Differential roles of the ubiquitin proteasome system (UPS) and autophagy in the clearance of soluble and aggregated TDP-43 species

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Summary
TARDBP (TDP-43) is the major pathological protein in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Large TDP-43 aggregates decorated by degradation adaptor proteins are seen in the cytoplasm of remaining neurons in patients post mortem. TDP-43 accumulation, and ALS-linked mutations within degradation pathways, implicates failed TDP-43 clearance as a primary disease mechanism. Here we report the differential roles of the ubiquitin proteasome system (UPS) and autophagy in the clearance of TDP-43. We have investigated the effects of UPS and autophagy inhibitors on the degradation, localisation and mobility of soluble and insoluble TDP-43. We find that soluble TDP-43 is degraded primarily by the UPS, while aggregated TDP-43 clearance requires autophagy. Cellular macroaggregates, which recapitulate many pathological features of patient aggregates, are reversible when both the UPS and autophagy are competent. Their clearance involves the autophagic removal of oligomeric TDP-43. We speculate that in addition to age-related decline, a second hit in the UPS or autophagy pathways drives the accumulation of TDP-43 in ALS and FTD. Therapies for clearing excess TDP-43 should therefore be targeted to a combination of these pathways.
Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are neurodegenerative diseases characterised by the deposition of pathological protein aggregates of TAR DNA binding protein (TDP-43)(Neumann et al., 2006). These ‘TDP-43 proteinopathies’ span a clinical spectrum from predominantly upper and lower motor neuron pathology in ALS, to predominantly cortical neuron pathology in FTD, to a mixed presentation of both. TDP-43 is a DNA- and RNA-binding protein which regulates mRNA processing and trafficking, the stress granule response, and microRNA biogenesis (Buratti et al., 2010; Colombrita et al., 2009; Gregory et al., 2004; Tollervey et al., 2011). A causative role for TDP-43 was confirmed by the finding that mutations in TDP-43 cause ALS, and account for ~3% of familial and 0.5% of sporadic cases (Lagier-Tourenne and Cleveland, 2009). In the absence of genomic mutation, wildtype TDP-43 is aggregated in the brain and spinal cord of 90% of ALS patients and in the brain of 60% of FTD patients (Neumann et al., 2006).

Evidence that dysregulation of TDP-43 can cause disease comes from many animal models. A neurodegenerative ALS-like phenotype can be generated by either overexpression or knockdown of TDP-43 in flies, fish and mice (Kabashi et al., 2009; Li et al., 2010; Schmid et al., 2013; Wils et al., 2010; Wu et al., 2012). Indeed, various strands of evidence implicate a failure of TDP-43 clearance in human ALS and FTD. Aggregated TDP-43 in ALS and FTD tissue is phosphorylated, ubiquitinated, and labelled by ubiquilins 1 and 2 (Deng et al., 2011; Neumann et al., 2006) and sequestosome 1/ p62 (Arai et al., 2003), which target proteins for degradation. Also, mutations in ubiquilin 2 (Deng et al., 2011), sequestosome 1/ p62 (Fecto et al., 2012), or another degradation pathway adaptor protein, valosin containing protein (VCP) (Watts et al., 2004), can cause ALS and FTD characterised by TDP-43 aggregation. Cellular studies indicate that primary mutation of TDP-43 also confers resistance to degradation (Ling et al., 2010).

Two of the major pathways for cellular protein degradation are the ubiquitin proteasome system (UPS) and macroautophagy. Autophagy has been shown to degrade soluble and aggregated protein substrates which are too large to enter the UPS pore (Verhoef et al., 2002). Ubiquitination may direct proteins toward either a UPS or autophagic fate, therefore ubiquitin pathology in human ALS and FTD tissue does not distinguish which pathway is implicated in
disease pathogenesis. The adaptor proteins sequestosome 1/ p62 and ubiquilin 2 which
decorate TDP-43 aggregates are also associated with both the UPS and autophagy (Lamark
and Johansen, 2009).

Determining the relative importance of these two protein degradation pathways in turning
over TDP-43 is challenging, due to the complex interplay between them, yet is crucial to
understanding the mechanisms underpinning TDP-43 accumulation in disease. While the role
of TDP-43 macroaggregation remains controversial, the cytoplasmic accumulation of TDP-43
is definitely linked to toxicity, and therapeutic strategies designed to enhance TDP-43
clearance are of great appeal. We therefore sought to investigate how TDP-43 protein
degradation is regulated and the potential for targeting degradative pathways as a treatment
for ALS and FTD.
Results

Establishing cellular models of TDP-43 proteinopathy

Stable cellular models of ALS and FTD based on doxycycline (DOX)-inducible expression of TDP-43 were generated in human neuroblastoma (SH-SY5Y) cells and human embryonic kidney (HEK293) cells. In both cell types, and in agreement with previous reports, following DOX induction with 1 µg/mL DOX, HA-tagged wildtype TDP-43 was predominantly nuclear, TDP-43 with a deleted nuclear localisation sequence (ΔNLS) was both nuclear and cytoplasmic, and C-terminal fragment TDP-43 (amino acids 181-414; CTF) was predominantly cytoplasmic (Fig. 1B,C). Nuclear localisation of ΔNLS TDP-43 is likely due to dimerisation with endogenous TDP-43, leading to co-import. Stable SH-SY5Y and HEK293 lines expressing EGFP-tagged TDP-43 WT, ΔNLS and CTF recapitulated these localisation profiles. The expression of TDP-43 in both SH-SY5Y and HEK293 lines was DOX dose-dependent (Fig. 1D,E, EC50; HEK293, 0.97 ng/mL; SH-SY5Y, 0.35 ng/mL).

Degradation of TDP-43

Next we sought to investigate relative degradation rates of the TDP-43 constructs. Because of its ability to autoregulate (Ayala et al., 2011), quantification of endogenous TDP-43 protein degradation cannot be performed by steady state analyses. We exploited the inducible nature of our cellular model to perform non-radioactive pulse-chase, in order to assay the relative degradation of HA-TDP-43 protein after removal of the DOX inducer. In all experiments HA-TDP-43 expression was induced for 24 h using 10 ng/mL DOX to achieve almost maximal expression.

Using HEK293 lines assayed over 72 h (Fig. 2A), wildtype TDP-43 was degraded with a half-life of 32.5 h. The 25 kDa CTF of TDP-43, which is predominantly cytoplasmic, was turned over far more rapidly, with a half-life of 11 h. TDP-43 ΔNLS, which is both nuclear and cytoplasmic, showed an intermediate rate of degradation, with a half-life of 24.4 h. Previous reports on untagged wildtype TDP-43 half life have ranged from 4 h to ≥34 h (Ling et al., 2010; Pesiridis et al., 2011; Watanabe et al., 2013).

In order to verify that the assay was not simply measuring dilution of HA-TDP-43 protein by cell division, or slow decay of DOX-induced transcripts, we performed a modified experiment over a shorter time course in SH-SY5Y lines (Fig. 2B), and used quantitative
PCR to assess transcript levels after DOX washout (Fig. 2C). SH-SY5Y cells have a doubling time of ~48 h, compared to ~24 h for HEK293. Also, protein levels were assayed over a 24 h period which began 24 h after DOX washout (see schematic Fig. 2B), to ensure that DOX-induced transcripts were not still present as a substrate for continued protein synthesis. Indeed, using real-time reverse transcriptase PCR, we verified that HA-TDP transcripts rapidly decayed after DOX washout (Fig. 2C). Published half-lives for endogenous TDP-43 transcript in various cell types range from 1.9 to 10.3 h (Ayala et al., 2011; Schwanhausser et al., 2011; Sharova et al., 2009). The three HA-TDP-43 proteins were turned over at very similar rates in SH-SY5Y lines to HEK293 lines, despite their differing cell division rates and the use of a shorter time course (t_{1/2}, HA-TDP WT, 29.2 h; HA-TDP ΔNLS, 16.6 h; HA-TDP CTF, 10.2 h).

