Human TDP43 is required for ALS-related annexin A11 toxicity in *Drosophila*

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Abstract. Genomics allows identification of genes and mutations associated with amyotrophic lateral sclerosis (ALS). Mutations in annexin A11 (ANXA11) are responsible for ~1% of all familial ALS and fronto-temporal dementia cases. The present study used the fruit fly, *Drosophila melanogaster*, to assess the mechanism of toxicity of ANXA11 mutants in residues that are conserved in the fly ANXB11 protein, the closest homolog to human ANXA11. In immune fluorescence, lifespan and negative geotaxis assays ANXA11 mutants, while displaying some degree of alteration in localization and function, did not exert any relevant organism toxicity in *Drosophila*. However, they showed a specific interaction with human TAR DNA-binding protein (TDP43). The present study illustrated that the ANXA11 mutants interact with human TDP43, but not the fly TAR DNA-binding protein-43 homolog (TBPH) or other ALS-associated genes such as super oxide dismutase 1, to shorten lifespan and increase negative geotaxis defects. This sheds light both on the mechanisms underlying ALS, further elucidating the intricate molecular network implicated in ALS and placing ANXA11 as a key player in its pathology, and on the complexity of using *Drosophila* as a model organism for researching genes in ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder, typically manifesting in adulthood, marked by the progressive loss of both upper and lower motor neurons. Despite ongoing gene therapy trials, effective pharmacological treatments for ALS remain elusive, and the mean survival is 2-5 years post-diagnosis (1,2). The genetic landscape of ALS is complex, with a vast array of sporadically occurring mutations with low penetrance, which poses challenges for the development of effective treatments (3,4).

Smith *et al* (5) discovered causative mutations in annexin A11 (ANXA11), a gene involved in calcium and phospholipid binding. Subsequent studies across diverse ethnic groups have confirmed the presence of causative ANXA11 mutations, solidifying the significance of ANXA11 in ALS pathology (6-16). The array of neurodegenerative conditions linked to ANXA11 mutations extends beyond ALS, encompassing frontotemporal dementia, multisystem proteinopathy, Paget's disease, muscular dystrophy, aphasia and oculopharyngeal muscular dystrophy (7,13,17-19).

The role of ANXA11 in neuronal biology has expanded since discovery of the association between ANXA11 mutations and familial and sporadic ALS (5). ANXA11 is associated with specific ALS-implicated pathways, such as disrupted liquid-liquid phase separation (LLPS) (20) and RNA and stress granule transport in the context of calcium biology (10,21,22). However, the exact role of ANXA11 and its relationship with other key ALS-linked proteins is yet to be fully understood.

Given that ANX serves critical roles in phospholipid-binding, cellular trafficking and autophagy and the presence of a highly conserved orthologous gene (ANXB11) in fruit flies (*Drosophila melanogaster)*, the present study developed a novel ALS model that overexpresses human ANXA11 mutations in *Drosophila*, a widely used model organism in ALS research (23-26). The present study aimed to assess the neurological phenotypes observed in this model and examine the interactions between ANXA11 and other ALS-associated genes.

Materials and methods

Drosophila stocks. A total of 2,535 were used in experiments. The following stocks were used *elav-Gal4*, *OK371-Gal4*,

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UAS-eGFP, *ubi-Gal80^{ts}* as previously described (27). Interfering RNA (IR) stocks *UAS-anxB11^{IR}* HMS01775, was obtained from Bloomington Drosophila Stock Centre (BDSC) and GD36186 was obtained from Vienna Drosophila Resource Center (VDRC)). *UAS*-TAR DNA-binding protein wild-type (*TDP43^{WT}* (human TDP43 Wild-Type) and *UAS-SOD1^{G85R}* (Human SOD1 Gly85Arg mutant) were a gift from Dr Jemeen Sreedharan (King's College London). *UAS-TBPH* was a gift from Dr Frank Hirth (King's College London).

