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Addition of exogenous SOD1 aggregates causes TDP-43 mislocalisation and aggregation

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Addition of exogenous SOD1 aggregates causes TDP-43 mislocalisation and aggregation

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Abstract
ALS is characterised by a focal onset of motor neuron loss, followed by contiguous outward spreading of pathology throughout the nervous system, resulting in paralysis and death generally within a few years after diagnosis. The aberrant release and uptake of toxic proteins including SOD1 and TDP-43 and their subsequent propagation, accumulation and deposition in motor neurons may explain such a pattern of pathology. Previous work has suggested that internalization of aggregates triggers stress granule formation. Given the close association of stress granules and TDP-43 we wondered whether internalisation of SOD1 aggregates stimulated TDP-43 cytosolic aggregate structures. Addition of recombinant mutant G93A SOD1 aggregates to NSC-34 cells was found to trigger a rapid shift of TDP-43 to the cytoplasm where it was still accumulated after 48 hours. In addition, SOD1 aggregates also triggered cleavage of TDP-43 in to fragments including a 25 kDa fragment. Collectively, this study suggests a role for protein aggregate uptake in TDP-43 pathology.

Introduction

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disorder characterized by the loss of both the upper and lower motor neurons in the brain and spinal cord respectively, resulting in the progressive paralysis of the muscles of speech, limbs, swallowing and respiration, due to the progressive degeneration of innervating motor neurons [1]. The neuropathology of all cases of ALS are characterised by disease-specific proteins, mutant and wild type alike; mislocalised and abnormally accumulated as misfolded, insoluble aggregates in the cytoplasm of afflicted motor neurons [2-4]. However, aggregation in ALS is not restricted to disease-specific proteins as more than 70 proteins, which have in common the biophysical property of being supersaturated, can be found in ALS deposits suggesting a collapse of protein homeostasis [5]. Proteinaceous inclusions, containing misfolded aggregated proteins, peptides and fragments, also occur in many other neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FTD), Huntington's disease (HD) [6]; and prion diseases, such as Creutzfeldt-Jakob disease, Kuru and fatal familial insomnia diseases [7, 8]. How misfolded proteins cause cellular dysfunction is unknown, one hypothesis is that exposed hydrophobic surfaces interact inappropriately with cellular components [9].

Mutations in several genes cause familial ALS (fALS) which account for 5-10% of total ALS cases and contribute to the development of sporadic ALS (sALS, 90% of ALS cases) [10]. In fALS, mutations in the gene encoding the Cu/Zn superoxide dismutase (SOD1), a ubiquitously-expressed homodimeric enzyme, results in the monomerisation [11, 12], misfolding and aggregation of this normally stable protein [13]. However, recently misfolded SOD1 species have been increasingly identified in non-SOD1 fALS and sALS cases [3, 14], suggesting that misfolded SOD1 may play a pathological role in all types of ALS [15].

In addition to SOD1, TAR DNA binding protein (TDP-43) has been identified as a major component of cytoplasmic inclusions in sALS, SOD1-negative fALS and ALS with dementia; as well as most common pathological subtype of frontotemporal dementia (FTD) with ubiquitinated inclusions [16-21]. However, aggregate formation pathways and final structures are likely different between SOD1 and TDP-43 [22]. Clinical and pathological overlap between forms of ALS and FTD has raised the possibility that there is a pathogenic mechanistic-link between these disorders and has prompted their reclassification as TDP-43-proteinopathies. The shared pathology includes neuronal cytoplasmic inclusions (NCIs), loss of the normal nuclear TDP-43, ubiquitination and hyperphosphorylation of TDP-43 and lastly formation of abnormal fragments of TDP-43 in post-mortem tissue [21, 23, 24]. However, TDP-43 is also found in inclusions in Machado-Joseph disease [25], spinocerebellar ataxia [26], Huntington's disease [27], Alzheimer's disease [28], inclusion body myositis [29] and Parkinson's disease [30], suggesting TDP-43 accumulation is not ALS or FTD specific. Of interest, AD associated Aβ has been implicated in triggering the

phosphorylation and cytosolic accumulation of pathogenic TDP-43 in rodent models and in brain autopsies from AD patients [31]. This may explain the presence of TDP-43 pathology in a proportion of AD cases [32]. Interestingly, the process of internalization of tau aggregates triggers stress granule formation and accumulation [33], and given that TDP-43 is associated with stress granule formation this may explain the accumulation of TDP-43 with other neurodegenerative diseases where aggregate propagation is involved in pathology.

