

Interactions between ALS-linked FUS and nucleoporins are associated with defects in the nucleocytoplasmic transport pathway

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ABSTRACT

Nucleocytoplasmic transport (NCT) decline occurs with aging and neurodegeneration. Here, we investigated the NCT pathway in models of ALS-fused in sarcoma (FUS). Expression of ALS-FUS lead to a reduction in NCT and nucleoporin (Nup) density within the nuclear membrane of human neurons. FUS and Nups were found to interact independently of RNA in cells, and to alter the phase separation properties of each other *in vitro*. FUS-Nup interactions were not localized to nuclear pores, but were enriched in the nucleus of control neurons versus the cytoplasm of mutant neurons. Our data indicate the effect of ALS-linked mutations on the cytoplasmic mislocalization of FUS, rather than on the physiochemical properties of the protein itself, underlie our reported NCT defects. An aberrant interaction between mutant FUS and Nups is underscored by studies in *Drosophila*, where reduced Nup expression rescued multiple toxic FUS-induced phenotypes, including abnormal nuclear membrane morphology in neurons.

INTRODUCTION

Nucleocytoplasmic transport (NCT) refers to the active transfer of macromolecules through nuclear pore complexes (NPC) embedded within the nuclear membrane. Each NPC is comprised of multiple copies of 30 different nuclear pore proteins called nucleoporins (Nups). Some Nups contain low-complexity domains with phenylalanine (F)/glycine (G) motifs that undergo phase separation¹, a process whereby macromolecules demix from aqueous solution and form assemblies through weak, non-covalent interactions². Phase separation of F/G-Nups into polymers and hydrogels may contribute to the selectivity filter of the NPC, as the central channel of the pore is enriched with F/G motifs that directly bind and guide nuclear transport receptor-bound cargo through the pore³.

NCT becomes compromised as a function of aging, particularly in post-mitotic cells such as neurons^{4,5}. In dividing cells, the nuclear membrane and NPC disassemble during mitosis, allowing for pore components to turnover and become replenished as needed before pore reassembly⁶. Conversely, in terminally differentiated cells, Nups represent some of the longest-lived proteins and are therefore vulnerable to age- and stress-induced damage⁷. For example, age-dependent changes in nuclear transport receptor expression correlate with nuclear pore deterioration and pore “leakiness”⁵. Further, certain Nups are highly susceptible to oxidative damage and stress-induced degradation in aged and stressed cells, which can lead to further NCT dysfunction^{4,8}.

The NCT pathway has also been implicated in age-dependent neurodegenerative disorders⁴. Notably, the NCT pathway recently emerged as a therapeutic target in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), two related and incurable disorders⁹. In the context of ALS/FTD caused by repeat expansions in *C9orf72*, a series of unbiased genetic screens identified components of the NCT pathway as modifiers of *C9orf72*-induced toxicity¹⁰⁻¹³. Nuclear membrane abnormalities and loss of

nucleocytoplasmic compartmentalization were subsequently observed in human *C9orf72* iPSC-derived neurons^{11,12,14}. Similar observations were reported in neurons expressing ALS-linked variants of TDP-43¹⁵ and of profilin 1¹⁶. Despite an emerging role of NCT in neurodegenerative disease pathogenesis, the mechanism(s) by which NCT-related defects arise in these disorders remain unclear⁹.

Herein, we examined the NCT pathway in models of disease-linked fused in sarcoma (FUS). FUS is an ALS/FTD-associated, RNA/DNA binding protein that undergoes nucleocytoplasmic shuttling for various cellular functions¹⁷. However, nuclear import of FUS is impaired when the nuclear localization sequence (NLS) is mutated in ALS, which abrogates binding between FUS and the nuclear transport receptor karyopherin- β /transportin-1¹⁸. As a result, mutant FUS accumulates in the cytoplasm where it is thought to exert a toxic gain of function that leads to ALS¹⁷. Cytoplasmic accumulation of pathological proteins has been shown to correlate with NCT dysfunction, potentially through sequestration of transport factors by pathological proteins¹⁹. In light of these observations, we investigated the NCT pathway in models of disease-linked FUS. We demonstrate loss of nuclear pore integrity and other phenotypes related to disrupted nucleocytoplasmic transport in multiple systems including isogenic human neurons and *Drosophila* models of FUS-induced toxicity. Interactions between FUS and Nups may underlie these phenotypes, as FUS interacts directly with Nups in cells and *in vitro*, where FUS and Nups alter the phase separation properties of each other. Importantly, reduced expression of Nups mitigates FUS toxicity *in vivo*. Our results indicate that NCT dysfunction is a feature of ALS-FUS, and that targeting aberrant FUS/Nup interactions may be a viable therapeutic strategy for FUS-mediated neurodegeneration.

RESULTS

Nucleocytoplasmic transport is impaired in human neurons expressing ALS-linked FUS.