Human ANXA11 transgenes were generated by PCR cloning the human open reading frames from previously described vectors (5) adding a Myc tag at the C terminal. PCR products were purified with QIAquick PCR purification kit (Qiagen GmbH), cut with *Xba*I and *Xho*I enzymes and cloned in the corresponding sites in the multi cloning site in pUAST-attB vectors (a gift from Dr Joe Bateman (King's College London)]. Presence of the correct ANXA11 mutation was validated by Sanger sequencing performed by Eurofins Genomics. Insertions were generated by injection in the *attP40 Drosophila* stocks at the Cambridge Fly Facility (Cambridge, UK) injection service. The following Oligos from Eurofins Genomics were used for PCR and cloning (restriction sites are underlined, Myc tag encoding sequence in lower case, *ANXA11* sequences in italics): Forward, 5'-GACTCGAG*ATGAGCTACCCTGGCTATCC*-3' and reverse, 5'-AGTCTA GATTAc agatc ctct tctgagatgagtttttgttc*GTCATTGCCACC ACAGATCTTCAGC*-3'.

Drosophila maintenance and husbandry. All fly stocks were routinely maintained at 18˚C in an incubator with 60% humidity and standard 12/12-h light cycle on a standard fly food mixture of yeast, agar and cornmeal with nipagin and propionic acid.

Lifespan analysis. Flies were monitored over the course of their life cycle to quantify death across genotypes as previously described (27). Briefly, newly eclosed flies were collected daily for 3 days and transferred to a 29˚C incubator in batches of 20 flies/vial (equal mix of males and females). Three times/week the flies were counted and transferred into a new vial with fresh fly food using CO_2 to anaesthetise the flies. The number of flies still alive was recorded each time and flies that escaped or were stuck in the food were censored (attributed a value of 0 on the Day). A dead fly was attributed the value 1 on the day of death.

Negative geotaxis assay. Flies were kept at 29˚C and age-matched female flies of the genotypes were placed into empty 70 mm tubes (10 flies/tube). When flies are tapped to the bottom of a vial, they immediately climb to the top of the vial due to their innate negative geotaxis abilities (27). To assess negative geotaxis, flies were tapped to the bottom of the vial after acclimatization and distance climbed by the flies in 2-min intervals over five trials was measured as previously described (27). The number of flies climbing to each cm increment was scored and a genotype mean was calculated for each time point across five trials. Flies that jumped or did not perform a vertical climb in one movement burst were excluded from that trial.

Immunohistochemistry. Whole mount larval ventral nerve cords were dissected from wandering third instar larva fixed in 4% paraformaldehyde (Sigma) for 45 min on ice, blocked for 1 h at room temperature (RT) in Phosphate Buffer Saline (PBS) complemented with 0.2% Tritox-X100 (Sigma) and 10% Normal Goat Serum (NGS, Gibco) and stained with a rabbit anti-GFP (1:300, cat. no. A11122; Thermo Fisher Scientific, Inc.) and a mouse monoclonal antibody against Myc tag (1:100, cat. no. 9E10; Roche Diagnostics, Ltd.). Additionally, flies were aged for 12 days at 29˚C, sacrificed and whole brains were dissected, fixed and blocked as aforementioned. The mouse primary antibody against TDP43 (used at 1:500) was a gift by Dr Jemeen Sreedharan (King's College London). Brains were imaged on a Nikon A1R inverted confocal microscope and analysis was performed in NIS Elements (Nikon 5.21). Secondary antibodies used were Alexa 555 anti-mouse and Alexa 488 anti-rabbit (Thermo fisher Scientific, Inc.; cat. nos. A21422 and A11008 and) at 1:200. All antibodies were diluted in Blocking solution. Primary antibodies were incubated over night at 4˚C, while secondary antibodies were incubated 1 h at RT. For TDP43 localization, data was normalised for cell area and expressed as a ratio of cytoplasmic TDP43 over nuclear TDP43.

