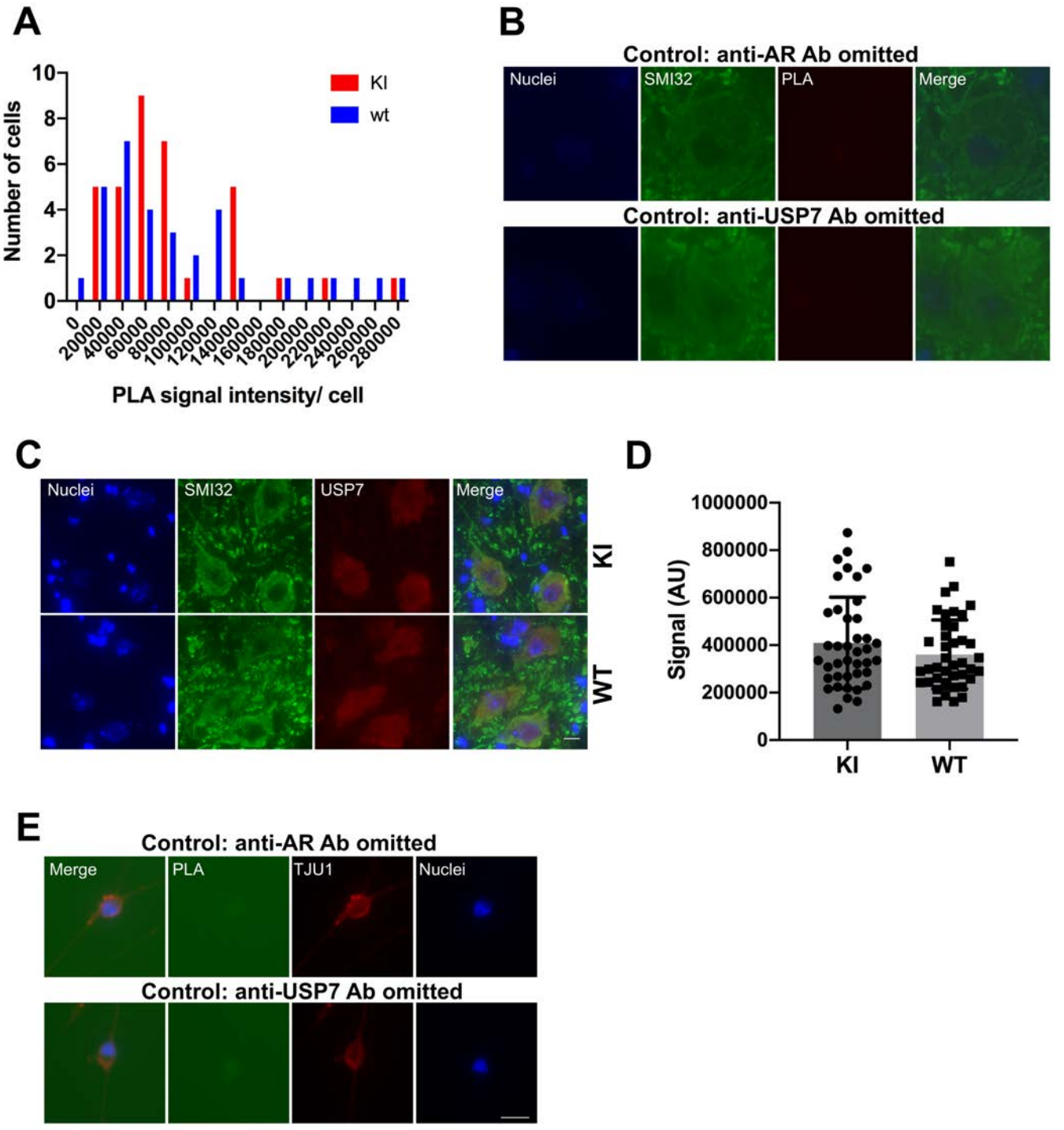


Figure S3, related to Figure 3. (**A**) Quantification of the USP7-AR interaction (see **Fig. 3C**) was performed by measuring PLA signal intensity per cell. Thirty-three motor neurons were evaluated per condition. (**B**) Technical controls for the PLA experiment to evaluate USP7-AR interaction in motor neurons from spinal cord sections in a KI SBMA mouse or a wild type mouse (see **Fig. 3C**) were carried out by omission of one antibody as indicated. (**C**) USP7 immunostaining in motor neurons from spinal cord sections of a 7-month-old KI SBMA or a wild type mouse. Forty motor neurons were evaluated per mouse. (**D**) Quantification of experiment in (**C**) was carried out as described in Methods. (**E**) Technical controls for the PLA experiment to evaluate USP7-AR interaction in iPS- derived motor neurons from SBMA patient (see **Fig. 3D**) were carried out by omission of one antibody as indicated.

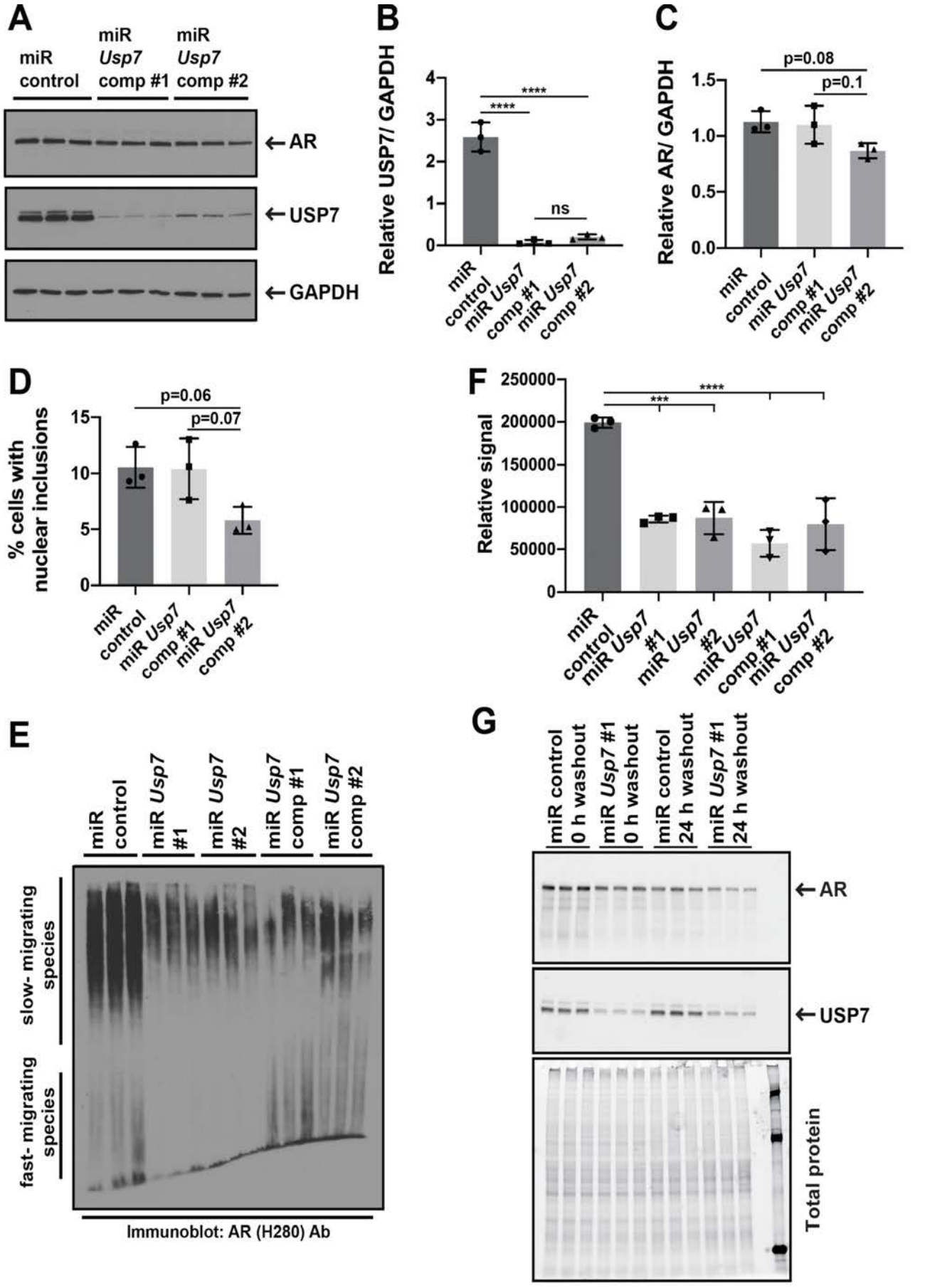


Figure S4, related to Figure 5. Complete knockdown of USP7 does not increase polyQ-expanded AR

aggregation in a cell model of SBMA. PC12 cells expressing AR112Q and constitutively expressing miRNAs targeting different regions of *Usp7* mRNA (miR *Usp7* #1 and miR *Usp7* #2) or non-targeting miRNA (miR control) were treated with 10 nM DHT for 48 hrs. (**A**) Cell lysates were analyzed for USP7 knockdown and AR levels; comp: complete (more than 90%) knockdown of USP7. (**B, C**) Quantification of USP7 and AR levels from **A**, respectively. (**D**) Percentage of cells with nuclear inclusions following 48 hrs treatment with 10 nM DHT. 500 cells were counted in triplicate per condition. Experiment was repeated 3 times. (**E**) Cell lysates from (**A**) were resolved on SDS-agarose followed by Western analysis with anti-AR antibody. (**F**) Quantification of the signal intensity of the slow migrating species from (**E**). Statistical significance was evaluated by one-way ANOVA with *post hoc* Tukey **p<0.01, ***p<0.001, ****p<0.0001. Experiments were repeated three times. Error bars represent SD. (**G**) AR level changes upon USP7 knockdown was analyzed by Western blot (see **Fig. 5F** for quantification).