Impaired NCT has been reported in multiple age-related neurodegenerative disorders⁹. To assess NCT in ALS-FUS iPSC-derived neurons, we examined the distribution of the Ras-related nuclear protein (Ran) between the nucleus and the cytoplasm, referred to as the Ran gradient, which establishes the directionality of normal NCT^{9,20}. Normally, Ran is localized in the nucleus, but changes to this gradient are often accompanied by compromised NCT^{9,21}. Human induced pluripotent stem cells (iPSCs) harboring the autosomal dominant ALS-FUS mutations, M511Nfs or R521G, were subjected to a protocol for motor neuron differentiation and processed for Ran immunofluorescence analysis (Figure S1). The FUS nuclear localization sequence (NLS) is abrogated by the M511Nfs mutation, leading to severe cytoplasmic mislocalization of the protein in cells and juvenile ALS-onset in humans. Mutations at residue R521 are the most common cause of adult-onset ALS-FUS²² and also disrupt the FUS NLS albeit to a lesser degree than M511Nfs (Figure S1). We observed a significant decrease in the nuclear-cytoplasmic (N/C) distribution of Ran in iPSC-derived motor neurons harboring the more severe

M511Nfs mutation compared to motor neurons derived from control or R521G iPSCs (Figures 1a-1c). We repeated this analysis on motor neurons derived from iPSCs that were gene-edited to correct the M511Nfs mutation (referred as M511Nfs*Cor) compared to isogenic mutant neurons²³. Again, the Ran N/C ratio was significantly lower in FUS M511Nfs neurons compared to FUS M511Nfs*Cor neurons in both ISL1/2-positive (Figures 1d and 1e) and -negative neurons (Figure 1f). A Western blot analysis revealed similar levels of Ran protein between FUS M511Nfs and M511Nfs*Cor neurons, suggesting the N/C change was due to a shift in the Ran gradient rather than altered protein levels (Figures 1g and 1h). Similarly, the Ran N/C ratio was significantly lower in FUS R495X neurons compared to the corresponding isogenic control (Figures S2 and S3). Therefore, expression of ALS-FUS is sufficient to disrupt the N/C Ran distribution in ALS patient-derived neurons, leading to higher levels of Ran in the cytoplasm.

Next, we assessed nuclear pore density within FUS M511Nfs and control neurons. In general, neurons exhibited variable nuclear morphologies and degrees of membrane invaginations when fixed. To facilitate immunofluorescence analysis of nuclear pores, neurons were semi-permeabilized prior to fixation, resulting in isolated and intact nuclei that were devoid of membrane invaginations²⁴. Neuronal nuclei were probed for nucleoporins (Nups), including the nuclear pore membrane protein 121 kDa (Pom121), a scaffold Nup and Nup62, a central channel Nup. Staining of control nuclei with both markers produced a robust and continuous ring of signal at the nuclear membrane. In contrast, there were numerous gaps in Pom121 and Nup62 signals around FUS M511Nfs nuclei (Figure 2a). This phenotype was quantified using a “cluster analysis” as illustrated in Figure 2b. Briefly, a continuous Nup distribution was assigned a cluster of one (i.e., no gaps). Gaps in nuclear pore signal resulted in a higher cluster number, reflecting a loss of continuous pore signal at nuclear membrane, but not necessarily a clumping of pores. FUS M511Nfs nuclei exhibited significantly more gaps (i.e., higher cluster numbers) for both markers compared to control nuclei (Figures 2c and 2d), although Pom121 and Nup62 did not always co-localize (Figure S4a). Nuclei probed for either marker were binned according to their cluster number in Figures 2e and 2f, confirming that a higher percentage of FUS M511Nfs nuclei exhibited more gaps in nuclear pore signal compared to control nuclei, and vice versa. This analysis could not accommodate additional markers, such as ISL1/2, for co-staining. In a Western blot analysis, total Nup protein levels were similar in mutant and control neurons (Figure S4b and S4c), although we cannot exclude the possibility of reduced Nup expression in a subset of cells with high cluster numbers.

Proper Ran and nuclear pore distributions are required for efficient NCT²⁰, raising the possibility that transport is also impaired by expression of ALS-FUS. To assess active transport, cultured neurons were transduced with a lentiviral construct encoding the fluorescent shuttling reporter, tdTomato, fused to a classical nuclear localization sequence (NLS) and nuclear export sequence (NES) at the N- and C- terminus, respectively. We previously showed that nucleocytoplasmic shuttling of this reporter is dependent on nuclear pore activity in neurons²⁵. Here, NLS-tdTomato-NES exhibited a diffuse expression pattern and was predominately localized to the nuclei of human neurons (Figure S5a). Fluorescence recovery after photobleaching (FRAP) was performed on nuclear tdTomato fluorescence signal. TdTomato fluorescence was bleached within the entire area of the nucleus, and the then recovery of this fluorescence

signal was monitored over a time course of 450 seconds (Figure S5b). Recovery of nuclear tdTomato fluorescence is therefore dependent on nuclear import of the tdTomato reporter from the cytoplasm. Complete nuclear fluorescence recovery was not observed in any FRAP experiment herein, likely because most of the reporter is expressed in the nucleus to begin with and eventually the entire cell becomes photobleached during the analysis (Figure S5a,b). Nonetheless, the overall tdTomato fluorescence recovery was significantly less efficient in FUS M511Nfs neurons compared to control neurons (Figure 2g) as demonstrated by the area under the recovery curve (Figures 2h). Further, the initial rates of the fluorescence recovery were significantly slower in mutant versus control neurons (Figure 2i). The reduced FRAP recovery in mutant neurons is not due to differential expression of the NLS-tdTomato-NES protein between lines (Figure S5c).

As nuclear export may also contribute to diminished NCT, tdTomato fluorescence was measured in the cytoplasm of the same neurons that underwent nuclear photobleaching. Nuclear export of photobleached tdTomato molecules effectively dilutes cytoplasmic tdTomato fluorescence, thereby diminishing cytoplasmic tdTomato fluorescence over time (Figure S5b). However, cytoplasmic tdTomato fluorescence did not decrease in mutant neurons, indicative of an export defect, to the same extent as in control neurons (Figure S5d). While differences in nuclear import and export were observed between aged (DIV 56-60) mutant FUS and control neurons, no differences were detected between young (DIV 18) mutant and control neurons (Figures S5e-h), suggesting the effect of mutant FUS on NCT may manifest with age, consistent with recent observations in ALS-C9orf72 human neurons ¹⁴.