Statistical analysis. All data are presented as mean ± SEM and were analysed using Microsoft Excel (office 365, www.microsopht.com) and GraphPad Prism 9 (www.graphpad.com). Lifespan was analysed with the Kaplan-Meyer log-rank (Mantel-Cox) test. One-way ANOVA with Dunnett's multiple comparisons post hoc test was used for comparison of ≥3 groups of normally distributed data. Kruskal-Wallis non-parametric analysis with Dunn's multiple comparisons test was used for comparison of ≥3 groups of non-normally distributed data. Two-way ANOVA with Dunnett's multiple comparisons post hoc test was used for negative geotaxis assays where ≥2 groups of normally distributed data were confounded by a third parameter (timepoints). $P<0.05$ was considered to indicate a statistically significant difference. All experiments have been performed at least three times.

Results

Drosophila ANXB11 is structurally similar to human ANXA11. Sequence comparison revealed that *Drosophila* ANXB11 was closely related to human ANXA11 than other *Drosophila* genes were, with two splicing isoforms expressing an ANX protein with a long N-terminal tail (Fig. 1A). While the N-terminus containing the human mutation hotspot (22) around aa36-40 is not conserved, other key residues mutated in ALS, such as G175 and R235, are also conserved in *Drosophila* (Fig. 1A).

The present study constructed overexpression transgenes for human ANXA11 with G175R and R235Q mutations, and a corresponding wild-type (WT) transgene. All transgenes were knocked into a well-characterised neutral genomic locus to guarantee similar expression levels and neutrality for insertional mutagenesis.

ANXA11 transgenes are expressed and mutants are enriched in the nucleus. The present study demonstrated the expression