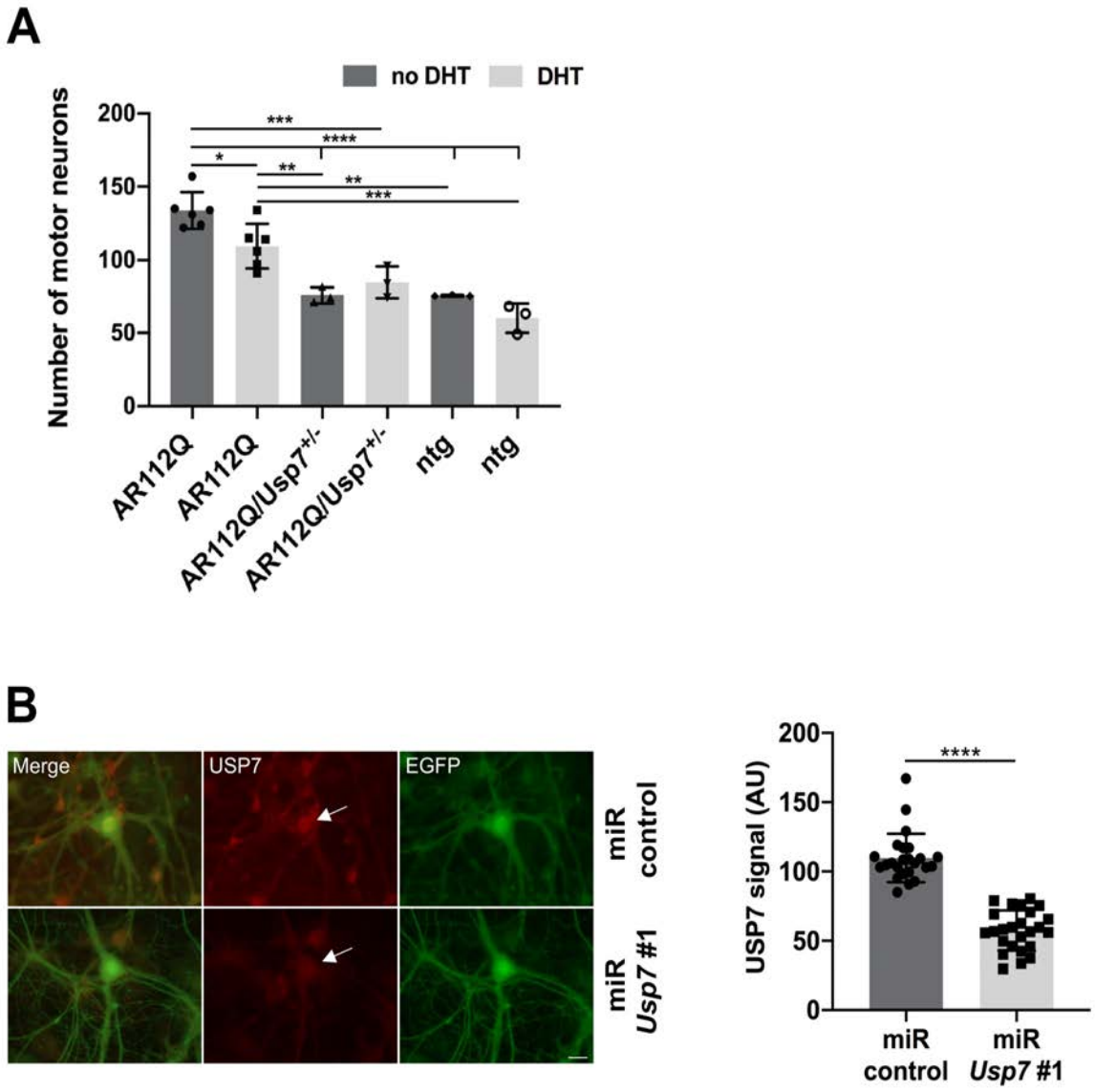


Figure S5, related to Figure 6. (**A**) Haploinsufficiency of *Usp7* rescues DHT induced toxicity in AR112Q-expressing motor neurons. Example of one experiment, in which dissociated spinal cord cultures from transgenic AR112Q (n=6), AR112Q/*Usp7*$^{+/-}$ (n=3) or non-transgenic (ntg) (n=3) mouse embryos were treated with 10 μM DHT or vehicle (ethanol) for 7 days and motor neurons were identified both by morphology and by immunostaining for neurofilament-heavy chain using SMI32 antibody. Motor neurons from 10 randomly selected fields were counted for each experimental condition. Statistical significance was evaluated by one-way ANOVA with *post hoc* Tukey *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars represent SD. (**B**) Dissociated spinal cord cultures from mouse embryos of wild type mice were infected with AAV1 expressing either miR control or miR *Usp7* #1 for 5 days, followed by ethanol treatment for additional 7 days. Levels of USP7 was evaluated in total of 24 or 25 motor neurons (from two biological replicates) treated with miR control or miR *Usp7* #1, respectively. Representative images are show on the left, with quantification on the right. The statistical significance was evaluated by one-tailed Student's t-test ****p<0.0001. Error bars represent SD.