In the current study, we examined whether exogenous recombinant SOD1 protein aggregates can induce and/or contribute to TDP-43 pathology, specifically its mislocalisation and aggregation. Upon incubation with large recombinantly formed SOD1 aggregates with NSC-34 cells, WT TDP-43 was found mislocalised to the cytoplasm of both mouse neuronal-like cells and human embryonic kidney cells. We also demonstrate that, upon addition of the SOD1 aggregates, fragments of TDP-43 can be observed in the cytoplasm of NSC-34 cells. Thus, we conclude that addition of recombinant SOD1 aggregates to the extracellular environment of neuron like cells in culture results in TDP-43 mislocalisation, aggregation and fragmentation.

Materials and Methods

Cell Lines

The mouse neuroblastoma x spinal cord hybrid cell line (NSC34 cells) [34] were routinely cultured in DMEM/F-12 supplemented with 10% (v/v) FBS and 2 mM GlutaMAX. Cells were maintained in an incubator at 37°C under a humidified atmosphere containing 5% (v/v) CO_2. Where pCMV6-AC-GFP expression vector containing WT TDP43 cDNA was obtained from Origene. pCMV6-AC-tGFP expression vector containing $TDP43^{WT}$ cDNA was obtained from Origene. TDP-tomato red (TdTomato) constructs were created by replacing the EGFP sequences in the SOD1-tGFP plasmids with tdTomato (by Genscript, USA). Cells were then incubated in DMEM-F-12 serum-free culture medium containing 2 μg WT TDP tomato red (TR) plasmid DNA and Lipofectamine 2000 for 5 h at 37°C under a humidified atmosphere containing 5% (v/v) CO_2. Cells were then washed once with serum free media and replenished with complete culture medium. Cells were then incubated for a longer period of time either for 19 h, 43 h or 67 h (24 h, 48 h, and 72 h in total, respectively).

Aggregation of WT and mutant G93A SOD1

WT and G93A SOD1 were expressed and purified from *E.coli* as previously outlined [35, 36]. SOD1 aggregation was performed *in vitro* as previously described [35]. Briefly, solutions of purified WT or G93A mutant SOD1 protein (1 mg/mL) in PBS were co-incubated with 20 mM dithiothreitol (DTT) and 5 mM ethylenediaminetetraacetic acid (EDTA) for 72 h at 37°C with shaking using a digital shaker (universal IKA® MS 3, 230 V) (Sigma, St. Louis, MO).

Treatment of transfected cells with SOD1

Cells were visualised prior to experimentation using an Eclipse TE2000 inverted microscope (Nikon, Tokyo, Japan) to confirm transfection and determine transfection efficiency. NSC34 cells were incubated with labelled biotinylated aggregates, or soluble (non-aggregated) WT and mutant G93A SOD1 proteins (20 μg/mL) or no protein as a control at 37°C/CO_2, for either 2 h or 72 h. Post incubation, cells were analysed either by confocal microscopy or FloIT. In the case where cells were analysed by confocal microscopy inclusions were counted manually and expressed as a proportion of transfected cells. To account for toxicity of the addition of aggregates the proportion of cells containing cytoplasmic $TDP\text{-}43^{WT}$ tdtomato was normalised to the amount of cell loss over 72 hours. To assess the toxicity of SOD1 to cells expressing $TDP\text{-}43^{WT}$ tdtomato constructs in NSC-34 cells over a time course, an IncuCyte® automated fluorescent microscope (Essen BioScience, USA) was used as previously described [37]. Briefly, NSC-34 cells were plated into 12-wells plates at a

confluency of 60% and were transfected 24 h post-plating. Cells were dissociated 24 h post-transfection and plated into 96-well plates at a confluency of 20% in phenol-red-free DMEM-F12 supplemented with 10% FBS. At least 3 images were acquired per-well at 2 h intervals for 72 h in both phase and red channels. The number of TDP-43^{WT} tdtomato positive cells at each time point for each transfection was normalized to the intial value determined in the first scan after plating. Then, the normalized values of the SOD1 treatments at each time point were divided by the normalized TDP-43^{WT} tdtomato alone data at the same time points to determine the relative cell viability. The proportion of cells containing cytoplasmic TDP-43^{WT} tdtomato for each treatment was then normalised to the relative viability at 72 h.