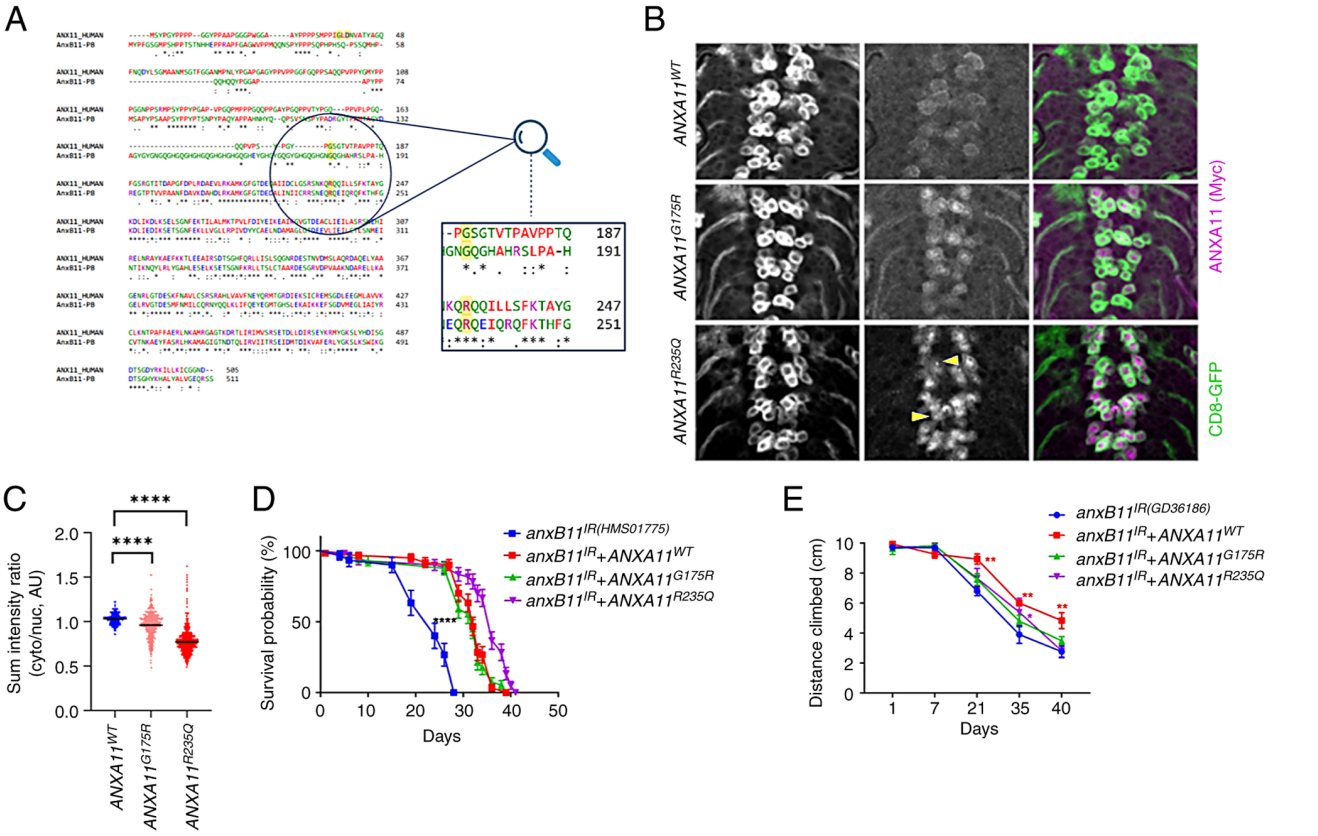

Figure 1. Comparison of human and *Drosophila* ANX11. (A) *Drosophila* ANXB11 is similar to human ANXA11. G175 and R235 residues are conserved, while the G38-D40 region is divergent. Numbers indicate the amino acids in the protein sequence. *Indicates identical aminoacids in the protein sequence; ***Indicates three identical aminoacids. (B) Mutant ANXA11 transgenes display nuclear expression in larval motor neurons. ANXA11^{R235Q} forms discernable aggregates (arrows). Scale bar, 10 mm. Magnification, x600. (C) Quantification of nuclear enrichment. *ANX11* transgenes rescue the phenotypes generated by endogenous *ANXB11* knockdown for (D) lifespan and (E) climbing distance. Kaplan-Meier (Log Rank) test. *P<0.05, **P<0.01, ****P<0.0001 vs. *anxB11^{IR}*. ANX, Annexin; IR, Interfering RNAi; WT, Wyld-Type; AU, Arbitrary Units; cyto, cytoplasm; nuc, nuclear.

of ANXA11 transgenes and notable enrichment of G175R and R235Q mutants around and within the cell nucleus in larval neurons (Fig. 1B and C). In addition, the R235Q mutant protein displayed notable aggregation (Fig. 1B), as reported in human cells (5).

ANXA11 transgenes rescue phenotypes generated by endogenous ANXB11 knockdown. Our previous study showed that knockdown of the fly ANXB11 gene decreases in lifespan and negative geotaxis (28). The introduction of human ANXA11 transgenes successfully rescued the short lifespan observed following pan-neuronal knock-down of the endogenous ANXB11 (Fig. 1D). When analysing the negative geotaxis due to motor neuron knockdown, however, the rescue was more limited, and only the WT A11 construct constantly improved negative geotaxis after 21 days (Fig. 1E).

Annexin A11 transgenes improve lifespan and negative geotaxis defects caused by the expression of TDP43, but not of Drosophila TBPH. Despite successful expression, altered subcellular localization and functional alterations of the ANXA11 transgenes, there were no obvious signs of organism-level toxicity when mutant ANXA11 transgenes were expressed in *Drosophila* neurons, both in terms of lifespan and negative geotaxis. Genetic interactions with other known ALS genes were assessed using transgenic flies expressing the human genes that display toxicity in *Drosophila*, such as TDP43 (24) and SOD1 (25).

ANXA11 transgenes markedly worsened lifespan and negative geotaxis deficits in *Drosophila* expressing TDP43, a protein associated with ALS pathology (Fig. 2A and B). *ANXA11* transgenes significantly enhanced the negative geotaxis defects cause by TDP43 after 11 days and made lifespan significantly shorter.

Negative geotaxis response and lifespan analysis showed that OK371-Gal4, TDP43 WT flies illustrate decreased negative geotaxis compared with the ANX mutants alone, which did not significantly affect negative geotaxis. When co-expressed with human TDP43, all *ANXA11* transgenes exacerbated the negative geotaxis defects seen with TDP43 alone (Fig. 2B). Annexin A11 transgenes further decreased lifespan compared with TDP43 (Fig. 2B). These effects were not observed in models expressing *Drosophila* TBPH, an ortholog of TDP43 essential for *Drosophila* motor neurons (26). TBPH had stronger negative geotaxis defects alone than when co-expressed with human ANXA11 (Fig. 2C) and ANXA11 did not affect TBPH lifespan (Fig. 2D).