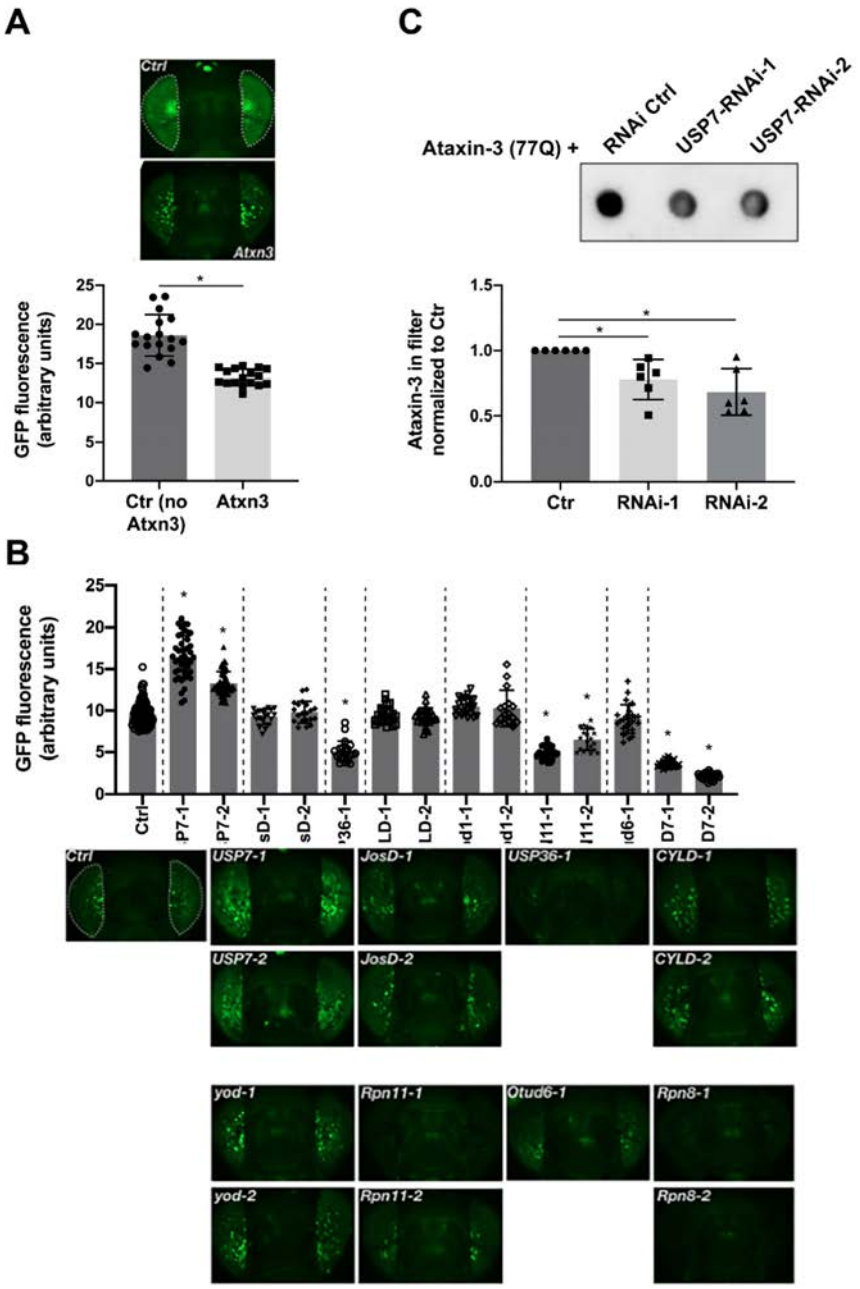


Figure S6, related to Figure 7. USP7 knockdown rescues SCA3 phenotype in a fly model. (**A**) Representative images of fly eyes expressing CD8-GFP or CD8-GFP and Ataxin-3 77Q. Normalized GFP fluorescence intensity for both fly lines is shown. Reduced GFP fluorescence is indicative of eye degeneration. Error bars represent SD. Statistical significance was determined using one-tailed Student's t-test, comparing Atxn3 eyes to Ctrl. (**B**) Normalized CD8-GFP fluorescence intensity for Ataxin-3 77Q expressing flies subjected to knockdown of the indicated gene. Error bars represent SD. Statistical significance was determined using one-tailed Student's t-test, comparing each RNAi line to its respective control. Representative images are presented below. (**C**) Effect of USP7 knockdown on Ataxin-3 77Q aggregation in a fly model of SCA3 was evaluated by filter-trap assay followed by western blot analysis with an anti Ataxin-3 antibody (see Methods). Representative Western blot image with quantification from 6 experiments is presented, with error bars representing SD. Statistical significance was determined using one-tailed Student's t-test, comparing each RNAi line with control.

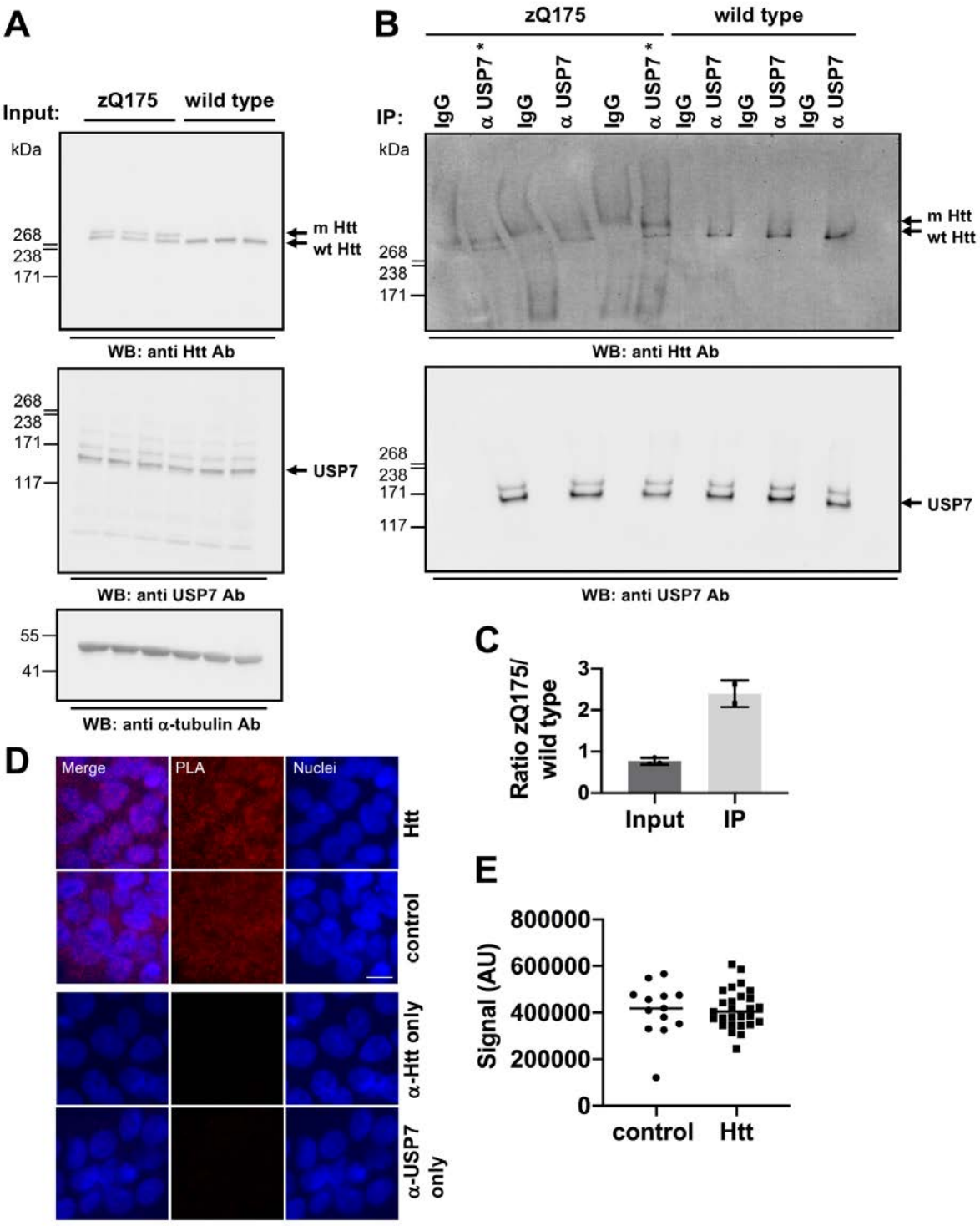


Figure S7, related to Figure 7. USP7 preferentially interact with polyQ-expanded huntingtin in a knock- in mouse model of Huntington's disease. Protein lysates from striata and cortex of three 6-months old zQ175 mice or three 6-months old wild type mice (**A**) were subjected to immunoprecipitation with an anti-USP7 antibody, followed by immunoblotting with anti-Htt or anti-USP7 antibodies (**B**). (**C**) The ratio of mutant to wild type huntingtin in lysates from three zQ175 mice (Input (**A**)) as well as from USP7-associated huntingtin (IP) (lanes marked with * were considered for quantification, the third sample was not used for quantification due to diffused signal). (**D**) Htt-USP7 interaction was evaluated in HD-patient derived iPS cells (expressing Htt 109Q) or control iPS cells (expressing Htt 21Q) by proximity ligation assay. Representative images are shown, with PLA intensity quantification in (**E**). Due to a large number of puncta

per cell and a high cell density, we presented signal intensity by measuring corrected total cell fluorescence (CTCF) (see Methods). Thirteen and twenty-seven images were evaluated for control and Htt, respectively. The number of cells per image varied from 39 to 156.

