Polyglutamine-expanded androgen receptor interferes with TFEB to elicit autophagy defects in SBMA

Constanza J Cortes1,10, Helen C Miranda1,2,10, Harald Frankowski1, Yakup Batlevi1, Jessica E Young2, Amy Le1, Nishi Ivanov3,4, Bryce L Sopher4, Cassiano Carromeu1, Alysson R Muotri1,2,5–8, Gwenn A Garden3,4 & Albert R La Spada1,2,5–9

Macropautophagy (hereafter autophagy) is a key pathway in neurodegeneration. Despite protective actions, autophagy may contribute to neuron demise when dysregulated. Here we consider X-linked spinal and bulbar muscular atrophy (SBMA), a repeat disorder caused by polyglutamine-expanded androgen receptor (polyQ-AR). We found that polyQ-AR reduces long-term protein turnover and impaired autophagic flux in motor neuron–like cells. Ultrastructural analysis of SBMA mice revealed a block in autophagy pathway progression. We examined the transcriptional regulation of autophagy and observed a functionally significant physical interaction between transcription factor EB (TFEB) and AR. Normal AR promoted, but polyQ-AR interfered with, TFEB transactivation. To evaluate physiological relevance, we reprogrammed patient fibroblasts to induced pluripotent stem cells and then to neuronal precursor cells (NPCs). We compared multiple SBMA NPC lines and documented the metabolic and autophagic flux defects that could be rescued by TFEB. Our results indicate that polyQ-AR diminishes TFEB function to impair autophagy and promote SBMA pathogenesis.

As postmitotic cells incapable of rapid self-renewal, neurons are exquisitely susceptible to different types of cell stress; hence, maintenance of protein quality control poses a special challenge for CNS cells1–2. Autophagy is dedicated to recovery from nutrient stress in non-neural cells, but in neurons it has been adapted to degrade misfolded proteins and dysfunctional organelles5. The current view of neuronal autophagy is that of a prosurvival response that counters aggregate-prone proteins, as basal autophagy is crucial for normal CNS function6,7 and autophagy can promote clearance of aggregate-prone proteins and improve disease in mouse and fly models of neurodegeneration8,9. The autophagy pathway involves the de novo synthesis of a double-membrane-bound structure, the autophagosome, that sequesters cytoplasmic contents and delivers them to the lysosome for degradation8. Studies done in yeast have shown that initiation and promotion of autophagy are regulated by a family called Atg genes that respond to specific signals8. In non-neural cells, nutrient deprivation drives the autophagy pathway (‘starvation-induced autophagy’), but in neurons, upregulation of ‘quality control’ autophagy occurs in response to accumulating aggregate-prone proteins that impair the ubiquitin–proteasome system10.

Neurons exhibit a high rate of basal autophagy, and lysosome-mediated autophagosome turnover occurs rapidly11,12. In neurodegenerative disease, autophagic vesicles often accumulate, indicating a pronounced impairment in neuronal autophagy. For example, in Alzheimer’s disease, immunoelectron microscopy analysis of patient brains reveals striking accumulations of autophagic vesicles in the cell bodies and axons of cortical neurons13. Similar alterations in the autophagy pathway have been reported in Huntington’s and Parkinson’s diseases14. This suggests that autophagy dysfunction is a common feature in neurodegenerative proteinopathies. However, whether autophagic vesicle accumulations reflect an initial neuroprotective response or result from defective autophagy induction and progression remains unclear. Indeed, autophagy itself is a target in neurodegeneration, as a number of disease proteins, such as huntingtin, α-synuclein and presenilins, are directly involved in key steps of the autophagy pathway, including cargo recognition, autophagosome–lysosome fusion and autophagosome cargo clearance14, suggesting that loss of autophagy function is a central feature of neurodegenerative disease.

In 2009, the coordinated lysosomal enhancement and regulation (CLEAR) gene network emerged as a nucleus-to-lysosome signaling axis that controls lysosome function15. The basic helix-loop-helix transcription factor EB (TFEB) acts as a master regulator of the CLEAR network, and its targets include genes that encode Atg proteins, vesicular trafficking proteins, lysosomal enzymes and lysosomal structural proteins15,16. Thus, TFEB is a key node in the transcriptional regulation of the autophagy–lysosome pathway, and numerous studies have documented that modulation of TFEB activity can ameliorate neurodegenerative disorders caused by polyglutamine-expanded androgen receptor (polyQ-AR). We found that polyQ-AR reduced long-term protein turnover and impaired autophagic flux in motor neuron–like cells. Ultrastructural analysis of SBMA mice revealed a block in autophagy pathway progression. We examined the transcriptional regulation of autophagy and observed a functionally significant physical interaction between transcription factor EB (TFEB) and AR. Normal AR promoted, but polyQ-AR interfered with, TFEB transactivation. To evaluate physiological relevance, we reprogrammed patient fibroblasts to induced pluripotent stem cells and then to neuronal precursor cells (NPCs). We compared multiple SBMA NPC lines and documented the metabolic and autophagic flux defects that could be rescued by TFEB. Our results indicate that polyQ-AR diminishes TFEB function to impair autophagy and promote SBMA pathogenesis.

1 Department of Pediatrics, University of California, San Diego, La Jolla, California, USA. 2 Department of Cellular & Molecular Medicine, University of California, San Diego, La Jolla, California, USA. 3 Center on Human Development & Disability, University of Washington, Seattle, Washington, USA. 4 Department of Neurology, University of Washington, Seattle, Washington, USA. 5 Department of Neurosciences, University of California, San Diego, La Jolla, California, USA. 6 Institute for Genomic Medicine, University of California, San Diego, La Jolla, California, USA. 7 Sanford Consortium for Regenerative Medicine, University of California, San Diego, La Jolla, California, USA. 8 Rady Children’s Hospital, San Diego, California, USA. 9 Division of Biological Sciences, University of California, San Diego, La Jolla, California, USA. 10 These authors contributed equally to this work. Correspondence should be addressed to A.R.L. (alaspada@ucsd.edu).

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disorders and lysosomal storage diseases characterized by neurological dysfunction\textsuperscript{15–18}. In agreement with this work, recent evidence has uncovered an important role for TFEB dysfunction in neurodegeneration. As a master regulator of the CLEAR network, interference with TFEB signaling can lead to impaired autophagy function and diminished lysosomal degradative capacity, undermining neuronal survival. Indeed, reduced PPAR\textgamma coactivator-1\textalpha induction of TFEB expression in Huntington’s disease transgenic mice contributes to the aggregate clearance defects, neurological abnormalities and neurodegeneration in this model\textsuperscript{17}. In Parkinson’s disease, \alpha-synuclein neurotoxicity may also involve TFEB dysregulation, as degenerating dopaminergic neurons accumulate TFEB in the cytosol, resulting in reduced autophagy-lysosome pathway function\textsuperscript{18}. Notably, these studies found TFEB upregulation to be sufficient to rescue neurodegenerative phenotypes, confirming that impaired TFEB action is key to Huntington’s and Parkinson’s disease pathogenesis and linking TFEB to the mechanistic basis of autophagy dysfunction in neurodegeneration.

Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy’s disease, is an X-linked inherited neuromuscular disorder characterized by lower motor neuron degeneration leading to weakness and atrophy of bulbar, facial and limb muscles. SBMA patients display signs of androgen insensitivity, with full disease penetrance restricted to adult males\textsuperscript{19}. The causative mutation in SBMA is a CAG trinucleotide repeat expansion in the first exon of the androgen receptor (AR) gene\textsuperscript{20}. This mutation encodes an extended polyglutamine (polyQ) stretch in the translated AR protein and thus defines SBMA as one member of a family of nine polyQ repeat disorders, including Huntington’s disease, dentatorubral-pallidolysian atrophy and six forms of spinocerebellar ataxia\textsuperscript{21}. AR is a transcription factor that controls the expression of androgen-responsive genes upon ligand-mediated nuclear translocation\textsuperscript{22}. In SBMA, disease pathogenesis is likely to involve two distinct pathways: gain-of-function toxicity due to production of misfolded polyQ-AR protein and loss or alteration of AR normal function\textsuperscript{23}.

In this study, we examined autophagy dysregulation in SBMA by analyzing in vivo and in vitro models, including a human SBMA stem cell model derived from induced pluripotent stem cells (iPSCs) reprogrammed from patient fibroblasts. These investigations revealed abnormalities of autophagic vesicle maturation and fusion with lysosomes in SBMA cell culture models, transgenic mice and iPSC-derived NPCs, and enabled us to link impaired autophagy pathway dysfunction to the onset of SBMA disease phenotypes. After demonstrating a physical and functional interaction between AR and TFEB, we detected polyQ-AR interference with TFEB transactivation and determined that TFEB dysregulation accounted for autophagic flux defects present in both SBMA motor neuron–like cells and patient NPCs. Our findings thus reveal altered autophagy onset as a crucial turning point in the SBMA pathogenic cascade and implicate TFEB as a target for therapeutic intervention in this motor neuron disease.

RESULTS

Polyglutamine-expanded AR protein impairs autophagic flux

To determine the effect of polyQ-expanded AR protein on proteostasis, we examined rates of long-lived protein turnover in hybrid cells derived from mouse embryonic spinal motor neurons (MN-1 cells) that stably express normal human AR with 24 glutamines (MN-1 AR24Q) or mutant AR with 65 glutamines (MN-1 AR65Q). Whereas short-lived protein turnover is primarily mediated by the ubiquitin-proteasome system (UPS), long-lived protein turnover is mainly attributable to the autophagy-lysosome pathway\textsuperscript{9}. To quantify long-lived protein turnover, we labeled newly synthesized proteins with the nonradioactive methionine analog L-azidohomoalanine and measured rates of protein degradation after L-azidohomoalanine washout. We found that MN-1 AR65Q cells displayed reduced long-lived protein turnover (Supplementary Fig. 1).

Functional autophagy progression—that is, autophagic flux—requires that autophagosomes fuse with lysosomes, thereby ensuring that engulfed cargoes are efficiently degraded. To directly assess autophagic flux in MN-1 cells, we used a tandem-tagged mCherry-EGFP–light chain-3 (LC3) vector. Upon autophagosome formation, LC3 is incorporated into autophagosome membranes and both fluorescent tags are active, producing yellow puncta in merged images. When autophagosomes fuse with lysosomes, vesicle pH becomes acidic, quenching the EGFP signal so that only mCherry fluorescence remains detectable. This acidification-dependent change in fluorescence emission can be used to monitor autophagosome maturation. When we tested the effect of AR protein expression on autophagic flux by transfecting MN-1 cells with the mCherry-EGFP-LC3 construct, we observed a marked increase in the number of autophagosomes per cell in both MN-1 AR24Q cells and MN-1 AR65Q cells, though autophagosome number was significantly higher in MN-1 AR65Q cells and autolysosome number was correspondingly lower in MN-1 AR65Q cells (Fig. 1a). To exclude altered expression of the mCherry-EGFP-LC3 vector as a possible explanation, we transfected a mouse embryonic fibroblast (MEF) cell line stably expressing mCherry-GFP-LC3 with AR25Q-BFP or AR125Q-BFP; we obtained similar results (Fig. 1b). Next, we directly measured autophagy degradation function by analyzing the flux through the pathway and measured changes in LC3 isoform II (LC3-II) levels relative to \beta-actin (Fig. 1c). When we analyzed LC3-II levels after ammonium chloride treatment, we observed reduced autophagic flux for MN-1 AR65Q cells (Fig. 1d), which indicates inefficient turnover of newly formed autophagosomes. Autophagic flux can also be assayed by measuring levels of p62, a protein adaptor of autophagic cargo to autophagosome membranes and marker of autophagy pathway activity\textsuperscript{24}. Inhibiting lysosome function with ammonium chloride increased p62 levels (Fig. 1e). Following ammonium chloride washout, p62 immunoblot analysis revealed that control MN-1 cells and MN-1 AR24Q cells were able to normalize p62 levels, suggesting competent lysosomal degradation of accumulated cargo. However, p62 levels remained elevated in MN-1 AR65Q cells (Fig. 1e), yielding impaired recovery after ammonium chloride treatment (Fig. 1f), indicating impaired autophagic flux. Taken together, these data indicate autophagy pathway dysfunction in the presence of polyQ-AR.

The autophagy pathway is blocked in symptomatic SBMA mice

To evaluate the physiological relevance of autophagic flux defects observed in MN-1 cells, we performed ultrastructural analysis of autophagy pathway status in lumbar motor neurons from AR YAC CAG100 (YAC AR100) transgenic mice and AR YAC CAG20 (YAC AR20) transgenic control mice\textsuperscript{25}. Male YAC AR100 mice recapitulate key features of SBMA, developing a progressive neuromuscular phenotype accompanied by motor neuron degeneration, while male YAC AR20 mice do not develop disease. We selected two time points for this study: a presymptomatic time point at which YAC AR100 mice do not display any neurological or cellular abnormalities (6 months) and a symptomatic time point by which YAC AR100 mice display a pronounced neuromuscular phenotype (14 months). To evaluate autophagosome maturation in SBMA mice, we generated electron micrographs of motor neurons from age-matched non-transgenic, YAC AR20 and YAC AR100 male littermates and reviewed these micrographs for the presence of autophagosomes and autolysosomes, using established morphological criteria (Supplementary Fig. 2).
Electron microscopy remains the gold standard for autophagy analysis, as it allows direct observation of autophagic vesicles at different stages of maturation. At 6 months, non-transgenic, YAC AR20 and YAC AR100 mice displayed comparable numbers of autophagosomes per motor neuron field, but YAC AR20 and YAC AR100 motor neurons contained greater numbers of autolysosomes than controls, with many AR100 motor neurons containing high numbers of autolysosomes (Fig. 2a). However, at 14 months of age, YAC AR100 motor neurons displayed numerous autophagosomes, in contrast to non-transgenic and YAC AR20 motor neurons, in which the autolysosome:autophagosome ratio was much greater (Fig. 2b).