FUS directly interacts with nucleoporins in cells and *in vitro*.

To gain insight into how mutant FUS disrupts NCT, we explored the possibility that FUS interacts directly with nuclear pore proteins. We considered proteins that were immunoprecipitated by FUS from HEK-293T cells and subsequently detected by a mass spectrometry (MS) analysis ²⁶. While most of the FUS-interacting proteins were other RNA-binding proteins, several nuclear pore associated proteins were detected, albeit in low abundance. We pursued Nup62 given the important role of this protein in NCT and prior reports that Nup62 becomes dysregulated in models of neurodegeneration ^{9,27}. This interaction was validated here through IP of both FUS and Nup62 in multiple ALS-FUS iPSC lines, including FUS P525L and an isogenic control ²⁸ (Figure 3a), and additional mutant FUS and control lines (Figure S6). The IP was performed with and without RNase, which was added when the lysates were applied to the antibody-bound beads (illustrated in Figure S6a). As opposed to RNA-dependent interactions of FUS ^{26,29}, the FUS/Nup62 interaction was enhanced in the presence of RNase (Figures 3b,c and Figure S6). RNase treatment likely removed RNA-dependent protein interactions of FUS in the IP reaction, thus increasing the opportunity for Nup62 to bind FUS (Figure S6a).

To expand on these findings, full-length FUS WT, full-length ALS-FUS M511Nfs and full-length Nup62 were recombinantly expressed and purified *in vitro* as fusion proteins containing an N-terminal maltose binding protein (MBP) solubility tag ³⁰ (Figure S7a). Due to the extreme aggregation propensity of recombinant Nup62 after liberation from the MBP tag, we elected to analyze the biochemical interaction of FUS and Nup62 while fused to MBP. Co-IP of MBP-FUS with MBP-Nup62 confirmed that these proteins directly and specifically interact even in the presence of the MBP tags (Figure S7b).

FUS and Nup62 alter the phase separation properties of each other.

In light of the functional implications of phase separation for both FUS² and Nup62³, we also considered whether one protein could affect the phase separation properties of the other. First, the phase separation of each protein alone was initiated with polyethylene glycol (PEG), a molecular crowding reagent. To monitor the phase separation of each protein using fluorescence microscopy, full-length MBP-FUS and full-length MBP-Nup62 were doped with Alexa-555 labelled MBP-FUS and Alexa-488 labelled MBP-Nup62, respectively. In the presence of PEG, MBP-FUS readily condensed into dynamic spherical droplets (Figure 4a, Figure S8, Supplemental Video 1), similar to prior reports³⁰⁻³². Under these conditions and over a 24-hour timeframe, there were no discernable differences in droplet formation or droplet morphology between MBP-FUS WT and MBP-FUS M511Nfs (Figure 4a and Figure S9), indicating that mutation of the FUS NLS does not affect the physicochemical properties of the protein with respect to droplet formation. In contrast to MBP-FUS, MBP-Nup62 assembled into non-spherical particles in the presence of PEG (Figure 4a). Neither MBP-FUS droplets nor MBP-Nup62 assemblies were formed under these conditions in the absence of PEG (Figure S10).

Next, we investigated whether MBP-Nup62 affects the phase separation of MBP-FUS and vice versa, by mixing MBP-FUS (either WT or M511Nfs) with MBP-Nup62. After combining equimolar amounts of the proteins, PEG was added to the mixture and droplet formation was monitored by fluorescence microscopy. Strikingly, MBP-FUS WT (Figure 4a) formed irregular shaped or amorphous assemblies *in lieu* of spherical liquid droplets when MBP-Nup62 was added to the mixture. Further, the appearance of MBP-Nup62 amorphous assemblies was accelerated and augmented by the presence of FUS (compare MBP-Nup62 alone to MBP-Nup62 + MBP-FUS at every time point in Figure 4a). Compared to the droplets formed by MBP-FUS, FRAP measurements of FUS/Nup62 amorphous assemblies appeared less dynamic with lower fluorescence recovery. However, these species tended to oscillate in solution (Supplemental Video 2), contributing to the variability of the FRAP recovery curves (Figure S8). Collectively, these results demonstrate a synergistic effect of Nup62 and FUS on the phase separation properties of the other, where co-mixing leads to non-spherical, less dynamic and less liquid-like species. These effects were specific for sequences encoded by FUS and Nup62, as addition of free MBP protein had no discernable effect on FUS (Figure S11a) or Nup62 (Figure S11b) condensation, although free MBP did co-localize with MBP-FUS droplets as reported³³. Results of the co-mixing reactions were the same, regardless of whether FUS WT (Figure 4a) or FUS M511Nfs (Figure S9) was mixed with Nup62. Apparently, NLS mutations in FUS do not alter the Fus/Nup interaction *in vitro*. We note that TEV-proteolytic cleavage of the MBP-tag also induced FUS droplet formation as reported³⁰, but we noticed that the TEV enzyme itself aggregated over time and was therefore viewed as a confounding factor for our co-mixing studies of MBP-FUS and MBP-Nup62.

Given that FUS and Nup co-IPs were enhanced in the presence of RNase (Figures 3 and S6), we performed the same co-mixing experiments in the presence of torula yeast RNA at a mass ratio of RNA:FUS:Nup62 $\approx$ 1.5:1:1. Strikingly, the presence of RNA prevented the formation of irregular shaped FUS/Nup assemblies. Instead, FUS condensed into spherical species, even though Nup62 was present (Figure 4b). Further,

some Nup signal co-localized within FUS droplets, suggesting the FUS/Nup interaction was not fully abrogated by RNA but rather might be occurring in the droplet phase (Figure 4b). Thus, RNA modulates FUS/Nup62 interactions.

