
















Inhibition of retrograde transport modulates misfolded protein accumulation and clearance in motoneuron diseases

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ABSTRACT

Motoneuron diseases, like spinal bulbar muscular atrophy (SBMA) and amyotrophic lateral sclerosis (ALS), are associated with proteins that because of gene mutation or peculiar structures, acquire aberrant (misfolded) conformations toxic to cells. To prevent misfolded protein toxicity, cells activate a protein quality control (PQC) system composed of chaperones and degradative pathways (proteasome and autophagy). Inefficient activation of the PQC system results in misfolded protein accumulation that ultimately leads to neuronal cell death, while efficient macroautophagy/autophagy-mediated degradation of aggregating proteins is beneficial. The latter relies on an active retrograde transport, mediated by dynein and specific chaperones, such as the HSPB8-BAG3-HSPA8 complex. Here, using cellular models expressing aggregate-prone proteins involved in SBMA and ALS, we demonstrate that inhibition of dynein-mediated retrograde transport, which impairs the targeting to autophagy of misfolded species, does not increase their aggregation. Rather, dynein inhibition correlates with a reduced accumulation and an increased clearance of mutant ARpolyQ, SOD1, truncated TARDBP/TDP-43 and expanded polyGP C9ORF72 products. The enhanced misfolded protein clearance is mediated by the proteasome, rather than by autophagy and correlates with the upregulation of the HSPA8 cochaperone BAG1. In line, overexpression of BAG1 increases the proteasome-mediated clearance of these misfolded proteins. Our data suggest that when the misfolded proteins cannot be efficiently transported toward the perinuclear region of the cells, where they are either degraded by autophagy or stored into the aggresome, the cells activate a compensatory mechanism that relies on the induction of BAG1 to target the HSPA8-bound cargo to the proteasome in a dynein-independent manner.

ARTICLE HISTORY

Received 4 November 2015
Revised 28 February 2017
Accepted 15 March 2017

KEYWORDS

aggregation; amyotrophic lateral sclerosis; autophagy; BAG1; BAG3; HSPB8; misfolded protein; proteasome; protein quality control

Introduction


Motoneuron diseases (MNDs) are neurodegenerative diseases (NDs) in which cortical and spinal motoneurons are primarily affected, although other cell types (e.g., glial or muscle cells) also contribute to onset and/or progression of disease.^{1–11} Some MNDs forms are associated with the presence of toxic proteins with aberrant conformations (misfolded proteins).^{12–15} Typical examples of MNDs associated with misfolded proteins toxicity are spinal and bulbar muscular atrophy (SBMA) and amyotrophic lateral sclerosis (ALS). SBMA is due to an expansion of the CAG repeat in exon 1 of the androgen receptor (*AR*) gene translated into an abnormally long polyQ tract in the AR protein (ARpolyQ).¹⁶ Interestingly, elongated polyQ exerts neurotoxicity

only after ARpolyQ activation by its natural ligand testosterone (T),¹⁷ which implies chaperones release and protein conformational changes for nuclear translocation.¹⁸ Protein misfolding is also relevant in ALS either in sporadic (sALS) or familial (fALS) forms, which are clinically indistinguishable. While fALSs represent only 10% of total ALSs, several fALS-responsible mutant proteins (e.g., SOD1 [superoxide dismutase 1, soluble], TARDBP/TDP-43 [TAR DNA binding protein], FUS, OPTN [optineurin], UBQLN [ubiquilin], etc.) have been found to aberrantly behave as wild-type (WT) forms also in most sALSs,¹⁹ suggesting that common pathogenic mechanisms contribute to both fALS and sALS.

To prevent misfolded protein toxicity, cells respond by enhancing the functions of the protein quality control (PQC)

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 Supplemental data for this article can be accessed on the [publisher's website](#).

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system. The PQC system comprises chaperone proteins (e.g., the heat shock proteins, HSPs) and degradative systems (e.g., the ubiquitin proteasome system, UPS; the different autophagic pathways, etc.). When poorly removed by the PQC system, misfolded proteins accumulate in cells forming different types of inclusions. Large cytoplasmic inclusions are generally thought to be protective, at least in their initial stages of formation.^{18,20-23} Inclusions also localize into the nucleus or even in neurites exerting toxicity by altering several pathways regulating neuronal functions.^{24,25} Inclusion formation proceeds through several steps. The process typically begins with the dynein and dynactin mediated transport of misfolded proteins escaping proteasomal degradation to the microtubule-organizing center (MTOC), where they form aggresomes.²⁶ Aggresomes could be removed by autophagy, but, when this system fails, aggresomes are gradually converted into insoluble inclusions.

Several multiprotein complexes assist the misfolded proteins transport to the MTOC. One example is a complex involving the HSPB8 (heat shock protein B8) chaperone, a small HSP family member. HSPB8 promotes autophagic removal of misfolded proteins, including ARpolyQ, HTT (huntingtin)-polyQ, ATXN3 (ataxin 3)-polyQ, mutant SOD1, full-length TARDBP (FL-TARDBP) and its ALS-associated fragments, in different ND models.²⁷⁻³³ HSPB8 acts together with BAG3 (BCL2-associated athanogene 3), a cochaperone of HSPA8/HSC70 (heat shock protein 8) and HSPA1A.^{28,29,34-37} All these proteins are induced when the proteasome is inhibited²⁷⁻²⁹ to route misfolded proteins to autophagy.^{36,38} The ubiquitin ligase STUB1/CHIP (STIP1 homology and U-box containing protein 1) assists this complex by ubiquitinating clients^{28,29,39} for their SQSTM1/p62 (sequestosome 1)-mediated targeting to phagophores, the precursors to autophagosomes.⁴⁰ BAG3 is a scaffold protein that plays a central role in autophagy.⁴¹⁻⁴⁵ With its proline-rich (PXXP) repeat motif it specifically bridges misfolded proteins to the dynein complex via the YWHA/14-3-3 protein, to allow their delivery to the MTOC for their autophagic degradation.^{28,29,39,42} Several misfolded proteins found in aggresomes are not ubiquitinated,⁴⁶ and BAG3 activity can also be independent from their ubiquitination status.⁴² Alternatively, aggresome formation is controlled by HDAC6 (histone deacetylase 6), which requires substrate ubiquitination; also HDAC6 interacts with dynein (through DCTN1/p150^{Glued} [dynactin 1]), but only after ubiquitination of substrates escaping UPS degradation,⁴⁷⁻⁵¹ and transports misfolded protein to MTOC.^{47,49,52-54} Both BAG3-mediated and HDAC6-mediated clients routing to aggresomes require dynein.²⁶

Notably, mutations in *DYNC1H1*, the gene coding for dynein (dynein cytoplasmic 1 heavy chain 1, the essential subunit of the cytoplasmic dynein complex) are linked to different neuronal diseases⁵⁵⁻⁶² involving autophagy alterations. Therefore, dynein impairment may have a role in neurodegeneration. Surprisingly, dynein activity deficiency correlates to improvement of motor function and prolonged survival of the transgenic SOD1^{G93A} fALS mice⁶³⁻⁶⁵ suggesting that the involvement of dynein in disease progression may be more complex than expected.

In this study, we analyzed the role of dynein on the clearance of misfolded ARpolyQ and of different proteins involved in ALS (human mutant SOD1, TARDBP, RAN translated polyGP from

C9ORF72). We found that depletion or chemical inhibition of dynein decreased the aggregation of misfolded proteins and enhanced their proteasomal degradation. Dynein inhibition was paralleled by an upregulation of *BAG1* (BCL2-associated athanogene 1) expression, which targets the HSPA8 and HSPA1A-bound clients to the UPS.^{38,66,67} Indeed, BAG1 overexpression enhanced misfolded protein degradation via the proteasome pathway even when dynein was inhibited. If BAG3 dynein-mediated transport of misfolded species to autophagy fails, an alternative pathway, which relies on BAG1 and the UPS, is activated to efficiently assist misfolded protein clearance. We postulate that the alteration of the rerouting system from autophagy to proteasome (and possibly vice versa) when one of the 2 degradative pathways is overwhelmed or impaired, rather than the alteration of the degradative pathway per se might be responsible for the accumulation of aggregate-prone proteins in MNDs.

Results

HSPB8 promotes the clearance of misfolded ARpolyQ

We initially validated the prodegradative effect of HSPB8 on aggregated forms of ARpolyQ generated by T treatment. Fig. 1A shows that mutant ARpolyQ resides in the cytoplasm in basal conditions and translocates to the nucleus after T treatment. In approximately one-third of total transfected cells^{20,68} (see also Fig. 4F) nuclear translocation was incomplete and the misfolded ARpolyQ fraction aggregated as inclusions visible by immunofluorescence analysis (IF; Fig. 1A), and quantifiable in Filter Retardation Assay (FRA; Fig. 1B; compare lane 1 and lane 2, without or with T, respectively) in agreement with previous reports.^{31,32,69} In basal conditions (T-untreated cells) ARpolyQ aggregation propensity is barely detectable and comparable to that of wild-type (WT) AR (see below and FRA in Fig. S1). The levels of insoluble WT AR (AR.Q23 in the absence or presence of T) and of T-untreated ARpolyQ (AR.Q46) were much lower than that found for T-activated ARpolyQ (Fig. S1, almost 2-fold increase over its control, untreated AR.Q46). We have already shown that the misfolded ARpolyQ accumulates upon T treatment, decreasing autophagy and leaving unaffected the proteasome,^{31,32,69} and have found that the chaperone HSPB8 favors its degradation by autophagy. In line, here we show that both the T-induced cytoplasmic inclusions (Fig. 1A) and the insoluble ARpolyQ fraction (Fig. 1B, upper inset and quantification in bar graphs) disappear when HSPB8 is overexpressed, and the effect was present, at a lower intensity, on the total soluble ARpolyQ protein identified by western blot (WB; Fig. 1B lower inset, quantification in Fig. S2). No major effects are exerted by HSPB8 on WT AR.³² Since HSPB8 requires BAG3,^{30,34,70} for SQSTM1 cargo insertion into phagophores at the MTOC,^{28,29,39} and BAG3 utilizes the dynein (and the YWHA/14-3-3 protein which cosegregates with ALS-protein aggregates⁷¹⁻⁷⁴)-mediated transport of the complex, we analyzed the role of dynein in HSPB8-mediated autophagic clearance of aggresomes.

We designed a specific siRNA recognizing *Dync1h1* (*Dync1h1* siRNA) that abolished dynein production in NSC34 cells (Fig. 1C and Fig. 1D), and then we evaluated its effects on GFP-tagged ARpolyQ (GFP-AR.Q39) stably expressed in

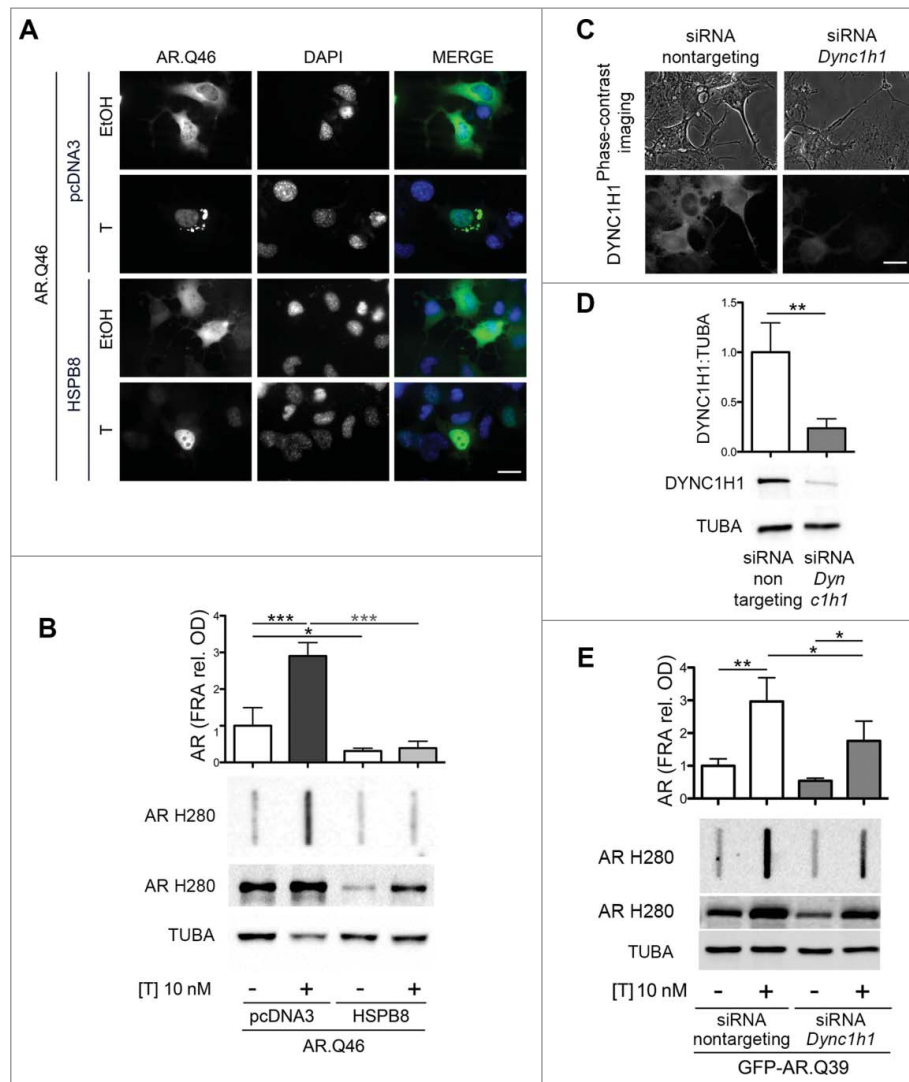


Figure 1. Autophagic clearance of ARpolyQ. NSC34 cells cotransfected with AR.Q46 and pcDNA3 or HSPB8, and treated with vehicle (ethanol, EtOH) or 10 nM T, for 48 h. (A) Immunofluorescence microscopy (IF) analysis (63x magnification) of AR (green); nuclei were stained with DAPI (blue); scale bar: 20 μ m. (B) WB and FRA show AR.Q46 total levels and AR.Q46 insoluble fraction respectively. TUBA was used as loading control. Bar graph represents the FRA mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD (* = $P < 0.05$, *** = $P < 0.001$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test). (C) Phase contrast imaging and IF performed on NSC34 cells transfected with *Dync1h1* siRNA show cell morphology and dynein distribution. Scale bar: 20 μ m. (D) WB analysis of NSC34 cells transfected with *Dync1h1* siRNA shows the levels of dynein heavy chain; TUBA was used as loading control. Bar graph represents the WB mean relative optical density measured over 3 independent biological samples for each condition ($n = 3$) \pm SD (** = $P < 0.01$, one-tailed unpaired Student *t* test). (E) FRA and WB of NSC34 stably expressing GFP-AR.Q39 and transfected with *Dync1h1* siRNA show AR.Q39 insoluble fraction accumulation after 10 nM T treatment and reduction after dynein silencing. Bar graph represents the FRA mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD (* = $P < 0.05$, ** = $P < 0.01$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test).