To quantify autophagy pathway progression in SBMA mice, we counted numbers of autophagosomes and autolysosomes per motor neuron field. At 6 months of age, autophagosomes were comparable in frequency, but they became markedly increased in motor neurons from YAC AR20 and YAC AR100 mice at 14 months (Fig. 3a). In 6-month-old mice, YAC AR100 motor neurons contained more autolysosomes than YAC AR20 motor neurons and significantly more than non-transgenic controls (Fig. 3b). At 14 months, YAC AR20 mice still showed proportionately more autolysosomes than non-transgenic controls, but autolysosome counts in YAC AR100 motor neurons were significantly decreased, appearing instead comparable to non-transgenic motor neurons (Fig. 3b). To evaluate autophagic flux, we reasoned that the ratio of autolysosomes to autophagosomes serves as a gauge of autophagosome maturation and autophagy pathway progression, so we compared autolysosome:autophagosome ratios in SBMA transgenic mice. We found that this ratio, which we designated the autophagy index, was increased in YAC AR100 motor neurons at 6 months of age (Fig. 3c). At 14 months of age, however, a significant reduction in the autophagy index was apparent in YAC AR100 motor neurons (Fig. 3c). The inability of YAC AR100 mice to maintain autophagy progression in the face of a persistent misfolded protein insult, as reflected by reduced autolysosome formation at the symptomatic stage, may thus contribute to the pronounced motor neuron disease and progressive neural dysfunction seen in SBMA mice.

Impaired TFEB action underlies SBMA autophagy dysfunction

We hypothesized that autophagy pathway dysfunction in SBMA may result from altered transcription, as transcription dysregulation is a common pathology in polyQ disorders involving disease proteins that result from altered transcription, as transcription dysregulation is a common pathology in polyQ disorders involving disease proteins that are transcription factors. This led us to examine whether polyQ-AR interacted with transcription factors such as TFEB. To determine whether this physical interaction has functional significance, we measured both AR25Q and AR125Q (Fig. 4a). However, at 14 months of age, YAC AR100 motor neurons containing high numbers of polyQ-AR retards autophagy flux. (a) MN-1 cells were transfected with mCherry-EGFP-LC3 vector, fixed and imaged after 24 h. Numbers of yellow puncta (autophagosomes) and red puncta (autolysosomes) per cell were counted. Autophagosomes: n = 3 independent experiments, F = 3.56; autolysosomes: n = 3 independent experiments, F = 20.13. One-way ANOVA with post hoc Tukey test. *P < 0.05, ***P < 0.001. n = 34 cells per genotype. (b) We transfected MEFs stably expressing mCherry-EGFP-LC3 with BFP-tagged AR25Q or AR125Q. Cells were imaged after 24 h, and yellow puncta (autophagosomes) and red puncta (autolysosomes) in AR-expressing (blue) cells were counted. Autophagosomes: n = 3 independent experiments, F = 20.65; autolysosomes: n = 3 independent experiments, F = 4.49. One-way ANOVA with post hoc Tukey test. *P < 0.05, **P < 0.01, ***P < 0.001. n = 33 cells per genotype. (c) MN-1 cells were imunoblotted for LC3 in the presence or absence of ammonium chloride to evaluate autophagic flux. A representative LC3 western blot is shown. All ratios were normalized to MN-1 WT cells at baseline, which was set to 1. (d) The ratio of LC3-II:actin for c determined by densitometry analysis using ImageJ. n = 3 independent experiments, F = 0.4501, one-way ANOVA with post hoc Tukey test. *P < 0.05. (e) MN-1 cells were treated with ammonium chloride to block lysosomal activity, and then ammonium chloride was removed and cells were allowed to degrade accumulated autophagy cargo during a 6-h recovery period. We performed p62 western blot analysis on MN-1 cells at baseline (-), after ammonium chloride treatment (+) and after the recovery period (Rec). All ratios were normalized to MN-1 WT cells at baseline, which was set to 1. (f) On the basis of the p62 values obtained in e, we calculated the efficiency of degraded accumulated autophagic cargo by dividing p62 at baseline by p62 at recovery and normalized the baseline/recovery values to baseline/recovery for MN-1 WT cells. n = 3 independent experiments, F = 5.45, one-way ANOVA with post hoc Tukey test. *P < 0.05, **P < 0.01. Data are presented as mean ± s.e.m. Scale bar, 20 μm. Individual P values and degrees of freedom are available in the Supplementary Methods Checklist.
the TFEB response element in the promoters of TFEB target genes.\(^1\)

We treated MN-1 cells bearing the 4X-CLEAR reporter with sucrose, which causes osmotic stress resulting in lysosomal activation via TFEB transactivation. Although sucrose yielded significant increases in 4X-CLEAR reporter activity in both control and AR24Q MN-1 cells, sucrose treatment did not elicit any transactivation response in MN-1 AR65Q cells (Fig. 4b). We then treated MN-1 cells with the lysosomal inhibitor ammonium chloride, which also promotes TFEB transactivation, and measured the expression of TFEB target genes. We observed expression increases for TFEB target genes in both control MN-1 and MN-1 AR24Q cells, as expected, but did not detect appreciable changes in the expression of these targets in MN-1 AR65Q cells (Fig. 4c). One noteworthy outcome of both the sucrose challenge and ammonium chloride treatment was the potent effect of normal AR24Q protein on induction of 4X-CLEAR activity and TFEB target genes, as AR24Q promoted significantly higher levels of 4X-CLEAR activity at baseline and upon sucrose challenge in comparison to MN-1 WT cells that do not overexpress AR (Fig. 4b), and AR24Q expression yielded much higher levels of induction for two of the TFEB target genes (Fig. 4c). These findings indicate that AR may act as a positive regulator of TFEB transactivation competence, suggesting that AR is a coactivator for TFEB. To assess the physiological relevance of these findings, we derived embryonic day (E) 13 motor neurons from YAC AR20 and YAC AR100 transgenic mice, and obtained both spinal cord samples and quadriceps muscle samples from symptomatic 14-month-old YAC AR100 transgenic