Cytoplasmic interactions between nucleoporins and FUS are enhanced in mutant FUS neurons.

While the co-IP studies and droplet assays did not demonstrate differential interaction between Nup62 and mutant versus wild-type FUS (Figures 3, S6 and S7), the nature of FUS/Nup interactions could depend on their localization within the cell. Immunofluorescence analyses demonstrated that wild-type FUS is predominately localized in the nucleus of cells whereas ALS-linked mutations in the NLS of FUS cause cytoplasmic mislocalization of the protein (Figures S1-S3 and 5a). However, there is no compartmentalization of cytoplasmic and nuclear-specific factors in the biochemical assays. Therefore, we investigated the FUS/Nup interaction *in situ*. Given that Nup62 is a F/G-Nup, and that FUS may generally interact with this class of proteins, we performed co-immunofluorescence analysis with an anti-F/G-Nup monoclonal antibody (mAb414)^{15,16} together with an anti-FUS antibody. Some mAb414 signal was also detected in the nucleus and the cytoplasm, the latter which may represent F/G-Nups associated with annulate lamellae (Figure 5a)^{6,34-36}.

The proximity ligation assay (PLA) was employed using the same FUS and mAb414 antibodies. The PLA generates a signal when two proteins are proximally located within ~40 nm of each other and thus reports on potential protein-interactions *in situ*. PLA signal was detected between FUS and F/G Nup proteins in both control and mutant neurons. *A priori*, we expected PLA signal at the nuclear membrane where the pore signal was diminished. However, PLA signal was not localized to the membrane, and was strikingly different between genotypes (Figure 5b). For control neurons, prominent FUS/mAb414 PLA signal was detected within nuclei, whereas the PLA signal was distributed between the nucleus and the cytoplasm of mutant neurons (Figure 5b). Quantification of total FUS/mAb414 PLA signal was significantly higher in M511Nfs neurons (Figure 5c), which could be due to higher endogenous FUS expression in mutant cells (Figure 3a and ^{23,37}). Equatorial planes of each cell were used to quantify the cytoplasmic-nuclear (C/N) ratio for FUS/mAb414 PLA signal (Figure 5d), which was significantly shifted toward the cytoplasm in mutant neurons compared to control (Figure 5e).

In addition to the pan F/G Nup mAb414 antibody, we also performed the PLA for FUS and individual F/G Nup proteins, including central channel Nups (Nup62 and Nup98) and a nuclear basket Nup (Nup153). Total PLA signals for these other Nups were lower than mAb414 and did not differ significantly between genotypes (Figures S12e, S12h and S12k). However, all three Nups produced a significantly higher C/N PLA ratio in FUS M511Nfs neurons (Figures 5f, 5g and 5h). Plotting PLA intensities in grey values revealed that the nuclear PLA signal was reduced in the mutant neurons relative to the control neurons for all Nup antibodies tested, although this did not reach statistical significance for the FUS/mAb414 PLA (Figures S12a, S12c, S12f and S12i). Further, there was a significant increase in cytoplasmic FUS/Nup PLA signal in the mutant neurons compared to the control for all Nups except Nup98 (Figures S12b, S12d, S12g and S12j). Taken

together, these data indicate that enhanced FUS/Nup interactions within the cytoplasm of ALS-FUS neurons is largely due to a shift in signal from the nucleus to the cytoplasm.

PLA was then used to detect potential interactions between nuclear pore proteins themselves using mAb414 and anti-Nup62 antibodies (Figure 5i). While the total PLA signal was not significantly different between genotypes (Figure S12n), there was a significant increase in the C/N ratio for the Nup62/mAb414 PLA in mutant compared to control neurons (Figure 5j). This result is consistent with a displacement of nuclear pore proteins from the nucleus and/or the nuclear pore complex in mutant FUS neurons (Figures S12l and S12m).

Nuclear abnormalities associated with ALS-linked FUS are rescued by knock-down of Nup62 *in vivo*

Our data suggests that interactions between ALS-linked FUS variants and nucleoporins could become pathological, and manifest in disease-relevant phenotypes within neurons. We therefore examined the effect of nucleoporin expression on FUS-induced toxicity in *Drosophila*^{38,39}. Targeted overexpression of FUS WT and disease-causing variants (R518K and R521C) in fly eyes caused significant eye degeneration characterized by depigmentation, ommatidial fusion and resultant waxy external eye phenotype (Figure S13a). Overexpression of Nup62 in this context exacerbated the eye degeneration in WT and mutant FUS flies (Figure S13a), whereas Nup62 overexpression on its own was well tolerated (Figure S13b) thus confirming the increased toxicity is not due to combined individual toxicities. In contrast, knocking down Nup62 using an RNAi line in FUS WT and mutant flies significantly rescued FUS-mediated eye degeneration (Figures S13a,c). In addition to Nup62, knocking down other nucleoporins including Nup54, Nup98 and Nup214 also rescued FUS toxicity (Figures S13-S15). RNAi-mediated knockdown of Nup37, Nup43, Nup153 and Nup205 in flies exhibited a partial rescue of the eye degeneration phenotype induced by FUS overexpression (Figure S15). These data illuminate multiple FUS/Nup interactions that influence cellular homeostasis *in vivo*.