FloIT assay

The FloIT assay was performed as in previously published work [38]. Briefly, NSC-34 cells were transiently transfected with TDP-43 (TDP-43^{WT} tdtomato and TDP-43^{G124A}-EGFP). Following transient transfection at 24h, 48 h and 72h in total, cells were harvested and washed using centrifugation. An aliquot of cells (2 x 10^5cells/0.15mL) were collected then analysed for transfection efficiency. The remaining cells (4 x 10^5cells/0.35mL) were washed as above and lysed prior to analysis in lysis buffer. Cell lysates were then incubated with RedDot2 (1:1000) at RT for 2 min. Events were collected using a LSRFortessa X-20 Cell Analyzer (BD Biosciences) (excitation 488 nm, emission collected with 525/50 band-pass filter and excitation 561, emission collected with 586/15 nm band-pass filter for EGFP and RedDot2, respectively). Lysates were firstly gated on forward and side scatter and then the fluorescence from RedDot2 were determined by flow cytometry. All parameters were set to $\log^{10}$ during acquisition from cell lysates. The forward scatter threshold was set to the minimum value (200 AU) to minimise the exclusion of small protein inclusions. Nuclei were identified and enumerated based on RedDot2 fluorescence and forward scatter and then excluded from further analysis. The remaining particles were analysed for the presence of inclusions based on GFP/ tdTomato fluorescence, forward scatter and comparison lysates prepared from cells expressing only the corresponding fluorescent protein. The number of inclusions in the population can be normalised to the number of nuclei, and reported as inclusions/100 transfected cells (iFloIT) according to the equation outlined in [38]. Analysis of all events was determined using FlowJo software (Tree Star, Ashland, OR).

Subcellular Fractionation assay of NSC34 cells

NSC34 cells were incubated with WT SOD1 in a soluble or aggregated form (20 μg/mL) in PBS for 2 h at 37°C/5% CO_2. Post incubation, the cells were washed three times with PBS (300 *x g* for 5 min) harvested using 0.5% trypsin and 5 mM EDTA and washed (500 *x g* for 5 minutes). The cells were washed three times with ice cold

PBS (500 x *g* for 3 minutes) for fractionation using a Subcellular Protein Fractionation Kit for Cultured Cells as per manufacturer's instructions (Thermo Fisher Scientific).

Protein concentration was determined using the BCA method. Supernatants (20 µg protein/lane) were separated under reducing conditions (5% β-mercaptoethanol) using Any kD Mini-PROTEAN TGX Stain-Free™ Precast Gels. Proteins were then transferred to nitrocellulose membranes using a Trans-Blot Turbo Transfer System (Bio-Rad). Total protein per lane was then imaged and measured with a Bio-Rad Criterion Stain Free Imager and Image Lab software. Membranes were blocked with heat denatured casein (HDC) for 1 h at 37°C. To test the purity of the separation, rabbit anti-EEA1 pAb (1:500), rabbit anti-Vimentin pAb (1:500) and mouse anti-actin mAb (1:5000), diluted in HDC/PBS for 1 h at 37°C were used to probe the ME, NE and PE fractions. Membranes were visualised using chemiluminescent substrate and Amersham Hyperfilm ECL (GE Healthcare). Images of films were collected using a GS-800 Calibrated Densitometer (Bio-Rad). The processing of films was achieved using GBX Developer and Replenisher and GBX Fixer and Replenisher (Kodak Australiasia, Collingwood, Victoria Australia). Images of the films were collected using a GS-800 Calibrated Densitometer (Bio-Rad).