![Figure 2](image1)

**Figure 2** SBMA mice display accumulations of autophagosomes and reduced autolysosome formation in degenerating motor neurons. (a, b) Electron micrographs of motor neuron perinuclear regions from age-matched non-transgenic (Nt), YAC AR20 and YAC AR100 transgenic mice before disease onset (a, 6 months of age) and after prominent neuromuscular and molecular pathology is apparent (b, 14 months of age). (a) At 6 months of age, occasional autophagosomes (yellow arrowheads) are noted in Nt, YAC AR20 and YAC AR100 at roughly equivalent frequency. Autolysosomes (red arrowheads) are much more common and are present in higher numbers in YAC AR20 and YAC AR100 motor neurons. (b) At 14 months of age, when YAC AR100 mice display signs of motor neuronopathy and molecular pathology, we observed many YAC AR100 motor neuron micrographs with frequent autophagosomes and autolysosomes (yellow arrowheads). Despite this increase in autophagosomes, autolysosomes were fewer in number at this age in YAC AR100 motor neurons, such that autophagosome numbers approached autolysosome numbers in YAC AR100 motor neurons. This was never the case for Nt motor neurons or YAC AR20 motor neurons. Main panels are at original magnification 2,200x and insets are at original magnification 3,700x.

![Figure 3](image2)

**Figure 3** Quantification of autophagic vesicle type uncovers impaired autophagy progression. (a) Mean number of autophagosomes per motor neuron field in electron micrographs from non-transgenic (Nt), YAC AR20 and YAC AR100 transgenic mice at 6 and 14 months of age, ± s.e.m. 6 months: n = 3 independent experiments, F = 0.63, one-way ANOVA with post hoc Tukey test. P = 0.534. 14 months: n = 3 independent experiments, F = 4.32, one-way ANOVA with post hoc Tukey test. *P < 0.05. (b) Mean number of autolysosomes per motor neuron field in electron micrographs from Nt, YAC AR20 and YAC AR100 transgenic mice at 6 and 14 months of age, ± s.e.m. 6 months: n = 3 independent experiments, F = 4.18, one-way ANOVA with post hoc Tukey test. *P < 0.05. 14 months: n = 3 independent experiments, F = 4.56, one-way ANOVA with post hoc Tukey test. *P < 0.05. (c) Mean autophagy index, dividing number of autolysosomes by number of autophagosomes for each motor neuron field, ± s.e.m. At 6 months, YAC AR100 motor neurons displayed a markedly increased autophagy index, n = 3 independent experiments, F = 129.81, one-way ANOVA with post hoc Tukey test. **P < 0.01. However, once YAC AR100 mice develop disease pathology at 14 months, the autophagy index for YAC AR100 motor neurons is significantly decreased; n = 3 independent experiments, F = 82.82, one-way ANOVA with post hoc Tukey test. **P < 0.01. Individual P values and degrees of freedom are available in the Supplementary Methods Checklist.
After RNA isolation, we interrogated gene expression for TFEB target genes in isolated YAC AR100 E13 motor neurons (Fig. 4d), but did not detect any differences in TFEB target genes in transgenic spinal cord lysates, likely because motor neurons comprise less than 5% of the cells in this sample type. Notably, as in MN-1 AR24Q cells, overexpression of normal AR in YAC AR20 motor neurons elicited robust induction of two TFEB target genes, comparable to or exceeding that in non-transgenic control motor neurons (Fig. 4d). RT-PCR analysis of quadriceps muscle, however, yielded evidence for dramatic upregulation of TFEB target genes in YAC AR100 mice (Supplementary Fig. 3), which is consistent with the results of studies of SBMA knock-in mice26 and indicates a
muscle-specific process of supraphysiological induction of TFEB in diseased muscle cells in SBMA. To determine whether TFEB transcription interference accounts for autophagy dysregulation in the SBMA MN-1 cell model, we transected control MN-1 WT cells, MN-1 AR24Q cells and MN-1 AR65Q cells with a blue fluorescent protein (BFP)-tagged TFEB expression vector or BFP empty vector, along with the 4X-CLEAR luciferase reporter. We noted marked increases in 4X-CLEAR reporter activity in all cases (Fig. 5a). To assess whether TFEB overexpression can rescue impaired TFEB transactivation, MN-1 AR65Q cells expressing the 4X-CLEAR reporter were exposed to starvation stress or to rapamycin or ammonium chloride treatment. MN-1 AR65Q cells expressing BFP-TFEB displayed increased induction of 4X-CLEAR reporter activity in response to all three TFEB inducers, as compared to untransfected MN-1 AR65Q cells transfected with BFP empty vector (Fig. 5b). In light of these results, we tested whether TFEB upregulation would rescue diminished autophagic flux in MN-1 AR65Q cells using the mCherry-GFP-LC3 assay. We began by transfecting MN-1 WT cells with the BFP-TFEB vector and observed increased numbers of autolysosomes, validating our TFEB construct and confirming the responsiveness of MN-1 cells to TFEB upregulation (Supplementary Fig. 4). Although MN-1 AR65Q cells expressing BFP empty vector exhibited similar frequencies of autophagosomes and autolysosomes to those of untransfected MN-1 AR65Q cells, MN-1 AR65Q cells expressing BFP-TFEB displayed improved autophagic flux (Fig. 5c,d). These results suggest that TFEB dysfunction may account for the impaired autophagy pathway progression observed in multiple SBMA models.

**AR functions as a coactivator of TFEB**

As our findings indicated that WT AR positively co-regulates TFEB, suggesting that AR could be a bona fide coactivator of TFEB, we chose to further explore the nature of the physical and functional interaction between AR and TFEB. Using a set of AR N-terminal and C-terminal deletion constructs (Fig. 6a), we co-transfected different AR constructs with TFEB in HEK293 cells and mapped the TFEB interaction domain on AR to its C-terminal 180 amino acids (Fig. 6b,c). As entry
of TFEB into the nucleus is required for transactivation of its target genes and inhibitory regulation of TFEB by phosphorylation restricts TFEB to the cytosol, we tested whether AR activation by its ligand affects TFEB subcellular localization. In MN-1 cells overexpressing AR with a normal glutamine tract, addition of the synthetic ligand R1881 resulted in localization of TFEB to the nucleus (Fig. 6f). This effect was AR-dependent, as MN-1 WT cells that do not overexpress AR did not exhibit this effect. Similarly, R1881 treatment of MN-1 65Q cells did not alter TFEB subcellular localization (Fig. 6f), indicating that polyQ-AR coactivation of TFEB is impaired.