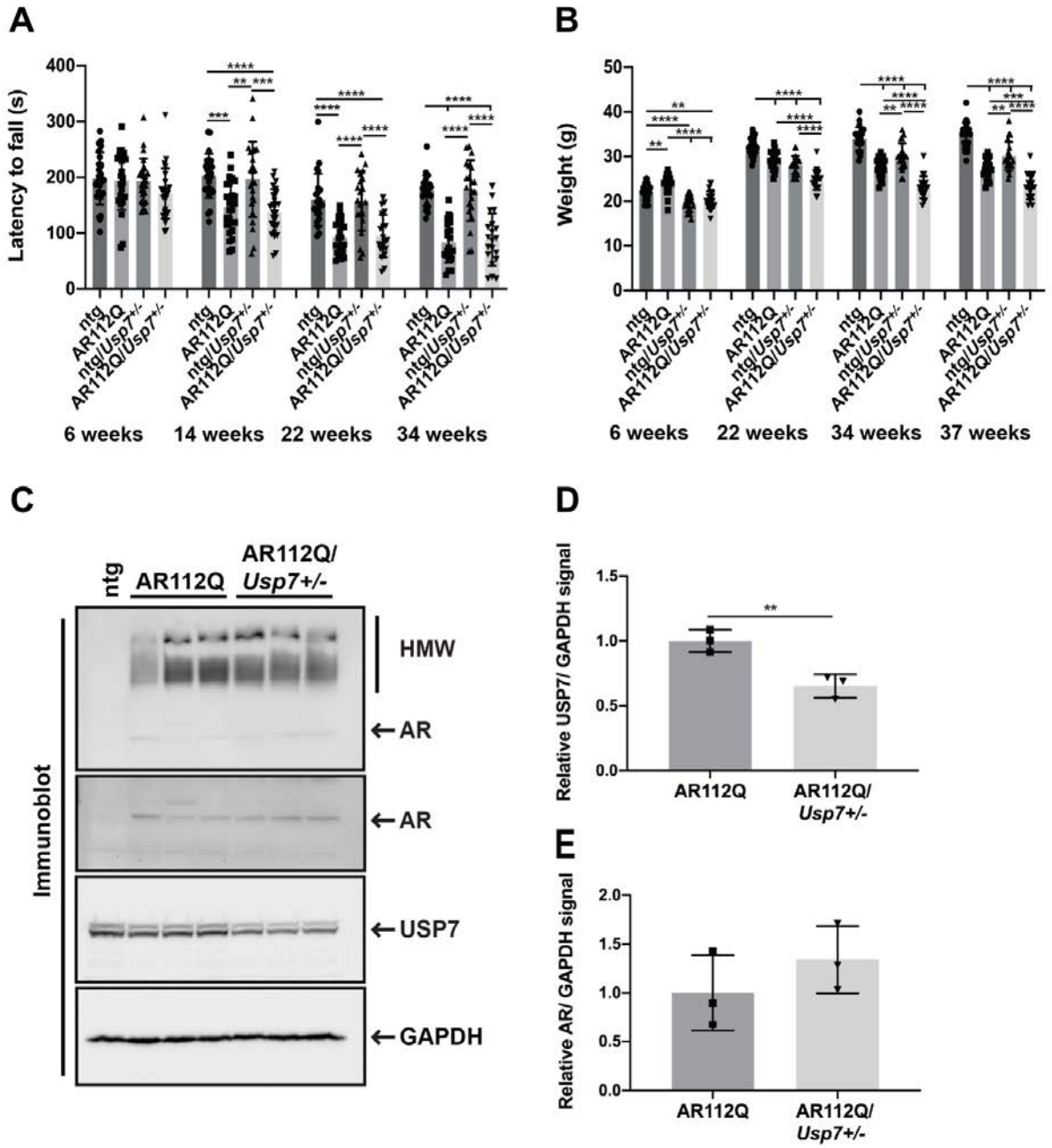


Figure S8, related to Figure 8. (**A**) AR112Q, AR112Q/*Usp7*$^{+/-}$, ntg/*Usp7*$^{+/-}$ and ntg male mice (n = 25 per cohort) were evaluated every 4 weeks on accelerating Rotarod starting at the age of 6 weeks. By the age of 14 weeks, AR112Q (n = 25) and AR112Q/*Usp7*$^{+/-}$ (n = 25) mice displayed rotarod deficits in comparison to ntg (n = 25) and ntg/*Usp7*$^{+/-}$ (n = 24) mice. This effect was also observed in mice at 22 weeks of age ((ntg (n = 22), AR112Q (n = 22), ntg/*Usp7*$^{+/-}$ (n = 21) and AR112Q/*Usp7*$^{+/-}$ (n = 20)) and in mice at 34 weeks of age ((ntg (n = 22), AR112Q (n = 21), ntg/*Usp7*$^{+/-}$ (n = 21) and AR112Q/*Usp7*$^{+/-}$ (n = 20)). (**B**) Mice were weighed every four weeks starting at 6 weeks of age. Ntg/*Usp7*$^{+/-}$ male mice and AR112Q/*Usp7*$^{+/-}$ male mice were significantly smaller than AR112Q male mice. Statistical significance was evaluated by one-way ANOVA with *post hoc* Tukey **p<0.01, ***p<0.001, ****p<0.0001. Error bars represent SD. (**C**) Western blot analysis of brain lysates from 18 week-old mice for the presence of AR and USP7. (**D, E**) Quantification of USP7 and AR protein levels from **C**. Statistical significance was evaluated by two-tailed Student' t-test **p<0.01. Error bars represent SD.

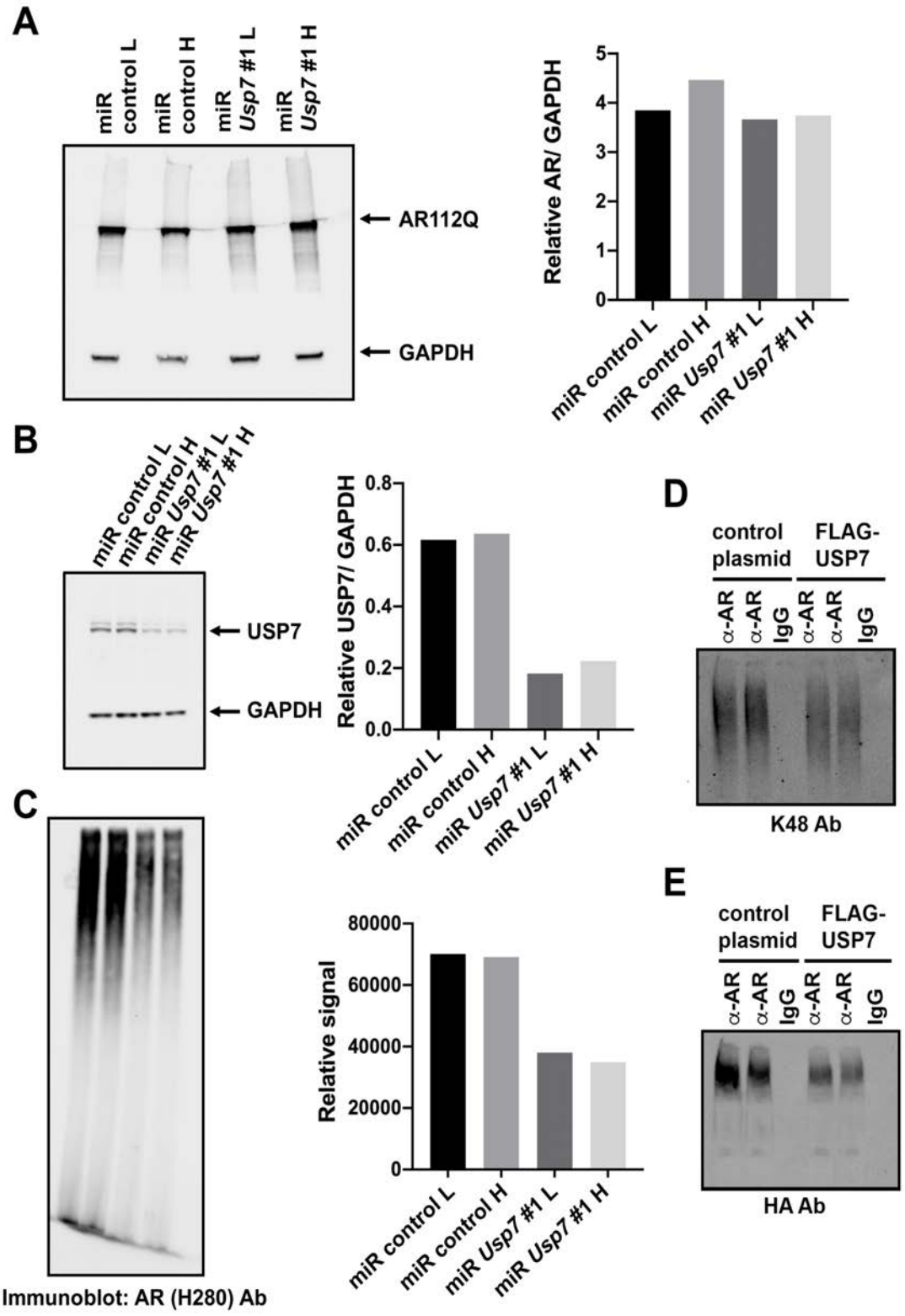


Figure S9, related to Figure 11 and Table S3. Protein lysates from experiment described in **Table S3** were resolved on SDS-PAGE, followed by immunoblot with (**A**) anti-AR antibody, (**B**) anti-USP7 antibody, or (**C**) resolved on SDS-agarose and immunostained with anti-AR antibody; quantifications are shown on the right. (**D, E**) Contrast enhanced images from **Fig. 11A**.