NSC34 cells in an inducible manner under the TetON promoter. Unexpectedly, after dynein downregulation, we found a significant reduction of the total amount of T-induced misfolded ARpolyQ insoluble species retained by the cellulose acetate membrane in FRA (Fig. 1E). In addition, a significant decrease of the total levels of ARs in WB was observed in dynein downregulated samples, both in the case of unactivated ARpolyQ (normally processed by the proteasome) and of T-treated ARpolyQ (see WB quantification in Fig. S2).

Effects of dynein impairment on autophagy in basal conditions or after autophagy activation

We next investigated whether dynein depletion correlated with a compensatory activation of autophagy. We initially evaluated

the effects of dynein impairment in basal autophagy conditions in NSC34 cells. We measured the intracellular distribution and levels of the 2 autophagy markers SQSTM1 and MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3). To our surprise, we found that in basal conditions, dynein downregulation does not alter the overall properties of these markers (Fig. 2A), suggesting that its impact on cargo delivery does not result in a compensatory activation of autophagy. However, since dynein may also regulate the fusion between the autophagosomes and lysosomes, we then further investigated the involvement of dynein on LC3 dynamics. To this purpose, we pharmacologically blocked the basal autophagic flux with NH_4Cl , and we found that the levels of the lipidated LC3-II form were similarly significantly increased by this treatment in all conditions tested (see quantification of LC3-II to TUBA and LC3-II to LC3-I ratio

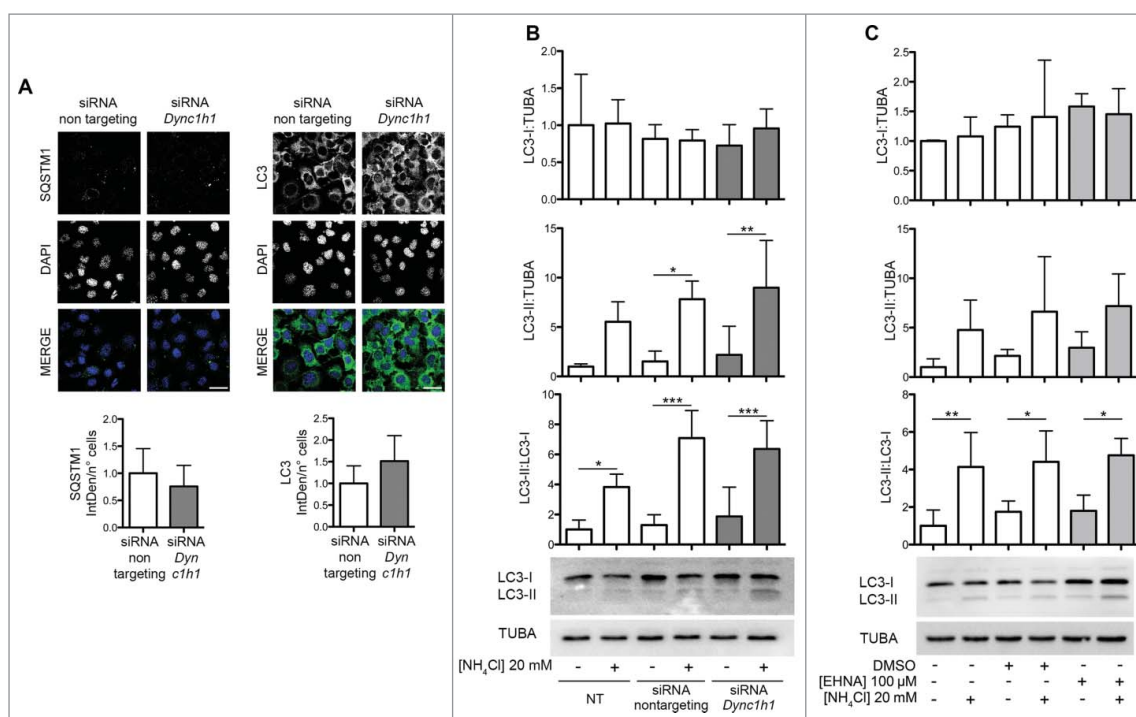


Figure 2. Effect of dynein-silencing on autophagy and ARpolyQ clearance. (A) Confocal microscopy analysis (40x magnification) performed on NSC34 cells transfected with *Dync1h1* siRNA shows SQSTM1 or LC3 distribution (green); nuclei were stained with DAPI (blue); scale bars: 50 μ m. Bar graphs represent the quantification of total green-fluorescence integrated density (IntDen) normalized on cells number. Six images were analyzed for each condition. (B) WB of LC3-I and LC3-II levels performed on NSC34 transfected with *Dync1h1* siRNA and treated with 20 mM NH₄Cl for the last 1.5 h. Nontargeting siRNA was used as control; TUBA was used as loading control. Bar graph represents the mean relative optical density computed over 3 independent biological samples for each condition (n = 3) \pm SD (* = $P < 0.05$, ** = $P < 0.01$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test); (C) WB of LC3-I and LC3-II levels performed on NSC34 treated with 100 μ M EHNA and/or 20 mM NH₄Cl for the last 1.5 h. Nontargeting siRNA was used as control; TUBA was used as loading control. Bar graphs represent the mean relative optical density computed over 3 independent biological samples for each condition (n = 3) \pm SD (* = $P < 0.05$, ** = $P < 0.01$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test).

in Fig. 2B and Fig. S2, respectively). The relatively low LC3-II accumulation in the presence of NH₄Cl and almost constant levels of LC3-I (compare basal vs activated autophagy in Fig. 3D) are supportive of low levels of basal autophagic activity in these cells. Overall, these results support preservation of basal autophagic flux even upon dynein knockdown and suggest that dynein has only a minor effect on the basal autophagic flux.

However, it is possible that, whereas *Dync1h1* siRNA is very efficient in each single cell, some cells may resist transfection and dynein downregulation may not involve the whole cell population. Moreover, ATP-independent activities of dynein have been described,⁷⁵ with possible impact on different intracellular pathways. This prompted us to adopt a second approach to block dynein-mediated transport. Therefore, we chemically and selectively blocked the ATP-dependent activity of dynein (specifically required for cargo delivery) with erythro-9-[3-(2-(hydro-synonyl)] adenine (EHNA).^{76,77} EHNA binds specifically to the ATP binding site of dynein inhibiting the ATPase and motor activities, selectively impairing the microtubule-based transport toward the MTOC. We initially compared the effects of EHNA with that of dynein-silencing in our NSC34 cells, by analyzing LC3-II levels in the presence or absence of NH₄Cl to block its lysosomal degradation (Fig. 2C and Fig. S2).

We found that the effects of dynein downregulation and of EHNA on the LC3 turnover and LC3-II to LC3-I ratio overlap in both conditions, supporting the view that dynein impairment has no effect on LC3 turnover in condition of basal autophagy (Fig. 2C and Fig. S3). At present, we are unable to explain why

and how autophagosomes assemble when autophagy is running at basal levels, and the relevance of dynein in this context. One possibility is that, even after siRNA or EHNA treatment, cells maintain a residual dynein activity, which may be sufficient to sustain the autophagosome formation at low levels, or other alternative dynein-independent pathways of cargo delivery may exist. It is also possible that in condition of basal autophagy, the fusion between autophagosomes and lysosomes (which is required to degrade LC3-II into the autolysosomes) is only partially sustained by dynein; the role of dynein may become more relevant when the flux is activated in response to specific stimuli. However, since misfolded proteins likely activate autophagy (and may also block autophagy),³² we analyzed the effects of dynein impairment when autophagy flux was pharmacologically induced. To this purpose, we compared the alteration induced by EHNA on autophagy in basal conditions or induced with trehalose (a MTOR-independent autophagy activator^{31,32,78-80}). First, EHNA alone had no effect on *SQSTM1* or on *LC3* mRNA expression levels (Fig. 3A and B, respectively). Trehalose induced the expression of *SQSTM1* and *LC3* mRNAs; but, dynein inhibition with EHNA counteracted the effects of trehalose on the expression of the 2 autophagy markers (Fig. 3A and B). This suggests that cells respond to dynein inhibition with a downregulation of autophagy activation (and/or possibly by activating alternative pathways? see later). The data were confirmed also with both IF (Fig. 3C) and WB (Fig. 3D and quantification in Fig. S3) analyses. Trehalose-treated cells were highly immunoreactive (Fig. 3C, upper panels); to appreciate the effects

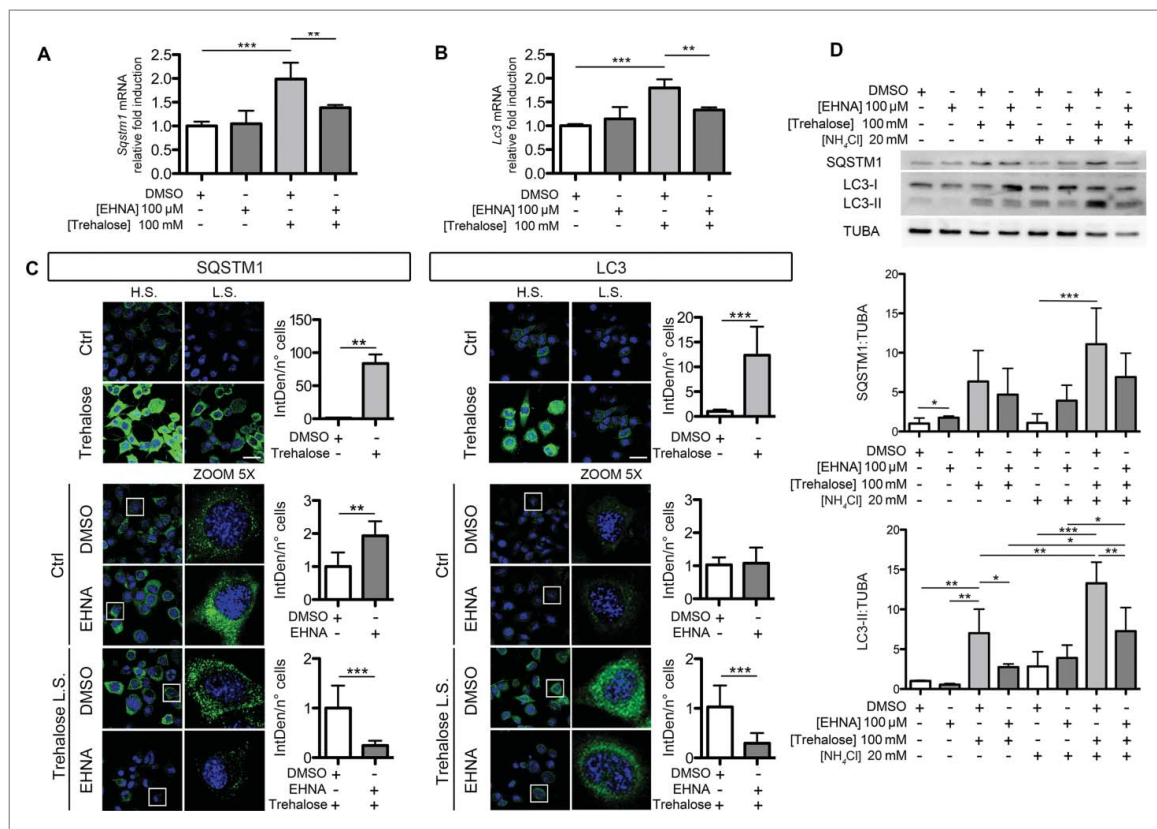


Figure 3. Autophagy modulation by EHNA treatment. NSC34 cells treated with DMSO, 100 μM EHNA and/or 100 mM trehalose for 48 h; (A, B) the bar graph represents *Sqstm1* and *Lc3* mRNA relative levels normalized with *Rplp0* mRNA levels. Analysis was conducted on 4 independent biological samples for each condition ($n = 4$) \pm SD (** = $P < 0.01$, *** = $P < 0.001$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test). (C) Confocal microscopy analysis shows SQSTM1 and LC3 localization (40x magnification); scale bars: 50 μm. Bar graph represents the quantification of total green fluorescence integrated density (IntDen) normalized on cells number. Six images were analyzed for each condition ($n = 6$) \pm SD (** = $P < 0.01$, *** = $P < 0.001$; one-tailed unpaired Student *t* test). Lower gain was used to acquire trehalose-treated cells (low sensitivity [L.S.]) compared to control (high sensitivity [H.S.]). (D) WB analysis shows SQSTM1 and LC3 levels; TUBA was used as loading control. Bar graphs represent the mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD of SQSTM1:TUBA (middle inset) and LC3-II:TUBA (low inset) ratio (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$; One-Way ANOVA followed by the Uncorrected Fisher LSD test).

of EHNA treatment (Fig. 3C, lower panels) images at a lower gain were captured (low sensitivity: L.S.). Indeed, trehalose increased endogenous SQSTM1 and LC3 protein levels (IF quantification per cells reported in bar graphs of Fig. 3C, upper panels, and WB analysis in Fig. 3D, with relative quantification in the bar graphs and in Fig. S3) and induced the classical distribution expected for the 2 markers when autophagy is activated (i.e., LC3-II punctated distribution) (Fig. 3C). In basal conditions, EHNA does not modify the endogenous LC3 levels (Fig. 3C, see also Fig. 2C) or its conversion to LC3-II (Fig. 3C and D, and quantification in the lower bar graphs and Fig. S3), while it significantly increased of almost 2-fold SQSTM1 immunoreactive levels in IF (Fig. 3C, left panels), and 1.7 fold in WB (Fig. 3D and S4). A difference has been found between the effects of dynein inhibition with EHNA and dynein downregulation with *Dync1h1* siRNA on SQSTM1 immunoreactivity quantified using confocal microscopy (compare Fig. 2A with Fig. 3C). However, as a small molecule, EHNA affects the entire pool of cells, while the siRNA only targets a fraction of them. Therefore, although both experimental approaches affect autophagy, differences can be observed in the 2 conditions. It is also possible that these differences are associated with the ATP-independent activities of dynein (see above and⁷⁵).

During trehalose-stimulated autophagy, we found that EHNA reduced the overall levels of the endogenous SQSTM1

and LC3 proteins (IF in Fig. 3C), in agreement with the observed transcriptional downregulation (Fig. 3A and B). Moreover, analysis of LC3-II degradation by addition of NH₄Cl, in cells with trehalose-stimulated autophagy (Fig. 3D and quantification in the lower bar graph and in Fig. S3), revealed that EHNA abolished the increase in LC3-II turnover induced by trehalose. These data suggest that dynein inhibition counteracts the formation of trehalose-induced LC3-II-positive autophagosomes, possibly by preventing their formation. Therefore, dynein inhibition with EHNA does not affect autophagy in basal conditions, but prevents the trehalose-stimulated formation of autophagosomes and this may in turn stimulate the activation of alternative pathways of degradation.