To further examine the ability of normal AR to promote TFEB transactivation, we transfected WT MN-1 cells with either an AR25Q-BFP expression construct or BFP empty vector in the presence of the 4X-CLEAR luciferase reporter. We observed significantly higher induction of 4X-CLEAR luciferase activity in AR25Q-expressing cells at baseline and upon ammonium chloride or rapamycin treatment (Fig. 6f). We then performed the opposite experiment and knocked down AR in MN-1 AR24Q cells, achieving at least 50% protein knockdown. We observed a moderate reduction in 4X CLEAR luciferase activity upon AR short hairpin RNA knockdown at baseline but noted a marked decrease in 4X CLEAR luciferase activity upon AR shRNA knockdown in rapamycin-treated MN-1 AR24Q cells (Fig. 6g). While the reduction in 4X-CLEAR reporter activity upon AR shRNA knockdown at baseline was only moderate, when we measured TFEB target gene expression in MN-1 24Q cells subjected to AR knockdown, we documented significant reductions in the expression of all tested TFEB targets (Fig. 6h). All of these studies suggest that AR functions as a TFEB coactivator.

TFEB rescues autophagy defects in an SBMA stem cell model

Using retroviral transduction as previously described, we reprogrammed primary fibroblasts from three different SBMA patients and three different healthy controls to iPSCs. We established at least three clone lines per patient (Supplementary Fig. 5) and confirmed that all derived clones demonstrated key characteristics of embryonic stem cells (Supplementary Fig. 6a–c). To determine whether our stem cell models retained disease-specific features, we sequenced the AR CAG repeat regions of 22 iPSC clones and their six progenitor fibroblast cell lines and confirmed that CAG repeat alleles remained virtually unchanged in size. We then generated NPCs by collecting neural rosettes from plated embryoid bodies, as previously described. NPC status was confirmed by detection of nestin and Sox2 (Supplementary Fig. 6d). To determine whether NPCs derived from SBMA patients retained disease-specific phenotypes, we performed filter trap assays, detecting accumulation of insoluble AR protein in SBMA NPC samples (Fig. 7a). Another established feature of SBMA is mitochondrial dysfunction, as expression of polyQ-AR in neuron-like cells yields mitochondrial membrane depolarization. To evaluate mitochondrial function in the NPC lines, we exposed the different NPC clones to JC-1 dye and assessed mitochondrial membrane potential as a function of red/green fluorescence intensity ratio (Supplementary Fig. 7). About twice as many SBMA NPCs as control NPCs contained depolarized mitochondria (Fig. 7b). This occurred at baseline, without subjecting NPCs to any insult, and was disease specific.

After confirming that SBMA-derived NPCs encode polyQ-expanded AR proteins resistant to degradation, we evaluated the NPC lines for autophagy pathway function with the mCherry-EGFP-LC3 vector. As generation of iPSC lines and NPC derivatives is often associated with a high degree of clonal variability, we examined autophagic flux in three different clonal lines per patient (Supplementary Fig. 5). SBMA NPCs had more autophagosomes than control NPCs (Fig. 7c,d). We calculated the autophagy index for control and SBMA NPCs and noted a roughly 50% reduction in the autophagy index for SBMA NPCs, confirming that autophagic flux is impaired in SBMA NPCs, in
agreement with SBMA cell culture and mouse models. When we measured the expression of TFEB target genes, we observed marked reductions in TFEB targets in SBMA NPCs (Fig. 7e). Coimmunoprecipitation experiments confirmed a physical interaction between TFEB and AR in both control and SBMA NPCs (Fig. 8a).

To determine whether decreased TFEB function contributes to autophagy dysregulation and mitochondrial dysfunction in SBMA NPCs, we tested whether TFEB overexpression could rescue these phenotypes. We began by transfecting control NPCs with BFP empty vector and BFP-TFEB. We noted a trend toward increased autolysosome formation and flux (Supplementary Fig. 8). We then transfected SBMA NPCs with BFP-TFEB, treated them with JC-1 dye and determined mitochondrial membrane polarization by analyzing red:green fluorescence intensity ratio. Upregulation of TFEB significantly reduced the percentage of SBMA NPCs with depolarized mitochondria (Fig. 8b). We also repeated the autophagic flux assay. TFEB overexpression promoted autophagic flux (Fig. 8c), yielding a significant reduction in autophagosomes and a modest increase in autolysosomes in SBMA NPCs (Fig. 8d). Calculation of the autophagy index yielded a fivefold increase for SBMA NPCs expressing TFEB. Hence, the effect of TFEB on autophagic vesicle profiles translated into a marked increase in the autophagy index for TFEB-expressing SBMA NPCs, demonstrating that TFEB overexpression virtually abolished the SBMA autophagic flux defect.

**DISCUSSION**

Autophagy has emerged as a key pathway in neurodegenerative disease, and it helps maintain normal neural function by degrading aggregate-prone proteins even when neurons are not exposed to mutant misfolded peptides or increased levels of altered conformers. Despite its clearly demonstrated protective actions, the capacity of the autophagy pathway for handling proteotoxic insults, such as polyQ disease proteins, is limited. Although autophagy is widely accepted as a crucial factor in the response of the CNS to misfolded protein stress, many unanswered questions remain. When in the course of a neurodegenerative proteinopathy does the autophagy pathway fail to operate properly? What step(s) in the autophagy pathway are the first to be blocked in neurodegenerative disease? With these key questions in mind, we studied autophagy dysfunction in a SBMA mouse model, an immortalized cell line model and a human iPS-based stem cell model. Our results offer insights into the mechanistic basis of autophagy dysfunction in SBMA and related neurodegenerative proteinopathies.

Our findings identify a block in the fusion of autophagosomes with lysosomes as a likely cause of the autophagy dysfunction in SBMA. Ultrastructural analysis of SBMA motor neurons indicated that induction of autophagy was intact, if not markedly increased, in the face of AR polyQ proteotoxic stress. Hence, in the case of AR polyQ stress, there is no evidence for reduced activation of the autophagy pathway, as shown for the related polyQ disorder Huntington’s disease, where sequestration of mammalian target of rapamycin (mTOR) by polyQ-huntingtin has been reported. Rather, AR neurotoxicity involves a block in autophagy pathway progression, similar to that observed in Alzheimer’s disease, where there is a striking accumulation of immature autophagic vesicles in patients’ dystrophic neurites. To delineate the basis of this effect, we considered the transcriptional regulation of the autophagy pathway, as transcriptional interference is a feature of polyQ diseases involving disease proteins that are transcription factors. One possibility, suggested by recent work on lysosome biogenesis, is that polyQ-expanded AR could interfere with TFEB-dependent transcription. As we and others have documented AR transcription dysregulation both in vitro and in vivo, and TFEB and AR share common sumoylation regulatory pathways and presumably cofactors, we undertook a thorough analysis of TFEB function in SBMA. Our results strongly suggest that TFEB dysregulation, mediated by an inhibitory interaction with polyQ-expanded AR, is responsible for autophagic flux impairments present in SBMA models.