Presentation of data and statistical analyses

Data is presented as the mean ± SD. ANOVA paired with Tukey's HSD multiple comparison post-test tests were used to analyse and compare differences between multiple treatments. Unpaired student's t-tests were performed for single treatment comparisons. Prism 5 for Windows (Version 5.01) (GraphPad Software, San Diego, CA) was used to generate these statistical analyses. Differences were defined as significant for $P < 0.05$.

Results

Transiently expressed TDP-43^{WT} –tdTomato is observed in the cytosol of NSC-34 cells upon treatment with recombinant SOD1 protein aggregates

TDP-43 redistribution and aggregation is observed in the cytoplasm of affected cells in a diverse set of neurodegenerative diseases. However, the factors responsible for TDP-43 mislocalisation and aggregation remain ambiguous. Both SOD1 and TDP-43 have been implicated in fALS and sALS pathogenesis, although whether SOD1 misfolding or aggregation contribute to TDP-43 pathology is yet to be established. Given Aβ aggregates can affect TDP-43 deposition [31], and uptake of tau aggregates causes stress granule formation [33], we tested whether exogenously added SOD1 aggregates have an effect on TDP-43 cytosolic mislocalisation and aggregation. Initially we followed cells transiently expressing WT TDP-43-tomato red (TDP-43^{WT} tr) in live cells. Post transfection, cells were incubated with either WT or mutant G93A SOD1 in both their aggregated and soluble state (**Fig. 1A**). The percentage of

cells containing cytosolic TDP-43 in foci that measured >1 μm per (this definition would include stress granules and inclusions) was determined by confocal microscopy. Cytosolic TDP-43 by this definition did not distinguish between cells where TDP-43^{WT} was cleared from the nucleus from TDP-43 that had accumulated in the cytosol even though some nuclear TDP-43^{WT} remained (**Fig. 1B**). Examples of TDP-43 morphology scored in this work are given in **Fig. 1B**. This included a granular appearance, large inclusions, with or without clearance of TDP-43 from the nucleus.

The addition of SOD1 protein aggregates to NSC-34 cells resulted in a rapid and significant increase in percentage of cells that contained cytosolic TDP-43^{WT} when treated with SOD1 aggregates (15.6±2%) compared to the controls (absence of protein treatment; **Fig. 2A**). However, there was also a significant increase in cells displaying mislocalised TDP-43^{WT} when incubated with soluble G93A SOD1 (10.4±2%) and soluble WT SOD1 (8.7±2%). Furthermore, little to no cells were observed to contain TDP-43^{WT} positive cytoplasmic inclusions without protein treatment after the 2 h incubation period. While cells incubated with aggregated SOD1 aggregates had more TDP-43^{WT} inclusions than those incubated with soluble G93A, no significant differences were detected between them. In contrast, cells treated with SOD1 aggregates had significantly more TDP-43^{WT} aggregates when compared to cells treated with soluble WT SOD1 (**Fig. 2A**).

Given TDP-43 translocation can be a rapid response to stress [39] that can be reversible [40], we next tested whether the TDP-43^{WT} mislocalisation associated with SOD1 aggregate treatment persisted for 72 h. After the extended incubation with SOD1 aggregates, a large number of cells still exhibited cytosolic TDP-43^{WT}, which appeared as larger clusters of aggregates or inclusion bodies, when assessed by confocal microscopy (**Fig. 2B**).

Incubation of aggregated SOD1 is resulted in a significantly higher percentage of transfected cells with miscolcalised TDP-43^{WT} compared to the control cells (**Fig. 2B**). In contrast to the 2 h experiment, there was no significant increase in cells displaying mislocalised TDP-43^{WT} when incubated with soluble G93A SOD1 and soluble WT SOD1. However, it is important to note that complete loss of normal nuclear staining was still not observed and that addition of SOD1 aggregates leads to a variety of aggregation states (examples shown in **Fig. 1B**).

Given that the above data relied on manual counting which can be biased by the images taken and used for counting, an unbiased and novel flow cytometric (FloIT) method was next employed to quantify TDP-43 aggregation after addition of SOD1 aggregates. Cells were transiently transfected with TDP-43^{WT}-tdTomato and incubated with human recombinant G93A mutant SOD1 aggregates for either 24, 48 or 72 h (**Fig. 2C**). At each time point, a significant increase in the number of TDP-43^{WT} inclusions were identified in cells incubated in the presence of mutant SOD1

aggregates compared to the corresponding cells in the absence of mutant SOD1 aggregates (**Fig. 2C**).