Dynein blockage reduces the ARpolyQ aggregation in an autophagy-independent manner

To test the role of dynein on ARpolyQ clearance we utilized different and complementary cell models of SBMA, based on neuronal cells stably expressing in an inducible manner or transiently expressing WT or mutant ARpolyQ. Based on data from literature of different laboratories, including ours,^{20,25,31,32,69,81-87} as control for ARpolyQ we utilized either the WT AR (with unexpanded polyQ) or the unactivated (unbound) ARpolyQ (which is not pathogenic). When the

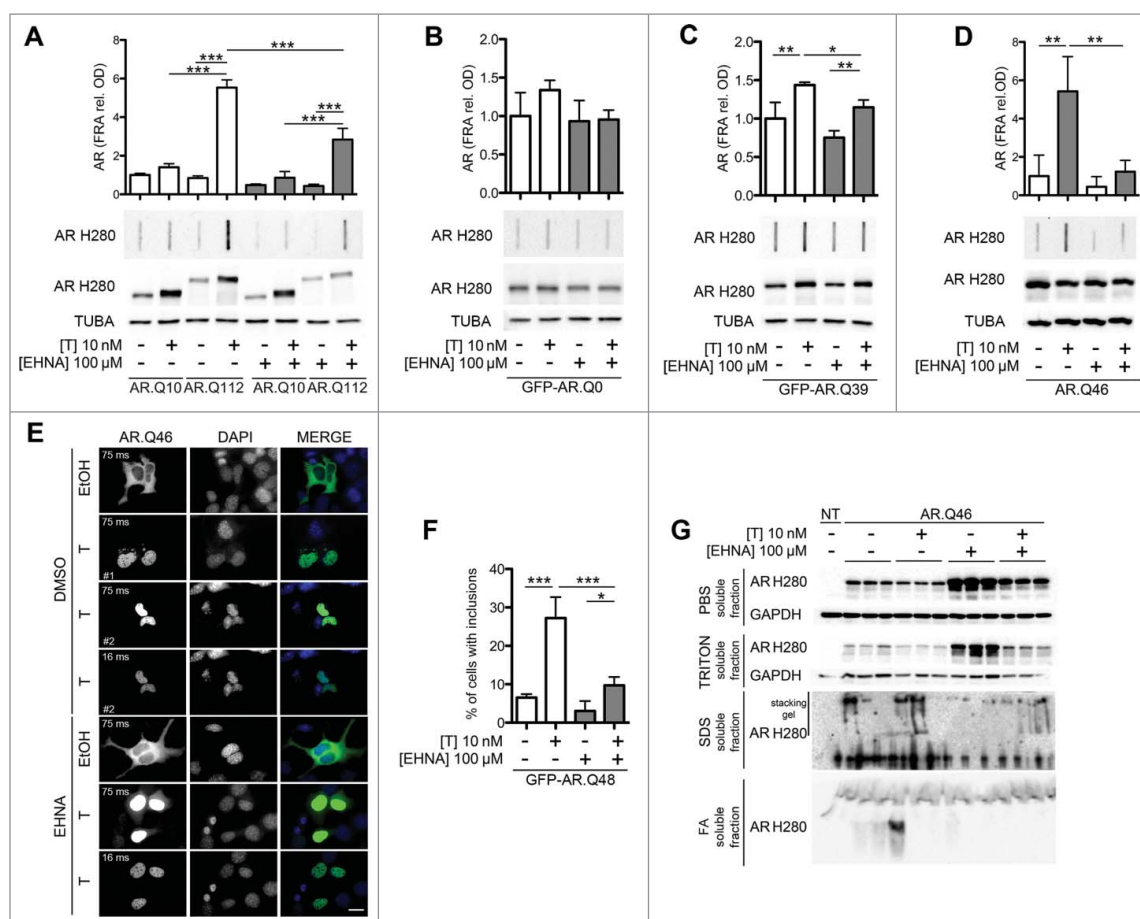


Figure 4. Effect of dynein ATPase activity-inhibition on ARpolyQ clearance. FRA and WB show ARpolyQ total levels and ARpolyQ insoluble fraction, respectively, of: (A) PC12 cells stably transfected with AR.Q10 and AR.Q112 induced with 1 μg/mL doxycycline for 72 h. Cells were treated with ethanol (EtOH) or 10 nM T and/or EHNA 100 μM for the last 48 h prior to analysis. TUBA was used as loading control and bar graphs represent the FRA mean relative optical density computed over 3 independent biological samples for each condition (n = 3) ± SD (*** = $P < 0.001$; One-Way ANOVA followed by the Uncorrected Fisher LSD test); (B) TR4 NSC34 cells stably transfected with GFP-AR.Q0 induced with 1 μg/mL doxycycline for 72 h; (C) TR4 NSC34 cells stably transfected with GFP-AR.Q39 induced with 1 μg/mL doxycycline for 72 h; (D) NSC34 cells transiently transfected with AR.Q46 for 48 h. Cells were treated with ethanol (EtOH) or 10 nM T and/or EHNA 100 μM for the last 48 h prior to analysis. (C and D) TUBA was used as loading control and bar graphs represent the FRA mean relative optical density computed over 3 independent biological samples for each condition (n = 3) ± SD (* = $P < 0.05$, ** = $P < 0.01$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test). (E) IF performed on NSC34 cells transfected with AR.Q46 and treated with 10 nM T or EtOH and 100 μM EHNA or DMSO for 48 h. AR was stained with AR H280 antibody (green), nuclei were stained with DAPI (blue) (63x magnification); scale bar: 20 μm. (F) Bar graph represents the mean percentage of transfected cells with inclusions computed over 3 independent biological samples for each condition (n = 3) ± SD (* = $P < 0.05$, *** = $P < 0.001$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test). Ten fields for each biological sample were analyzed. (G) WB analysis of PBS, Triton X100, SDS and FA lysates fractions of NSC34 cells transfected with AR.Q46 and treated with 10 nM T or EtOH and 100 μM EHNA or DMSO for 48 h. GAPDH was used as loading control.

experimental design was particularly complex, we compared the “neurotoxic” ligand activated ARpolyQ, with the “not neurotoxic” unactivated (nonpathogenic) ARpolyQ.^{18,20,25,32,69,86-88}

We initially used rat pheochromocytoma PC12 cells stably expressing AR.Qn (n = 112, and WT n = 10, developed and kindly provided to us by Dr. Diane Merry, Thomas Jefferson University, Philadelphia, PA, USA⁸⁹) with a very long polyQ tract highly prone to aggregate into the nucleus.^{31,90} Using these models, in FRA (Fig. 4A), we found that T-induced misfolded AR.Q112 insoluble species formation, while EHNA reduced this accumulation (Fig. 4A). EHNA had no effects on T-untreated AR.Q112 or on WT AR (AR.Q10), which do not form insoluble inclusions (Fig. 4A). We then used mouse motoneuronal NSC34 cells stably expressing GFP-AR.Qn (n = 39 and control n = 0), where the mutant polyQ has a size that causes SBMA, but with late onset and low progression rate, and it is in the very low range of expansions identified in patients.⁹¹⁻⁹³ AR.Q39 does not form intracellular inclusions

visible in IF (not shown), but it forms low amount of insoluble species detectable in FRA (Fig. 4C). As expected, control AR.Q0 does not form insoluble species, even after T treatment (Fig. 4B). Because of its shorter polyQ size, the AR.Q39 is likely to be less prone to misfold than AR.Q112. It is conceivable that AR.Q39 may have a milder impact than AR.Q112 on the degradative system (compare Fig. 4A and C); but, after its T activation, AR.Q39 is still mostly processed by autophagy (see also later in Fig. 5A, 3-MA-treated samples). Also in this case, EHNA significantly reduced the formation of T-induced misfolded AR.Q39 insoluble species to levels similar of that found in untreated AR.Q39 (Fig. 4C). No effects were seen on AR.Q0 (Fig. 4B). Interestingly, EHNA was also active on preformed inclusions (EHNA added 24 h after T treatment and maintained for other 24 h, Fig. S4), suggesting that these insoluble species are still dynamic at this stage.

Next, we enhanced the stressful conditions for motoneuronal cells by overexpressing proteins with transient

transfection experiments, and used a polyQ tract of an intermediate size (Q46, which falls in the middle range of the pathological polyQ size described in SBMA patients⁹¹⁻⁹³). The aim was to verify whether, by producing a large amount of misfolded ARpolyQ insoluble species, the effect of

dynein-mediated transport blockage was still present. As shown in Fig. 4D, overexpression of AR.Q46 correlated with an accumulation of a large fraction of T-induced misfolded ARpolyQ-insoluble species. No accumulation was observed in untreated AR.Q46 (Fig. 4D; WB quantification in

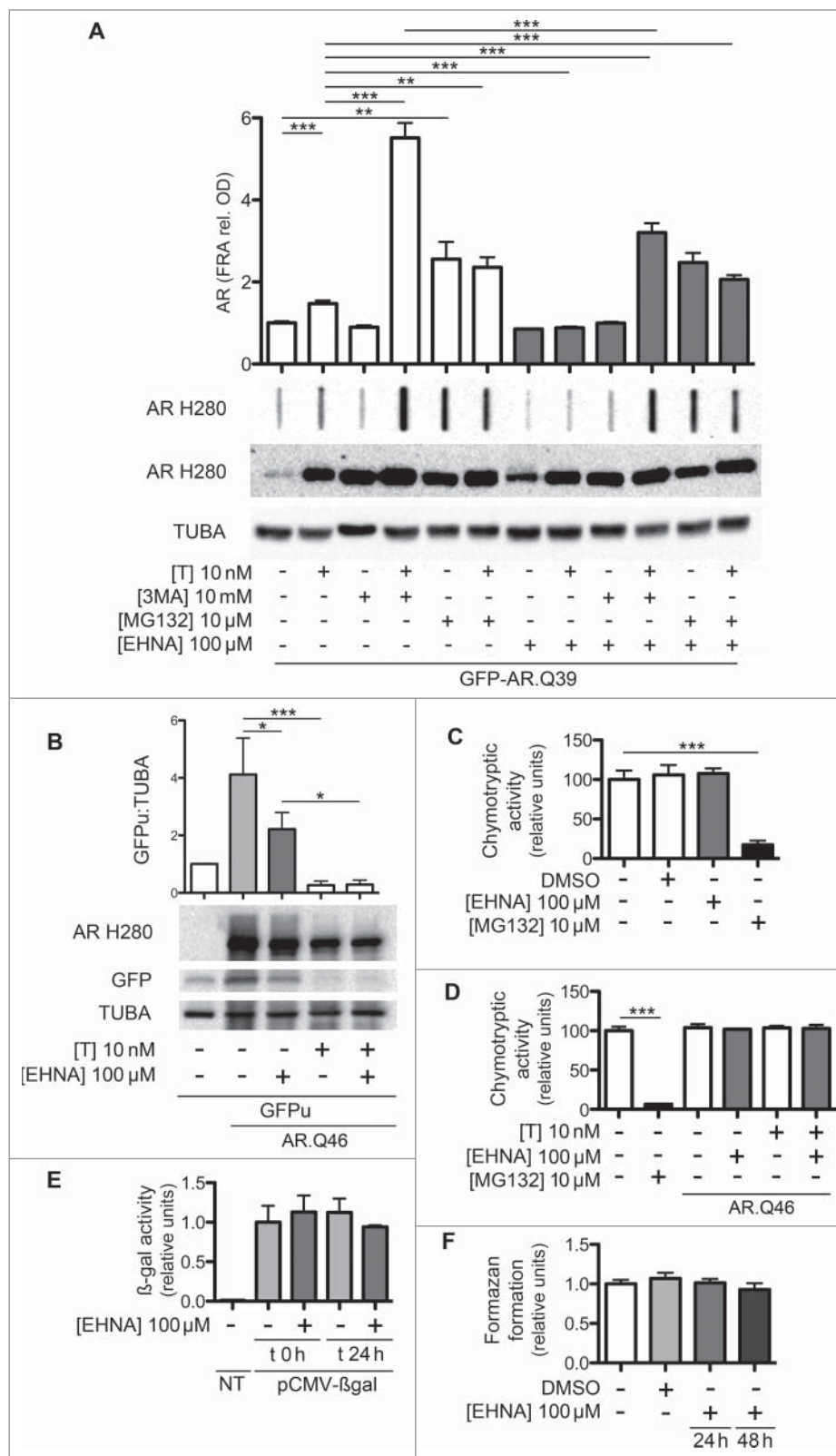


Figure 5. (For figure legend, see page 1287).

Fig. S3). The induction of ARpolyQ-insoluble species was similar (6-fold over control, Fig. 4A and D) in transiently transfected AR.Q46 (which aggregates in the cytoplasm,^{20,25,31,32,69,86,87} and Fig. 4E) and in stably transfected AR.Q112 (which aggregates in the nucleus^{24,89,90}). Even with the overexpressing conditions, dynein inhibition with EHNA reduced the accumulation of T-induced misfolded ARpolyQ-insoluble species to levels comparable to those found in untreated ARpolyQ (Fig. 4D). Dynein inhibition with EHNA partially affected also the overall levels of the various AR.Qn utilized in these experiments (see WBs in 4A, 4C and 4D and quantified in S4). In parallel, with the reduced aggregated forms of the various AR.Qn, the decrease (even if relatively small) of the overall T-treated ARpolyQ levels in WB, which comprises all SDS-soluble species (therefore, including the cytoplasmic ARpolyQ aggregates evaluated in IF), could be indicative of an increased clearance of the entire pool of the misfolded ARpolyQ fraction prior to its aggregation (see below). Another possibility is that by preventing ARpolyQ concentration and aggregation, chaperones might have a larger time-window to properly fold the mutant protein. This increased efficiency of chaperone activity might enhance the fraction of ARpolyQ detected in WB. Unfortunately, the relative contribution between clearance and correct folding of the mutant ARpolyQ allowed by dynein blockage is unpredictable and modifications of this balance explain why we observed some fluctuation of the total levels of ARpolyQ in WB after EHNA treatment.

Next, using IF microscopy we examined the effects of dynein inhibition on the formation of intracellular inclusions of mutant AR.Q46 in NSC34 cells (Fig. 4E). In line with our previous observations, the presence of ARpolyQ cytoplasmic aggregates (present only in about 30% of total transfected cells, Fig. 4F) correlated with a decreased nuclear bioavailability of the AR protein (Fig. 4E, see second panel row).²⁰ In cells treated with EHNA, only a few cells still contain aggregates, and in general, AR is free to migrate into the nucleus (Fig. 4E, see sixth and seventh panel rows) at levels comparable to that observed in EHNA-untreated T-treated cells with no cytoplasmic aggregates, in which ARpolyQ normally translocated into the nucleus (Fig. 4E, see third and fourth panel rows). Thereby, EHNA treatment counteracted the formation of cytoplasmic ARpolyQ inclusions induced by T (Fig. 4E), and this resulted in a higher localization of diffuse AR in the nucleus

(Fig. 4E, compare different exposure times utilized). Quantification data, performed using GFP-tagged ARpolyQ, showed that EHNA treatment almost completely counteracted the formation of inclusion in NSC34 cells after T treatment, since the total number of inclusions found after dynein inhibition was similar to that found in control (T-untreated) cells (Fig. 4E, F).

The WB in Fig. 4D, which represents the average of cells with or without aggregates, shows that the overall levels of soluble AR remains similar in the 2 conditions, apart from the fraction that is insoluble (as observed in FRA of Fig. 4D), which is indeed counteracted by EHNA.