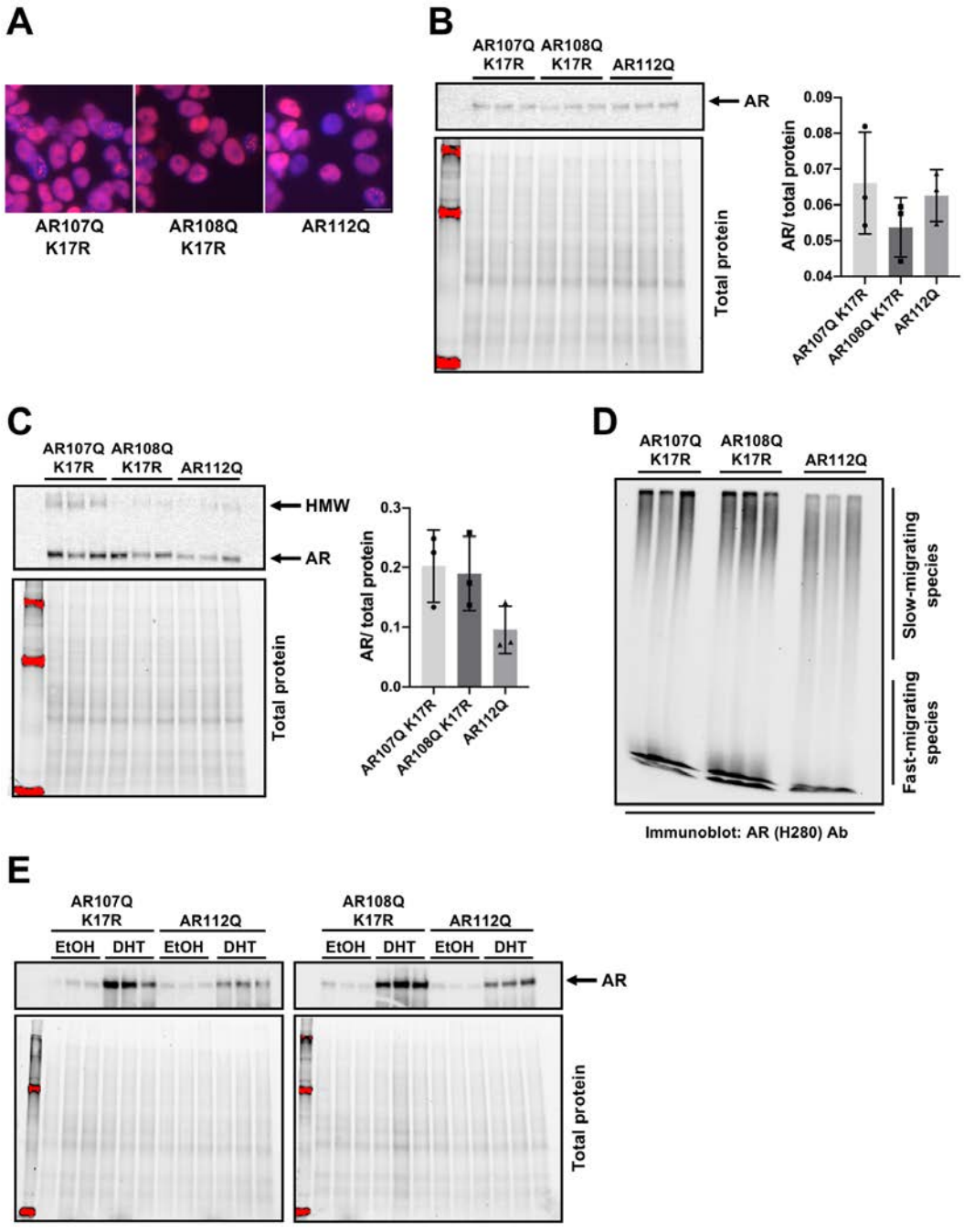


Figure S10, related to Figure 11 and Table S3. (**A**) Representative images of PC12 cells expressing AR112Q, AR107Q K17R or AR108Q K17R that were treated with DOX and DHT for 48 hrs followed by immunofluorescence with anti-AR antibodies to determine the percentage of cells with inclusions (see **Fig. 11D**). (**B**) PC12 cells expressing AR112Q, AR107Q K17R or AR108Q K17R were treated with DOX (but no DHT) for 24 hrs to evaluate overall AR expression. Protein lysates were analyzed by SDS-PAGE followed by Western blot analysis with anti-AR antibody, with quantification shown on the right. (**C**) Cell lysates from (**A**) were analyzed by SDS-PAGE followed by immunoblot with anti-AR antibody (with quantification on the right) or (**D**) by SDS-AGE followed immunoblot with anti-AR antibody. (**E**) To evaluate AR turnover, cells expressing AR112Q, AR107Q K17R, or AR108Q K17R were treated with DOX for 48 hrs to induce AR expression. Following DOX washout, cells were treated with cycloheximide and either DHT or vehicle (EtOH) for additional 24 hrs. AR levels were analyzed by western blotting with quantification shown in **Fig. 11E**).

Supplemental Methods

Cell culture

Tet-on pheochromocytoma-derived rat PC12 cells expressing human full length androgen receptor harboring expanded polyQ tract (AR112Q) or non-expanded polyQ tract (AR10Q) (1) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin/streptomycin, 200 µg/mL hygromycin, and 100 µg/mL G418 sulfate. All experiments were performed using charcoal-stripped serum to remove serum-derived androgens. AR expression in cells was induced with 500 ng/mL doxycycline (Dox, Clontech) and AR activation was stimulated by treatment with 10 nM DHT (Sigma Aldrich). HEK293T cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin/streptomycin. All cells were maintained at 37°C, 10% CO_2.

Creation of PC12 cell lines with USP7 knockdown

Two sets of oligonucleotides (forward and reverse for each, Mmi461 and Mmi1954) containing miRNA sequences against *Usp7*, as well as non-targeting control sequences (obtained from Invitrogen), were annealed and cloned into pcDNA™ 6.2-GW/ EmGFP-miR (BLOCK-iT™), containing a blasticidin resistance gene, according to the manufacturer's recommendations. Inducible PC12 cells expressing AR112Q were stably transfected with these plasmids. Stable transformants were selected with (5 µg/mL) blasticidin. Single colonies were screened for USP7 levels by Western blotting using USP7 antibody (ab157132, Abcam).

Creation of PC12 cell lines expressing FLAG-USP7

PC12 cells expressing AR112Q were transfected with pCI-neo FLAG-HAUSP to express FLAG-USP7 under CMV promoter (pCI-neo FLAG HAUSP was a gift from Bert Vogelstein (Addgene plasmid # 16655; http://n2t.net/addgene:16655; RRID:Addgene_16655) or pBABE control plasmid using Lipofectamine 2000 (Invitrogen). Stable transfectants were selected with 10 µg/mL puromycin. Single colonies were screened for constitutive expression of FLAG-USP7 by immunoblotting using an anti-FLAG antibody (PA 1-984B, Thermo Scientific). Site-directed mutagenesis (QuikChange II, Stratagene) was

used to mutate FLAG-USP7 to the FLAG-USP7 C223S catalytic mutant. The mutation was confirmed by sequencing.

Creation of mutant AR cell lines

PC12 cells expressing poly-Q expanded AR K17R mutants were constructed as follows. Mutation of Lys-17 to arginine was performed by site-directed mutagenesis (QuikChange II, Stratagene) of pTRE-AR112Q. The mutation as well as CAG length were sequence verified. Tet-On PC12 cells (Clontech, Mountainview, CA) were transfected with mutant pTRE-AR plasmid and a hygromycin-resistance-conferring pTK-Hygro plasmid and selected with 200μg/mL hygromycin. Single colonies were isolated and screened for AR protein by inducing AR with 1μg/mL doxycycline. Genomic DNA was isolated from positive clones and sequenced to verify the mutation and CAG repeat length. The appropriate amount of doxycycline required to induce comparable AR levels was determined (see **Fig. S9B**).

PC12 cell toxicity assay

DHT-induced toxicity was quantified as described previously (2). Briefly, PC12 cells expressing AR112Q and either miR control or miR *Usp7* #1 were treated with 500 ng/mL doxycycline in the presence of 10 nM DHT or ethanol (vehicle) for 12 days. The percentage of dead cells was determined by counting the number of Trypan blue-positive cells in at least 200 cells per experimental condition. Three independent experiments were performed at least in triplicate. Experimenter was blinded to the experimental conditions.

Co-immunoprecipitation and immunoprecipitation assays

PC12 cells were pelleted in PBS and lysed in a lysis buffer (20 mM HEPES-NaOH, pH 7.9, 100 mM NaCl, 2 mM $MgCl_2$, and 0.1% Igepal CA-630 supplemented with EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH), 10 mM NaF, 200 μM Na_3VO_4, 10 nM okadaic acid, and 0.1% phenylmethylsulfonyl fluoride (diluted from a saturated stock solution prepared in isopropanol)). Lysates were incubated on ice for 30 min and cleared by centrifugation. Protein concentration was determined using a DC protein assay (Bio-Rad). One mg of lysate (at 1 mg/mL concentration) was incubated with 2 μg of an anti-AR antibody (BD Biosciences) or control mouse IgG (Santa Cruz Biotechnology) (crosslinked to Dynabeads M-270 Epoxy (Invitrogen)) overnight at 4°C with rotation. Similar immunoprecipitation

reactions were set up with an anti-USP7 antibody (ab157132, Abcam) or goat IgG (Santa Cruz Biotechnology). Bead-bound proteins were washed 3-4 times with lysis buffer and eluted with Laemmli buffer, resolved on SDS-PAGE, followed by immunoblot analysis.

Dissected frozen brains or spinal cords of two 10-week old SBMA male transgenic mice were pulverized and lysed in a lysis buffer containing 20 mM TRIS-HCl, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, and 0.1% Igepal CA-630 supplemented with EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH), 10 mM NaF, 200 μM Na_3VO_4, 10 nM okadaic acid, 0.1% phenylmethylsulfonyl fluoride (diluted from a saturated stock solution prepared in isopropanol) (as above) and 5 mM N-ethylmaleimide. Lysates were incubated at 4°C for 40 min with rotation and cleared by centrifugation. Two milligrams of brain or spinal cord lysate (at 4 mg/mL and 3 mg/mL concentration, respectively) were incubated with 10 μg of anti-USP7 antibody (ab157132, Abcam) or goat IgG (Santa Cruz Biotechnology) (crosslinked to Dynabeads M-270 Epoxy (Invitrogen) overnight with rotation. Beads were washed 3 times with lysis buffer and eluted with Laemmli buffer, resolved on 7.5% SDS-PAGE, followed by immunoblot analysis with anti-AR (H280, Santa Cruz Biotechnology) or anti-USP7 antibodies (ab157132, Abcam).