Involvement of the UPS and autophagy in the degradation of TDP-43

Having determined that TDP-43 degradation rates were consistent between cell lines and time courses, we next analysed the pathway/s by which TDP-43 was being degraded using HEK293 lines. Inhibitors of the ubiquitin proteasome system (UPS) or autophagy were included during the final 48 h of the DOX washout period when protein degradation occurs (for schematic see Fig. 3A). Inhibitors were used in favour of genetic approaches in order to achieve temporal control and homogeneity across the cell population.

These assays examined the total complement of TDP-43, of which ~90% was detergent-soluble (Fig. 4D,E). Degradation of all three TDP-43 species was inhibited to varying degrees by the panel of inhibitors tested (Fig. 3A). The autophagy inhibitor 3MA significantly inhibited the degradation of CTF TDP-43 (p≤0.05) and to a lesser extent wildtype and ΔNLS TDP-43. 3MA showed minimal effect on the autophagy reporters p62 and LC3 at the time point chosen for assay of TDP-43 levels (48 h 3MA), however shorter time courses of 3MA treatment revealed autophagy inhibition had occurred (Fig. 3B). The autophagosome-lysosome fusion inhibitor bafilomycin only minimally inhibited degradation of each TDP-43 species, despite effectively inhibiting autophagy as evidenced by LC3-II accumulation. In contrast to the moderate effects of autophagy inhibitors, the UPS inhibitor MG132 significantly inhibited the degradation of all three TDP-43 constructs (Fig. 3A, wildtype TDP-43 p≤0.05, ΔNLS p≤0.05, and CTF TDP-43 p≤0.001). The related UPS inhibitor epoxomicin also caused significant accumulation of wildtype, ΔNLS and CTF TDP-43 (all
p≤0.05). The inhibition of degradation by both pathways (combined 3MA and MG132) did not inhibit the degradation of any of the protein constructs any more than MG132 alone. Activators of the autophagy pathway were used to validate these findings as the addition of trehalose and LiCl during DOX washout significantly accelerated the degradation of CTF TDP-43 (Fig. 3C).

**UPS, but not autophagy inhibition, induces cytoplasmic TDP-43 macroaggregates**

We sought to assess the phenotypic consequences of TDP-43 accumulation under either autophagic or UPS inhibition. Parallel biochemical and immunocytochemical studies were carried out in SH-SY5Y cells as their large cytoplasm was more amenable to imaging than HEK293 cells.

To investigate the localisation and accumulation of TDP-43, its expression was induced with 1 µg/mL DOX in stable SH-SY5Y cells in the presence of inhibitors of autophagy or the UPS for 48 h. For both wildtype and ΔNLS, inhibition of the UPS, but not autophagy, caused TDP-43 accumulation resulting in the formation of macroaggregates (Fig. 4A). The UPS inhibitor MG132 induced the formation of large, cytoplasmic aggregates in around 20% of cells (WT, 17.4 ± 1.3% (224 cells counted); ΔNLS, 25.6 ± 3.4% (368 cells counted)). Epoxomicin also induced TDP-43 macroaggregates. In contrast, neither of the autophagy inhibitors, 3MA or bafilomycin, induced the formation of ubiquitinated TDP-43 macroaggregates. Interestingly, co-application of MG132 and 3MA more robustly generated aggregates in both HA-TDP WT- and HA-TDP ΔNLS-expressing cells.

As shown in figure 1B,C, the HA-TDP CTF was expressed at extremely low levels in both SH-SY5Y and HEK293 cells and was barely detectable by immunocytochemistry, likely due to its rapid degradation (as shown in Fig. 2A,B). Therefore, the localisation of the HA-TDP-43 CTF was not investigated further. We have found an equivalent EGFP-tagged CTF to form aggregates under UPS but not autophagy inhibition, similar to wildtype or ΔNLS TDP-43, however the physiological relevance of the EGFP-tagged fragment is unclear given that we and others (Li et al., 2011) find its steady state levels to far exceed that of CTFs with small tags.
To examine more sensitively the conditions under which ubiquitinated TDP-43 accumulates, we performed co-immunoprecipitation after treatment with 3MA or MG132, under native or denaturing conditions (Fig. 4B). In HEK293 HA-TDP-43 WT stable lines transfected with FLAG-Ub, then induced with 1 µg/mL DOX alone, immunoprecipitation with anti-HA antibody yielded a small amount of FLAG-Ub, which ran as high molecular weight smears (>100 kDa). Induction with DOX plus 3MA for 24 h resulted in a small increase in the co-immunoprecipitation of FLAG-Ub by HA-TDP-43 WT, while 24 h MG132 treatment gave a more striking increase. This finding, which arose under non-denaturing immunoprecipitation conditions which preserve protein complexes, could reflect HA-TDP-43 complexation with molecules which are ubiquitinated, rather than direct ubiquitination. Indeed, using denaturing conditions (lysate boiled with 0.5% SDS to denature non-covalent interactions) a direct FLAG-Ub interaction with HA-TDP-43 was observed following MG132 treatment, but not under basal or 3MA-treated conditions. These findings indicate that ubiquitinated TDP-43 accumulates predominantly when the UPS is blocked.

TDP-43 aggregates in human ALS and FTD were initially characterised as non-amyloid (Cairns et al., 2007), however recent studies have found TDP-43 aggregates to be amyloid in at least a subset of cases (Bigio et al., 2013; Robinson et al., 2013). We used thioflavin T staining to determine whether the TDP-43 aggregates induced by UPS inhibition were amyloid. UPS inhibition induced the formation of multiple small amyloid aggregates, which were sometimes studded within TDP-43 positive macroaggregates, however the TDP-43 macroaggregates themselves were negative for thioflavin T (Fig. 4C). These findings suggest the macroaggregates are amorphous and non-amyloid.

We next investigated the relationship between macroaggregates detected by immunocytochemistry, and detergent-insoluble species on western blot; described by Neumann and colleagues as one of the hallmark features of ALS and FTD (Neumann et al., 2006). Sequential biochemical fractionation of TDP-43 proteins (generated by 48 h induction of stable SH-SY5Y cells with 1 µg/mL DOX) demonstrated that under basal conditions, HA-tagged wildtype or ΔNLS TDP-43 showed a similar biochemical profile to endogenous TDP-43, with a small proportion (7-21%) soluble in the low salt fraction, the majority of the protein in the triton-soluble fraction (72-89%), and a minority of the protein soluble only in sarkosyl or urea (4-11%) (Fig. 4D,E). The HA-tagged CTF TDP-43 used in this study was...
almost completely soluble in low-salt and triton fractions (>99%). Given its low expression levels, lack of aggregate formation and high basal solubility, the solubility of CTF TDP-43 was not investigated further.

Using a simplified solubility protocol previously validated to isolate insoluble aggregated TDP-43 (Winton et al., 2008), we analysed the effect of UPS or autophagy inhibition on the solubility of wildtype and \( \Delta \text{NLS} \) TDP-43 (Fig. 4F,G). As for the immunocytochemical experiments, TDP-43 expression in stable SH-SY5Y cells was induced with 1 \( \mu \)g/mL DOX in the presence of inhibitors of autophagy or the UPS for 48 h. The effect of UPS inhibitors on total TDP-43 levels (lysate) were more subtle than in figure 3A, likely due to the differing experimental paradigm—application of inhibitors during washout in figure 3A versus application of inhibitors with continued overexpression in Fig. 4F,G. Due to the low proportion of cells forming aggregates, we could not detect an increase in TDP-43 in the insoluble fraction for conditions which yielded immunodetectable macroaggregates. However, combined UPS and autophagy inhibition (3MA and MG132) was shown to increase the levels of insoluble high molecular weight TDP-43, indicating that macroaggregates are composed of insoluble oligomeric TDP-43. Also of note, even under experimental conditions where TDP-43 appears diffuse by immunocytochemistry (Veh, 3MA, Baf), a significant amount of monomeric TDP-43 was RIPA-insoluble.