Based on the observation reported above, we performed fractionation experiments using buffers characterized by increasing detergent power to analyze which particular insoluble fraction of ARpolyQ was mainly affected by EHNA treatment. The WB analysis on the solubilized pellets (see detailed scheme in Fig. S5), obtained after fractionation, revealed that cotreatment with EHNA and T increased the total levels of detergent-soluble AR.Q46, as compared to single treatment with T (Fig. 4G). This effect is possibly due to a shift between a phosphate-buffered saline (PBS)-Triton-insoluble fraction (likely associated with accumulating ARpolyQ in IF and FRA, and evaluated in the SDS-soluble fraction, (third inset)) to the PBS-Triton-soluble fraction of ARpolyQ (Fig. 4G [upper 2 insets]). Indeed, dynein inhibition increased the overall ARpolyQ content in NSC34 cell lysates (particularly in the PBS soluble fraction), and it also led to the complete removal of the ARpolyQ species present in the SDS-formic acid (FA) fractions (Fig. 4G). Some variability was found in the SDS/FA fractions obtained from T-treated NSC34 and AR.Q46 cells not exposed to EHNA. This is likely due to biological variations in the transition from a dynamic SDS-soluble (likely aggregates, Fig. 4G, third inset) to a stable SDS-insoluble (likely inclusions, Fig. 4G, fourth inset) status of the ARpolyQ aggregating materials, in cells. This transition may occur in very few samples, since in our experimental condition the amount of SDS-insoluble species is negligible. Notably, since EHNA should prevent ARpolyQ concentration and thus reduce aggregation of misfolded species, it also prevents the accumulation of all the species detected after fractionation. Collectively, these data suggest that EHNA prevents misfolded ARpolyQ accumulation into insoluble species, possibly by reducing their concentration, a process favoring protein-protein interaction and aggregation.

Figure 5. (see previous page) Dynein ATPase activity inhibition promotes ARpolyQ clearance via UPS. (A) NSC34 cells stably transfected with GFP-AR.Q39, treated with 10 nM T, 10 mM 3MA and/or 100 μ M EHNA for the last 48 h and/or 10 μ M MG132 for the last 16 h. WB shows ARpolyQ levels and FRA shows ARpolyQ insoluble fraction. Bar graph represents the FRA mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD (** = $P < 0.01$; *** = $P < 0.001$; one-tailed unpaired Student t test). (B) NSC34 cells transfected with GFPu reporter and/or ARpolyQ and treated with 10 nM T and/or 100 μ M EHNA. WB shows ARpolyQ and GFPu bands; TUBA was used as loading control. Bar graph represents the GFP WB densitometric analysis of 3 independent samples for each condition ($n = 3$) \pm SD (* = $P < 0.05$; *** = $P < 0.001$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test); (C) Proteasome chymotryptic activity assay performed on NSC34 treated with DMSO or 100 μ M EHNA and 10 μ M MG132 used as negative control. Bar graph represents the mean relative fluorescence normalized on control group and computed over 4 independent biological samples for each condition ($n = 4$) \pm SD (*** = $P < 0.001$, One-Way ANOVA followed by the Uncorrected Fisher LSD test). (D) Proteasome chymotryptic activity assay performed on NSC34 transfected with AR.Q46 and treated with 10 nM T, DMSO or 100 μ M EHNA and 10 μ M MG132 used as negative control. Bar graph represents the mean relative fluorescence normalized on control group and computed over 4 independent biological samples for each condition ($n = 4$) \pm SD (*** = $P < 0.001$, One-Way ANOVA followed by the Uncorrected Fisher LSD test). (E) β -galactosidase assay performed on NSC34 cells transfected with pCMV- β and treated with DMSO or 100 μ M EHNA for 24 or 48 h after transfection. Bar graph represents the mean relative absorbance normalized on transfected cells treated for 48 h and computed over 6 independent biological samples for each condition ($n = 6$) \pm SD (Two-Way ANOVA, not significant). (F) MTT assay performed on NSC34 cells treated with DMSO or 100 μ M EHNA for 24 or 48 h. Bar graph represents the mean relative absorbance normalized on untreated group and computed over 6 independent biological samples for each condition ($n = 6$) \pm SD (One-Way ANOVA, not significant).

Dynein inhibition reroutes ARpolyQ misfolded species from autophagy to proteasomal degradation

Autophagy is the main degradative pathway for the disposal of aggregates, which can inhibit proteasome activities by clogging the proteasome. Since dynein modulation does not impair basal autophagy, but maintains ARpolyQ in a soluble (likely monomeric) state compatible with proteasomal clearance, we investigated whether autophagy or proteasome inhibition affect the antiaggregating activity of EHNA. Fig. 5A shows that in AR.Q39 cells, when dynein is functional, both the inhibition of autophagy, with the inhibitor 3-methyladenine (3MA), or of the proteasome, with the inhibitor MG132, increased the levels of T-induced misfolded ARpolyQ insoluble species retained on the membrane by FRA. These species are considerably more abundant after 3MA than MG132 treatment, supporting that autophagy is the main degradative pathway activated by the cells to clear mutant ARpolyQ. Notably, in the case of the stably expressed AR.Q39, we found that this misfolded protein is characterized by a low tendency to aggregate (than with overexpressed AR.Q46 or stably expressed AR.Q112), possibly with low impact on autophagy flux. Indeed, after pharmacological autophagy blockage, T-induced misfolded AR.Q39 insoluble species accumulated at levels similar to those found in stably expressed AR.Q112 (compare with Fig. 4A; WB quantification in Fig. S3) or in transiently expressed AR.Q46 (compare with Fig. 4D; WB quantification in Fig. S3) after T treatment (about 6-fold over the corresponding untreated ARpolyQ). Therefore, the polyQ size may influence both the rate of misfolded AR autophagic clearance and the intensity of autophagy impairment associated with the presence of insoluble species.

Conversely, in NSC34-expressing AR.Q39 cells cotreated with T and EHNA, we found that inhibition of autophagy with 3MA had a lower impact on the accumulation of misfolded ARpolyQ insoluble species (Fig. 5A, compare lane 4 and lane 10; WB quantification in Fig. S6), suggesting that other degradative pathways, including the proteasome, may become more relevant to ensure ARpolyQ disposal. Indeed, when the proteasome was inhibited by MG132, the effect of EHNA was reduced (Fig. 5A), suggesting that proteasome, but not autophagy, is required to clear misfolded ARpolyQ insoluble species when their transport and concentration to MTOC is inhibited. Our hypothesis is that, in basal conditions, the proteasome with its narrow barrel-shape cannot properly digest misfolded ARpolyQ insoluble aggregate-prone species and these are targeted for degradation to autophagy in a dynein-dependent manner. Instead, the inhibition of misfolded protein transport might delay their aggregation, which requires protein-protein interaction, a step largely favored by the concentration of proteins achieved via dynein-mediated transport to specific intracellular sites (e.g., the MTOC or other sites where autophagosomes could be generated). The inhibition of misfolded protein concentration will maintain the ARpolyQ into soluble (likely) monomeric forms digestible by the proteasome.

To test this hypothesis, we used the proteasome reporter GFPu, which consists of the GFP protein tagged with a short degron (CL1) that routes the protein to the proteasome for degradation.^{86,94,95} Inhibition of the proteasome activity will result in GFPu accumulation into the cells.^{31,32,69,87} We have already

shown, and confirmed here (Fig. 5B and quantification in Fig. S6), that the unactivated ARpolyQ, normally processed by the proteasome, may compete with GFPu for degradation, leading to a reduced GFPu clearance; instead, the formation of T-induced misfolded ARpolyQ insoluble species correlated with proteasome desaturation (and enhanced GFPu clearance). In addition, we found that dynein inhibition with EHNA improved the clearance of GFPu in all conditions tested, proving that misfolded protein transport blockage has no effect on proteasome activity (Fig. 5B). Moreover, EHNA treatment does not alter the proteasome chymotryptic activity (Fig. 5C), even in presence of control or T-treated ARpolyQ (Fig. 5D). We then verified whether dynein inhibition with EHNA perturbs the global-mediated transcriptional (or the translational) activity of NSC34 cells (please note that the expression of all WT and mutant genes utilized in this study are under the control of the CMV promoter). As control, we evaluated the CMV-mediated transcription of the classical *lacZ* reporter gene measuring the encoded protein levels by testing the enzymatic β -galactosidase activity. In NSC34 cells, we found that the β -galactosidase activity was not affected by treatment with EHNA (Fig. 5E) indicating that dynein inhibition does not impair transcriptional regulation of CMV regulated genes. In addition, we evaluated whether EHNA treatment has effects on cell survival by evaluating cell viability using the MTT assay. No differences in the levels of mitochondrial formazan formation were observed between control and EHNA-treated NSC34 cells (Fig. 5F), suggesting that at this concentration EHNA has no toxic effects on immortalized motoneuronal cells. Together these results exclude that the reduced levels of misfolded ARpolyQ insoluble species observed after EHNA treatment are a consequence of reduced protein synthesis and/or increased cell death. Instead, combined with the higher rate of the proteasome-mediated degradation of the GFPu reporter, these results support the hypothesis that cells treated with EHNA promote the degradation of mutant ARpolyQ with a mechanism that is dynein- and autophagy-independent, and seems to be proteasome-dependent.

So far, our data indicate that inhibition of dynein-mediated delivery of misfolded ARpolyQ species prevents their accumulation and aggregation in motoneuronal cells. Dynein inhibition does not increase proteasomal activity, but rather it might facilitate misfolded ARpolyQ species recognition by the proteasome, a process that requires them to remain into the monomeric status. It is known that chaperones targeting misfolded proteins to autophagy for degradation (such as the HSPB8-BAG3 complex associated with HSPA8- and HSPA1A-bound substrates^{27-29,38,96}) are generally upregulated when the proteasome is impaired. We asked whether EHNA treatment may activate an alternative chaperone response. We focused on BAG1, which, like BAG3, is a member of the BAG family of nucleotide exchange factors. In alternative to the HSPB8-BAG3 complex, BAG1 targets HSPA8- and HSPA1A-bound substrates to the proteasome for disposal.^{38,66,67,97} Interestingly, after EHNA treatment, both *Hspb8* (Fig. 6A) and *Bag3* (Fig. 6B) mRNA levels remained unchanged in NSC34 cells, while *Bag1* expression increased to over 2.5-fold supporting our working hypothesis (Fig. 6C). We next tested the functional

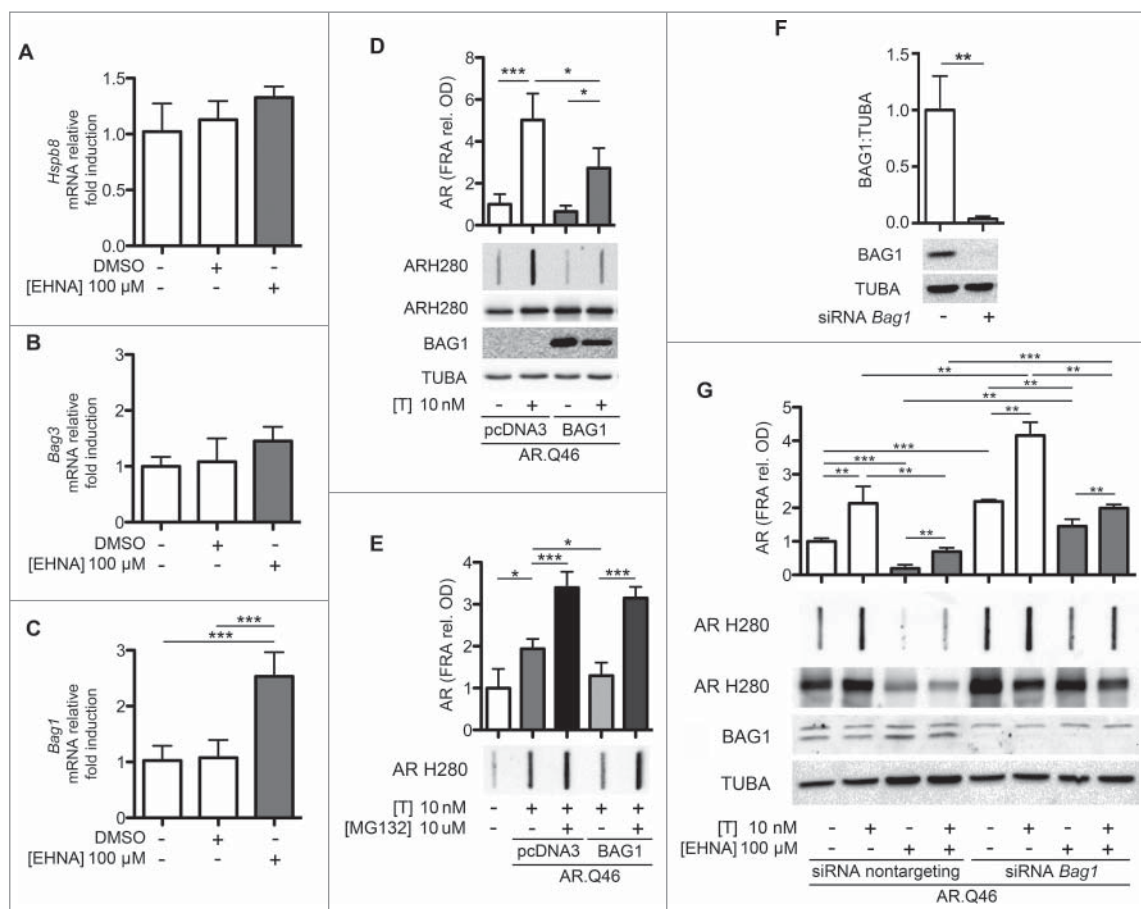


Figure 6. BAG1 upregulation reduces ARpolyQ accumulation. (A to C) NSC34 cells treated with DMSO or 100 μ M EHNA for 48 h. The bar graphs represent *Hspb8* (A), *Bag3* (B) and *Bag1* (C) mRNA levels normalized with *Rplp0* mRNA levels. Four independent biological samples for each condition were analyzed ($n = 4$) \pm SD (*** = $P < 0.001$; One-Way ANOVA followed by the Uncorrected Fisher LSD test). (D) NSC34 cells overexpressing AR.Q46 alone or coexpressing BAG1 and treated with T for last 48 h. FRA shows insoluble AR-polyQ levels (upper panel) and bar graph represents the FRA mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD (* = $P < 0.05$, *** = $P < 0.001$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test). WB (lower panel) shows AR.Q46 and BAG1 protein levels. (E) NSC34 cells expressing AR.Q46 alone or coexpressing BAG1 and treated with 10 nM T for last 48 h and/or 10 μ M MG132 for last 16 h. FRA shows insoluble AR-polyQ levels, bar graph represents the FRA mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD (* = $P < 0.05$, *** = $P < 0.001$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test and one-tailed unpaired Student *t* test for control condition). (F) WB analysis of NSC34 cells transfected with *Bag1* siRNA shows the levels of BAG1; TUBA was used as loading control. Bar graph represents the WB mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD (** = $P < 0.01$, one-tailed unpaired Student *t* test). (G) FRA and WB of NSC34 transfected with AR.Q46 and with *Bag1* siRNA shows AR.Q46 insoluble fraction; TUBA was used as loading control. Bar graph represents the FRA densitometric analysis of 3 independent biological samples for each condition ($n = 3$) \pm SD (** = $P < 0.01$; *** = $P < 0.001$; one-tailed unpaired Student *t* test).

significance of BAG1 upregulation on misfolded ARpolyQ clearance upon inhibition of dynein-mediated transport. Overexpression of BAG1 significantly reduced the accumulation of ARpolyQ retained on the cellulose acetate membrane (FRA, Fig. 6D; WB quantification in Fig. S6). Since BAG1 targets HSPA8- and HSPA1A-bound clients to the proteasome for degradation, we also evaluated whether the BAG1 prodegradative function depends on a functional proteasome. In agreement with previous reports,⁹⁷ we found that proteasome inhibition prevented the BAG1 prodegradative activity on T-inducible misfolded ARpolyQ insoluble species in NSC34 cells (Fig. 6E). Therefore BAG1, which is upregulated upon dynein inhibition with EHNA, enhances the proteasome-mediated degradation of ARpolyQ. This will limit ARpolyQ aggregation induced by its “concentration” mediated by dynein. In fact, protein “concentration” is required for protein selfassociation if it is combined with insufficient autophagy for proper disposal. To further test this hypothesis, we downregulated endogenous *Bag1*

expression with a specific siRNA in NSC34 cells (Fig. 6F). Depletion of *Bag1* in NSC34 cells expressing mutant ARpolyQ led to an increase in the levels of soluble and aggregated AR.Q46 species, both in absence and presence of T, supporting that BAG1 participates in ARpolyQ clearance. Depletion of *Bag1* also significantly decreased the antiaggregation function of EHNA, since aggregated AR.Q46 accumulated at higher levels as compared to control cells (Fig. 6G, compare lanes 3, 4 with lanes 7, 8; WB quantification in Fig. S6). Notably, in agreement with our previous reports demonstrating that “nontoxic” and soluble T-untreated AR.Q46 is mainly processed by the proteasome,^{69,86,87} *Bag1* silencing increased the accumulation of unactivated AR.Q46.