Frozen striata and cortex of HD or wild type mice were pulverized, mixed and lysed in a lysis buffer containing 20 mM HEPES-NaOH, pH 7.9, 100 mM NaCl, 2 mM $MgCl_2$, and 0.1% Igepal CA-630 supplemented with 2x Halt Protease and Phosphatase inhibitors (Thermo Fisher Scientific), 10 nM okadaic acid, 20 mM N-ethylmaleimide, and 0.1% phenylmethylsulfonyl fluoride (diluted from a saturated stock solution prepared in isopropanol). Lysates were incubated at 4°C for 40 min with rotation and cleared by centrifugation. Six hundred micrograms of lysate (at 2 mg/mL concentration) was incubated with 5 μg of anti-USP7 antibody (ab157132, Abcam) or goat IgG (Santa Cruz Biotechnology) (crosslinked to Dynabeads M-270 Epoxy (Invitrogen) overnight with rotation. Bead-bound proteins were washed 3 times with lysis buffer and bead-bound proteins were eluted with Laemmli buffer, resolved on 3-8% TRIS-acetate gel, followed by immunoblot analysis with anti-Huntingtin (ab109115, Abcam) (this antibody recognizes human Huntingtin sequence within aa 1-100) or anti-USP7 (ab157132, Abcam) antibodies.

Quantitative proteomics

For quantitative proteomics experiments, the proteomes of PC12 cells expressing AR112Q or

AR10Q were differentially labelled by growing the cells in medium (containing dialyzed FBS (Fisher Scientific PI88212), DMEM (PI89985) and dialyzed horse serum) supplemented with heavy isotope-labeled amino acids $^{13}C^{15}N$-lysine ("Lys-8") (P189988, Fisher Scientific) and $^{13}C^{15}N$-arginine ("Arg-10") (P189990, Fisher Scientific), or light $^{12}C^{14}N$-lysine (L8662, Sigma Aldrich) and $^{12}C^{14}N$-arginine (A8094, Sigma Aldrich) ("Lys-0" and "Arg-0, respectively) amino acids for at least 5 generations, respectively (schematic **Fig. S1**). A second experiment was carried out in reverse, with cells expressing AR112Q and AR10Q grown in light and heavy SILAC medium, respectively. Protein lysates (as described above) were mixed in a 1:1 ratio, followed by immunoprecipitation of AR with either an anti-AR (G122-434, BD Biosciences) or a polyQ-expanded and conformation-*specific* antibody (3B5H10, gift from Steven Finkbeiner, Gladstone Institute of Neurological Disease and UCSF) for each growth condition. For the third experiment, cells expressing AR112Q and AR10Q were grown in heavy and light SILAC medium, respectively. In this experiment, immunoprecipitation of AR was performed with 3B5H10 antibody. Immunoprecipitated proteins were digested with trypsin using the FASP method (in-solution digest on filters (3)) and analyzed by LC-MS/MS on a Q Exactive Plus mass spectrometer. MS/MS spectra were searched against a custom UniProt Rat database using MaxQuant 1.5.2.8. The proteins were identified and further filtered by removing the common contaminants and low confidence proteins (proteins identified by < 2 nonredundant (total razor+unique) peptides and < 2 total ratio count). The LC-MS/MS analyses were performed at the Proteomics Facility of the Wistar Institute in Philadelphia, PA (http://www.wistar.org/our-science/shared-facilities/proteomics-facility). Based on heavy/light ratio for AR peptides obtained by mass spectrometry analysis, the fold enrichment of the AR interacting proteins was corrected for the differences in recovery of AR using the anti-AR antibody.

For the ubiquitinome analysis, PC12 cells inducibly expressing AR112Q and constitutively expressing miR *Usp7* #1 or non-targeting miR control (see **Fig. 5**) were differentially labelled by growing the cells in SILAC medium supplemented with heavy isotope-labeled amino acids $^{13}C^{15}N$-lysine ("Lys-8") and $^{13}C^{15}N$-arginine ("Arg-10") (described in **Table S3** and **Fig. S9** as H), or light $^{12}C^{14}N$-lysine and $^{12}C^{14}N$-arginine ("Lys-0" and "Arg-0, respectively) (described in **Table S3** and **Fig. S9** as L) amino acids for at least 5 generations, respectively (as described above). The efficiency of incorporation of heavy amino acid

was measured by mass spectrometry to be greater than 96% (data not shown). Cells were treated with 10 nM DHT for 48 hrs and 10 μM MG132 for the last 6 hrs prior to harvest. Cells were lysed with a buffer composed of 8 M urea, 50 mM Tris-HCl pH 8.0, and 1 mM EDTA, supplemented with 1x Halt Protease and Phosphatase Inhibitor cocktail (Thermo Fisher Scientific), PMSF (1:1000 dilution from saturated stock in isopropanol), 10 nM okadaic acid and 5 mM N-ethylmaleimide. Equal amounts of protein lysates from both cell types (10 mg each) were mixed in 1:1 ratio and the ubiquitinome analysis was performed at Proteomics Facility of the Wistar Institute in Philadelphia, PA.

AR ubiquitination analysis

For AR ubiquitination studies, HEK293T cells were transfected with pcDNA3.1 3x NLS AR111Q, pRK5-HA-Ubiquitin-WT (from Ted Dawson (Addgene plasmid #17608; http://n2t.net/addgene:17608; RRID:Addgene_17608)), pCI-neo FLAG HAUSP (or pcDNA 3.1 GFP), respectively, using the calcium phosphate method or with Lipofectamine 2000 (Thermo Fisher Scientific). Cells were treated with 10 nM DHT for 48 hrs, with 10 μM MG132 added for the last 6 hrs prior to harvest. Cells were washed with PBS, lysed in 20 mM HEPES-NaOH, pH 7.9, 150 mM NaCl, 1 mM EDTA, 1% SDS with 2x Halt Protease and Phosphatase Inhibitor cocktail (Thermo Fisher Scientific), 10 nM okadaic acid and 20 mM N-ethylmaleimide (Acros Organics) and boiled for 10 min. Cooled lysates were then sonicated using a Branson cup sonifier, diluted 10 fold with dilution buffer (20 mM HEPES-NaOH, pH 7.9, 150 mM NaCl, 2 mM EDTA, 0.4% Triton X-100 supplemented with 1x Halt protease and phosphatase inhibitor cocktail, 10 nM okadaic acid and 20 mM NEM), followed by addition of Pierce Universal Nuclease for Cell Lysis (88701, Thermo Fisher Scientific) according to manufacturer's instructions and incubated for 1 hr at 4°C with rotation, and the lysates were cleared by centrifugation. One mg of cell lysate (at 0.5 mg/mL concentration) was incubated with 4 μg of anti-AR antibody (BD Biosciences) or control mouse IgG (crosslinked to Dynabeads M-270 Epoxy (Invitrogen)) overnight at 4°C with rotation. Beads were washed three times with dilution buffer (5 min each), followed by 2 washes (5 min each) with 20 mM HEPES- NaOH, pH 7.9, 1 M NaCl, 1 mM EDTA, 1% Igepal CA-630 with 1x Halt protease and phosphatase inhibitor cocktail, 10 nM okadaic acid and 20 mM N-ethylmaleimide). Immunoprecipitated proteins were resolved by 4-20% SDS-PAGE (Invitrogen) followed by immunoblotting to detect K48-linked ubiquitin chains with anti-K48 linkage

specific antibody (4289S, Cell Signaling Technology). The immunoblot was then stripped of the primary antibody and re-probed with an anti-HA tag antibody (Santa Cruz Biotechnology) to detect total ubiquitin, and finally re-stripped and re-probed with an anti-AR antibody (H280, Santa Cruz Biotechnology).

Immunostaining

PC12 cells expressing AR112Q under various experimental conditions were plated on poly-D-lysine coated glass coverslips and treated with 500 ng/mL doxycycline and 10 nM DHT for 48 hrs. Cells were fixed with 4% paraformaldehyde, immunostained with an anti-AR antibody (H280, Santa Cruz Biotechnology) or anti-USP7 (ab157132, Abcam), and visualized and imaged using a Leica DMR Fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) and iVision Mac® or ProgRes® software. To evaluate the percentage of cells with nuclear inclusions, cells from various experimental conditions were immunostained with an anti-AR antibody (H280, Santa Cruz Biotechnology). Five hundred cells were examined per cover slip with 3 cover slips per experimental condition. Each experiment was repeated 3 times. Experimenter was blinded to the experimental conditions.