ANXA11 has no genetic interaction with human SOD1 in Drosophila. To determine if the modulating effect of ANXA11 transgenes occurred in other ALS-associated genes, the

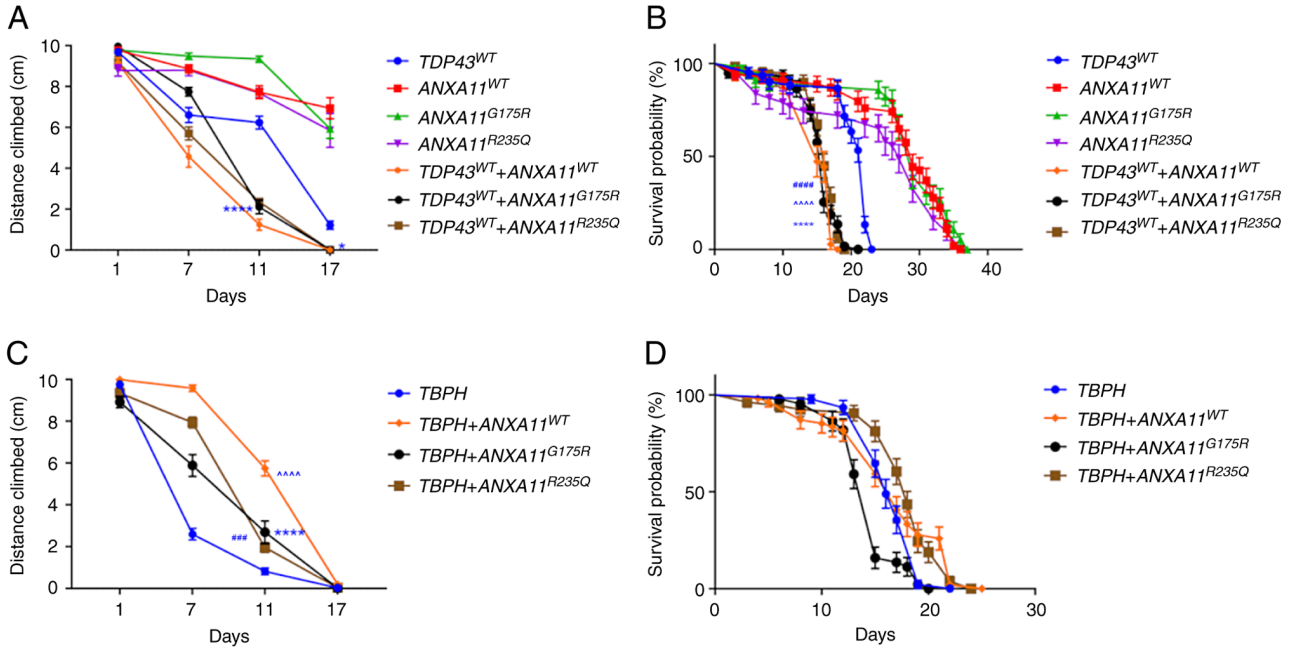

Figure 2. ANXA11 transgenes enhance lifespan and negative geotaxis defects caused by the expression of TDP43, but not of *Drosophila* TBPH. (A) TDP43-expressing flies illustrate decreased negative geotaxis compared with the ANX mutants alone, which do not significantly affect negative geotaxis. When co-expressed with human TDP43, all *ANXA11* transgenes exacerbated the negative geotaxis defects induced by TDP43 alone. *P= 0.0487, ****P<0.0001 vs. TDP43 WT. (B) ANXA11 transgenes worsen the lifespan shortening induced by TDP43. ****P<0.0001 vs. TDP43 + ANXA11 WT, ($^{\#\#\#\#}$P<0.0001 vs. G175R, ($^{\wedge\wedge\wedge\wedge}$P<0.0001 vs. R235Q)). (C) TBPH induces stronger negative geotaxis defects alone than when co-expressed with ANX at Day 11. $^{\#\#\#}$P=0.0006 vs. TBPH + ANXA11 G175R and ****P<0.0001 vs. TBPH + ANXA11 R335Q or ($^{\wedge\wedge\wedge\wedge}$P<0.0001 vs. ANXA11 WT. (D) ANX did not affect TBPH lifespan. ANX, Annexin; IR, Interfering RNAi; WT, Wild-Type; TDP43, TAR DNA binding Protein 43; TBPH, TAR DNA-binding protein-43 homolog.