Based on our findings we propose that, when the cells cannot transport misfolded ARpolyQ to the autophagosomes or aggresomes for its dynein-dependent autophagy-mediated degradation, they induce the expression of BAG1 to target these species to the proteasome for clearance.

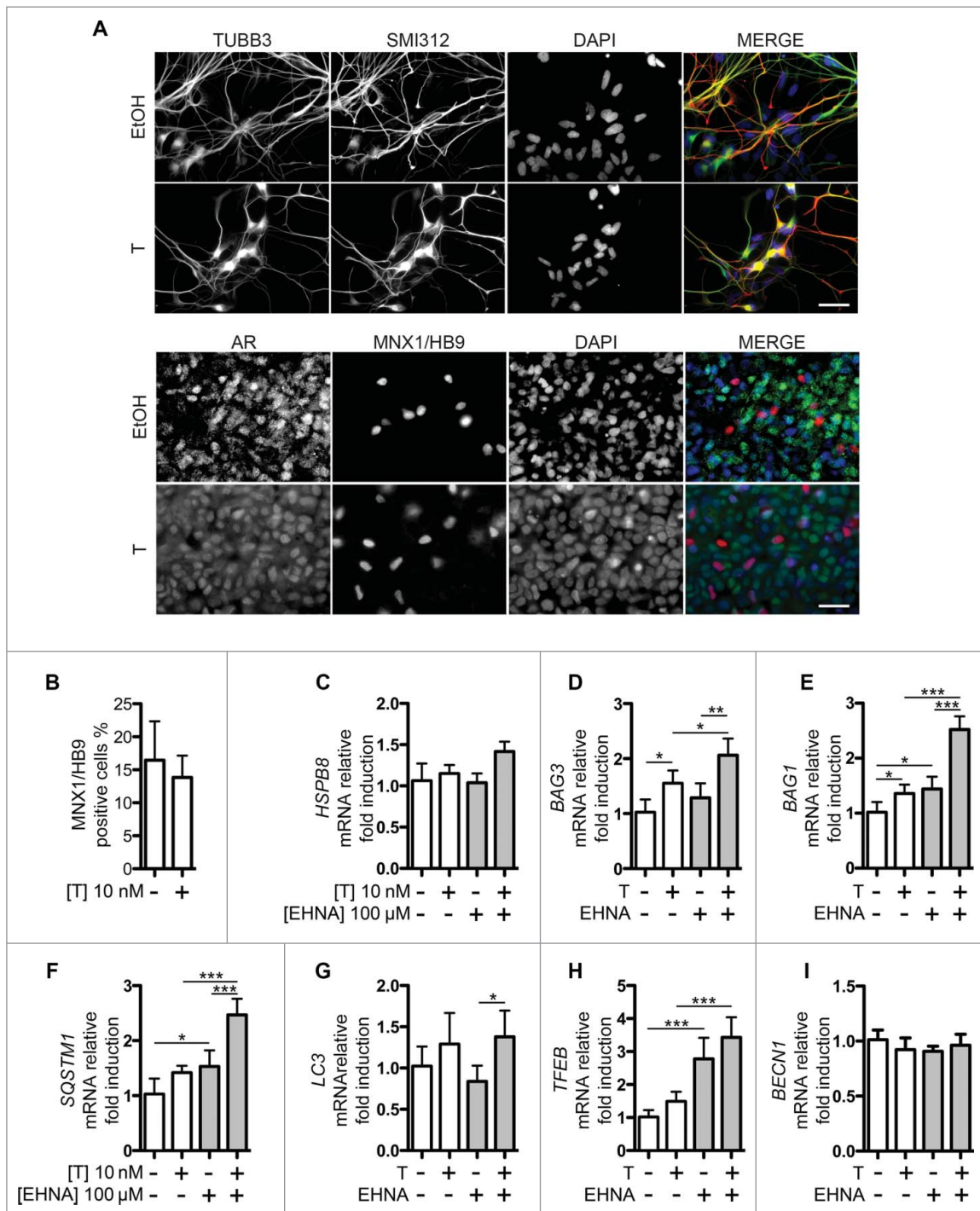


Figure 7. EHNA effects on neuronal and motoneuronal cells obtained by differentiation of human iPSCs generated from SBMA patients. (A) IF analysis of neuronal and motoneuronal cells obtained by differentiation of human iPSCs generated from SBMA patients and treated with ethanol or 10 nM T (40x magnification). TUBB3 (green) and SMI312 (red) (upper inset), AR (green) and MNX1/HB9 (red) (lower inset). Nuclei were stained with DAPI (blue); scale bars: 30 μ m. (B) Bar graph represents the percentage of MNX1/HB9-positive cells; 6 fields for each condition were analyzed (n = 6) \pm SD (one-tailed unpaired Student *t* test, not significant). (C to I) RealTime PCR analyses performed on iPSCs differentiated to neuronal cells treated with 10 nM T and/or 100 μ M EHNA for last 48 h. The bar graphs represent *HSPB8* (C), *BAG3* (D), *BAG1* (E), *SQSTM1* (F), *LC3* (G), *TFEB* (H) and *BECN1* (I) mRNA levels normalized with *RPLP0* mRNA levels. Four independent samples for each condition were analyzed (n = 4) \pm SD (* = *P* < 0.05, ** = *P* < 0.01, *** = *P* < 0.001; Two-Way ANOVA followed by the Uncorrected Fisher LSD test).

Rerouting of misfolded ARpolyQ in SBMA patient-derived iPSCs differentiated to neuronal and motoneuronal cells

To extend our observations to a more reliable model that better recapitulates the conditions present in human motoneurons of SBMA patients, we used a cell model alternative to NSC34 or

PC12 cells, the motoneuronal cells obtained by differentiation of human induced pluripotent stem cells (iPSCs) to neuronal cells generated from SBMA patients. These cells have been cultured both in basal condition or exposed to T to induce ARpolyQ misfolding and toxicity. Differentiation of iPSCs was performed using the protocol described by Grunseich et al.⁹⁸

(who developed the SBMA iPSCs), which allows to produce bona fide neuronal cells (all positive for neuronal markers including TUBB3 and the axonal specific marker SMI312; Fig. 7A) and enriched of a variable population of motoneuronal cells which ranges from 15% to 23% (positive for motor neuron and pancreas homeobox 1 (MNX1/HB9) marker, Fig. 7A and quantification in Fig. 7B) of the total neuronal population. No glial cells were observed using the differentiation protocol adopted here.⁹⁸ All iPSCs differentiated to neuronal cells from SBMA patients were also positive for AR expression, which translocated to nucleus upon T treatment (Fig. 7A). No major morphological variation was observed for neuronal and motoneuronal markers after T treatment (Fig. 7A and B).

Interestingly, after EHNA treatment, *HSPB8* mRNA levels remained unchanged in iPSCs differentiated to neuronal cells (both untreated or T-treated) (Fig. 7C); *BAG3* expression was mildly induced by T and to a lower extent by EHNA (Fig. 7D). EHNA treatment increased the expression of *BAG1* of almost 1.5-fold and 2.5-fold in untreated and T-treated iPSCs differentiated to neuronal cells, respectively (Fig. 7E), further supporting that *BAG1* upregulation represents a physiological stress response upon dynein inhibition. We next evaluated the impact of EHNA on the expression of key autophagy proteins in iPSCs differentiated to neuronal cells. After dynein inhibition (both in the absence and in the presence of T), the mRNA levels of *SQSTM1* and *TFEB* (transcription factor EB) were increased (Fig. 7F, H), while *LC3* levels remained unmodified after EHNA treatment (Fig. 7G). Notably, this upregulation of *TFEB* and *SQSTM1* could be a cell attempt to restore an altered autophagic flux or to improve the intracellular lysosomal activity when transport of cargo to the autophagosomes is blocked. Conversely, *BECN1* remained unchanged by dynein inhibition in iPSCs differentiated to neuronal cells (Fig. 7I). Combined these results strongly support that *BAG1* is a crucial physiologically activated PQC player.

Effects of dynein inhibition on the clearance of other misfolded proteins associated with motoneuron diseases

We next wanted to investigate whether the effects of dynein inhibition with EHNA described above are specific for the mutant misfolded ARpolyQ or are also exerted on other mutant proteins responsible for another motoneuron disease similar to SBMA, the ALS. We selected 2 proteins that have been associated with ALS: i) the SOD1 enzyme (mutated in about 20% of fALS),⁹⁹ and ii) a truncated form of TARDBP (TARDBP^{ΔC}, found in some fALS cases). Both mutant SOD1^{G93A} and TARDBP^{ΔC} misfold and accumulate into cytoplasmic aggregates; as shown for ARpolyQ^{31,32} their autophagic clearance can be enhanced by the HSPB8-BAG3 complex.^{28,29,32,96} In 2 different models of ALS, SOD1^{G93A} transiently (Fig. 8A; WB quantification in Fig. S6) or inducible stably transfected (Fig. 8B) motoneuronal NSC34 cells, EHNA treatment resulted in a reduction of the total amount of misfolded mutant SOD1 insoluble species accumulating on cellulose acetate in FRA. Similar results were obtained in ALS models generated using the TARDBP^{ΔC} mutant (Fig. 8C; WB quantification in Fig. S7), demonstrating that dynein inhibition also affects the accumulation of the truncated fragment of TARDBP involved in ALS. As

in the case of ARpolyQ, EHNA activity requires a functional proteasome activity, since its inhibition with MG132 prevented (even if not completely, see below) the clearance of mutant SOD1 (Fig. 8D; WB quantification in Fig. S7) and TARDBP^{ΔC} (Fig. 8E; WB quantification in Fig. S7), observed after dynein inhibition. Conversely, EHNA exerted its prodegradative effect on these 2 ALS-associated proteins when autophagy was inhibited by 3-MA treatment (Fig. 8D and E; WB quantification in Fig. S7).

Finally, we wanted to further extend these observations to other forms of proteinaceous aggregates also involved in motoneuron diseases, but structurally totally distinct from the misfolded proteins studied above. We analyzed one out of 5 potentially neurotoxic dipeptides generated by RAN-translation from the *C9ORF72* transcript containing an expanded exanucleotide repeat (GGGGCC)_n linked to several fALS (and some sALS) cases and in many cases of frontotemporal dementia.¹⁰⁰⁻¹⁰⁶ To produce the peptide selected (the dipeptide [glycine-proline], polyGP) we used a synthetic DNA in order to avoid multiple dipeptide production and/or (GGGGCC)_n-associated RNA toxicity.¹⁰⁷ The polyGP is not produced in basal physiological conditions and it is likely that the polyGP has no propensity to acquire a natural conformation. It is expected that its structure is likely to be unrelated to the aberrant conformation generated by the mutant misfolded proteins. Despite this, also the polyGP is prone to aggregate, and accumulates in neurons into intracellular insoluble inclusions. The polyGP is cleared by the PQC system,¹⁰⁷ an aspect that we confirmed in immortalized motoneurons, where both proteasome or autophagy inhibition (with MG132 or 3MA, respectively) increased the accumulation of insoluble polyGP species evaluated with FRA (Fig. 9; WB quantification in Fig. S7). In line with our previous data, dynein inhibition led to a reduction of polyGP insoluble species even when autophagy is blocked with 3MA. In the case of polyGP, the antiaggregant effect of EHNA was not completely counteracted by the cotreatment with the proteasome inhibitor MG132, even if more than 50% of total insoluble polyGP species accumulated with proteasome blockage. PolyGP is characterized by a very high proteasomal turnover. Indeed, we found a 30-fold increase of insoluble species accumulating with MG132 treatment. Since MG132 only inhibits the chymotryptic proteasomal activity,^{108,109} it is likely that the polyGP will be processed also using the postacidic or tryptic activities of the proteasome, which may explain the partial inhibitory effect obtained by treatment with MG132. Alternatively, as already postulated above, other degradative pathways (such as chaperone mediated autophagy [CMA]) could be involved in the polyGP clearance, as well as for ARpolyQ, mutant SOD1, TARDBP in which proteasome inhibition did not always correlate with a full blockage of EHNA effects.