Seven μm spinal cord sections were fixed in 4% paraformaldehyde for 20 minutes, then washed in PBS and treated with TrueBlack® Liposfuscin Autofluorescence Quencher (Biotium). Sections were immunostained with SMI32 antibody (1:1000, Sternberger Monoclonals) and anti-mouse IgG_1 594 (Invitrogen, 1:200), and visualized and imaged as above. Images were analyzed using ImageJ (NIH). The experimenter was blinded to the experimental conditions.

Seven μm spinal cord sections were fixed as described above. Sections were immunostained with anti USP7 antibody (ab157132, Abcam) and counterstained with SMI32 antibody, visualized and imaged using EVOS M7000 system (ThermoFisher Scientific). Images were analyzed using ImageJ (NIH). USP7 signal intensity was evaluated by calculating corrected total cell fluorescence (CTCF) (CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)) in each cell. Forty motor neurons were evaluated per condition. The experimenter was blinded to the experimental conditions.

Proximity ligation assays

PC12 cells expressing AR112Q and either non-targeting control or USP7-targeting miRNA were plated on poly-D-lysine coated glass coverslips and treated with 500 ng/mL doxycycline and 10 nM DHT for 24 hrs. Duolink® PLA Fluorescence was performed with primary antibodies against total AR (AR-318-CE, Leica Biosystems) and Ub (DAKO) according to the manufacturer recommended protocol (DUO92008, Sigma-Aldrich). Images were acquired with the same exposure time and the number of puncta per cell was counted, with 120 cells being analyzed per condition. The experiment was repeated 3 times.

In order to evaluate USP7-AR interaction, PC12 cells expressing AR10Q or AR112Q were treated with 500 ng/mL doxycycline and ethanol or with 500 ng/mL doxycycline and 10 nM DHT for 48 hrs. Duolink® PLA Fluorescence was performed with primary antibodies against total AR (AR-318-CE, Leica Biosystems) and USP7 (ab190183, Abcam). Images were acquired with the same exposure time and the number of puncta per cell was counted in 100 cells per experimental condition. The experiments were done in triplicate. Subsequently, the slides were immunostained with anti-AR antibody (H280, Santa Cruz Biotechnology) to visualize total AR.

To evaluate Huntingtin-USP7 interaction, we used HD-patient derived iPS cells (CS09iHD-109n1, Cedars Sinai) and control iPS cells (CS14iCTR-21n3, Cedars Sinai). The growth and maintenance of these cells is done at Jefferson Stem Cell and Regenerative Neuroscience Center. Cells were grown in 96 well plate. Duolink® PLA Fluorescence was performed with primary antibodies against Huntingtin (ab109115, Abcam) and USP7 (ab157132, Abcam) as described above. Due to a large number of puncta per cell and a high cell density, we presented signal intensity by measuring corrected total cell fluorescence (CTCF) (CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)) of the image taken (only area with the PLA signal was considered for quantification) and divided by the number of cells per image (based on nuclear staining). Thirteen and twenty-seven images were evaluated for control and Htt, respectively. The number of cells per image varied from 39 to 156.

AR-USP7 interaction was also evaluated in knock-in mouse model of SBMA. Seven μm spinal cord sections from 7-month-old knock-in (KI) SBMA or wild type male mice were fixed in 4% paraformaldehyde for 20 minutes followed by 3 washes in PBS. PLA fluorescence was performed with anti-AR antibody

(clone 3K11, ZRB1150, Sigma-Aldrich) and anti-USP7 antibody (ab157132, Abcam) as described above, followed by staining with SMI32 antibody (Sternberger Monoclonals). Slides were treated with TrueBlack® Liposfuscin Autofluorescence Quencher before analysis. Thirty-three motor neurons were evaluated per mouse. Due to a large number of puncta per cell, presented PLA signal intensity by calculating corrected total cell fluorescence (CTCF) (CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)) per each cell.

USP7-AR interaction in iPS-derived motor neurons from an SBMA patient was evaluated by PLA using anti-AR Ab (H280, Santa Cruz Biotechnology) and anti-USP7 (ab157132, Abcam), followed by staining with anti- Beta III tubulin antibody (ab 52623, Abcam).

Images were taken either with Leica DMR Fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) and iVision Mac® or ProgRes® software or EVOS M7000 system (ThermoFisher Scientific). Images were analyzed using ImageJ (NIH). For all PLA experiments, experimenter was blinded to the experimental conditions.

Immunoblot analysis

Cells were lysed in SDS lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2% SDS with protease inhibitors) and sonicated. Protein concentration was determined by DC protein assay (Bio-Rad). Ten or twenty μg of lysates was separated by electrophoresis on 10% SDS-PAGE and transferred onto 0.45 μm PVDF membrane (Immobilon-P). The membrane was blocked for 1 hr at room temperature with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20 containing 5% milk solids (all washes were performed in this buffer). After overnight incubation at 4°C with primary antibody, the membrane was washed and incubated for one hour with peroxidase-conjugated secondary antibody (anti-mouse AP308P Millipore; anti-rabbit AP307B, Millipore; anti-goat sc-2020 Santa Cruz Biotechnology). Data were visualized using the ECL system (Thermo Fisher) using ChemiDoc MP (Bio-Rad) or exposure to X-ray film.

Antibodies used include: AR (H280, Santa Cruz Biotechnology), USP7 (ab157132, Abcam), HA (Santa Cruz Biotechnology), α-tubulin (21445, Cell Signaling Technology), K48-ubiquitin (4289S, Cell

Signaling Technology), GAPDH (10R-G109A, Fitzgerald), Ataxin-3 (1H9; Millipore MAB5360), and Huntingtin (ab109115, Abcam).

SDS-agarose gel electrophoresis (SDS-AGE)

PC12 cells were lysed in SDS lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2% SDS with protease inhibitors) and sonicated. Twenty to fifty μg of protein lysate was diluted in non-reducing Laemmli sample buffer, boiled for 5 minutes and electrophoresed through a 1% agarose gel containing 0.1% SDS (in 375 mM Tris-HCl, pH 8.8), at 40-50 V at 4°C. Lysates were then transferred onto PVDF membrane at 200 mA for 3 hrs using a semi-dry transfer apparatus (Owl HEP Semidry Electroblotting Systems, Thermo Scientific), followed by immunoblot analysis using an anti-AR antibody (H280, Santa Cruz Biotechnology).

AR turnover experiments

PC12 cells expressing AR112Q and either miR control or miR *Usp7* #1 were treated with DOX for 48 hrs to induce AR expression. At this point, doxycycline was washed out and cells were either collected and lysed (0 h washout) or treated with 10 nM DHT and 50 μg/mL cycloheximide for additional 24 hrs (24 h washout) (**Fig. 5F** and **Fig. S4G**). Cell lysates were resolved on a stain-free SDS-PAGE (BioRad), followed by immunoblot with anti-AR antibody (H280, Santa Cruz Biotechnology). AR signal was quantified relative to the total protein signal. AR turnover in PC12 cells expressing AR112Q, AR108Q K17R or AR107Q K17R was determined in a similar manner, except that after DOX washout, the cells were treated with 50 μg/mL cycloheximide and either 10 nM DHT or EtOH for additional 24 hours and cells were collected at that point (**Fig. 11E** and **Fig. S10E**). Each experiment was performed in triplicate and the experiment was repeated 3 times.

Analysis of mouse motor function

Mice expressing full length human AR112Q under transcriptional control of the prion protein (PrP) promoter (4) were crossed with *Usp7*$^{+/-}$ mice (5) to create four cohorts of male mice: AR112Q (n = 25), AR112Q/*Usp7*$^{+/-}$ (n = 25), non-transgenic/*Usp7*$^{+/-}$ (ntg/*Usp7*$^{+/-}$, n = 25) and non-transgenic (ntg, n = 25). At 18-weeks of age, 3 mice were removed from each cohort for pathology and biochemical analyses. Motor function of mice from all four cohorts was assessed using an accelerating (4–40 rpm over 10 min) rotarod

(UgoBasile). Rotarod training was initiated at 6 weeks of age and consisted of 4 trials per day for 3 consecutive days; with the results (latency to fall) from the third day used for analysis. Mice were tested once every 4 weeks (4 trials per mouse with at least one hour between trials).

Grip strength was evaluated using a grip strength meter (Columbus Instruments), which measured the maximum force (kg) exerted by a mouse as it was pulled across a wire grid by its tail. Grip strength of all paws was measured once every four weeks starting at 6 weeks of age. Six measurements were recorded for each animal, the highest and lowest values excluded, and the remaining 4 values averaged.

The beam-walking test measured the time required to cross an 80-cm long and 12-mm wide beam elevated 50 cm from the ground. Mice at 33 weeks of age were tested for 3 consecutive days (3 trials per mouse). The shortest duration to cross the beam from the third day was used for analysis. All mice received a gentle nudge to encourage them to start walking along the beam. If the mouse stopped in the middle of the beam, it received one additional nudge.