Exogenous recombinant SOD1 aggregates induce the cytosolic mislocalisation and fragmentation of endogenous mouse TDP-43 in naive NSC-34 cells.

Previous work suggests that the accumulation of the truncated TDP-43 fragment CTF25 is a pathological feature of TDP-43 proteinopathies [21]. Since this fragment has been suggested to play a critical role in ALS pathogenesis, we investigated whether adding aggregated SOD1 to NSC-34 cells had any effect on the formation of CTF25. We have previously shown that exogenously added recombinant SOD1 is capable of entering into NSC-34 cells [41], escaping into the cytosol [42] and causes ER stress [43]. To begin to investigate the effect of SOD1 aggregates on endogenous TDP-43 location and truncation, a subcellular fractionation assay and western blot was carried out on NSC-34 cell lysates (**Fig. 3**).

TDP-43 location was then investigated by immunoblotting of the cytosolic, membrane (ER/Golgi), nuclear and cytoskeletal supernatant fractions (**Fig. 3**). In the SOD1 aggregate-treated fractions (**Fig. 3A**), TDP-43 was detected as a range of bands but predominantly as distinct bands at 43 kDa and ~25 kDa in all fractions to some degree. In contrast, in untreated controls and cells treated with soluble SOD1, TDP-43 was found predominantly in the nuclear fraction (**Fig. 3B,C**). In samples treated with aggregates, there were also bands found in the membrane fraction at approximately 40 kDa and 18 kDa. The additional 40 kDa band was also detected in the nuclear (N) and cytoskeletal (P) fractions at much lower levels. Interestingly, after treatment with SOD1 aggregates the full length mouse TDP-43 was predominantly cytosolic and associated with the membrane fraction suggesting a rapid movement from the nucleus after SOD1 aggregate addition. This was in contrast to the work presented above which shows that only a proportion of TDP-43^{WT}-tdTomato becomes cytosolic after SOD1 incubation. This is likely to be due to the fact that we are overexpressing human TDP-43 in that system. To further confirm the purity of the fractions, control immunoblotting using an anti-actin, anti-EEA1 and anti-vimentin antibody was performed (**Fig. 3D**). These bands were detected in the membrane fraction for EEA1 (180 kDa), and cytoskeleton fraction for vimentin (54 kDa), while actin (42 kDa) acted as a loading control and could be found in all fractions.

Discussion

Mutations in the TDP-43 gene are found in sporadic and familial ALS, implicating TDP-43 as a contributing factor to disease [44, 45]. TDP-43 has previously been reported to spontaneously form aggregates that resemble TDP-43 deposits in degenerating neurons in ALS FTD-U patients [46]. Furthermore, previous studies

have reported that in the TDP-43 protein sequence, the C-terminal domain is critical for spontaneous aggregation and that TDP-43 is intrinsically aggregation prone [46].

Consistent with misfolded SOD1 detected in sALS, previous work has demonstrated that WT SOD1 misfolding can be propagated cell to cell [41], at least partly due to protein aggregate uptake via macropinocytosis, subsequent stress and protein aggregation [42, 43, 47]. Further, internalization of tau aggregates was found to stimulate stress granule formation and accumulation [33]. Here we have shown that exogenously added large SOD1 protein aggregates are capable of inducing the cytoplasmic mislocalisation and accumulation of TDP-43 in the motor neuron-like cell line (NSC- 34). Furthermore, the present study demonstrates that the addition of large SOD1 aggregates induces rapid fragmentation of TDP-43 in the cytoplasmic fraction of NSC-34 cells. Collectively, this suggests that exogenous protein aggregates are capable of stimulating TDP-43 pathology through an unknown mechanism(s), resulting in their redistribution, fragmentation and aggregation, similar to what has been observed in most cases of ALS [15, 23, 48, 49].