We investigated whether reducing nucleoporin levels could also rescue FUS-induced defects in cell types that are relevant to ALS. To this end, we used previously published fly lines that overexpress RFP-tagged FUS-WT and mutant FUS-P525L⁴⁰ in ventral nerve chord (VNC) neurons (Figure 6a). FUS P525L is an ALS-linked variant that also results in the eye-degeneration phenotype, which is partially rescued with Nup62 RNAi (Figure S16a). FUS WT distributes primarily into punctate structures within the nucleus (Figure 6a, top). In contrast, FUS P525L exhibits a granular expression pattern, distributed between the nucleus and cytoplasm (Figure 6a, bottom). Due to a lack of antibodies available for *Drosophila* Nup62, we were unable to determine Nup62 subcellular distribution. VNC neurons were stained with Lamin C to assess nuclear morphology (Figure 6a), revealing significantly more FUS-P525L-expressing neurons with nuclear invaginations compared to FUS WT (Figure 6b). Nuclear invaginations appear as wrinkled membranes and are indicative of membrane disruptions¹⁰. Again, Nup62 knockdown significantly ameliorated the nuclear morphology defects caused by FUS-P525L expression (Figure 6b). The protective effect of Nup62 knock-down is not caused by changes in FUS expression or localization, as the nuclear levels and the nucleo-cytoplasmic (N/C) ratio of FUS-P525L was unchanged upon Nup62 knockdown (Figures 6c and 6d). Further, Nup62 knockdown did not affect FUS-P525L puncta

(Figures S16b, c), which may represent RNA granules or complexes of a non-pathological nature.

Finally, we probed for nuclear morphology abnormalities in post-mortem human ALS-FUS cases with either the H517Q or R521C mutation (Supplemental Table 1). Immunohistochemistry (IHC) of these ALS-FUS cases showed instances of abnormal nuclear morphology associated with cytoplasmic FUS inclusions. In contrast to the round shape of a control nucleus containing nuclear FUS signal (brown), some mutant FUS nuclei deviated from a round or circular shape. These neurons also exhibited cytoplasmic mislocalization of FUS (Figure 6e, top panel). To better visualize this, immunofluorescence for FUS with DAPI counterstain was analyzed using confocal microscopy, which further resolved the unusually misshapen nuclear morphology in FUS inclusion-bearing cells (Figure 6e, bottom panel). In a separate IHC analysis, we probed the FUS R521C case, which contains pathological FUS inclusions⁴¹ for Nup62. Obvious Nup62 inclusions were not detected in this case. Although this case also showed some lower motor neurons with misshapen nuclear Nup62 staining, at least a few similar cells were seen in the normal controls (Figure S17). Taken together, nuclear abnormalities appear to be a feature of FUS-mediated ALS, a phenotype that is attenuated by targeting Nup62 expression.

Modulation of Nup62 expression rescues FUS-induced toxicity *in vivo*.

Using the above *Drosophila* models of FUS-induced toxicity, we assessed whether knockdown of Nup62 in fly neurons could also provide a survival benefit. The RFP-tagged FUS animals did not survive to the stage of eclosion. To circumvent this issue, a pan-neuronal conditional expression driver (ElavGS-gal4) was used to induce transgene expression in Day-1 adults. Indeed, Nup62 RNAi significantly improved the survival of both WT and P525L-expressing flies (Figure S18). In contrast to the RFP-tagged FUS animals, *Drosophila* models of FUS WT, R518K and R521C constitutively expressing FUS under a motor neuron driver survived to adulthood, albeit with shortened survival compared to non-transgenic animals. Animals expressing ALS-linked FUS variants exhibit shortened survival curves compared to FUS WT animals (Figure 7a-d), particularly in the case of FUS R521C mutant animals. While Nup62 RNAi significantly improved survival in all fly lines, the protective effect of Nup62 knockdown was most pronounced in FUS R521C flies (Figure 7c). Further, mutant FUS animals exhibit crumpled wing morphologies, which may reflect locomotor dysfunction during eclosion⁴². This phenotype was also ameliorated with Nup62 RNAi (Figure 7e). Collectively, our data demonstrate a synergistically toxic interaction between mutant FUS and Nup62 *in vivo*.

DISCUSSION

Emerging evidence indicates that the NCT pathway may play a global role in neurodegenerative disease, having been implicated in Alzheimer's disease, FTD, ALS, Huntington's disease, and others^{4,9-11,15,16,27}. Across multiple model systems for these disorders, defects in the NCT pathway can manifest as aberrant nucleocytoplasmic compartmentalization of transport factors, "leaky pores", impaired NCT, reduced NPC density, and irregular nuclear envelope morphology⁹. Here, we uncovered NCT-related

phenotypes in models of ALS-linked FUS. Of note, the reduction in Ran N/C ratio observed here for FUS M511Nfs neurons is comparable to the decrease reported for ALS-C9orf72 human neurons ¹¹. In the case of mutant FUS, the magnitude of the NCT-related defect may correlate with disease severity in human ALS. For example, the Ran gradient was disrupted in neurons expressing FUS M511Nfs or R495X, variants that cause early onset and aggressive disease in humans, but not in neurons expressing FUS R521G ^{22,23}. As our studies were carried out on a laboratory time frame with endogenous FUS expression, certain phenotypes may not be manifest for moderate variants but could become detrimental with age and *in vivo*.

While NCT abnormalities have been associated with multiple forms of ALS, the underlying mechanism(s) remain ambiguous ⁹. Dipeptide repeat sequences derived from C9orf72 directly associated with nucleoporins *in vitro* and appeared to “clog” nuclear pores ⁴³. However, direct obstruction of NCT by these peptides was not recapitulated in cell culture assays, including in iPSC-derived motor neurons ⁴⁴. Our data also do not support direct obstruction of NPCs by mutant FUS. Results of our PLA between FUS and various Nups revealed interaction signals in the nucleus and the cytoplasm, but not localized to the nuclear membrane where NPCs reside. In fact, the localization of the PLA signals was strikingly different between mutant and control neurons, implicating cytoplasmic interactions between mutant FUS and Nups in our observed NCT-related defects (Figure S19).