**TDP-43 aggregates induced by UPS- or dual UPS/autophagy-inhibition resemble those seen in human disease**

We next characterised TDP-43 macroaggregates in terms of their labelling by molecules that target them for degradation. Given that dual inhibition of the UPS and autophagy increased the levels of insoluble TDP-43 and enhanced macroaggregation more than UPS inhibition alone, we compared the labelling of aggregates induced by dual inhibition with those induced by UPS inhibition alone.

We first characterised the basal pattern of staining in untreated SH-SY5Y cells of several degradation adaptor proteins; ubiquilin 1/2 (UBQLN) and sequestosome 1/p62 (p62), which are seen in aggregates in human ALS and FTD, and polyubiquitin chains with different internal lysine linkages (K48 and K63)(Fig. 5A). These adaptors were each localised diffusely in untreated cells, but colocalised with aggregates of wildtype (Fig. 5B,C) or \( \Delta \text{NLS} \)
TDP-43 (Fig. 5D,E) which had been induced with 1 µg/mL DOX plus either MG132 with
3MA, or MG132 alone. While 3MA enhanced the recruitment of HA-TDP-43 WT to
perinuclear aggregates induced by MG132 treatment, it did not alter the labelling profile of
the aggregates (Fig. 5 intensity plots).

**TDP-43 macroaggregates are cleared upon the restoration of UPS function**

We next sought to investigate the handling of insoluble TDP-43 specifically. We used
1 µg/mL DOX plus MG132, which acts reversibly, to induce the formation of insoluble
TDP-43 WT or ΔNLS cellular aggregates, and then we followed the fate of those aggregates.

1 µg/mL DOX was maintained in the medium during washout to ensure our findings
reflected TDP-43 aggregate handling in the face of continued expression of TDP-43. End
point imaging showed that for both WT (Fig. 6A) and ΔNLS TDP-43 (Fig. 6B), cells given a
48 h washout period after MG132 treatment were devoid of TDP-43-positive and p62-
positive aggregates, in stark contrast to cells fixed directly after the MG132 treatment. The
biochemical correlate of this was a reduction in detergent-resistant high molecular weight
TDP-43 following MG132 washout (Fig. 6C,D). These findings may indicate selective
survival of cells without TDP-43 aggregates, or that aggregated TDP-43 is either refolded or
degraded following restoration of UPS function.

We therefore examined the clearance of aggregates in live cells, using stable SH-SY5Y cells
transfected with EGFP-tagged TDP-43. We verified that replacement of the N-terminal HA-
tag with EGFP did not alter the time course of degradation of TDP-43 by performing
doxycycline pulse chase (Fig. 6E, EGFP-TDP WT $t_{1/2}$ 29.3 h, HA-TDP WT $t_{1/2}$ 29.2 h). We
also verified that EGFP-tagged TDP-43 was functional, as evidenced by its ability to
downregulate endogenous TDP-43 (Ayala et al., 2011)(Fig. 6F). We next performed live cell
imaging of cells with MG132-induced aggregates of EGFP-TDP ΔNLS, which indicated that
most aggregate-laden cells died over a 15 h washout period (69.3 ± 5.6%), and also that
aggregates were rapidly cleared by the surviving cells (Fig. 6G and movie 1). Due to the low
fluorescence intensity of EGFP-TDP WT aggregates, their clearance could not readily be
measured in living cells.

**TDP-43 aggregate clearance is due to enhanced mobility of lower order aggregated
species, rather than clearance of macroaggregated TDP-43**
Previous work on other aggregate-prone proteins indicates that some aggregates (e.g. juxta-nuclear quality control aggregates, JUNQ) are mobile, readily exchange with the diffuse pool, and can be cleared by the UPS (Ben-Gedalya et al., 2011; Kaganovich et al., 2008). To determine whether this was true for TDP-43, we performed fluorescence recovery after photobleaching (FRAP) on diffuse and macroaggregated EGFP-TDP ΔNLS (Fig. 7A) using stable SH-SY5Y cells. FRAP measures both the proportion of a given protein pool which is mobile, and the rate of mobility, by examining the migration of fluorescent proteins back into a region which has been bleached of fluorescence.

We examined cells induced to express EGFP-TDP-43 using 1 µg/mL DOX alone; or induced to form aggregates with 1 µg/mL DOX plus MG132; or induced to form aggregates then MG132 washed out, and cells left in fresh media containing 1 µg/mL DOX without MG132 for 8-10 h. Firstly, by bleaching diffuse cytosolic EGFP-TDP ΔNLS we found that the proportion of diffuse TDP-43 protein which was mobile was reduced by inhibition of the UPS, both in cells devoid of aggregates and more strikingly in cells which contained large aggregates (Fig. 7B,C. Percentage of TDP-43 which was mobile: DOX only, 69 ± 0.9%; MG132 without aggregates, 64 ± 1% p≤0.01; MG132 with aggregates, 48 ± 1.6% p≤0.001). Furthermore, we found that TDP-43 within the aggregates was almost completely immobile, with bleached portions of aggregates remaining dark for the duration of FRAP imaging and not visibly exchanging with the diffuse pool (Fig. 7A-C, Percentage of TDP-43 which was mobile: 2.7 ± 0.1% p≤0.001). Following 8-10 h washout of MG132, there was a significant increase in the proportion of diffuse TDP-43 which was mobile in cells still containing large aggregates, suggesting mobilisation or clearance of previously immobile species (Percentage of TDP which was mobile: 48 ± 1.6% before and 61 ± 0.7% after washout p≤0.001). However, direct bleaching of macroaggregated TDP-43 showed this species was not mobilised, as there was no increase in the mobile fraction after washout.

Next we examined the rate of recovery (the speed of molecular movement), for mobile species of TDP-43 protein (Fig. 7D). Diffuse TDP-43 in vehicle-treated cells showed rapid recovery (t₁/₂= 3.4 s). Diffuse TDP-43 in cells exposed to UPS inhibition but without aggregates showed a similar rate of recovery (t₁/₂= 3.6 s), while TDP-43 in cells with aggregates showed significantly slower recovery (t₁/₂= 6.9 s). A decrease in fluorescence recovery rate indicates increased binding, or increased size of the TDP-43 particle - consistent
with oligomerisation of TDP-43. Following 8-10 h washout of MG132, there was an increase in the rate of recovery of diffuse TDP-43 in cells with large aggregates ($t_{1/2} = 6.9$ s before vs. 4.0 s after washout), suggesting the removal or dissociation of slow-moving species.

**TDP-43 aggregate clearance requires autophagy**

Our findings to date suggested that during washout, slow-moving and immobile species were either resolved into mobile/ faster-moving species, or were removed directly. Oligomeric or microaggregated species cannot be cleared directly by the UPS, and their clearance may require aggregate autophagy, or “aggrephagy” (Lamark and Johansen, 2012; Overbye et al., 2007). Therefore, in order to test whether aggregate clearance involved autophagy, we first determined whether cells with TDP-43 aggregates contained autophagosomes, the double-membraned effector of autophagy. Cells transfected with the autophagosome marker mCherry-GFP-LC3 showed a significant increase in the number of autophagosomes (GFP-positive puncta) in cells induced to form TDP-43 aggregates (1 µg/mL DOX plus MG132), compared to controls (Fig. 8A,B, Autophagosomes per cell: WT, 36 ± 8 MG132 vs. 2 ± 1 DOX $p \leq 0.01$; ΔNLS, 31 ± 9 MG132 vs. 6 ± 2 DOX $p \leq 0.05$).