An association between misfolded and/or aggregated SOD1 and TDP-43 mislocalisation has been previously suggested. For example, a study has reported that TDP-43 cytoplasmic mislocalisation and deposition into ubiquitin immunoreactive inclusions were observed in lower motor neurons of end stage mutant SOD1 transgenic mice [50]. In addition to this, misfolded human WT SOD1 has been observed in association with a cytosolic accumulation of mutant TDP-43 in TDP-43-fALS and WT TDP-43 in sALS [15]. It has been suggested that mutant TDP-43 may indirectly induce the propagation of WT SOD1 misfolding in fALS and sALS [15]. Given that the aggregation of TDP-43 and SOD1 appear to be distinct processes with structurally and morphologically discrete aggregates [22], it is unlikely that one will seed aggregation of the other in a conventional manner and therefore must be explained by an alternate mechanism.

Cytoplasmic accumulation of WT TDP-43 as a local response to injury or cell stress has been previously described in sALS [40]. In addition to this, misfolded and aggregated SOD1 can induce ER stress and dysfunction and stress granule formation in cell culture [51], rodent ALS models at symptom onset and disease end stage and human ALS patients [52-56]. There is evidence that the uptake of extracellular misfolded WT and mutant G93A SOD1 into human and mouse neuronal cells causes disruptions to protein transport between the ER and Golgi apparatus, resulting in ER stress, Golgi fragmentation, and subsequent apoptotic cell death [43]. ER stress is activated when proteins accumulate within the ER lumen, thus triggering the unfolded protein response (UPR), which in turn may lead to cellular apoptosis if unresolved [57]. There is a strong link between ALS pathology and ER stress *[58]* and it has been proposed that prolonged ER stress leads to motor neuron death in ALS *[58]*. While generally the unfolded protein response activated by ER stress is a protective response able to rescue cells from proteotoxicity, in the ALS context this can lead to

further aggregation of ALS associated proteins such as TDP-43 *[59]*. Taken together, these data are consistent with the idea that ER stress caused by uptake and maintenance of SOD1 aggregates may trigger TDP-43 mislocalisation and accumulation. In the case that uptake of protein aggregates into neurons causes TDP-43 mislocalisation and inclusion formation, it may be possible to inhibit this process by suppressing the uptake of aggregates. This may be possible by utilising molecular chaperones or manipulating macropinocytosis pathways.

TDP-43 mislocalisation is a common response to a variety of stressors including oxidative stress [60], heat shock stress [61], and proteosomal stress [62]. Indeed extranuclear accumulation of WT TDP43 has been shown to be a common pathology in sALS, FTD and Alzheimer's disease, and therefore may be a general consequence of cellular stress [32]. Interestingly, exogenously applied Aβ amyloid aggregates associated with Alzheimer's disease have also been shown to induce TDP-43 mislocalisation [31] suggesting that uptake of any aggregate regardless of which protein they are made from might be enough to trigger TDP-43 accumulation. This widespread predisposition of various types of neurons to undergo cytosolic accumulation of TDP-43 may be a consequence of the generally low protesostasis capacity of neurons [63].

Conclusion

In conclusion, the work presented here presents findings to suggest that aggregates made from SOD1 are able to induce TDP-43 mislocalisation and rapid fragmentation consistent with TDP pathology observed in sporadic disease. However, the exact mechanism(s) for this observation were not determined. It is likely that the stress induced by aggregate uptake was enough to trigger the mislocalisation of TDP-43. However, it should be noted that a high concentration (20 μg/ml) of large SOD1 protein aggregates were used in the current study to induce TDP-43 pathology in cell lines. This concentration is higher than that used in a number of similar studies [64, 65]. Future investigations into the mechanism(s)/role of SOD1 aggregates on the formation of pathological TDP43 are therefore warranted.

Author Contributions

R. Zeineddine performed experiments, interpreted and analysed data, as well as wrote the initial manuscript. N.E. Farrawell and I. A. Lambert-Smith performed experiments and analysed data. J.J. Yerbury designed experiments, interpreted and analysed data, and wrote and edited the manuscript.