Discussion

Here, we analyzed how the inhibition of the dynein-mediated retrograde transport of misfolded proteins to MTOC affects their intracellular aggregation and clearance. Our approach was based on previous data showing that misfolded protein clearance is enhanced by their interaction with the complex HSPB8-BAG3,^{28,29} a complex that, in conjunction with HSPA8 and

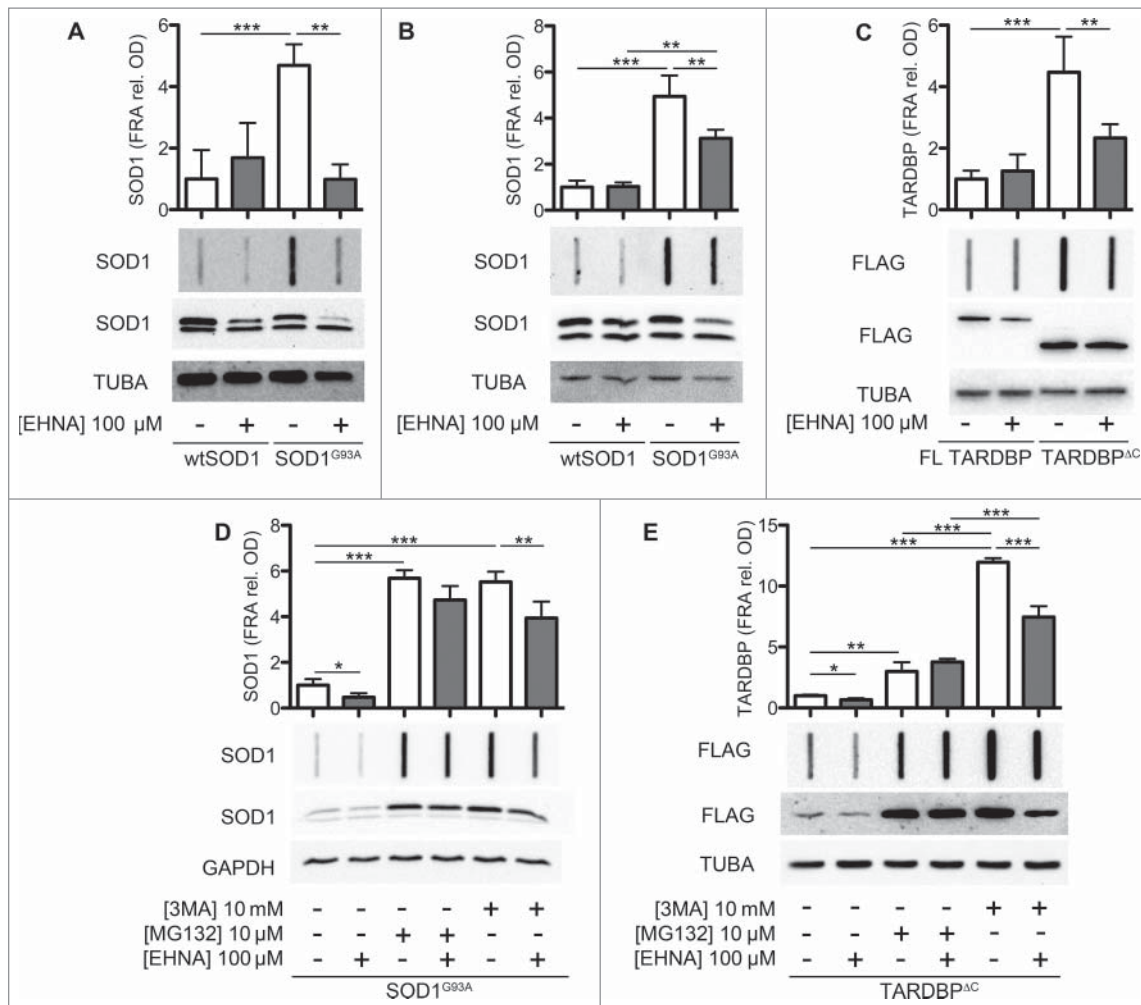


Figure 8. Effects of dynein inhibition on SOD1 and TARDBP insoluble species. (A) NSC34 cells expressing wtSOD1 and SOD1^{G93A} treated with DMSO or 100 μ M EHNA for last 48 h. FRA shows SOD1^{G93A} accumulation compared to wtSOD1 and SOD1^{G93A} reduction after 100 μ M EHNA treatment. WB shows total level of transfected SOD1, TUBA was used as loading control. (B) NSC34 stably transfected with wtSOD1 or SOD1^{G93A} induced with doxycycline and treated with DMSO or 100 μ M EHNA for last 48 h. FRA shows SOD1^{G93A} accumulation compared to wtSOD1 and SOD1^{G93A} reduction after 100 μ M EHNA treatment. WB shows total level of transfected SOD1, TUBA was used as loading control. (C) NSC34 expressing FL and TARDBP ^{Δ C} treated with DMSO or 100 μ M EHNA for last 48 h. FRA shows TARDBP ^{Δ C} accumulation compared to FL TARDBP and TARDBP ^{Δ C} reduction after 100 μ M EHNA treatment. WB shows total level of transfected TARDBP, TUBA was used as loading control; (A to C) Bar graphs represent the FRA mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD (** = $P < 0.01$, *** = $P < 0.001$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test). (D and E) NSC34 expressing SOD1^{G93A} (D) or TARDBP ^{Δ C} (E) and treated with DMSO, 100 μ M EHNA and/or 10 mM 3MA for the last 48 h and 10 μ M MG132 for the last 16 h. FRAs show SOD1^{G93A} (D) or TARDBP ^{Δ C} (E) accumulation after 3MA and MG132 treatments. EHNA treatment counteracts 3MA activity but not MG132 activity. WBs show total level of transfected SOD1 (D) or TARDBP ^{Δ C} (E), TUBA or GAPDH was used as loading control. Bar graphs represent the FRA mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$; Two-Way ANOVA followed by the Uncorrected Fisher LSD test and one-tailed unpaired Student t test for control conditions).

HSPA1A, recruits STUB1 to allow misfolded substrate ubiquitination and SQSTM1-mediated insertion into autophagosomes at the MTOC.³⁹ BAG3 interacts with the YWHA/14-3-3 protein and dynein, which transports the complex (misfolded protein-HSPB8-BAG3-HSPA8-STUB1) to the MTOC.^{73,74} Given this evidence, we were expecting that dynein inhibition correlated with an overall increased accumulation of insoluble and aggregated misfolded proteins inside the cells. Instead, in motoneuronal cells, we found that depletion or chemical inhibition of dynein decreased the aggregation of misfolded proteins maintaining them in a soluble state competent for proteasomal degradation. In fact, upon dynein inhibition, all misfolded proteins we analyzed were preferentially targeted to the proteasome for degradation, while autophagy inhibition had no or mild impact on their total levels and aggregation. Dynein inhibition did not impair basal autophagy, which appeared to be

very limited in basal conditions in our cells. Conversely, dynein inhibition reduced activation of autophagy stimulated by trehalose, mostly through changes at the transcriptional level.

Misfolded proteins species altered autophagy, but dynein blockage reduced their disposal via autophagy. Under these conditions, the autophagy markers, HSPB8 and BAG3 were not induced, while BAG1 was upregulated. BAG1 is a HSPA8 cochaperone which both assists HSPA-mediated protein folding (by enhancing its activity as nucleotide exchange factor),^{38,66,67} and targets HSPA8 client proteins to proteasomal degradation^{66,97,110-113} (and under specific circumstances even to CMA¹¹⁴). Our results suggested that BAG1 represents a crucial player by routing misfolded proteins to proteasome when their transport to the MTOC is blocked. In line, overexpression of BAG1 in cells with deficient or inhibited dynein allowed a more efficient proteasomal degradation of misfolded species

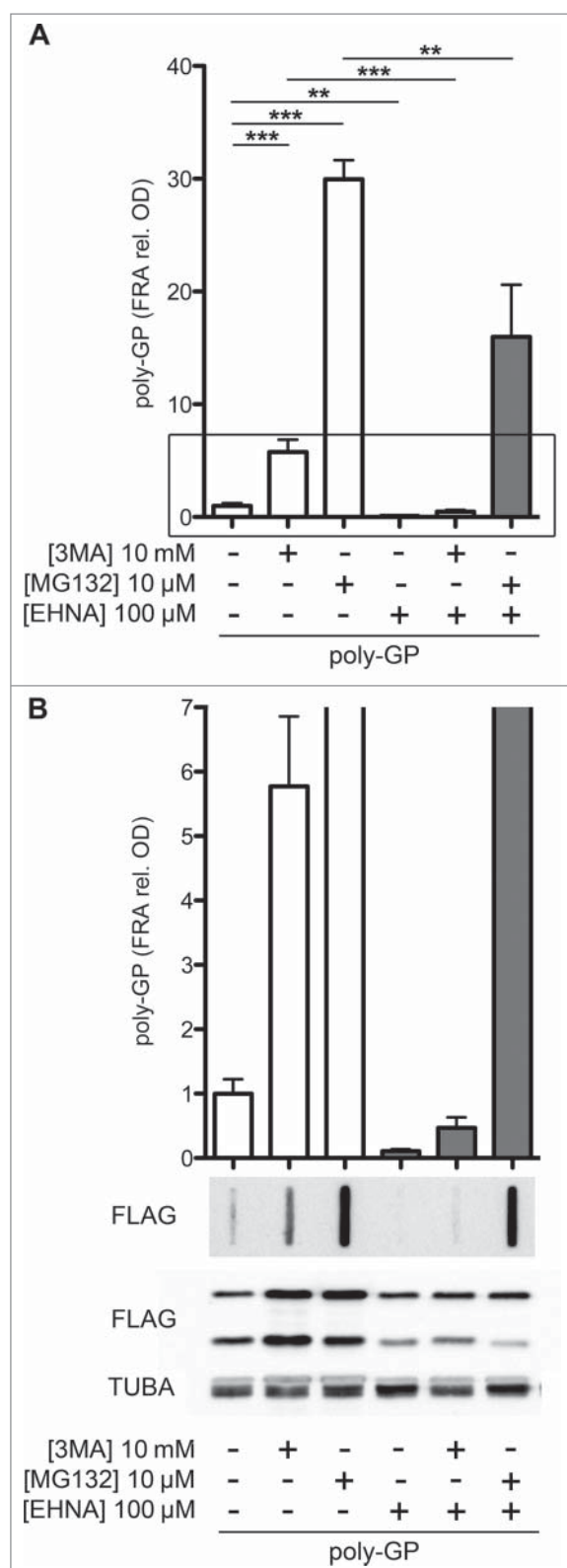


Figure 9. Effects of dynein inhibition on poly-GP insoluble fraction. (A) NSC34 cells expressing poly-GP (100 repeats) treated with 10 mM 3MA and/or 100 μ M EHNA for the last 48 h prior to analysis and/or 10 μ M MG132 for the last 16 h prior to analysis. FRA shows poly-GP insoluble fraction. Bar graphs represent the FRA mean relative optical density computed over 3 independent biological samples for each condition ($n = 3$) \pm SD (** = $P < 0.01$; *** = $P < 0.001$; one-tailed unpaired Student t test); (B) the portion shown in A by the rectangle has been magnified to show the differences between short bars. WB shows total level of transfected poly-GP; TUBA was used as loading control.

that were accumulating in a monomeric, or small oligomeric and soluble forms, rather than as aggregates because of the inhibition of their concentration at the MTOC (a process that facilitates aggregation).

Conversely, because proteasome inhibition leads to upregulation of BAG3 and HSPB8, which reroute (poly)-ubiquitinated and misfolded clients toward autophagy for degradation,^{28,29,38} here we propose that, in cells where dynein-mediated transport is inhibited, BAG1 upregulation and its mediated rerouting of misfolded substrates to the proteasome represent an alternative mechanism activated by cells to maintain protein homeostasis. Molecularly, this may be explained by the fact that BAG1 possesses an ubiquitin-like domain for targeting of the HSPA8-bound client to the proteasome, while BAG3 has no ubiquitin-like domains,^{37,115-117} but its interaction with YWHA/14-3-3-dynein permits the transport of misfolded proteins to the MTOC for autophagy clearance.^{70,73,74} Therefore, when misfolded proteins cannot be efficiently transported to autophagosomes, the cells upregulate an alternative chaperone system, that ensures enhanced proteasomal misfolded proteins clearance preventing their aggregation. In line, pharmacological autophagy blockage did not alter the effect of dynein inhibition on misfolded protein clearance. The prevention of misfolded protein concentration also might reduce protein-protein interaction, which is essential for aggregation, favoring the maintenance of the monomeric status of the protein. This might allow a larger time window for proper protein folding, since BAG1 also acts as a nuclear exchange factor for HSPA, enhancing and optimizing its chaperone activity and this acts alternatively to protein rerouting to the proteasome. This may explain why we occasionally found variations in the total levels of soluble ARpolyQ (measured in WB), as compared to the levels of insoluble ARpolyQ (detected with FRA), after EHNA treatment. Even if both folding and degradation processes may coexist, our data suggest that the role of degradation prevails on the refolding. Here we propose that dynein inhibition requires 2 steps: 1) control of aggregation and 2) routing of soluble clients toward the proteasome (or to proper folding) in a BAG1-assisted manner. In fact, under these conditions, insertion of aggregate-prone proteins into an aggresome is blocked^{46,118} and the aggregate-prone substrates are maintained in a state that is competent for degradation by the proteasome.¹¹⁹

Notably, also preformed (but still dynamic) aggregates can be redissolved into soluble proteasomal digestible proteins, since EHNA was also effective when added 24 h after ARpolyQ inclusions induction with T. The rerouting system here described might also have impact on nuclear inclusions. Indeed, we showed that also using an ARpolyQ with a very long size (Q112), which is capable of generating nuclear aggregates, resembling those typically found in SBMA patients,^{24,25} dynein blockage correlated with a reduced accumulation of insoluble species, possibly associated with enhanced proteasomal clearance taking place both in nuclear and cytoplasmic compartments.

Altogether, these data shed light on the importance of dynein-mediated transport in the intracellular routing of misfolded proteins to their preferred degradative system, and are in line with previous observations showing that the disruption of microtubules with nocodazole inhibits aggresome formation

in cells expressing the exon 1 of mutant HTT (huntingtin) or ARpolyQ.^{46,118,120} These studies also showed that prevention of aggregates formation correlates with an increased toxicity of the 2 misfolded proteins analyzed (HTT-polyQ and ARpolyQ), supporting the notion that aggresomes may have a protective activity in their earlier stages of formation.²⁰⁻²² In our study, a less invasive and more specific approach was used to block misfolded protein concentration at the MTOC, where aggresomes are formed.^{121,122}

Therefore, it is likely an alteration of the fine equilibrium of protein routing that may affect the release or accumulation of neurotoxic species, rather than the impairment or overwhelming of a single degradative pathway. These pathways may also include CMA. Indeed, BAG1, by interacting with HSPA8, might also direct misfolded proteins to CMA for clearance,^{114,119,123} Unfortunately, no selective inhibitors are available for CMA and we were unable to evaluate its contribution in the degradation of misfolded species after dynein impairment.

A second aspect that emerges from the data here presented is that the role of dynein in mediating the transport of autophagosomes to lysosomes to allow their fusion may be secondary to the cargo delivery to the MTOC. In fact, we did not find major problems in misfolded protein clearance even if autolysosome formation was impaired in parallel to transport blockage. As stated above the activity of this pathway is made irrelevant by the BAG1-mediated routing of misfolded proteins to the proteasome (and eventually CMA), and the role of dynein in cargo transport to phagophores precedes that in autophagosome and lysosome fusion.