Clasping tendency of 30-week old mice was determined by suspending the animal by the tail for 30 seconds. Scoring criteria used were: 0 (no clasping), 1 (one limb touching the abdomen for more than 15 sec, or two limbs touching the abdomen for less than 15 sec), 2 (two limbs touching the abdomen for more than 15 sec), and 3 (four limbs touching the abdomen for more than 15 sec). Significance was determined using one-way ANOVA and *post-hoc* Tukey analysis for all the motor function assessments.

For all experiments, the experimenter was blinded to the mouse genotype.

Dissociated spinal cord cultures

Dissociated spinal cord cultures were established as previously described (2). Briefly, spinal cords were dissected from embryonic day 13.5 PrP-AR112Q transgenic, AR112Q/U*sp7*$^{+/-}$, ntg/*Usp7*$^{+/-}$ and non-transgenic (ntg) mice, were dissociated with trypsin, and plated on poly-D-lysine coated coverslips in 24-well plates. Cultures were grown in glia-conditioned media containing minimal essential medium, 3% charcoal-stripped horse serum, 35 mM $NaHCO_3$, 0.5% dextrose, 1% N3, and 10 nM 2.5S nerve growth factor for 3 weeks. Cultures were then treated with either ethanol (vehicle) or 10 μM DHT for 7 days. Motor

neurons were identified by the presence of unphosphorylated neurofilament-heavy chain (SMI32 immunoreactivity) and morphology. Experimenter was blinded to the experimental conditions.

Spinal cord cultures from embryonic day 13.5 knock-in AR113Q or wild type mice, were initiated as described above. After 3 weeks, cultures were infected with AAV1 miR control or AAV1 miR *Usp7* #1 for 5 days, followed by treatment with 10 μM DHT or ethanol (vehicle) for 7 days. Infectivity was >98% as judged by EGFP signal. Motor neurons were identified by the presence of unphosphorylated neurofilament-heavy chain (SMI32 immunoreactivity) and morphology. USP7 knockdown was evaluated by immunostaining with anti-USP7 Ab (ab157132, Abcam) of wild type neuronal cultures infected either with AAV1 miR control or AAV1 miR *Usp7* #1 and treated with ethanol. USP7 intensity in motor neurons was evaluated from two replicates of the experiment (total of 24 or 25 motor neurons were evaluated from cultures infected with AAV1 miR control and AAV1 miR *Usp7* #1, respectively).

Generating adeno-associated virus

The *cis*-adeno-associated viral (AAV) plasmid (serotype 1), with CMV enhancer and chicken β-actin promoter, was digested with EcoRV and BamH1 and the ends blunted with Klenow. pcDNA™ 6.2-GW/ EmGFP-miR (BLOCK-iT™) plasmid containing miR control or miR *Usp7* #1 (construction of these plasmids described above) were digested with XhoI and partially digested with DraI, the ends filled in with Klenow, then ligated to the cis plasmid vector. Generated plasmids were co-transfected into HEK293T cells with the trans (H21) and adenovirus helper (pfΔ6) plasmids using calcium phosphate. Four days after transfection, the cells were harvested and the AAV1 miR control or AAV1 miR *Usp7* #1 viruses were purified using AAVproR purification kit (Takara Bio USA).

Drosophila stocks and assays

Fly husbandry was conducted in diurnal, controlled environments at 25°C and ~60% humidity. All offspring were heterozygous for driver (GMR-Gal4) and UAS transgenes (AR, Ataxin-3, RNAi, as indicated in figures and legends). Fly media was supplemented with EtOH or DHT (5 mM final concentration). The UAS-AR52Q transgenic line was a generous gift of Dr. J. Paul Taylor, St. Jude's Children's Research Hospital. GMR-Gal4 was Bloomington *Drosophila* Stock (BDSC) #8121. UAS-CD8-GFP was stock number

5130 from BDSC. UAS-Ataxin-3(77Q) was described previously (6, 7). RNAi lines were as follows: USP7-1: Stock number 18231 from the Vienna *Drosophila* RNAi Center (VDRC). USP7-2: #110324 from VDRC. JosD-1: #7113 from VDRC. JosD-2: #108379 from VDRC. Usp36: #11152 from VDRC. Cyld-1: #15340 from VDRC. Cyld-2: #101414 from VDRC. Rpn11-1: #19272 from VDRC. Rpn11-2: #33662 from BDSC. Rpn8-1: #108573 from VDRC. Rpn8-2: # 26183 from VDRC. Otud6b: #105469 from VDRC. Controls for RNAi were the background lines used to generate the RNAi transgenics. For histology, adult fly wings and proboscises were removed and bodies were fixed overnight in 2% glutaraldehyde/2% paraformaldehyde in Tris-buffered saline supplemented with 0.1% Triton X-100. Fixed bodies were then dehydrated in a series of 30, 50, 75, and 100% ethanol/propylene oxide solution, subsequently embedded in Poly/Bed812 (Polysciences), then sectioned at 5 μm. Sections were finally stained with toluidine blue and imaged in an Olympus BX53 microscope at 20X magnification. Measurement of separation of the lamina from the retinal array was conducted with ImageJ by drawing a line perpendicular to the lamina towards the bottom-most portion of the array, at the point where the distance was longest visually. For western blotting, dissected fly heads were homogenized in hot SDS buffer, boiled, sonicated, centrifuged at room temperature and loaded onto SDS-PAGE gels.

Filter-trap assay

Ten dissected adult fly heads per group were mechanically disrupted in 200 μL NETN lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40), supplemented with protease inhibitor cocktail (PI; Sigma-Aldrich S-8820). Lysates were diluted with 200μL PBS containing 0.5% SDS, sonicated at 50% for 15 seconds, and centrifuged at 4,500 x g for 1 minute at 4˚C. One hundred μL of the resulting supernatant was further diluted with 400 μL PBS, and 30 μL of each sample was filtered-vacuumed using a Bio-Dot apparatus (Bio-Rad) through a 0.45 μm nitrocellulose membrane (Schleicher & Schuell) that was pre-incubated with 0.1% SDS in PBS. Membrane was rinsed twice with 0.1% SDS in PBS, blocked in 5% milk in TBS-Tween 20 and incubated with HRP-conjugated, anti-Ataxin-3 antibody (mouse monoclonal 1H9; Millipore MAB5360) for Western blotting.

HD mouse model

Heterozygous zQ175 male and female mice expressing a knocked-in chimeric human/mouse exon

1 containing approximately 190 CAG repeats within the endogenous murine *HTT* gene were used in this study (8). These zQ175 mice were descendants from Jackson Laboratories animals maintained on a C57B6/J background. Mice were genotyped using primers specific for human HTT. Animals were housed in enriched, temperature-controlled environments with a 12-hr light/dark cycle. Food and water were provided ad libitum. Six-months old animals were anesthetized with ketamine (100 mg/kg) and Xylazine (10 mg/kg) and were perfused with cold saline. Their striatum and cortex were promptly collected and flash frozen in liquid nitrogen. Animal's euthanasia was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at The Children's Hospital of Philadelphia.

Differentiation of SBMA patient iPSCs to lower motor neurons

SBMA patient-derived iPSCs, previously transfected with an hNIL cassette (9), were acquired from Dr. K.H. Fischbeck and differentiated as described (9, 10). Briefly, patient-derived iPSCs were plated on Matrigel-coated 10-cm dishes at $1.5x10^6$ cells per dish in mTeSR1 (Stem Cell Technologies) + 10μM ROCK inhibitor. After 24 hours, cells were induced to differentiate by addition of Neuronal Induction Media (DMEM F12 with HEPES, 1X N2 supplement (Gibco), 1X non-essential amino acids (Gibco), 1X L-glutamine (Gibco), 10μM ROCK inhibitor (Tocris), 2ug/mL doxycycline (Sigma), 1X Compound E (Calbiochem)). After 48 hours in Neuronal Induction Media, cells were re-plated in a 96-well polymer-bottom, PDL- and laminin-coated tissue culture dish at $1x10^4$ cells/well in Neuronal Induction Media. 24 hours after re-plating, Neuronal Induction Media was replaced with Motor Neuron Media (Neurobasal medium (Gibco), 1X B27 supplement (Gibco), 1X N2 supplement (Gibco), 1X non-essential amino acids (Gibco), 1X L-glutamine (Gibco), 1ug/mL laminin (Gibco), 10μM ROCK inhibitor (Tocris), 1X Compound E (Calbiochem), 2X CultureOne (Gibco), 2ug/mL doxycycline (Sigma)). On day 7 post-induction, 72 hours after switching into Motor Neuron Media, cells were treated with 10 μM dihydrotestosterone (DHT) for 24 hours.

Statistical analysis

For multiple comparisons, statistical significance was determined by one-way analysis of variance (ANOVA) with *post-hoc* Tukey test. Comparisons between two experimental conditions were done by one-

tailed Student's t-test, unless otherwise indicated. For comparison of distributions, statistical significance was determined by the Kolmogorov-Smirnov test. A P value less than 0.05 was considered significant. Data were analyzed using Prism 7 (GraphPad).

Supplemental References

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