We next examined the impact of inhibiting autophagy on aggregate clearance, by performing live imaging. Cells were induced to form aggregates of EGFP-TDP ΔNLS using 1 µg/mL of DOX plus MG132, and inhibitors of autophagy were added during the washout period (Fig. 8C). As discussed, the majority of cells with aggregates died over the course of imaging. Aggregate-containing cells which died had significantly greater aggregate load than those that lived (Av. aggregate int. intensity 7328 ± 1661, vs. 1961 ± 522 in cells which lived, $p \leq 0.01$, T-test with Welch's correction). For cells which survived the period of imaging, we plotted the relationship between the load of aggregated protein and the rate of clearance from the cell (Fig. 8D). In the absence of autophagy inhibitors, we found a positive linear relationship between aggregated protein load and time taken for clearance (Fig. 8D, Veh). When autophagy inhibitors were present, macroaggregates were still disassembled into smaller, discrete aggregates, suggesting an autophagy-independent disaggregation step (Fig. 8D, Baf, and movie 2). However, these aggregates then persisted in the cells. The slope of the line relating initial aggregate load with clearance time therefore flattened in the presence of autophagy inhibitors (Fig. 8D,E, relative slope 3MA, 0.74; Baf, 0.15). A strong dependence of aggregate clearance upon autophagy was shown by aggregates which completely failed to
be cleared over the 15 h time course in the presence of bafilomycin, regardless of their size (Fig. 8D, dashed line).
Discussion

A number of genetic causes of ALS and FTD-TDP converge upon the pathways involved in TDP-43 protein degradation. Mutations in TDP-43 itself confer resistance to degradation (Kabashi et al., 2008; Rutherford et al., 2008; Sreedharan et al., 2008), while mutations in sequestosome 1/p62, ubiquilin 2, and VCP (Deng et al., 2011; Fecto et al.; Watts et al., 2004), disrupt the autophagy and UPS pathways, both of which are involved in the degradation of TDP-43. However, the relative contribution of these pathways to maintaining TDP-43 proteostasis has been unclear. Previous studies provide conflicting evidence, finding that wildtype TDP-43 was degraded predominantly by the UPS (Caccamo et al., 2009; Crippa et al., 2010; Wang et al., 2010; Zhang et al., 2010), or predominantly by autophagy (Urushitani et al., 2010), and none have specifically examined the degradation of aggregated TDP-43. Here we demonstrate that the degradation of the soluble complement of full-length TDP-43 is mediated primarily by the UPS, while aggregated TDP-43 requires autophagic clearance.

As is the case for many proteins, cellular TDP-43 can exist as several different species with variable appearance microscopically, and variable solubility and mobility profiles. We find evidence for soluble monomeric, soluble oligomeric, insoluble microaggregated, and insoluble macroaggregated forms of TDP-43. Soluble monomer appears diffuse, is detergent-soluble and shows fast FRAP. Soluble oligomer also appears diffuse and is detergent-soluble but shows slow FRAP. Insoluble microaggregates are detectable in cells with diffuse-appearing TDP-43 as detergent-insoluble species which are immobile by FRAP. Insoluble macroaggregates are evident only in UPS-treated cells, as immunodetectable entities composed of detergent-insoluble high molecular weight species which show no FRAP mobility.

Our proposed model for the existence of these species under different conditions is outlined in Fig. 9. Normally, in the presence of a functional UPS, the removal of soluble monomer serves to drive the equilibrium sufficiently to the left to favour small soluble species, and to preclude the formation of macroaggregates. Therefore, in both cell culture models and tissue from human controls, only a small proportion of TDP-43 is detergent-insoluble basally (Neumann et al., 2006). Conversely, UPS blockade drives the accumulation of slow-moving oligomers, and insoluble/immobile TDP-43 species- which may appear diffuse (microaggregates) or clearly aggregated (macroaggregates). The clearance of
macroaggregates involves fragmentation into “bite-sized” pieces, which is autophagy-independent, and then the removal of lower order species by autophagy. Oligomers, microaggregates or both may be substrates for autophagy, as autophagy blockade favours the continued existence of fragmented macroaggregates.

It has previously been shown that inhibition of the UPS can induce aggregates of full-length TDP-43 (Kim et al., 2012; van Eersel et al., 2011). However, to our knowledge, full-length TDP-43 has never been shown to aggregate in cells under conditions of autophagy inhibition alone. Similarly in vivo, ALS with TDP-43 proteinopathy can be modelled in mice by knockout of a proteasome subunit (Rpt3), but not by knockout of a key molecule in autophagy induction (Atg7)(Tashiro et al., 2012). This supports a model whereby the levels of soluble TDP-43 are predominantly regulated by the UPS, and that the accumulation of these species can lead to the “nucleation” of aggregates. Thus impairment of proteasomal degradation in cells recapitulates TDP-43 accumulation, aggregation and inclusion formation which occurs in man, while subsequent impairment of autophagy prevents the removal of aggregated TDP-43.

There has been fierce scientific debate amongst neurodegeneration researchers as to whether visible protein macroaggregates are the ‘toxic species’, are protective, or are epiphenomena associated with, but not causative of disease. The emerging consensus is that macroaggregates may be a “sink” for the true toxic species which are predominantly lower order oligomers with greater mobility and surface area, and thus enhanced potential for aberrant interactions (Caughey and Lansbury, 2003; Haass and Selkoe, 2007). Macroaggregates can however act as a surrogate to assess levels of these oligomeric species. It is likely that the large TDP-43 macroaggregates we report here are “aggresomes”, a congregation of smaller aggregates actively transported via microtubules to perinuclear regions (Kopito, 2000). Aggresome formation is dependent on several molecules whose genomic mutation is linked to ALS and FTD including; VCP, p62 and dynein/dynactin (Johnston et al., 2002; Puls et al., 2003; Seibenhener et al., 2004), as well as the ubiquilin 2-related molecule, ubiquilin 1 (Heir et al., 2006). Aggresomes are thought not only to sequester smaller aggregates to minimise their toxicity, but also to coordinate aggregate removal via autophagy- known as “aggrephagy” (Lamark and Johansen, 2012). Aggrephagy is therefore emerging as a key target for drug discovery in ALS/FTD (Thomas et al., 2013).
We show for the first time that full-length TDP-43 macroaggregates are labelled by both K48- and K63-linked polyubiquitin chains. Ubiquitin chains with these different linkages are thought to direct proteins towards different fates, due to the specificity of binding partners for specific linkages. K48-linked polyubiquitin chains are associated with UPS degradation (Xu et al., 2009). In contrast, K63-linked chains are associated with an autophagic fate. The autophagic adaptor protein p62 is selective for K63-linked chains (Seibenhener et al., 2004), and p62 in turn acts to recruit LC3-positive autophagosomes (Pankiv et al., 2007). Ubiquilin 2 can bind proteins labelled with either type of ubiquitin chain linkage (Rothenberg et al., 2010). The presence of both types of chain on TDP-43 aggregates may be consistent with a model where K48-ubiquitin-labelled substrates are not able to be degraded by the UPS, and are subsequently labelled with K63-ubiquitin for aggrephagy (Yamamoto and Simonsen, 2010).

An autophagic mode of clearance of aggregated TDP-43 echoes that of other neurodegenerative disease proteins. Soluble fragments of wildtype huntingtin are degraded proteasomally, but aggregated expanded repeat fragments are not accessible to the proteasome and are degraded by autophagy (Qin et al., 2003). Mutant androgen receptor, the protein which causes SBMA, is toxic to flies, and this toxicity is enhanced when autophagy is blocked together with the UPS (Pandey et al., 2007).

While autophagy is inducible in response to a heavy burden of aggregated proteins (Ding et al., 2007; Iwata et al., 2005; Laussmann et al., 2011; Rideout et al., 2004), in our model autophagy induction was unable to clear TDP-43 macroaggregates in the face of continued UPS inhibition. Dual UPS/autophagy inhibitor treatment revealed that autophagy had served to reduce aggregated TDP-43 burden seen with UPS inhibition alone, but complete aggregate clearance only occurred after UPS inhibitor washout. Although we used low dose, chronic UPS inhibition to attempt to more closely mimic the slow degenerative process in man, the degree of UPS impairment likely far exceeds that which could be caused by aging or genetic mutations detected in some ALS and FTD cases. Therefore, activation of autophagy remains a promising therapeutic approach to treating ALS and FTD in man. Indeed, autophagy activation has been shown to clear α-synuclein inclusions in primary postmitotic neurons in spite of continued moderate inhibition of the UPS using lactacystin (Rideout et al., 2004).
And excitingly, in a mouse model of FTD based on TDP-43 overexpression, rapamycin (an inhibitor of mTOR, which therefore activates (de-represses) autophagy) was used to induce clearance of TDP-43, increasing motor neuron survival and motor function (Wang et al., 2012).