In addition to demonstrating polyQ-expanded AR interference with TFEB transactivation, our data suggest that AR can normally interact with TFEB to promote its function. We found evidence of an interaction between normal-Q-length AR and TFEB in both overexpression systems and under endogenous conditions, and we report enhanced TFEB signaling and increased autophagy pathway activity when normal AR protein is overexpressed. When we mapped the TFEB...
interaction domain on AR, we localized the interaction domain to the C-terminal 180 amino acids. This region of AR comprises the bulk of the AF-2 activation domain, a highly conserved region that serves as a binding pocket for steroid receptor coactivator (SRC) proteins. These findings suggest that AR and TFEB may normally exist in the same protein complex and that AR and TFEB may require shared coactivator proteins for optimal transactivation function. Hence, AR activation may promote TFEB transactivation through recruitment of certain coactivator proteins, but, upon polyQ tract expansion, mutant AR may no longer maintain the proper dynamics of protein-protein interactions within the complex, resulting in diminished TFEB transactivation competence. Altered TFEB protein-protein interactions in the presence of polyQ-expanded AR in the cytosol may thus block TFEB nuclear localization by preventing a translocation factor from properly interacting with TFEB, even when TFEB is dephosphorylated and primed for nuclear entry. Future studies aimed at defining the nature of the cytosolic TFEB protein complex in the presence of polyQ-expanded AR may reveal the TFEB interactors responsible for its translocation and how inclusion of polyQ-expanded AR in the TFEB protein complex interferes with TFEB subcellular localization. Such a model of polyQ-expanded AR negatively altering the function of a protein with which it normally interacts is fully consistent with emerging views of polyQ disease pathogenesis, wherein studies of spinocerebellar ataxia type I have shown that polyQ-expanded ataxin-1 is less capable of promoting Capicua function than its normal-Q-length counterpart, resulting in reduced Capicua transcriptional repressor activity. As AR normally interacts with many transcription co-regulators and studies of a SBMA fly model indicate that polyQ-expanded AR may promote neurotoxicity by reducing the function of the co-regulators with which it interacts, reduced availability of a coactivator protein shared by AR and TFEB may result in decreased TFEB transactivation function in SBMA. Our findings thus underscore the importance of the normal functions of disease-associated proteins in delineating the mechanistic basis of the neurodegeneration in polyQ repeat disorders.

While induction of autophagy is initially protective in a primary neuron model of SBMA toxicity, and, correspondingly, overexpression of p62 beginning before disease onset can ameliorate motor phenotypes and neuropathology in SBMA transgenic mice, our studies indicate that inhibition of autophagosome-lysosome fusion ultimately occurs in SBMA, impairing turnover of long-lived proteins and compromising cellular proteostasis. Analysis of SBMA mice revealed that properly functioning autophagy was evident at early time points in presymptomatic mice but that, with disease progression, autophagy became dysfunctional and disease ensued. Our results also indicate that autophagy dysregulation differs between different cell types, as polyQ-AR inhibited TFEB function in the neuronal milieu but can actually promote TFEB transactivation in skeletal muscle, which may yield excessive autophagy that can contribute to muscle atrophy. As altered muscle physiology may initiate SBMA disease pathogenesis, understanding the connection and potential cross-talk between autophagy dysregulation in skeletal muscle and motor neurons will be an important goal of future research.

Modulation of autophagy is being touted as a potential therapy for neurodegenerative proteinopathies, but the timing of autophagy activation and the ability of an intervention to enhance autophagic clearance must both be carefully considered when designing treatment regimens. Indeed, further induction of dysfunctional autophagy may actually exacerbate cellular toxicity and, paradoxically, accelerate disease progression. Deconstructing the specific defects in the autophagy pathway in neurodegenerative proteinopathies and determining the impact of autophagy activation on documented alterations are required before therapies can be envisaged. In our SBMA NPC model, we documented significant downregulation of TFEB target genes in comparison to controls and found that induction of TFEB activity completely reversed metabolic and autophagic flux phenotypes observed in SBMA-derived NPCs. In light of these results, TFEB is an attractive candidate for therapy development efforts. In addition to elevating the status of TFEB for therapy development, we created a representative stem cell model of SBMA that will permit further study into the basis of disease pathogenesis. Another therapeutic application of the SBMA NPC model will be to develop it as a tool for identifying compounds that can correct autophagic flux defects, as high throughput screening for very minor changes (one or two repeats) in CAG allele size in iPSCs and NPCs upon reprogramming. Hence, our results, and those of another group working independently, confirm that CAG repeat length alteration is an infrequent event in SBMA stem cell modeling, and when it occurs, the observed changes are minor. In addition to tracking the sizes of CAG repeat alleles, we also examined SBMA iPSC-derived lines for disease-specific phenotypes. To do this, we performed filter trap assays and documented increased insoluble misfolded protein accumulations in NPC lines from SBMA patients. We also measured mitochondrial membrane potential and noted that SBMA-derived NPCs were twice as likely as control NPCs to contain depolarized mitochondria, in agreement with studies of MN-1 cells and mouse pheochromocytoma cell line 12 overexpressing polyQ-AR. As the process of reprogramming can introduce genetic and molecular alterations to the resultant cell lines and thereby yield spurious phenotypes, we generated SBMA stem cells from three different, unrelated SBMA patients and three different controls, and we produced three unique iPSC clones per human fibroblast line to yield a collection of 18 different clonal lines. Although production and characterization of so many lines required considerable effort, analysis of multiple independent clones from multiple patients and controls is necessary to insure the validity of observed disease-specific phenotypes. Using this approach, we found evidence for SBMA-specific reductions in autophagic flux in NPCs, consistent with observations in MN-1 cells and SBMA transgenic mice. We then documented the ability of TFEB to improve mitochondrial membrane polarization and relieve the autophagic flux blockage present in SBMA NPCs. During conditions of autophagic flux inhibition, autophagic cargo (including depolarized mitochondria) accumulate, leading to increased reactive oxygen species production and decreased lysosome function. Thus, upregulation of TFEB and activation of the CLEAR signaling network likely rescues the block in autophagic flux by promoting autophagy-lysosome pathway activity and facilitating the clearance of defective mitochondria. Although iPSC-derived neural modeling documented accumulations of autophagic vesicles and other abnormalities in a study of Parkinson's disease, to our knowledge, this is the first time that dynamic studies of autophagy pathway function in an iPSC-derived neural lineage have yielded a quantifiable defect in autophagic flux. Hence, this work provides a proof of principle for future efforts aimed at dissecting autophagy abnormalities in neurological diseases using a stem cell modeling approach.
compounds capable of rescuing the autophagy pathway block in this model could be pursued in a high content format. Compounds that favor autophagosome-lysosome fusion would be plausible therapies not only for SBMA but also for related disorders characterized by impaired autophagy pathway progression.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.J.C., H.C.M., H.F., Y.B., A.R.M., G.A.G. and A.R.L.S. designed the experiments. C.J.C., H.C.M., H.F., Y.B., J.E.Y., A.L., N.I., B.L.S., C.C. and G.A.G. performed the experiments. C.J.C., H.C.M., H.F., Y.B., J.E.Y., A.L., N.I., B.L.S., C.C. and G.A.G. analyzed the experiments. C.J.C., H.C.M., H.F., Y.B., J.E.Y., A.L., N.I., B.L.S., C.C. and G.A.G. analyzed the experiments. C.J.C., H.C.M., H.F., Y.B., J.E.Y., A.L., N.I., B.L.S., C.C. and G.A.G. performed the experiments. C.J.C., H.C.M., H.F., Y.B., J.E.Y., A.L., N.I., B.L.S., C.C. and G.A.G. analyzed the experiments. C.J.C., H.C.M., H.F., Y.B., J.E.Y., A.L., N.I., B.L.S., C.C. and G.A.G. performed the experiments. C.J.C., H.C.M., H.F., Y.B., J.E.Y., A.L., N.I., B.L.S., C.C. and G.A.G. analyzed the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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DNA constructs. The AR-Q(n) expression constructs (where ‘n’ is the number of CAG repeats) have been described previously47. The mCherry-EGFP-LC3 construct was kindly provided by Dr. T. Johansen. The empty EBFP2 construct was obtained from Addgene, and from it we derived BFP-TFEB, BFP-AR25Q and BFP-AR125Q vectors. The TFEB expression construct was obtained from Origene. We generated a 4X-CLEAR element driven by the HTK promoter by subcloning into the PGL3 firefly luciferase vector (Promega).