These data apparently contrast with the observations obtained in transgenic mice carrying missense point mutations in the cytoplasmic dynein heavy chain. Heterozygous mice are characterized by progressive motoneuron loss, but without accumulation of proteinaceous material in affected cells. Homozygous mice suffer motoneuronal degeneration with accumulation of Lewy-like inclusion bodies.⁶⁰ In addition, cytoplasmic dynein is activated by the ubiquitously expressed dynactin, and mutation in the DCTN1/p150^{Glued} subunit of dynactin, which prevents dynein activation, causes specific motoneuron loss, with the appearance of DCTN1/p150^{Glued} protein aggregates¹²⁴⁻¹²⁶ (see also¹²⁷ for an extensive review on dynein mutations in human and mice). However, different mice in which dynein and dynactin functions are altered (either because of dynein mutations (Legs at odd angles),^{65,128} or because of neuron-specific expression of Bicaudal D2 N-terminus¹²⁹) causing an ALS-like phenotype, have improved life span when crossed with transgenic SOD1^{G93A} mice.^{65,128,129} The phenotype of these double transgenic mice is also ameliorated when compared to the single transgenic mice expressing the mutant SOD1 protein. Therefore, while dynein mutations per se are deleterious to motoneurons, it is possible that dynein may contribute to exacerbate the adverse effects exerted by mutant misfolded proteins on motoneurons. It is likely that this adverse effect of dynein is due to a routing of misfolded proteins at the MTOC (which escape proteasome-mediated degradation) that induces aggregation and possibly autophagic flux blockage.

While these data may not be readily translated into possible therapeutic approaches, they could help to better understand the molecular mechanisms at the basis of misfolded proteins

clearance and the way that motoneurons select specific pathways of degradation for a given protein.

Materials and methods

Chemicals

Erythro-9-(2-Hydroxy-3-nonyl) adenine hydrochloride (EHNA; Sigma-Aldrich, E114), Z-Leu-Leu-Leu-al (MG132; Sigma-Aldrich, C2211), trehalose (Sigma-Aldrich, T9531), 3-methyladenine (3MA; Selleckchem, S2767), ammonium chloride (Euroclone, EMR089500).

Plasmids and siRNA

The plasmids coding for AR.Q23, AR.Q46, GFP-AR.Q48 and pCI-HSPB8 are routinely used in our laboratory and they have been previously described.^{20,30-32} The pcDNA/HA-BAG1 plasmid coding for the protein BAG1 was kindly provided by Prof. Harm H. Kampinga (University of Groningen, Groningen, The Netherlands).^{67,130} The plasmid expressing the proteasome function reporter GFPu was kindly provided by Prof. Ron Kopito (Stanford University, Stanford, CA, USA).⁹⁴ The pCMV- β plasmid, encoding β -galactosidase (Clontech, 6177-1) was used to evaluate EHNA effect on total protein expression levels. The pcDNA3-wtSOD1 and pcDNA3-SOD1^{G93A} plasmids expressing human wtSOD1 or mutant SOD1^{G93A} were kindly provided by Dr. Caterina Bendotti (Mario Negri Institute, Milano, Italy).¹³¹ pFLAG-FL TDP-43 (TARDBP), and pFLAG-TDP-43^{ΔC} (TARDBP^{ΔC}) plasmids, expressing FLAG-tagged full-length human TARDBP and an N-terminal truncated at residue 261 (lacking the C-terminal region), respectively, were kindly provided by Dr. Emanuele Buratti (International Center for Genetic Engineering and Biotechnology, Trieste, Italy).^{132,133} pCMV-Flag-polyGP-V5-His plasmid encoding the polyGP dipeptide was kindly provided by Prof. Daisuke Ito (Keio University School of Medicine, Tokyo, Japan).¹⁰⁷ The pcDNA3 (Life Technologies, V790-20) plasmid was used to normalize for transfected plasmid DNA amount. The pEGFPN1 (Clontech, U55762) plasmid was used to evaluate transfection efficiency in experiments involving transient transfections. pcDNA5/TO-GFPAR.Q0 and pcDNA5/TO-GFPAR.Q39 plasmids were used to obtain stable and inducible SBMA cell models and were constructed by cloning the sequence coding for GFPAR.Q0 and GFPAR.Q39 into *NheI* and *XbaI* sites of pcDNA5/TO (Life Technologies, V103320), previously modified with insertion of *BamHI* and *NheI* sites.

To silence endogenous *Dync1h1* and *Bag1* expression we used custom siRNA duplex: [5'-GGGAAAAGCUAGAGAGAAUUU-3' (sense)], [5'-AUUCUCUCUAGCUUUACCCUU-3' (antisense)] for *Dync1h1* and [5'-AAGAAGAGGUUGAGUAAAUUU-3' (sense)], [5'-UUUAACUCAACCUCUUCUUUU-3' (antisense)] for *Bag1* (Dharmacon, Thermo Scientific Life Sciences Research).

Cell cultures and transfection

The immortalized motoneuronal cell line NSC34^{134,135} is routinely used in our laboratory and has been transfected with Lipofectamine (Life Technologies, 18324020)-TRF (transferrin;

Sigma-Aldrich, T8158), as previously described,^{20,25,136} using 0.6 μ g of plasmid DNA, 4 μ L of TRF solution and 2 μ L of Lipofectamine (amount for 1 well of a 12-well multiwell plate). siRNA transfection was performed with Lipofectamine 2000 (Life Technologies, 11668019), using 40 pmol of target RNA and following the manufacturer's instructions.

Inducible stably transfected NSC34-GFPAR.Q(n) cell lines were obtained by transfecting pcDNA5/TO-GFPAR.Q(n) into TR4 NSC34 cell line stably transfected with pcDNA6/TR encoding for tetracycline repressor, kindly provided by Dr. Enrico Garattini (Mario Negri institute, Milan, Italy), and were routinely maintained as described previously.¹³⁷

Rat adrenal pheochromocytoma (PC12) cells stably transfected with the plasmid encoding AR.Q10 and AR.Q112 express AR under the control of a Tet-On promoter, responsive to 1 μ g/mL of doxycycline. PC12-AR.Q(n) cells have been produced and kindly provided by prof. Diane Merry (Thomas Jefferson University, Philadelphia, PA, USA) and were routinely maintained as described by Walcott et al., 2002.⁸⁹

iPSCs were obtained in the lab of Dr. Kennet Fischbeck (by Dr. Christopher Grunseich) at NIH, Bethesda, MD, USA from fibroblasts of SBMA patients (SB6MP2 clone) and generated by POU5F1/Oct4, KLF4, SOX2 and MYC/c-Myc transfection. iPSCs were cultured in Essential 8 medium (Life Technologies, A1517001) and differentiated into motoneurons as described previously.⁹⁸ Briefly, iPSCs were grown to 80% confluency, then digested with collagenase IV (Life Technologies, 17104019), scraped off of the dish, and replated into ultra-low attachment dishes (Corning, 3262) in KSR (Life Technologies, 10828028)-based medium, with 20 ng/ml FGF (ORF genetics, 01-A01110), 20 μ M ROCK-I (Selleckchem, S1049), 10 μ M SB431542 (Stemgent, 04-0010), 0.2 μ M LDN193189 (Stemgent, 04-0074), to allow embryo bodies (EBs) formation. EBs were transitioned to a KSR-free medium at d 3. Retinoic acid (1 μ M; Sigma-Aldrich, R2625) was added at d 5 to direct cell differentiation into the rostral spinal cord phenotype. Smoothed agonist (1 μ M; Merck Millipore, 566660) and 0.5 μ M purmorphamine (Caymanchem, 10009634) were added at d 7 to ventralize the differentiating population. The EBs were dissociated at d 14 and plated at 150,000 cells/well on 12-well multiwell plates for RNA extraction or at 50,000 cells/well on 24-well multiwell plates for immunofluorescence (IF). Plates were coated with poly-D-lysine (Sigma-Aldrich, P7405) and laminin (Life Technologies, 23017015). Differentiated-motoneurons were allowed to grow for an additional 10 d in neurobasal medium (Life Technologies, 21103049) with 25 μ M glutamate (Sigma-Aldrich, 1446600), 0.4 μ g/mL ascorbic acid, 10 ng/ml GDNF (PeproTech, 167450-10), 10 ng/ml CNTF (PeproTech, 167450-13), 1 μ g/mL laminin, B-27 (Life Technologies, 17504044), N2 (Life Technologies, 17502048), nonessential amino acids (Euroclone, ECB3054D), and pen/strep/glutamine (Euroclone). Motoneuronal cells were treated with ethanol or 10 nM T and/or 100 μ M EHNA for the last 48 h prior to analysis.

Stably transfected NSC34-wtSOD1 and NSC34-SOD1^{G93A} cell lines have been produced and kindly provided by Prof. Maria Teresa Carri (Università Roma Tor Vergata, Roma, Italy). These cell lines express MYC-tagged human wtSOD1 or SOD1^{G93A} and were routinely maintained as described by Ferri and colleagues.¹³⁸

In the experiments involving steroid hormone treatments, the serum was replaced with charcoal-stripped serum, to eliminate endogenous steroids.^{25,139,140} The effects of treatments (T, EHNA, 3MA, MG132, etc.), for each mutant protein considered, were analyzed on 3 (4 for the RT-qPCR analysis) independently cultured, transfected and treated samples (considered as independent variables (n)).

mRNA expression analysis

NSC34 cells were plated at 180,000 cells/well (4 wells for each condition to be tested; n = 4) in 6-well multiwell plates, allowed to growth for 24 h and then treated with 100 μ M EHNA and/or with 100 mM trehalose, or with DMSO (used as negative control). Forty-eight h after treatments, cells were harvested and centrifuged 5 min at 100 \times g at 4°C; the pellets were resuspended in 300 μ L of TRI Reagent (Sigma-Aldrich, T9424) and total RNA isolated according to manufacturer's instructions. RNA quantification was carried out by absorbance at 260 nm. Total RNA (1 μ g) was treated with DNase I (Sigma-Aldrich, AMPD1), and reverse transcribed into cDNA using the High-Capacity cDNA Archive Kit (Life Technologies, 4368813) according to the manufacturer's protocol. All primers for real-time PCR were designed using the program Primer 3.

The primers were synthesized by MWG Biotech (Ebersberg, Germany) with the following sequence: *Map1LC3-B*: 5'-CGT CCT GGA CAA GAC CA-3' (forward), 5'-CCA TTC ACC AGG AGG AA-3' (reverse); *Sqstm1*: 5'-AGG GAA CAC AGC AAG CT-3' (forward), 5'-GCC AAA GTG TCC ATG TTT CA-3' (reverse); *Rplp0*: 5'-GGT GCC ACA CTC CAT CAT CA-3' (forward), 5'-AGG CCT TGA CCT TTT CAG TAA GT-3' (reverse); *Hspb8*: 5'-ATA CGT GGA AGT TTC AGG CA-3' (forward), 5'-TCT CCA AAG GGT GAG TAC GG-3' (reverse); *Bag3*: 5'-ATG GAC CTG AGC GAT CTC A-3' (forward), 5'-CAC GGG GAT GGG GAT GTA-3' (reverse); *Bag1*: 5'-GAA ACA CCG TTG TCA GCA CT-3' (forward), 5'-GCT CCA CTG TGT CAC ACT C-3' (reverse); *HSPB8*: 5'-AGA GGA GTT GAT GGT GAA GAC C-3' (forward), 5'-CTG CAG GAA GCT GGA TTT TC-3' (reverse); *BAG1*: 5'-TTT AGA GCA GCC CGA GTG AT-3' (forward), 5'-GAC AGC AGA CAG CCA ACA AA-3' (reverse); *BAG3*: 5'-GGG TGG AGG CAA AAC ACT AA-3' (forward), 5'-AGA CAG TGC ACA ACC ACA GC-3' (reverse); *SQSTM1*: 5'-CCA GAG AGT TCC AGC ACA GA-3' (forward), 5'-CCG ACT CCA TCT GTT CCT CA-3' (reverse); *MAP1LC3B*: 5'-CAG CAT CCA ACC AAA ATC CC-3' (forward), 5'-GTT GAC ATG GTC AGG TAC AAG-3' (reverse); *TFEB*: 5'-CAA GGC CAA TGA CCT GGA C-3' (forward), 5'-AGC TCC CTG GAC TTT TGC AG-3' (reverse); *BECN1*: 5'-ATG CAG GTG AGC TTC GTG TG-3' (forward), 5'-CTG GGC TGT GGT AAG TAA TGG A-3' (reverse); *RPLP0*: 5'-GTG GGA GCA GAC AAT GTG GG-3' (forward), 5'-TGC GCA TCA TGG TGT TCT TG-3' (reverse).

The evaluated efficiency of each set of primers was close to 100% for both target and reference gene. Real-time PCR was performed using the CFX 96 Real-Time System (Bio-Rad, Hercules, CA, USA) in a 10- μ L total volume, using the iTaq SYBR Green Supermix (Bio-Rad, 1725124), and with 500 nM primers. PCR cycling conditions were as follows: 94°C for 10 min, 40 cycles at 94°C for 15 sec and 60°C for 1 min. Melting curve

analysis was performed at the end of each PCR assay as a control for specificity. Data were expressed as Ct values and used for the relative quantification of targets with the $\Delta\Delta\text{Ct}$ calculation.^{141,142} To exclude potential bias due to averaging, data have been transformed through the equation $2^{-\Delta\Delta\text{Ct}}$ to give N-fold changes in gene expression, all statistics were performed with ΔCt values. Each experiment was carried out with 4 independent samples ($n = 4$).

Western blot (WB) analysis and filter retardation assay (FRA)

NSC34 or PC12 cells were plated in 12-well multiwell plates at 80,000 cell/well (3 wells for each condition to be tested; $n = 3$). 24 h after plating, cells were transfected as previously described or induced with 1 $\mu\text{g}/\text{mL}$ doxycycline, and AR activation was induced by 10 nM T treatment for 48 h. Inhibition of dynein ATPase activity was performed by 100 μM EHNA for 48 h. In experiments involving autophagy blockage or induction, 10 mM 3MA for the last 48 h, 20 mM NH_4Cl for the last 1.5 h or 100 mM trehalose for the last 48 h were added to the cells. Proteasome inhibition was performed by 10 μM MG132 treatment for the last 16 h (overnight treatment). Seventy-two h after plating, cells were harvested and centrifuged 5 min at $100 \times g$ at 4°C ; the cell pellets were resuspended in PBS (Sigma-Aldrich, P4417) added of the protease inhibitor cocktail (Sigma-Aldrich, P8340) and homogenized using slight sonication to lyse cells and nuclei as previously described.^{86,139} Total proteins were determined with the bicinchoninic acid method (BCA assay; Euroclone, EMP014500).