In contrast to most interactions of FUS ^{26,29}, FUS and Nup interact directly and more strongly in the absence of RNA. Relatively high levels of RNA in the nucleus favor liquid droplet formation of various RNA binding proteins, including FUS. Conversely, RBPs exhibit poor solubility in cellular environments with low RNA concentrations, such as the cytoplasm ^{32,45}. Here, FUS liquid droplet formation was perturbed by Nup62, leading to the formation amorphous assemblies. In this regard, the effects of Nup62 on FUS contrast starkly with the solubilizing effects of nuclear transport receptor karyopherin- β ⁹. Further, RNA inhibited the formation of FUS/Nup62 amorphous assemblies. These data support a model whereby FUS and FUS/Nup complexes are in the soluble, liquid phase within the nucleus, but enter a less dynamic (e.g., gel-like) phase in the cytoplasm. This model explains how FUS variants can differentially interact with Nups *in situ*, but not necessarily *in vitro* (Figure S19). For isolated proteins, the effects of Nup62 on the phase separation of FUS, and vice versa, were indistinguishable between WT and ALS-linked FUS. Consistent with a report of other NLS-FUS variants, the LLPS behavior of isolated WT and FUS M511Nfs were the same in our studies ³¹. Collectively, these data indicate the mislocalization of mutant FUS into the cytoplasm, an environment with relatively low RNA levels, rather than a physiochemical property of the mutant protein itself accounts for the differential interaction of mutant FUS and Nups compared to FUS WT (Figure S19).

Aberrant mutant FUS/Nup interactions may underlie the observed NCT-related defects in our human neurons. Upregulated FUS expression in mutant FUS human neurons may exacerbate the formation of mutant FUS/Nup cytoplasmic complexes ²³. Expression of mutant FUS also led to enhanced cytoplasmic localization of the Nup proteins, as determined by our PLA. Interactions between FUS and Nup62 have the potential to form

amorphous assemblies reminiscent of insoluble oligomers or small aggregates. Despite being long-lived, Nups are robustly synthesized proteins, a fraction of which remain in the cytoplasm until their insertion into NPCs by a piecemeal mechanism in post-mitotic cells⁷. Insoluble cytoplasmic FUS/Nup62 complexes could physically trap Nup62 and other Nups in the cytoplasm (Figure S19). These species could also be targeted by the proteasome thereby prohibiting proper Nup assembly into NPCs. Diminished nuclear pore signal in our ALS-FUS neurons may account for the reduced nucleocytoplasmic transport capacity in these cells. Indeed, a recent study demonstrated that overexpression of POM121 restored normal expression of certain nucleoporins, which in turn ameliorated NCT defects in ALS-C9orf72 human neurons¹⁴. Therefore, it seems logical that the number of functional NPCs would correlate with nucleocytoplasmic capacity.

The toxic nature of the mutant FUS/Nup interaction is underscored by results from *Drosophila* models of acute FUS toxicity. While overexpression of FUS is toxic, overexpression of Nups exacerbated this toxicity, possibly by driving the formation of toxic FUS/Nup complexes (Figure S19). Further, over-expressed ALS-linked FUS in fly motor neurons caused more severe phenotypes than FUS WT (e.g., higher incidence of both nuclear membrane distortions and crumpled wings, as well as shortened survival). In all experiments, knock-down of Nups attenuated toxic FUS-mediated phenotypes *in vivo*. We speculate that FUS/Nup interactions may become toxic *in vivo* in acute models of toxicity because endogenous levels of RNA cannot mitigate aberrant FUS/Nup phase transitions (Figure 4). It follows that knocking down one of the toxic interaction partners (e.g., Nups) reduces the opportunities for FUS/Nup complexes to form, thereby attenuating toxic FUS-mediated phenotypes in animals.

We cannot exclude indirect contributions of mutant FUS NCT decline. For instance, mutant FUS has been shown to activate stress-response pathways^{23,46}. Nuclear pore integrity and the nucleocytoplasmic distribution of transport factors are affected by elevated levels of cellular stress, including reactive oxygen species and excitotoxicity^{8,47}. The results of our PLA data also reveal interactions between FUS and Nups in the nucleus of control cells. Given that both FUS and Nups are involved in nuclear functions unrelated to NCT, such as DNA repair and transcription^{17,48}, we speculate that normal biological functions could rely on nuclear FUS/Nup interactions. By extension, reduced FUS/Nup interactions in the nucleus could contribute to NCT-related defects through a loss-of-function mechanism. FUS WT/Nup interactions warrant further investigation, also in light of our results showing that knock-down of Nups attenuates toxicity induced by FUS WT overexpression in flies.

Selective inhibitors of exportin-1 (SINE) are currently being tested in humans with ALS. While these inhibitors modulate nuclear export, thus preventing aberrant misexpression of proteins in the cytoplasm, they may also exert therapeutic effects through other mechanisms⁴⁹. Interestingly, genetic downregulation of exportin-1 was neuroprotective in a *Drosophila* model of FUS overexpression⁵⁰, consistent with our results showing protective effects of Nup knockdown in two different FUS *Drosophila* models. Taken together, our results implicate impaired NCT in FUS-mediated ALS and support the NCT pathway as a viable therapeutic target for multiple causes of neurodegeneration.