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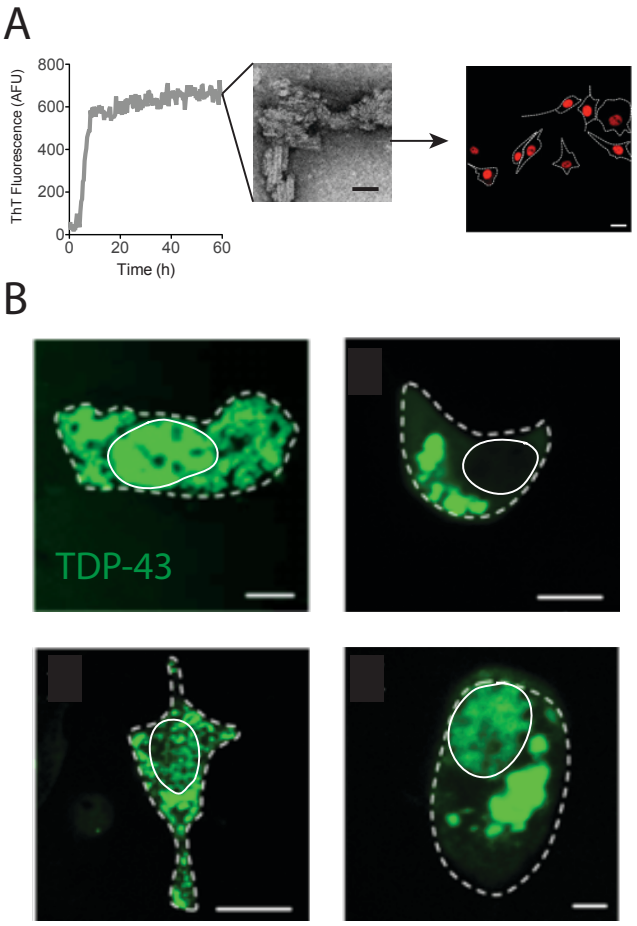

Figure 1. Treatment of NSC-34 cells with large SOD1 protein aggregates. (A) Schematic diagram of experimental outline; recombinant human SOD1 was aggregated (followed by ThT fluorescence), or not, and then added to NSC-34 cells expressing TDP-43^{WT}-TR. TEM bar represents 50 nm. (B) TDP-43 mislocalisation was initially determined by manually counting fluorescent foci larger than 1 μm; various examples of TDP-43 structures scored as mislocalised are shown here. Bars represent 10 μm.