WB was performed on 7.5% (to detect AR protein), 12% (to detect SOD1 and TARDBP proteins) or 15% (to detect LC3 and SQSTM1/p62 proteins) SDS-polyacrylamide gel electrophoresis loading 15 μg of total proteins. Samples were then electrotransferred to nitrocellulose (Bio-Rad, 1620115) or PVDF (polyscreen transfer membrane; Amersham, 10600023) using a semidry transfer apparatus (Trans-Blot TurboTM Transfer System; Bio-Rad). The membranes were treated with a blocking solution containing 5% nonfat dried milk powder (Euroclone, EMR180500) in Tris-buffered saline with Tween 20 (0.01%; Sigma, P1379) (TBS-T; Tris base 20 mM, NaCl 140 mM, pH 7.6) for 1 h and then incubated with one of the following primary antibodies: (a) rabbit polyclonal AR-H280 (dilution 1:3,000) to detect wtAR and ARpolyQ (Santa Cruz Biotchnology, sc-13062); (b) rabbit polyclonal anti-LC3A/B (dilution 1:4,000; Sigma-Aldrich, L8918); (c) rabbit polyclonal anti-SQSTM1 (dilution 1:4,000; Abcam, ab91526); (d) rabbit polyclonal anti-dynein heavy chain (dilution 1:1,000; Santa Cruz Biotchnology, sc-9115); (e) rabbit polyclonal anti-GFP HRP conjugated (dilution 1:15,000; Vector Laboratories, MB-0712); (f) rabbit polyclonal anti-HA (dilution 1:1,000; Santa Cruz Biotchnology, sc-7392) to detect overexpressed BAG1; (g) rabbit polyclonal anti-BAG1 (dilution 1:1,000; Santa Cruz Biotchnology, sc-939) to detect endogenous BAG1; (h) rabbit polyclonal Cu/Zn SOD (1:1,000; Enzo Life Science, ADI-SOD-100) to detect SOD1; (i) mouse polyclonal anti-flag M2 (dilution 1:1,000; Sigma-Aldrich, F1804) to detect TARDBP; (j) mouse monoclonal anti-TUBA (dilution 1:4,000; Sigma-Aldrich, T6199); (k) rabbit polyclonal anti-GAPDH (1:4,000; Santa

Cruz Biotchnology, sc-25778). Immunoreactivity was detected using the following secondary peroxidase-conjugated antibodies: goat anti-rabbit (dilution 1:20,000; Santa Cruz Biotchnology, sc-2004); goat anti-mouse (dilution 1:20,000; Santa Cruz Biotchnology, sc-2005). Signals were revealed by chemiluminescence detection kit reagents (ClarityTM Western ECL Blotting Substrate; Bio-Rad, 170-5060). The same membranes were subsequently processed with different antibodies to detect the levels of different proteins in the same sample, after stripping for 20 min at room temperature (StripABlot; Euroclone, EMP100500).

FRA was performed using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad). Six μg of the total SOD1, TARDBP and polyGP proteins or 1.5 μg of the total AR.Q(n) were filtered through a 0.2- μm cellulose acetate membrane (Whatman, 100404180). Slot-blot were probed as described for WB.

A ChemiDoc XRS System (Bio-Rad, Hercules, California, USA) was used for the image acquisition of WB and FRA. Optical density of samples assayed with WB or FRA was detected and analyzed using the Image Lab software (Bio-Rad). Statistical analyses have been performed using the relative optical densities defined as the ratio between optical densities of each independent biological sample ($n = 3$) and the mean optical density of control samples.

Protein fractionation

Protein fractionation of NSC34 transfected with AR.Q46 was modified from Koyama et al.¹⁴³ The cells were harvested and centrifuged 5 min at $100 \times g$ at 4°C , then resuspended in PBS containing protease inhibitor cocktail and homogenized using slight sonication. The homogenates were centrifuged at $16,000 \times g$ for 10 min at 4°C . Subsequently, the supernatants were collected as the PBS-soluble fraction. The pellets were resuspended in 1% Triton X-100 (TX)-PBS and homogenized using slight sonication. After centrifugation at $16,000 \times g$ for 10 min at 4°C , the supernatants were collected as the TX-soluble fraction. Next, the pellets were resuspended in 5% SDS (Fisher Molecular Biology, FS0109)-PBS and homogenized using slight sonication. After centrifugation at $16,000 \times g$ for 10 min at 4°C , the supernatants were collected as the SDS-soluble fraction. Next, the pellets were resuspended in 88% formic acid/PBS and homogenized using slight sonication. After centrifugation at $16,000 \times g$ for 10 min at 4°C , the supernatants were collected as the FA-soluble fraction. (See also Fig. S5 in the supplementary materials). Protein concentrations of PBS and TX fractions were measured by a BCA protein assay (Euroclone, EMP014500) and 15 μg were loaded for SDS-polyacrylamide gel electrophoresis. For SDS and FA fractions (10 μL) were loaded for SDS-polyacrylamide gel electrophoresis. All fractions were analyzed as described for WB.

Microscopy analyses on NSC34 cells

NSC34 cells were plated in 12-well multiwell plates containing coverslips at 70,000 cells/well density, transiently transfected with the plasmid coding for AR.Q46 or HSPB8 or with *Dync1h1* siRNA as previously described. AR activation was induced by 10 nM T treatment for 48 h, inhibition of dynein

ATPase activity was performed by treatment with 100 μM EHNA for 48 h. In experiments involving autophagy induction, 100 mM trehalose was added to the cells for the last 48 h. Then, the cells were fixed and processed as previously described.⁹⁵

SBMA patient-derived iPSCs cells, differentiated to neuronal cells as described above, were plated in 24-well multiwell plate containing coverslips coated with laminin and poly-D-lysine at 50,000 cell/well density, and allowed to grow and differentiate to motoneuronal cells for 10 days. AR activation was induced by 10 nM T treatment for the last 48 h prior to fixation. Inhibition of dynein ATPase activity was performed by treatment with 100 μM EHNA for the last 48 h prior to fixation. Then the cells were fixed and processed as previously described.⁹⁸

The following primary antibodies were used to analyze protein distributions in NSC34: rabbit polyclonal AR-H280 antibody (1:500; Santa Cruz Biotechnology, sc-13062) in TBS-T with 5% nonfat milk, rabbit polyclonal anti-LC3 antibody (1:500, Sigma-Aldrich, L8918) in TBS-T with 5% nonfat milk, and rabbit polyclonal anti-SQSTM1 antibody (1:500; Abcam, ab91526) in TBS-T with 5% nonfat milk. The following primary antibodies were used to analyze protein distributions in differentiated SBMA patient-derived iPSCs: rabbit polyclonal ARN20 antibody (1:200; Santa Cruz Biotechnology, sc-816) in 3% BSA (Sigma, A7030)-PBS-0.1% Tween 20; mouse monoclonal MNX1/HB9 antibody (1:200; DSHB, 81.5C10) in 3% BSA-PBS-0.1% Tween 20; rabbit polyclonal TUBB3 (1:200; Cell Signaling Technology, 5568) in 3% BSA-PBS-0.1% Tween 20; mouse monoclonal SMI312 antibody (1:1000; Sternberg Monoclonals Inc., SMI312) in 3% BSA-PBS-0.1% Tween 20. Secondary antibodies were: Alexa Fluor 488 anti-rabbit (1:1000; Life Technologies, A11070) and Alexa Fluor 594 anti-mouse (1:1000; Life Technologies, A11072) in 3% BSA-PBS-0.1% Tween 20. Cells were stained with DAPI to visualize the nuclei. Images were acquired with i) Axiovert 200 microscope (Zeiss Instr., Oberkochen, Germany) equipped with FITC/TRITC/DAPI filters and combined with a Photometric Cool-Snap CCD camera (Roper Scientific, Trenton, NJ, USA), phase contrast image was used to evaluate the morphology. Images were processed using the Metamorph software (Universal Imaging, Downingtown, PA, USA). ii) LSM510 Meta system confocal microscope (Zeiss, Oberkochen, Germany) and images were processed with the Aim 4.2 software (Zeiss).

Proteasome activity

Proteasome chymotryptic assay was performed as described by Allen et al.¹⁴⁴ NSC34 cells were plated in 6-well multiwell plates at 180,000 cells/well (4 wells for each condition to be tested; $n = 4$). Where indicated, cells were transfected with plasmid coding for AR.Q46, as described above, and treated with 10 nM T for 48 h, 100 μM EHNA for 48 h, 10 μM MG132 for the last 16 h, or DMSO for 48 h. Cells were washed with ice-cold PBS and then harvested and centrifuged at $100 \times g$ for 5 min, at 4°C. Pellets resuspended in 0.3 mL of proteasome extract buffer (20 mM Tris HCl, pH 7.4, containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM ATP [Sigma, A1852], 20% v/v glycerol, and 0.04% v/v Nonidet P-40 [Sigma, 98379]) were homogenized by 10 passages through a 21-gauge needle, and then centrifuged at $12,000 \times g$ for 15 min at 4°C, saving the

supernatant. Total proteins were determined with BCA protein assay. Proteasome assay reaction mixtures consisted of 50 mM HEPES-KOH, pH 8.0, containing 5 mM EGTA, 100 μg of cell protein extract per mL of assay reaction. Chymotryptic proteasome substrate conjugated with amidomethylcoumarin (AMC), the peptide N-Suc-LLVY-AMC (Sigma, S6510), was added to the mix at 50 μM and incubated at 37°C for 45 min. The resulting fluorescence was measured at 340 nm excitation and 460 nm emission using a plate spectrofluorimeter (Enspire, Perkin Elmer, MA, USA).

Cell viability assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich, M2128)-based cell proliferation assay (MTT assay) was carried out on NSC34 cells 24 or 48 h after treatment with 100 μM EHNA, and performed in 24-well multiwell plates at 45,000 cells/well (6 wells for each condition to be tested; $n = 6$). MTT solution was prepared at 1.5 mg/mL in DMEM without phenol red and was filtered through a 0.2- μm filter. Then, the culture medium was removed from the plate and 300 μL of MTT solution was added into each well. Cells were incubated for 30 min at 37°C with 5% CO₂, 95% air and complete humidity. After 30 min, 500 μL of 2-propanol was added into each well and the precipitates were suspended. The optical density (OD) of the wells was determined using a plate reader at a wavelength of 550 nm.

β -galactosidase assay

NSC34 cells were plated in 24-well multiwell plates at density of 40,000 cells/well (6 wells for each condition to be tested; $n = 6$) and transfected with 0.4 μg of pCMV- β gal plasmid. 24 h after transfection the cells were lysed in 250 μL of lysis buffer (Promega, E194A) and 100 μL of samples were added to 750 μL of assay buffer (60 mM Na₂HPO₄ 40 mM NaH₂PO₄ 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol, pH 7.0), in presence of 4 mg/mL of β -galactosidase substrate o-nitrophenyl- β -D-galactopyranoside (ONPG; Sigma-Aldrich, N1127) and incubated at 37°C until yellow color appearance. Then, 500 μL of 1M Na₂CO₃ were added and 200 μL of the final solution were transferred into a 96-well plate and 420-nm absorbance was evaluated using Enspire plate spectrofluorimeter (PerkinElmer).

Statistical analysis

In all figures, data are presented as mean \pm SD. Statistical analysis has been performed by using the one-tailed unpaired Student *t* test to compare data between 2 groups, One-Way ANOVA to compare more than 2 groups of data, and Two-Way ANOVA to compare the effect of 2 different independent variables (see figure legends for details). When ANOVA resulted to be significant, we performed an Uncorrected Fisher LSD *post hoc* test (and the one-tailed unpaired Student *t* test when the variances between groups were highly different) (see figure legends for details) for multiple comparisons. Computations were done with the PRISM (ver. 6.0h) software (GraphPad Software, La Jolla, CA, USA).

Abbreviations

3MA	3-methyladenine
ALS	amyotrophic lateral sclerosis
AR	androgen receptor
BAG1	BCL2-associated athanogene 1
BAG3	BCL2-associated athanogene 3
C9ORF72	chromosome 9 open reading frame 72
CMA	chaperone-mediated autophagy
DYNC1H1	dynein cytoplasmic 1 heavy chain 1
EHNA	erythro-9-[3-2-(hydroxynonyl)]adenine
FA	formic acid
FALS	familial ALS
FRA	filter-retardation assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GP	dipeptide (glycine-proline)
HDAC6	histone deacetylase 6
HSP	heat shock protein
HSPA	heat shock protein A
HSPA1A	heat shock protein 1A
HSPA8/HSC70	heat shock protein 8
HSPB8	heat shock protein family b (small) member 8
IF	immunofluorescence analysis
IPSCS	induced pluripotent stem cells
MAP1LC3/LC3	microtubule-associated protein 1 light chain 3
MNDS	motoneuron diseases
MNX1/HB9	motor neuron and pancreas homeobox 1
MTOC	microtubule-organization center
ND	neurodegenerative disease
PQC	protein quality control
SALS	sporadic ALS
SBMA	spinal bulbar muscular atrophy
SOD1	superoxide dismutase 1, soluble
SQSTM1/p62	sequestosome 1
STUB1/CHIP	stip1 homology and u-box containing protein 1
T	testosterone
TARDBP/TDP-43	tar dna binding protein
TFEB	transcription factor eB
TUBA/ α -TUBULIN	tubulin, alpha
TUBB3/ β III-TUBULIN	tubulin, beta 3 class III
UPS	ubiquitin-proteasome system
WB	western blot
WT	wild type.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments


The authors are grateful to Dr. Kennet Fischbeck (NINDS-NIH, Bethesda, USA) for having provided the iPSCs derived fibroblast of SBMA patients and controls; to Dr. Enrico Garattini (Mario Negri Institute, Milan, Italy) for TR4 NSC34 cells; to Prof. D. Ito (Department of Neurology, Keio

University School of Medicine, Tokyo, Japan) for the pCMV-Flag-polyGP-V5-His encoding the poly-GP; to Prof E. Buratti (International Center for Genetic Engineering and Biotechnology, Trieste, Italy) for the pFLAG-FL TDP-43, and pFLAG- Δ C TDP-43; to Prof. Diane Merry (Thomas Jefferson University, Philadelphia, PA, USA) for the PC12-AR.Q112; to Prof. R. Kopito (Stanford University, Stanford, CA, USA) for the reporter plasmid GFPu; to Prof. H.H. Kampinga (University of Groningen, Groningen, The Netherlands) for the pcDNA/HA-Bag1; to Dr. A. Boncoraglio for the fruitful discussion.

Funding

The following grants are gratefully acknowledged: Fondazione Telethon, Italy (n. GGP14039 to A.P.); Fondazione Cariplo, Italy (n. 2014-0686 to A. P. and S.C.); Fondazione AriSLA, Italy (n. ALS_HSPB8 to A.P. and S.C.; ALS_Granulopathy to A.P. and S.C.); Association Française contre les Myopathies, France (AFM Telethon n. 16406 to A.P.); Regione Lombardia (to A.P.); Università degli Studi di Milano e piano di sviluppo UNIMI - linea B (to P.R.); Italian Ministry of Health (MinSal) (n. GR-2011-02347198 to V.C. and S.C.); Joint Program on Neurodegenerative Diseases (JPND, CureALS), EC (to A.P. and S.C.); Fondazione regionale per la ricerca biomedica (FRRB) (TRANS_ALS), Regione Lombardia, Italy (to A. P.); MIUR Rita Levi Montalcini (to S.C.); Italian Ministry of University and Research (MIUR), PRIN - Progetti di ricerca di interesse nazionale (n. 2015LFPNMN to A.P. and S.C.); European Molecular Biology Organization (EMBO), short-term fellowship (n. 537 - 2015 to R.C.)

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