MATERIALS AND METHODS

Additional methods details can be found in the accompanying Life Sciences Reporting Summary.

iPSC culture, characterization and spinal motor neuron differentiation

iPSC culture maintenance, characterization and differentiation were performed as previously described in ²³. Before differentiation, iPSCs were confirmed pluripotent by immunofluorescence analysis with OCT4 and SOX2 pluripotency markers as described on our previous report ²³ (Figure S2a). In brief, an embryoid body based small molecule approach ⁵¹ was adapted to differentiate iPSCs to spinal motor neurons, consistently yielding around 40% of ISL1/2 positive motor neurons (Figure S1b). Neuron cultures were maintained in motor neuron medium for 6-8 weeks (unless stated otherwise) and media was half-changed every 3-4 day. Cultures were treated with 1 μ M AraC (Sigma, C6645) to eliminate dividing progenitors at DIV 4. A detailed description of the media components and differentiation protocol used here has been described by us previously ²³.

iPS cell engineering of FUS R495X lines

The FUS R495X line and isogenic control lines were generated as follows. Euploid (46;XY) KOLF2.1 iPSC cell line was derived from KOLF2-C1 cells ⁵² in which a 19 base pair deletion in exon 5 of ARID2 was repaired by gene editing. The culture and editing of KOLF2.1 cells was performed essentially as described ⁵². The FUS R495X single nucleotide variant (C>T) was generated using a guide RNA that overlaps codon 495 of FUS and a 100-nt single-stranded oligonucleotide (ssODN) donor template. Briefly, pre-assembled Cas9 RNP (2 μ g IDT HiFi V3 Cas9 and 1.6 μ g Synthego synthetic modified sgRNA) and 200 pmol ssODN (Alt-R™ HDR Ultramer; IDT) were added to 20 μ l P3 buffer containing 1.6×10^5 Accutase dissociated iPSCs and nucleofected in a 16-well Amaxa 4D cuvette (Lonza; Primary Cell P3, pulse code 'CA137'). Cells were cultured for three days under cold shock conditions (32°C/5%CO₂) and in the presence of Revitacell (1X; ThermoFisher) and HDR enhancer (30 μ M; IDT) for the first 24 and 48 hrs, respectively. Upon reaching confluence, cells were dissociated and plated at low density to obtain single cell-derived clones. Clones were screened by Sanger sequencing of PCR amplicons to identify the desired homology-directed repair events in FUS.

PCR primer (forward): 5'- GGGAACATAGGGGAATGGGAATATG. PCR primer (reverse): 5'- TGGTCACTTTTAATGGGAACCAGAG.

Sanger sequencing primer (forward): 5'- TCTTTTGTCTAGGGGGTAAC. Sanger sequencing primer (reverse): 5'- TCCACCTAGCCCTCAAATG

Immunofluorescence microscopy for iPSC-derived neurons

Cells plated on the coverslips were fixed with 4% paraformaldehyde (Fisher Scientific, AAA1131336) for 15 min and permeabilized with 1% Triton X-100 (Sigma, T9284) for 10 min at room temperature. Coverslips were blocked with PBSAT buffer (1% BSA and 0.5% Triton X-100 in PBS) for 1 hour and probed with primary antibodies as follows: 1:1000 mouse anti-OCT4 (R&D Systems, MAB17591-SP) ²³; 1:1000 mouse anti-SOX2 (R&D Systems, MAB2018-SP) ²³; 1:200 rabbit anti-Ran (Bethyl Laboratories, A304-297A) ¹⁶; 1:500 rabbit anti-FUS (Proteintech, 11570-1-AP) ²³; 1:500 mouse anti-Islet 1,2

(Development Studies Hybridoma Bank, 39.4D5)²³; 1:400 rat anti-Nup62 (Abcam, ab188413)⁴⁷; 1:200 rabbit anti-Pom121 (Proteintech, 15645-1-AP)¹⁶; 1:2000 mouse anti-mAb414 (Abcam, ab24609)^{16,57}; 1:200 rat anti-Nup98 (Abcam, ab50610)^{14,57}; 1:200 mouse anti-Nup153 (Abcam, ab24700)⁸. After washing, secondary antibodies were diluted at 1:2000 and incubated for 1 hour at room temperature as described⁵³. Conjugated Alexa Fluor-647 anti-Tuj1 antibody (BioLegend, 657405)²³ at 1:400 dilution was used followed by secondary antibody incubations. Cells were counterstained with DAPI and coverslips were mounted with ProLong Gold anti-fade reagent (Invitrogen, P36930).

Image acquisition and processing for iPSC-derived neurons

All fluorescence signals for iPSC-derived neurons were imaged with a Leica DMI 6000 inverted fluorescent microscope equipped with APO 63X/ 1.4 NA oil immersion lens and a filter set for fluorophores in Cy3, Cy5, GFP, and DAPI channels (center/ band width, nm: EX 545/39, 620/60, 470/40, 360/40; EM 605/75, 700/75, 525/45, 470/40). Images were collected with Leica DFC365 FX camera (6.45 μ m pixel size) using AF6000 Leica Software v3.1.0 (Leica Microsystems). Whole cell, 12-bit stacks images with 0.2 μ m step size were acquired (50-70 planes). Immersion oil (Leica, 11513859) with 1.518 refractive index at room temperature was applied to the lens. Coverslips were mounted with ProLong Gold anti-face reagent (Invitrogen, P36930) with a refractive index of 1.46. All images were acquired with identical microscope settings within individual experiments. Brightness and contrast were adjusted equally for all images and cropped insets were generated in the same manner among all the experiments to facilitate visualization of representative cells. For any image adjustment, identical settings were always applied to all cells, irrespective of genotype. Neurons that were clumped, overlapping or obscured by debris were excluded from the quantification.