Cell culture. All cell culture lines were grown in complete DMEM (Life Technologies) with 10% FBS and 5 mM glutamine. Motor neuron–like (MN-1) AR24Q and AR65Q cells have been described previously48. Mixed primary motor neuron cultures were prepared from E13 WT, YAC AR20 or YAC AR100 mice, as previously described49. All cell line and primary neuron transfections used Lipofectamine 2000, following the manufacturer’s protocol (Life Technologies). Cells were treated with rapamycin 2 µM or ammonium chloride 20 mM, as indicated. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Briefly, for MN-1 cell transfection assays, MN-1 cells were transfected with the 4X-CLEAR reporter promoter construct, and CMV-promoter Renilla luciferase vector was used as the internal control. For AR knockdown, MN-1 AR24Q cells were transfected with AR shRNA (Santa Cruz, sc-39204) or scrambled control construct using the manufacturer’s protocol. Twenty-four hours after transfection, cells were treated overnight with indicated compounds and collected according to the manufacturer’s instructions. Firefly and Renilla luciferase activity was measured using Glomax 96 microplate luminometer (Promega), and values were normalized to those of untreated MN-1 WT cells. All luciferase reporter assays were performed in triplicate or quadruplicate.

iPSC derivation and NPC experimentation. Fibroblasts were collected from dermal biopsies from three male SBMA patients of European ancestry between the ages of 55 and 65 years old and three male healthy donors of European ancestry between the ages of 38 and 65 years old upon informed consent in compliance with UCSD-approved IRB protocol #130377Z. Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) FBS and 5% (v/v) antibiotics (penicillin and streptomycin, 10,000 U/ml). Low-passage fibroblasts were reprogrammed as previously described52. Briefly, POU5F1 (OCT4), MYC, KLF4 and SOX2 human cDNAs were obtained from Addgene and used in a retroviral system to infect fibroblasts52,53. Two days after infection, fibroblasts were transferred onto irradiated mouse embryonic fibroblasts (Chemicon) with hESC medium (Sigma) with DMEM/F12 medium, enriched with 0.5× N2, 0.5× B27 and FGF2. Primary antibodies used were Nanog (1:250, R&D Systems AF1997), Lin28 (1:250, R&D Systems AF3757), human nestin (1:100, Chemicon mab5326), Sox2 (1:250, Cell Signaling 2748) and Oct4 (1:250, Santa Cruz sc-635509). Images were taken using a Zeiss laser-scanning inverted confocal microscope and/or a Zeiss confocal microscope, and, for mCherry-EGFP-LC3 analysis, blinded observers counted vesicle numbers in the mCherry (red), GFP (green) or merged (yellow) channels for 25–50 cells per condition. All experiments were done in triplicate or quadruplicate.

Mitochondrial membrane potential assay. The mitochondrial membrane potential assay was conducted using image analysis in the NC-3000 (Chemometec, Denmark) system, which automatically detects cells with collapsed mitochondrial membrane potential. Briefly, 1 × 10⁶ cells were stained with JC-1 for 20 min. Stained cells were centrifuged and washed twice with PBS. Cells were then stained with DAPI to determine viability. Celluar JC-1 monomers and aggregates are detected as green and red fluorescence, respectively, with mitochondrial depolarization revealed as a decrease in red-green fluorescence intensity ratio. After staining, cells are loaded into either of two types of Chemometec slides: the two-chamber NC-Slide A2 or the eight-chamber NC-Slide A8. Samples were analyzed using the NC-3000 system and the amount of blue, green and red fluorescence of individual cells was quantified. The intensity of red and fluorescence is shown in a scatter plot, all experiments were done in triplicate and gating analysis, based on the control, was equal for all conditions.

DNA extraction and CAG repeat sizing. Genomic DNA extraction was performed using DNeasy Kit (Qiagen). Amplification of the CAG repeat site in the AR gene was performed using Fast Start PCR master mix kit (Roche). For the reaction, 100 ng of DNA were used as template with 10 mM primers, forward (5′ TCC AGA ATC TGT TCC AGA GCG TGC 3′) and reverse (5′ GCC ATG AAG GTT CCT GCT CAT CAT 3′). PCR products were sequenced using reverse primer, and the quality of generated sequences was analyzed by Geospiza (http://www.geospiza.com/finchtv/).