As we age, the efficiency of both the UPS and autophagy decline (Gamerdinger et al., 2009; Tydlacka et al., 2008; Zhou et al., 2003). However, in aged individuals without ALS or FTD, TDP-43 aggregates do not commonly accumulate. In ALS and FTD, TDP-43 inclusions form and remain at the end of life, leading us to speculate that in addition to normal aging, there is enduring secondary impairment of the UPS or autophagy, promoting the accumulation of misfolded species and preventing their removal. Augmenting UPS function may help to restore TDP-43 proteostasis, and where UPS impairment persists, autophagy activation may reduce aggregated TDP-43 protein burden. Therefore, identifying molecules that selectively enhance each of these processes, and which can be used in combination to reduce TDP-43 accumulation, is a valid therapeutic strategy.
Materials and methods

Plasmids

Constructs are shown schematically in Fig. 1A. These constructs allow doxycycline-inducible expression of tagged (HA or EGFP) TDP-43 wildtype (WT), nuclear localisation sequence-deleted (ΔNLS (Δ82-98)) or C-terminal fragment (CTF (181-414)). All constructs were generated by Gateway cloning (Invitrogen, Carlsbad, CA). AttB-flanked PCR products were subcloned into pDONR221 using BP clonase II, then into pDEST30 using LR clonase II according to manufacturer’s instructions (Invitrogen). N-terminal HA.11-tagged TDP-43 WT, ΔNLS and CTF were amplified from existing constructs (Nishimura et al., 2010). N-terminal EGFP-tagged TDP-43 WT was first generated using pEGFP-C1 (XhoI/ BamHI) then subcloned into Gateway. EGFP-tagged TDP-43 ΔNLS was generated by site-directed mutagenesis of the WT construct in pDONR (Quikchange II, Stratagene). Primer sequences are available on request.

All constructs were verified in-house by DNA sequencing using Big-Dye® Terminator v1.1 (Life Technologies Ltd., Paisley, UK) on an ABI3130 genetic analyser (Applied Biosystems Pty Ltd, Warrington, UK). Sequence chromatograms were analysed using Geneious version 5.4.6 (Biomatters, Auckland, NZ). All primers were obtained from Sigma-Aldrich, Dorset, UK. mCherry-GFP-LC3 plasmid (Pankiv et al., 2007) was kindly supplied by Dr. Terje Johansen (University of Tromsø, Norway). FLAG-Ub plasmid (Tan et al., 2008) was kindly gifted by A/Prof. Lim Kah-Leong (National University of Singapore).

UPS and autophagy modifying drugs

3MA (used at 10 mM), bafilomycin (400 nM) and lithium chloride (10 mM) were purchased from Acros Organics (Geel, Belgium). MG132 (0.5 µM), epoxomicin (100 nM) and rapamycin (0.2 µg/mL) were purchased from Cayman Chemicals (Cambridge, UK). Trehalose (100 mM) was purchased from Sigma-Aldrich.

Mammalian cell line culture, transfection, plating and induction

Stable, tetracycline-inducible (Tet-ON), clonal cell lines were generated in human neuroblastoma SH-SY5Y cells (CRL-2266; ATCC, Middlesex, UK) and human embryonic kidney HEK293 (T-REx HEK293, #R710-07, Invitrogen) using the T-REx system. Briefly, stable clonal lines transfected with pcDNA6/TR (encoding a constitutively expressed Tet-
repressor protein) were transfected with pDEST30-TDP-43 constructs using Lipofectamine 2000, selected using 600 µg/mL geneticin, and clonally isolated.

SH-SY5Y cells were maintained in DMEM/F12 + Glutamax supplemented with 10% FBS (Tet-free, S0115T, BioChrom AG, Berlin, Germany) and 100 U/mL penicillin/ 100 µg/mL streptomycin. HEK293 (stable Tet-ON) and HEK293T cells were maintained in DMEM + Glutamax supplemented with 10% FBS (Tet-free for Tet-ON lines) and 100 U/mL penicillin/ 100 µg/mL streptomycin. All cells were maintained at 37°C, 5% CO₂.

Transient transfection of mCherry-GFP-LC3 into stable SH-SY5Y lines was performed using Lipofectamine 2000, as per manufacturer’s instructions. Transient transfection of FLAG-Ub into stable HEK293 lines was performed using Fugene HD (Promega, Southampton, UK) as per manufacturer’s instructions. For experimental analyses, SH-SY5Y lines were plated at 75,000 cells/cm², HEK293 lines were plated at 25,000 cells/cm², and TDP-43 expression was induced using 1 µg/mL DOX, unless otherwise stated. All reagents were purchased from Invitrogen unless otherwise stated.

**Immunofluorescence and imaging**

For immunofluorescent analyses, SH-SY5Y lines were plated on untreated 13 mm coverslips (1.5 thickness). HEK293 lines were plated on poly-D-lysine-treated coverslips. After treatment, cells were fixed in 4% paraformaldehyde (PFA, VWR International Ltd., Leicestershire, UK) for 10 min and rinsed with phosphate-buffered saline (PBS). Cells were incubated with primary antibody overnight at 4°C, then with fluorescent secondary antibodies for 6 h at room temperature, each diluted in PBS with 0.2% Triton-X100 and 1% serum (goat or donkey as appropriate). For thioflavin T staining, cells were stained for 10 mins with freshly prepared and filtered 0.1% thioflavin T (Acros Organics) in PBS and rinsed. After immunodetection, coverslips were mounted onto slides using fluorescence mounting medium (Dako, Carpenteria, CA) and left to harden overnight.

Antibodies used for immunofluorescence were TDP-43 (mouse: sc-100871, 1:500, Santa Cruz Biotechnology, Inc., Heidelberg, Germany; rabbit: 1078-2-AP, 1:500, ProteinTech Europe, Manchester, UK), HA.11 (mouse: MMS-101P, 1:1000, Covance, Leeds, UK; rabbit: #3724, 1:1000, Cell Signaling Technology, Beverley, MA), P62 (#610833, 1:1000, BD
Biosciences, San Jose, CA), ubiquitin (Z0458, 1:200, Dako UK Ltd., Ely, UK), K48-linked ubiquitin (#05-1307, 1:500, Millipore, Temecula, CA), K63-linked ubiquitin (#05-1308, 1:500, Millipore), Ubiquilin 2 (sc-100612, 1:2000, Santa Cruz Biotechnology, Inc.). Invitrogen Alexafluor goat secondary antibodies (anti-mouse 488, A11001; anti-rabbit 568, A11011) or Jackson Immunoresearch donkey secondary antibody (anti-mouse 649, 715-495-150) were used at 1:500.

Wide-field imaging was performed using a Zeiss Axiovert S100 (Carl Zeiss Ltd., Hertfordshire, UK) with a 63x/NA 1.25 Plan Neofluar oil immersion objective, fitted with a CoolSnap EZ digital camera (Photometrics, Tucson, AZ). Confocal images were acquired using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Buckingham, UK) using a 63x/NA 1.4 Plan Apo oil immersion objective, using a pinhole of 95.6 µm (Airy 1). Images (1024×1024 pixels) were acquired at 400 Hz with line averaging of 8, using Leica LAS-AF software, and 405 nm, 488 nm and 633 nm laser lines.

Line intensity profiles were quantified using the color profiler plugin (Dimiter Prodanov) in ImageJ (version 1.45e, NIH, Bethesda, USA, http://rsb.info.nih.gov/ij/) on merged RGB images for a representative experiment. GFP-LC3 autophagosomes were quantified using the Granularity tool in Metamorph (v. 7.7, Molecular Devices, Wokingham, UK) and granules normalised to the number of transfected cells by manual counting of GFP-positive cells. 113-622 granules were counted in 20-50 transfected cells in a representative experiment.