association between ANXA11 and SOD1 (a gene implicated in familial forms of ALS) (25) was assessed. There was no discernible genetic interplay between A11 and G85R mutant SOD1 (Fig. S1).

Localization of human TDP43 is affected by ANXA11 overexpression and its nuclear increase is modulated by the mutations in ANXA11. To determine the potential molecular mechanism underlying the interaction with human TDP43 of the ANXA11 transgenes, the present study assessed expression of TDP43 in the adult fly brain. Overexpression of ANXA11 altered the typical distribution of TDP43, increasing its nuclear accumulation (Fig. 3A and B), which may interfere with key nuclear functions.

Furthermore, the extent of TDP43 nuclear accumulation was decreased by G175R and R235Q mutations in ANXA11 compared with WT ANXA11, indicating that ALS-mutations in ANXA11 may cause promote cytoplasmic localisation of TDP43 compared with WT ANXA11 (Fig. 3B). These varying degrees of TDP43 nucleo-cytoplasmic localization suggested that the link between ANXA11 and TDP43 may be sensitive to the structural changes induced by these mutations either by gain or loss of function.

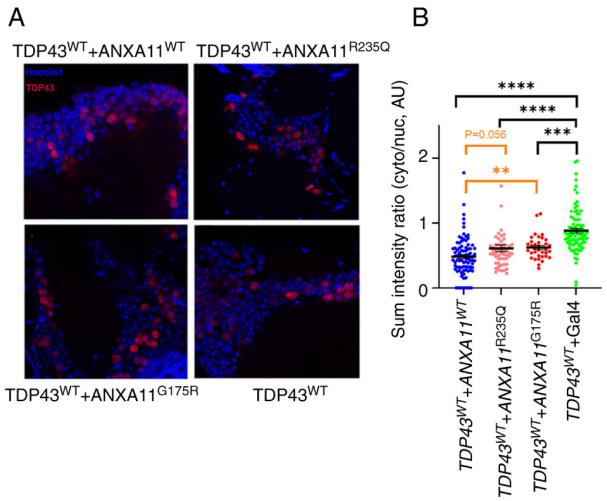

Figure 3. Localization of human TDP43 is affected by *ANX11* overexpression and its nuclear increase is modulated by the mutations in *ANXA11*. (A) Expression of human TDP43 (red) in the brain cells of the fly, showing nuclear (blue) localisation. Scale bar, 10 mm. Magnification, x600. (B) In the presence of ANXA11 there is an increased presence of TDP43 in the nucleus **P<0.01, ***P=0.0002, ****P<0.0001. ANX, annexin; WT, Wild-Type; AU, arbitrary Units; cyto, cytoplasm; nuc, nuclear; TDP43, TAR DNA binding Protein 43.

Discussion

The precise mechanism by which ANXA11 causes ALS is unknown, but progress has been made in understanding the role of ANXA11 as a molecular tether for axonal RNA transport, which is impaired by ALS-associated ANXA11 mutations (21). ANXA11 mutations are reported in patients with sporadic ALS, causing dysregulation of intracellular Ca^{2+} homeostasis and stress granule dynamics (10,21).

Drosophila has been widely used as a model organism for ALS research (23-26) due to high conservation in ALS genes and ease of genetic manipulation that allows study

of interactions between different genes. The present study constructed a novel *Drosophila* model for studying the role of ANXA11 in ALS pathogenesis.