RT-PCR analysis. Total RNA from MN-1 cells, E13 motor neurons or SBMA mice was extracted using the Trizol method. cDNA was generated using SuperScript III First-Strand Synthesis System (Life Technologies). For NPCs, total RNA extraction from EBs or NPCs was performed using the RNeasy kit (Qiagen). cDNA was generated with the High Capacity reverse transcription kit (Life Technologies). Quantification of mRNA was performed using an Applied Biosystems 7500 Real Time Sequence Detection System with ABI Assays-on-Demand primers and TaqMan-based probes. ABI TaqMan primer and probe set designations are given in Supplementary Table 1. Relative fold change expression levels were calculated and normalized to controls, and all experiments were done in triplicate.

Immunoblotting analysis. Cells were harvested in RIPA lysis buffer (10 mM Tris, pH 7.5, 0.1% SDS, 1% DDOC, 0.01% TX-100, 150 mM NaCl) and homogenized...
by passing 5 times through a 26.5-gauge syringe. Fifty micrograms of protein lysate were run on Bis-Tris gels (Life Technologies) and transferred to PVDF membranes (Millipore) using a semi-dry transfer system (Life Technologies). The membranes were blocked with 5% nonfat dried milk at room temperature for 1 h and then probed with rabbit anti-LC3 antibody (1:1,500, Novus #2220), rabbit anti-p62 antibody (1:1,000, MBL #PM045), rabbit anti-AR (1:2,000, Santa Cruz H280 sc-13062), rabbit anti-TFEB (1:1,000, Cell Signaling #4240BC) or mouse anti-β-actin antibody (1:2,000, Abcam #8226) in 5% BSA at 4 °C overnight. After washing, membranes were incubated with HRP secondaries (1:5,000, Santa Cruz sc-2005 (anti-mouse), sc-2004 (anti-rabbit)) in 5% milk, PBS-T for 1 h at room temperature. After treatment with enhanced ECL chemiluminescence (Upstate), the membranes were visualized by autoradiography. Filter trap assays were performed as described previously17. Cellulose acetate membranes (Whatman) were blotted overnight with constant agitation at 4 °C in anti-AR antibody N-20 (Santa Cruz sc-816; 1:500), followed by horseradish peroxidase–conjugated secondary antibody (1:5,000; Santa Cruz sc-2004), and then visualized using ECL chemiluminescence. For the subcellular fractionation, the PVDF membranes were analyzed using the Odyssey western blotting system (Li-Cor). The membrane was blotted overnight in constant agitation at 4 °C with anti-TFEB (1:500; Cell Signaling 4240S), anti-histone H3 (1:5,000; Cell Signaling 3638S) and anti-Lamp1 (1:1,000; Cell Signaling 3243S) followed by IRDye secondary antibody (1:10,000; Li-Cor anti-mouse 680RD 926-68072, anti-rabbit 800CW 926-32213, anti-goat 680RD 926-68074) and then visualized with the Odyssey infrared imaging system (Li-Cor). All densitometry was performed using NIH ImageJ software. For figures containing cropped gel blots, we have provided full-length blots (Supplementary Fig. 9).

Ultrastructural analysis. Male non-transgenic, YAC AR20 or YAC AR100 mice (C57BL6/J background) (n = 3 or 4 per cohort) were sacrificed at 6 months and 14 months of age, and lumbar cord regions were processed as previously described37. Semi-thin (1 μm) sections were stained with Richardson’s and examined by light microscopy to assure cytological tissue quality. Thirty-nanometer sequential sections were collected on coated copper or nickel 200-mesh grids and then stained with lead citrate and uranyl acetate for contrast. Motor neurons were mapped and photographed. Each section yielded an average of 7 motor neurons with 3 sections per individual. The sections were taken from 3 different blocks and thus represented the L3–L5 region of the lumbar spinal cord. With a Philips TEM/CM 10 electron microscope, 50–60 motor neurons per cohort were photographed, scanned into Adobe Photoshop and examined by three independent observers, blinded to experimental conditions, who had been trained to identify autophagic vesicles and autolysosomes on the basis of established criteria and sample images from the literature. Mice were kept in standard dark/light cycle conditions and housed under standard requirements of rodent IACUC guidelines (3 or 4 individuals per cage). If isolation was required due to fighting, singly housed animals were provided enrichment. All animal experiments were performed in accordance with institutional IACUC guidelines at the University of Washington and UCSD.

Long-lived protein turnover assay. We labeled nascent protein populations in MN-1 cells with the nonradioactive methionine analog 1-azidohomoalanine (AHA) (Invitrogen) and chased with unlabeled medium. Cell lysates were collected after 48 and 100 h for long-term protein clearance analysis. Briefly, equal amounts of lysate were run on a western blot, and AHA-labeled protein populations were detected by biotin-alkyne reaction. The amount of labeled protein per lane per time point was quantified by densitometry (ImageJ), and linear fit curves over time were determined for each cell line. The slope of each curve was determined to be the rate of lysosomal-mediated long-lived protein turnover.

Statistical analysis. No statistical methods were used to determine sample sizes, but our samples sizes are similar to or exceed those reported in previous publications28,41,50. For the iPSC experiments, 10 to 12 clones were generated, and we randomly selected three different clones for each individual line to compensate for the high degree of clonal variability. A total of 9 clones for the control group and 10 clones for the SBMA group were analyzed in the autophagic flux assay, which is well in excess of previous publications in the field44. Autophagic flux data collection and analysis were performed in a manner blinded to the conditions of the experiment. For experiments using mice, no randomization was required, as groups were generated on the basis of genotype status. All transgenic male animals were analyzed and their genotypes determined twice. Data distribution was assumed to be normal. All data were prepared for analysis with standard spreadsheet software (Microsoft Excel). All errors bars shown in the figures are s.e.m. Statistical analysis was done using Microsoft Excel, GraphPad Prism v5 or the VassarStats website (http://vassarstats.net/). For ANOVA analysis involving multiple sample comparisons, we performed post hoc testing to discriminate significance relationships. For t-test analysis, we applied one-tailed tests for independent samples.

A Supplementary Methods Checklist is available.

48. Brooks, B.P. et al. Characterization of an expanded glutamine repeat androgen receptor in a neuronal cell culture system. Neurobiol. Dis. 3, 313–323 (1997).
49. Malik, B. et al. Absence of disturbed axonal transport in spinal and bulbar muscular atrophy. Hum. Mol. Genet. 20, 1776–1786 (2011).
50. Bailey, C.K., Andriola, I.F., Kampinga, H.H. & Merry, D.E. Molecular chaperones enhance the degradation of expanded polyglutamine repeat androgen receptor in a cellular model of spinal and bulbar muscular atrophy. Hum. Mol. Genet. 11, 515–523 (2002).