**Doxycycline pulse-chase assay**

Degradation rates of the various TDP-43 constructs were investigated by doxycycline (DOX) pulse-chase as described previously (Ravikumar et al., 2002). Briefly, TDP-43 expression was induced for 24 h using 10 ng/mL DOX to achieve almost maximal expression (Fig. 1D,E). For washout, cells were rinsed twice in fresh DOX-free media. For initial characterisation of degradation rate in HEK293 cells, samples were harvested just before DOX washout (0 h) and 24, 48, and 72 h after washout (Figure 2A). For validation of degradation rates in SH-SY5Y cells, samples were harvested 24, 28, 32 and 48 h after washout (Figure 2B). To investigate the degradation pathways for TDP-43 in HEK293 cells, samples were harvested just before DOX washout (0 h), then inhibitors, activators or vehicle controls were added after 24 h of washout, and samples taken after a further 48 h (Figure...
Cells were harvested into 1x SDS load buffer (Laemmli buffer (Laemmli, 1970) without bromophenol blue or reducing agents) and boiled for 10 min before freezing at -20°C. Wells receiving MG132, epoxomicin or bafilomycin were plated at 2x density for degradation assays to reduce toxicity (Bar et al., 2004). Degradation time course data were fitted to a one-phase exponential decay and plotted using GraphPad Prism 6.02 (GraphPad Software, San Diego, CA).

**Quantitative PCR**

Total RNA was extracted and DNase-treated using the RNeasy Lipid Tissue Mini Kit (Qiagen, West Sussex, UK) and cDNA was reverse transcribed using oligo(dT) primers with SuperScript® III First-Strand Synthesis SuperMix (Life Technologies). qPCR was performed using FASTStart SYBR Green Mastermix on a 7900HT Fast Real-Time PCR System (Applied Biosystems) running SDS v. 2.3 software. The primers to detect HA-TDP WT and ΔNLS transcripts were TACCCATACGATGTTCCAGATTAC and GCATGCAGAATTCCTTCTACC, HA-TDP CTF transcripts were quantified using the same HA-specific forward primer with the reverse primer CCCGTACTGAGAGAAGAACT. GAPDH was used as a reference transcript using the primers CAGCCTCAAGATCATCAGCA and GGCATGGACTGTGGTCATGAG. PCR cycling conditions were: Activation, 10 min at 95 °C; Cycling, 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C for 40 cycles; followed by the thermal denaturation protocol. Expression levels of HA-TDP transcripts relative to GAPDH transcripts (reference), with normalization to no DOX (control), were determined using the Pfaffl method (Pfaffl, 2001).

**Solubility fractionation**

Sequential biochemical fractionation of cellular proteins was performed as described previously for ALS and FTD brain homogenates (Sampathu et al., 2006), with several modifications for cells. Pellets from the previous step were resuspended in the following volumes relative to the LS volume: TX, 200%; Sark, 30%; Urea; 40%. Centrifugation was performed at 14,000 rpm/ 30 mins/ 4°C. The myelin flotation buffer step was omitted. RIPA/urea solubility fractionation was performed as described by Winton and colleagues (Winton et al., 2008), using centrifugation at 14,000 rpm/ 30 min/ 4°C and omitting sonication. The final (urea) pellet was resuspended in 10% of the original lysis volume.
**Western blotting and densitometry analysis**

Protein concentrations were quantified using the BioRad DC Protein Assay (BioRad, Hemel Hempstead, UK) and equivalent protein was loaded for each sample (HEK293, 5 µg; SH-SY5Y, 10 µg). For solubility experiments, whole lysate protein was quantified, and the equivalent liquid volume of the soluble and insoluble fractions loaded. Gels were transferred onto nitrocellulose using the iBlot (Invitrogen), stained with Ponceau S, then blocked in TBS with 0.05% Tween 20 (TBS-T) with 5% non-fat dried milk (NFDM, Sigma-Aldrich) for 30 min. Blots were probed overnight at 4°C with primary antibody, then for 3 h at room temperature with secondary antibodies (all in TBS-T plus 1% NFDM). Blots were scanned on the Li-Cor Odyssey gel scanner (Li-Cor Biotechnology, Cambridge, UK). Blots were then reprobed (without stripping) for loading control proteins using the same antibody conditions and scanning protocol.

Antibodies used for blotting were TDP-43 (mouse: sc-100871, 1:1000), HA.11 (#3724, 1:1000), Histone H3 (H0164, 1:10,000, Sigma-Aldrich), P62 (#610833, 1:1000), GAPDH (G9545, 1:1000, Sigma-Aldrich), GFP (mouse: sc-9996, 1:1000, Santa Cruz), LC3B (#2775, 1:500, Cell Signaling Technology), Dylight fluorescent secondary antibodies (35521, Goat anti-mouse 680 nm, 1:5000; 35568, Goat anti-rabbit 700 nm, 1:10,000, Fisher Scientific UK Ltd., Leicestershire, UK).

Blot images in TIF format were quantified using the gel analyzer tool in ImageJ. Integrated band intensities were normalized to band intensities of loading controls, and also to relative input for solubility assays.

**TDP-43 aggregate clearance assays**

Clearance of TDP-43 aggregates was assessed by RIPA/urea solubility fractionation and immunofluorescence (HA-tagged constructs), and live cell imaging and fluorescence recovery after photobleaching (FRAP)(EGFP-tagged constructs). For all aggregate clearance assays, stable SH-SY5Y TDP WT or ΔNLS cells were plated at 100,000 cells/cm² and left to recover overnight. TDP-43 expression was induced with DOX for 24 h, then aggregate formation induced using DOX with 0.5 µM MG132 for a further 48 h. For washout experiments cells were then washed and left in fresh media containing DOX without MG132, in the presence or absence of inhibitors as for times periods as stated.
**Live cell imaging**

Live imaging was used to assess whether EGFP-TDP ΔNLS aggregates were cleared. Stable SH-SY5Y cells were plated on Hi-Q4 dishes (Ibidi GmbH, Germany). Immediately before imaging, cells were washed and left in fresh media without MG132 containing DOX and either vehicle (0.05% DMSO), 3MA (10 mM), or bafilomycin (400 nM).

Epifluorescent and phase images (1280×960) of selected cells with aggregates were acquired twice per hour for 15 h using a BioStation IM-Q (Nikon UK Ltd., Surrey, UK) fitted with a 20x/ NA 0.5 Plan Fluor objective, and maintained at 37°C, 5% CO₂. Image sets were analyzed using ImageJ. Aggregate load was defined as the total integrated density (area x intensity) of all aggregates in a given cell, with aggregate boundaries determined by intensity thresholding. Clearance time was defined as the time at which aggregates were indistinguishable from diffuse TDP-43.

**Fluorescence recovery after photobleaching**

FRAP experiments were performed to investigate the mobility of TDP-43 protein. Stable SH-SY5Y EGFP-TDP WT or ΔNLS cells were plated on 18 mm coverslips (1.5 thickness). To investigate protein mobility after MG132 washout, cells were washed and left in fresh media containing DOX but without MG132, for 8-10 h before FRAP.

FRAP was performed using a Nikon A1 plus laser scanning confocal microscope fitted with an environmental chamber maintained at 37°C (Solent Scientific, Segensworth, UK). Confocal images were acquired using a 60x/ NA 1.4 Apo oil immersion objective, a confocal pinhole of 34.8 µm (Airy 1.2), and using a 488 nm laser line. FRAP was performed using NIS-Elements AR software (v. 4.00.04). Images of 512×512 pixels were acquired. Five pre-bleach frames were acquired with a laser power of 1-3%, before bleaching EGFP-TDP-43 (70 msec, 50% argon laser) using a square region of interest (ROI) of 2 µm². Fluorescence recovery was followed for 1 minute, with a laser power of 1-3% to minimise acquisition bleaching.