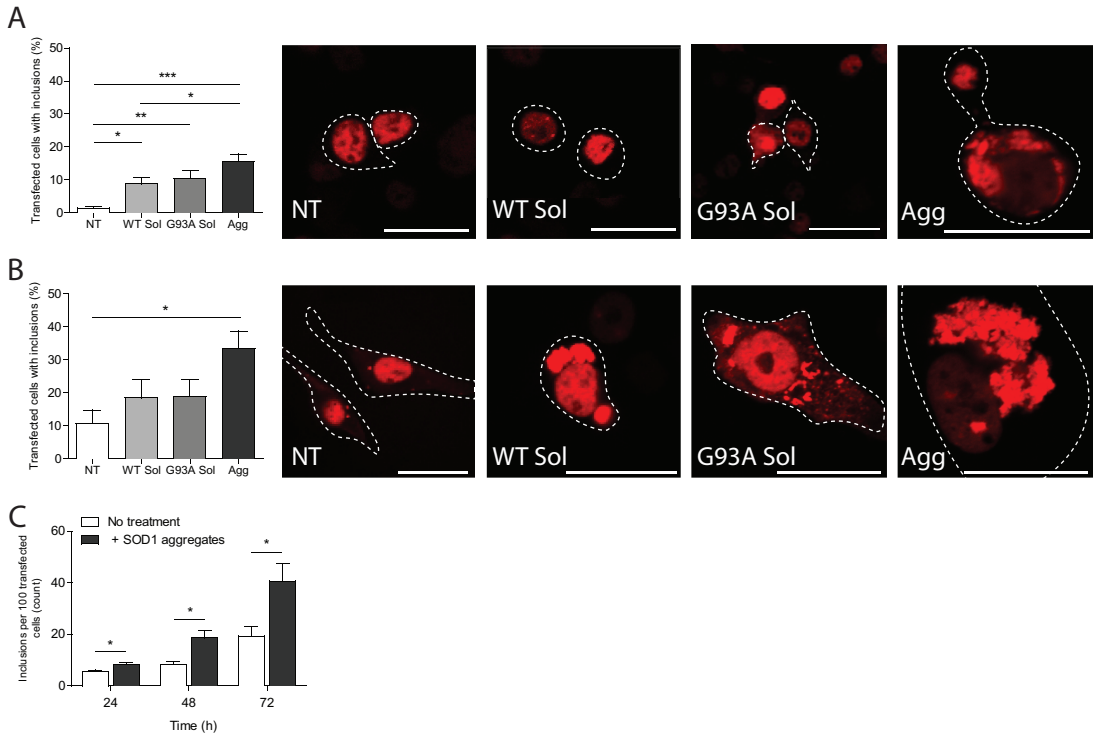

Figure 2. Exogenous recombinant SOD1 aggregates induce TDP43 mislocalisation and aggregation. The percentage of NSC34 cells containing TDP43^{WT} positive aggregates was assessed by the number of cells containing mislocalised TDP43 into foci that measured >1µm per treatment including both TDP43^{WT} cleared from the nucleus and TDP43^{WT} that had accumulated in the cytosol even though some nuclear TDP43^{WT} remained, as determined by Image J. Results shown for 2 h (A) and 72 h (B) as means ± SD, $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Example Confocal images of TDP-43 pathology from respective time points. Bars represent 25 µm. (C) FloIT analysis of cell lysates. Following transfection (24, 48 and 72 h), adherent NSC-34 cells transiently transfected with TDP-43^{WT} were co-cultured with 20 µg/ml and aggregated G93A SOD1 for indicated time intervals at 37°C. Cells were harvested for supernatant analysis and then lysed. Cell lysates were incubated with RedDot2 for 2 min at RT. Transfection efficiencies, the number of inclusions and nuclei were determined by flow cytometry and results means ± SD, $n = 3$, *P < 0.05 compared to corresponding control (no protein treatment).

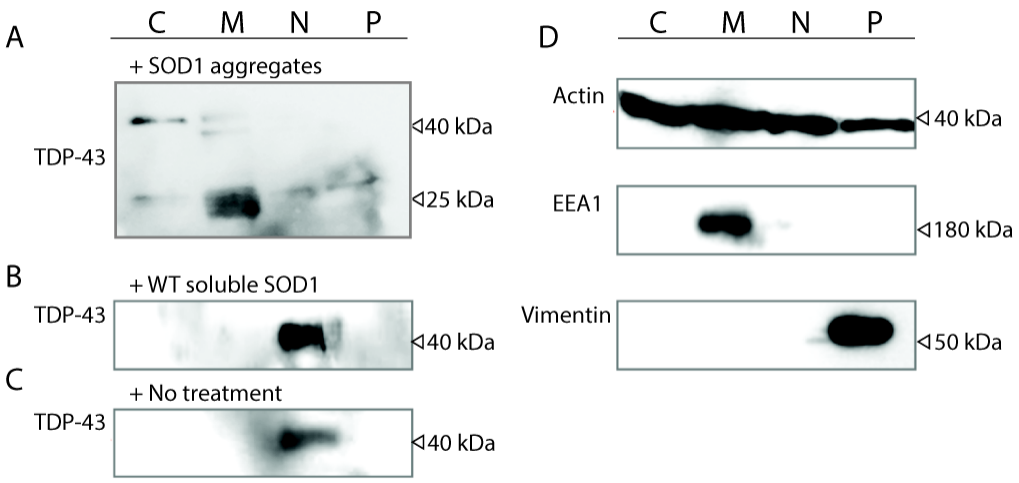

Figure 3. Exogenous recombinant SOD1 proteins induce TDP43 fragmentation and cytosolic mislocalisation in NSC34 cells. Cytoplasmic extract (C), membrane extract (ER/Golgi) (M), nuclear extract (N) and pellet extract (cytoskeleton) (P) fractions by centrifugation from NSC-34 cells treated with either (A) SOD1 aggregates or (B) WT SOD1 soluble (20 µg/mL) or (C-D) no added protein were separated by SDS PAGE under reducing conditions, transferred to nitrocellulose membrane and incubated with anti-TDP43, anti-actin, anti-EEA1 or anti-vimentin Abs (as indicated).