The present study demonstrated functional conservation between fly ANXB11 and human ANXA11 and altered subcellular localisation of ANXA11 mutants. The phenotypical rescue suggests that the human ANXA11 transgenes not only are expressed but retain sufficient structural and functional similarity to the *Drosophila* ANXB11, allowing them to compensate for its loss. The more consistent rescue by WT ANXA11 also suggests that the G175R and the R235Q mutations interfered, at least partially, with full ANXA11 functionality in *Drosophila*.

Comparative enrichment of the ANXA11 mutant proteins in the nucleus suggests that mutations may impair nuclear-cytoplasmic shuttling or result in a potential toxic impairment of nuclear function.

ANXA11 mutants did not exert organism toxicity in *Drosophila* but can only enhance the toxicity of human TDP43, specifically, but not that of fly TBPH or human SOD1. Despite TBPH being an ortholog of TDP43, the absence of enhancement of TBPH's impact on lifespan and negative geotaxis by ANXA11 transgene expression suggests a specific interaction between human A11 and TDP43 that is not conserved with its *Drosophila* counterpart. This could be attributed to differences in protein-protein interactions, post-translational modification or cellular localization between human TDP43 and *Drosophila* TBPH and may be the reason why ANXA11 transgenes did not exert significant toxicity in *Drosophila*.

The lack of interactions with SOD1 has implications for ALS heterogeneity at a genetic and molecular level. ALS is a multifactorial disease with diverse genetic contributors (1). The absence of a genetic link between these two ALS-associated genes confirms distinct pathogenic pathways for SOD1-associated ALS.

This also has implications for the development of ALS models and the interpretation of previous studies (24-26): It indicates that models based on overexpression or mutation of *ANXA11* may not be suitable for studying mechanisms related to $SOD1^{G85R}$ pathology. This is crucial for ensuring the accuracy and relevance of research models to specific subtypes of ALS.

Thus, the present study demonstrated a novel and potentially deleterious interaction between ANXA11 and TDP43 in ALS pathology. The redistribution of TDP43 subcellular localization suggested a potential regulatory role of *ANXA11* in the cellular trafficking or localization of TDP43, which could be critical in understanding the pathological mechanisms of ALS. The modulation of TDP43 localization by ANXA11 and the mutation-dependent nature of this interaction opens novel avenues for exploring the molecular basis of ALS. It suggests that alterations in protein trafficking and localization are a key aspect of disease progression as a result of nuclear pathology in ANXA11-associated ALS and other types of neurodegenerative diseases (28,29).

The N-terminus of ANX A11 binds and traffics RNA granules with mutations, impairing LLPS (10,21). ANXA11 mutations may potentially impair nuclear RNA dynamics and TDP43 function at an early stage of the disease process (30). In post-mortem tissue staining of ANXA11 mutant cases, partial axonal co-localisation of phosphorylated TDP43 and immunoreactive ANXA11 has been observed (5,12). Furthermore, patients with multisystem proteinopathy harbouring a D40Y mutation exhibit ANXA11/TDP43 cytoplasmic co-localisation in muscle (18). To the best of our knowledge the association between endogenous nuclear ANXA11 (or N and C terminal mutants) and TDP43 in neurons has yet to be established.

It is presently unclear whether the specificity of the ANXA11-TDP43 axis is a fundamental molecular and mechanistic difference between fly TBPH and human TDP43 or lack of full conservation between human ANXA11 and fly ANXB11 in terms of their association with TDP43 molecules. This is a limitation of the present study, which is also limited by the use of exogenous transgenes overexpressing the protein of interests. A knock in model for ANAX11 and or TDP43/TBPH is required when attempting to use *Drosophila* as a model for ALS genes whose toxicity is associated with TDP43, such as ANXA11. It is essential to study the ANXA11-TDP43 axis in other organisms to validate if the relationship is preserved or if this is a human-specific factor. Furthermore, understanding how specific ANXA11 mutations affect TDP43 localization in human neurons and other model organisms could provide insight into the heterogeneity of ALS symptoms and progression.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JB, RH, DM and MY performed experiments. JB, RH, BS and MF designed the experiments. MF and JB wrote the manuscript. JB, RH and MF confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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