Data analysis was performed using ImageJ with the Bio-Formats plugin (Linkert et al., 2010). Mean intensity at each time point was measured for the bleached ROI as well as ROIs in the background, and in a neighbouring unbleached cell to correct for acquisition bleaching. FRAP recovery curves for the bleached ROI were calculated by first subtracting the
background at each time point and correcting for acquisition bleaching, then normalising to
pre-bleach and bleach using the equation  $\text{Recovery} (%) = \frac{I_t - I_0}{I_i - I_0} \times 100$

where $I_t$ is the fluorescence intensity of the ROI at a given time, $I_i$ is the fluorescence
intensity of the ROI immediately after bleaching, and $I_0$ is the average fluorescence intensity
of the ROI before bleaching. Data were fitted to a one-phase exponential decay and plotted
using GraphPad Prism 6.02.

Statistical analysis

Data handling and graphical representations were performed using Microsoft Excel unless
otherwise stated. Statistical analyses as described in the figure legends were performed using
GraphPad Prism 6.02. One-way ANOVA was performed after Bartlett's testing for equal
variance. For both one- and two-way ANOVA, Bonferroni post-tests were used to compare
selected data sets. Two-tailed t-tests were performed after F testing for equal variance, and
Welch's correction applied where stated. Pearson correlation test was performed after
D'Agostino & Pearson normality testing. Statistical significance was set at $p \leq 0.05$ (*$p \leq 0.05$;
**$p \leq 0.01$; ***$p \leq 0.001$). All figures were prepared using Adobe Photoshop CS3.
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Figure legends

**Figure 1. Establishing cellular models of TDP-43 proteinopathy.**

A. Schematic of TDP-43 constructs used in this study. B,C. Immunocytochemical analysis of TDP-43 expression and localisation in stable SH-SY5Y (B) and HEK293 (C) cell lines induced to express HA-TDP-43 constructs by 48 h induction with DOX, showing nuclear localisation (wildtype, WT), cytoplasmic localisation (C-terminal fragment, CTF) or both (ΔNLS). Scale bar= 10 µm. D,E. Cell-based ELISA validation of DOX dose-dependent expression of HA-TDP WT in stable SH-SY5Y (D) and HEK293 (E) lines after 48 h induction with DOX (EC50; HEK293, 0.97 ng/mL; SH-SY5Y, 0.35 ng/mL).

**Figure 2. Degradation of TDP-43.**

A. Degradation of HA-TDP-43 proteins in stable HEK293 cells as assessed by DOX pulse-chase. TDP-43 CTF was degraded far more rapidly than TDP-43 ΔNLS or TDP-43 WT (t1/2; HA-TDP WT, 32.5 h; HA-TDP ΔNLS, 24.4 h; HA-TDP CTF, 11 h). B. Degradation of HA-TDP-43 proteins in stable SH-SY5Y cells over a shorter time course, and allowing a delay after washout for clearance of mRNA transcripts. HA-TDP protein degradation rates closely reflected those in HEK293 cells (t1/2; HA-TDP WT, 29.2 h; HA-TDP ΔNLS, 16.6 h; HA-TDP CTF, 10.2 h). C. Degradation of HA-TDP-43 mRNA transcripts in stable SH-SY5Y cells assessed by quantitative reverse transcriptase PCR. Transcripts for all three constructs decayed rapidly after DOX washout and had reached minima by 8 h (representative experiment shown, n=2). A-C. Arrowheads on schematics show the time points at which samples were taken.

**Figure 3. Involvement of the UPS and autophagy in the degradation of TDP-43.**

A. Relative HA-TDP-43 remaining in stable HEK293 cells at 72 h post-DOX washout with inhibitors of autophagy or the UPS included during the last 48 h. Veh, vehicle; 3MA, 3-methyl adenine; Baf, bafilomycin; MG, MG132; Epo, epoxomicin. The -DOX control lane represents the amount of HA-TDP-43 present at the 72 h washout point without expression having been induced (“leakage”). All lanes for each constructs are from the same blot spliced as shown. B. Western blot of LC3 levels after short treatments with the autophagy inhibitors 3MA and bafilomycin reveal LC3-I and II accumulation with 3MA, and LC3-II accumulation with bafilomycin. C. Relative HA-TDP-43 remaining in stable HEK293 cells subject to DOX pulse-chase with activators of autophagy included during the last 24 h of the 48 h chase
period. Veh, vehicle; Rapa, rapamycin; Tre, trehalose; LiCl, lithium chloride. Bars represent means ± SEM. Asterisks represent significant differences between vehicle and inhibitor-treated cells (Two-way ANOVA, Bonferroni post-test).

**Figure 4. UPS, but not autophagy inhibition, induces the formation of detergent-resistant cytoplasmic aggregates of TDP-43.**

A. Immunocytochemical analysis of stable SH-SY5Y lines induced to express HA-TDP-43 constructs by 48 h induction with DOX in the presence of various inhibitors of the UPS or autophagy. When used alone, UPS inhibitors but not autophagy inhibitors induced the formation of ubiquitin (Ub)-positive TDP-43 macroaggregates (arrow heads). Scale bar= 10 µm. B. Immunoprecipitation of HA-TDP-43 WT from HEK293 stable lines transfected with FLAG-Ub, then induced with 1 µg/mL DOX alone or together with 3MA or MG132 for 24 h. Under non-denaturing conditions which preserve complexes (left) HA-TDP WT co-immunoprecipitated with an increased amount of FLAG-Ub (smear >100 kDa) under treatment with 3MA, and a greater amount again with MG132. Immunoprecipitation of denatured lysate (right) showed that HA-TDP-43 which was directly and covalently linked to FLAG-Ub only accumulated following MG132 treatment. C. TDP-43 aggregates are non-amyloid. Stable SH-SY5Y lines were induced to express HA-TDP-43 constructs by 48 h induction with DOX in the absence or presence of MG132. In MG132-treated cells, thioflavin T staining detected amyloid aggregates which were independent of macroaggregates of HA-TDP-43 (dashed lines). MG132 treatment of parent SH-SY5Y cells induced macroaggregates (dashed line) with low levels of endogenous (Endo) TDP-43, likely due to autoregulation of TDP-43 protein levels. Scale bar= 10 µm. D,E. Four step fractionation of stable SH-SY5Y lines induced to express HA-TDP-43 constructs by 48 h induction with DOX. WT, upper open arrow; ΔNLS, middle closed arrow; CTF, lower closed arrow. The CTF was largely soluble in the low salt (LS) fraction (TX, triton X-100; Sark, sarkosyl). Note that pellets from each step were resuspended in different volumes to ensure detection of all fractions. Relative concentrations of fractions are: Lys 1, LS 1, TX 0.5, Sark 3.33, Urea 2.5. Bars represent means ± SEM. F,G. Solubility fractionation of the cells treated as in (A), showing lysate (L), RIPA-soluble (R) and urea-soluble (U) fractions of HA-TDP-43 WT (F) and ΔNLS (G). Urea fractions are concentrated 10-fold. Combined UPS and autophagy inhibitor treatment (同时也 increased the amount of insoluble high molecular weight
(HMW) TDP-43, particularly for TDP-43 ΔNLS (G). Veh, vehicle; 3MA, 3-methyl adenine; Baf, bafilomycin; MG, MG132; Epox, epoxomicin.

Figure 5. TDP-43 aggregates induced by UPS- or dual UPS/autophagy-inhibition resemble those seen in human disease.

A. Immunocytochemical analysis of untreated SH-SY5Y cells shows diffuse localisation of degradation pathway proteins ubiquilin 1/2 (UBQLN) and p62, and K48- and K63-linked forms of ubiquitin (Ub). Scale bar= 25 µm. B-E. Immunocytochemical analysis of stable SH-SY5Y lines induced to express HA-TDP-43 by 48 h induction with DOX in the presence of UPS or combined UPS/autophagy inhibition. B. HA-TDP WT formed cytoplasmic macroaggregates (arrowheads) following treatment with DOX plus the proteasome inhibitor MG132 in stable SH-SY5Y cells. These aggregates were positive for post-translational modifications (PTMs) including K48- and K63-linked ubiquitin, ubiquilin 1/2 and p62. C. Combined treatment with DOX plus MG132 plus the autophagy inhibitor 3MA enhanced recruitment of HA-TDP WT to macroaggregates, which were positive for the same markers as with MG132 treatment alone. D. HA-TDP ΔNLS also formed cytoplasmic aggregates following 48 h treatment with DOX plus MG132 in stable SH-SY5Y cells. Like HA-TDP WT, these aggregates were positive for K48- and K63-linked ubiquitin, ubiquilin 1/2 and p62. D. Combined treatment with DOX plus MG132 plus 3MA did not markedly alter recruitment of HA-TDP ΔNLS to macroaggregates. Scale bar= 10 µm.

Figure 6. TDP-43 macroaggregates are cleared upon the restoration of UPS function.

Immunocytochemical analysis of stable SH-SY5Y lines induced to form HA-TDP-43 WT (A) or HA-TDP-43 ΔNLS (B) aggregates by 48 h induction with DOX plus MG132 then fixed, or MG132 washed out and cells left for a further 48 h. Scale bar= 10 µm. C,D. Solubility fractionation of stable HA-TDP-43 WT (C) or HA-TDP-43 ΔNLS (D) SH-SY5Y lines, either induced with DOX; induced to form aggregates by 48 h treatment with DOX plus MG132; or induced to form aggregates, then MG132 washed out and cells left for a further 48 h. Lysate (L), RIPA-soluble (R) and urea-soluble (U) fractions. Urea fractions are concentrated 10-fold. MG132 increased the proportion of high molecular weight insoluble TDP-43 (HMW) and this was reversible upon MG132 washout (48 h). E. Validation that EGFP-tagged TDP-43 WT is degraded over the same time course as HA-TDP-43 WT in stable SH-SY5Y cells (t1/2; HA-TDP WT, 29.2 h; EGFP-TDP WT, 29.3 h). F. Validation that
both HA- and EGFP-tagged TDP-43 WT (gray arrows) are functional and can autoregulate the level of endogenous TDP-43 (black arrow) in stable SH-SY5Y cells. Black graph bars represent endogenous TDP-43 and gray bars represent exogenous TDP-43. G. Live cell imaging of stable SH-SY5Y lines induced to form EGFP-TDP-43 ΔNLS aggregates by 48 h induction with DOX plus MG132 then washed out and followed for a further 15 h. In cells which cleared aggregates, aggregates fragmented before clearance. Scale bar= 10 µm.

**Figure 7. TDP-43 aggregate clearance is due to enhanced mobility of lower order aggregated species, rather than mobility of macroaggregated TDP-43.**

A. Confocal images acquired during fluorescence recovery after photobleaching (FRAP). EGFP-TDP-43-ΔNLS showed rapid FRAP recovery for diffuse cytosolic forms (Cyt TDP), but no recovery for aggregated forms (Agg TDP), indicating that macroaggregated TDP-43 is immobile and unlikely to readily exchange with the diffuse pool. Scale bar= 10 µm. B. FRAP recovery curves, with bleach at t=0, and 100% representing pre-bleach fluorescence intensity. The fraction of cytosolic TDP-43 which was mobile was slightly decreased in cells treated with MG132 but devoid of aggregates (Cyt TDP -Agg), and greatly decreased in cells containing an aggregate (Cyt TDP +Agg). C. Fraction of TDP-43 which was mobile under various conditions. The decrease in mobile fraction in cells treated with MG132 and containing an aggregate (Cyt TDP +Agg) was rescued after 8-10 h washout (wash). There was no mobilisation of macroaggregated TDP-43 upon washout. D. The rate of FRAP recovery (given as K=ln(2)/t_{1/2}) for mobile species under various conditions. FRAP rate was significantly decreased in cells treated with MG132 and containing an aggregate (Cyt TDP +Agg), which was reversed after washout. Bars represent means ± SEM. Asterisks represent significant differences between control and treated cells, or as shown (One way ANOVA, Bonferroni post-test).

**Figure 8. TDP-43 aggregate clearance requires autophagy.**

A. Autophagosomes in cells containing TDP-43 aggregates. Stable SH-SY5Y lines were transfected with mCherry-GFP-LC3, then induced to form HA-TDP-43 WT or ΔNLS aggregates by 48 h induction with DOX plus MG132. B. Quantification of GFP-LC3 autophagosomes per cell in cells treated as per A. Bars represent means ± SEM. Asterisks represent significant differences from control (Unpaired t-tests with Welch’s correction). C. Live cell imaging of stable SH-SY5Y lines induced to form EGFP-TDP-43 ΔNLS aggregates.
by 48 h induction with DOX plus MG132 then washed out and followed for a further 15 h in
the presence of bafilomycin (Baf). Aggregates fragmented but were not cleared. D.
Regression analysis of the relationship between aggregate load (integrated intensity of
aggregate) and clearance time in the presence or absence of autophagy inhibitors during
washout (wo)(Veh, vehicle; 3MA, 3-methyl adenine; Baf, bafilomycin; Pearson’s r=0.72
(Veh wo), 0.70 (3MA wo), 0.23 (Baf wo)). Small aggregates were cleared rapidly in the
absence but not presence of autophagy inhibitors. In Baf wo cells a distinct population of
aggregates which were not cleared by the end of imaging (scored as 15 h) can be seen
(Dashed line). E. Relative rates of clearance of EGFP-TDP-43 ΔNLS aggregates according to
the slopes of the lines shown in D. Note that the slope for Baf wo includes the “non-clearing”
population. Non-significant by one way ANOVA.

Figure 9. Working model for differential degradation of TDP-43 species.
Upper panel. Working model for TDP-43 accumulation. Under normal conditions, TDP-43
exists as several different species with variable appearance (diffuse/ macroaggregated),
solubility, and mobility (fast/ slow). When the UPS is functional, removal of soluble
monomer drives the equilibrium in favour of small soluble species, and precludes the
formation of macroaggregates. Conversely, UPS blockade drives the accumulation of
oligomers and insoluble species, where insoluble species include “microaggregated” and
macroaggregated TDP-43; microaggregates appearing visually diffuse but immobile by
FRAP, and macroaggregates visible by immunofluorescence and immobile. Lower panel.
Working model for TDP-43 clearance after macroaggregate formation. Under normal
conditions macroaggregates are cleared via the induction of autophagy, which removes
oligomeric and microaggregated species, monomer is cleared by the UPS. Autophagy
blockade prevents the removal of oligomer and microaggregates, although fragmentation of
macroaggregates still occurs.

Movie 1. TDP-43 macroaggregate clearance.
Live cell imaging of stable SH-SY5Y lines induced to form EGFP-TDP-43 ΔNLS aggregates
by 48 h induction with DOX plus MG132 then washed out and followed for a further 15 in
the presence of vehicle. Large aggregates fragmented and were cleared. Each frame of the
video represents 1 h of imaging, total 15 h.
Movie 2. TDP-43 macroaggregate clearance requires autophagy.

Live cell imaging of stable SH-SY5Y lines induced to form EGFP-TDP-43 ΔNLS aggregates by 48 h induction with DOX plus MG132 then washed out and followed for a further 15 in the presence of bafilomycin. Large aggregates fragmented but fragments persisted under autophagy inhibition. Each frame of the video represents 1 h of imaging, total 15 h.
E

HA-TDP WT

MG+DOX 48 h washout

HA-TDP ΔNLS

MG+DOX 48 h washout

C

HA-TDP WT

DOX MG132 48 h w/o

+ DOX w/o

L R U L R U L R U

D

HA-TDP ΔNLS

DOX MG132 48 h w/o

+ DOX w/o

L R U L R U L R U

F

Untransfected
HA-TDP
EGFP-TDP

DOX

HA/ GFP

GAPDH

Relative TDP-43/GAPDH

UT HA-TDP EGFP-TDP

G

EGFP-TDP ΔNLS MG+ DOX then washout

4h 5h 6h 7h

8h 9h 10h 11h

12h 13h 14h 15h