Image analysis and quantification for iPSC-derived neurons

Ran nuclear-cytoplasmic ratio analysis: After immunostaining, fluorescent signals of the whole cells were collected using z-stacks and the maximum projected images from the center 5 planes were created for nuclear-cytoplasmic Ran ratio analysis. Nuclear and cytoplasmic areas were defined using DAPI and anti-Tuj1 (reactive for class III beta-tubulin) fluorescent signals respectively. Number of cells used for this analysis: pooled controls, n=151; M511Nfs, n=117; R521G, n=63, M511Nfs*Cor, n=103; M511Nfs, n=90 cells. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications^{11,16}. Data collection and analysis were not performed blind to the conditions of the experiments.

Nuclear pore cluster analysis: Neurons were semi-permeabilized with ice-cold permeabilization buffer (80 mM PIPES pH 6.8, 5 mM EGTA, 1 mM MgCl₂, 0.5% Triton X-100) on ice for 3 min and washed three times with DPBS (Corning, 21-031-CV). Coverslips were fixed and immunostained as described above. Fluorescent signals of the whole nuclei were collected using z-stacks and the center plane image (with the widest DAPI staining) was exported for the following image processing. Fluorescent signals were thresholded using the same parameter within experiments (differentiations) and

binary images were created for cluster analysis using ImageJ software. Numbers of areas represent numbers of clusters. Number of nuclei used for this analysis: M511Nfs*Cor, n=152, M511Nfs, n=144 nuclei. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications¹⁴. Data collection and analysis were not performed blind to the conditions of the experiments.

PLA analysis: After PLA staining, fluorescent signals of the whole cells were collected using z-stacks and the maximum projected images from the center 10 planes were created for nuclear-cytoplasmic PLA ratio analysis. Nuclear and cytoplasmic areas were defined using DAPI signals and phase contrast respectively. Maximum projected images from the whole cells were created for total PLA intensity measurements. Soma boundaries were defined using phase contrast images. Number of cells used for this analysis: M511Nfs*Cor, n=69; M511Nfs, n=68 cells. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications^{54,55}. Data collection and analysis were not performed blind to the conditions of the experiments.

Fluorescence recovery after photobleaching (FRAP)

For FRAP analysis in iPSC-derived neurons, cells plated on coverslips were transduced with high titer lentivirus expressing NLS-tdTomato-NES shuttling reporter at DIV 3 (MOI= 5). The tdTomato shuttling reporter construct was a kind gift from Dr. Martin Hetzer (Salk Institute, USA) and re-cloned to a lentiviral vector as described in²⁵. Phenol-free neurobasal medium (Invitrogen, 12348-017) was used in motor neuron medium. At DIV 55-60, coverslip was transferred to a 12mm coverslip chamber (Bioscience Tools, CSC-12) and FRAP procedures as previously described⁵³ were performed. In brief, experiments were carried out on a Leica SP5 II laser scanning confocal microscope using 40x 1.3 NA water immersion objective. For every neuron, one pre-bleached image was collected and tdTomato signals in the whole nucleus were bleached for 10 iterations of 100% 561nm laser power. Recovery of the fluorescence signals was monitored by collecting 150 post-bleached images spaced at 3 sec intervals. The fluorescence intensity was measured by drawing regions of interest in the bleached area (whole nucleus), the whole cell, and in background area without cells at each time point using AF6000 Leica Software. Because the entire field of view including the nucleus and the cell soma are exposed to continuous pulses of light, gradual photobleaching precludes a full FRAP recovery. Datasets were generated in excel and analyzed using easyFRAP-web tool⁵⁶ (version 1.8, <https://easyfrap.vimnet.upatras.gr>). To account for global bleaching during image acquisition, each post-bleached fluorescence intensity was normalized to the fluorescence intensity of an unbleached area at each timepoints. Fluorescence recovery curves were plotted using GraphPad Prism v8.1.2. Each time points represented means $\pm$ s.e.m.. Exponential one-phase association curve fitting was used to calculate area under the curve as described^{11,16,57}. Approximately 20 neurons were imaged per line per differentiations. Neurons at DIV18-22 and DIV 56-60 were considered as young and old neurons, respectively. Three independent neuron differentiations were performed. Data collection and analysis were not performed blind to the conditions of the experiments.

For FRAP analysis of co-mixing reactions with recombinant proteins, experiments were carried out on the same scope as above but with a 60x oil objective. One prebleach image was collected and Alexa555 signal in bleach region (~0.7-0.9 μm diameter) was bleached for 8 iterations (1.293 s each) using 561nm laser at 100% intensity. After bleaching, 300 images were acquired every 1.293s. Fluorescence intensity was measured in the bleach region, an unbleached region, and a background region outside droplet/amorphous assembly at each time point using AF6000 Leica Software. Fluorescence recovery curves were generated after correcting for background signal and photofading using a custom Python script based on equations described in easyFRAP-web tool ⁸⁴ (<https://easyfrap.vimnet.upatras.gr>). Fluorescence recovery curves were plotted using GraphPad Prism v8.1.2.

Western blotting

Cells were lysed with RIPA buffer (Westnet, BP-115-500) supplemented with fresh protease inhibitors (Roche, 11873580001). Lysates were incubated on ice for 15 min and cleared through centrifugation (16000 g) for 15 min at 4°C. Supernatants were collected and protein concentration were determined using BCA assay kit (Thermo Scientific Pierce, 23227). Western Blotting analysis were performed as described ²³. After blocking, blots were incubated with primary antibodies overnight at 4°C. Primary antibodies were used as follows: mouse anti-FUS (1:200, Santa Cruz, SC-373698; validated in Supplemental Figure 7); rabbit anti-Nup62 (1:1000, Bethyl Laboratories, A304-942A; validated in Supplemental Figure 7); mouse anti-Tuj1 (1:1000, BioLegend, 801201); mouse anti-maltose binding protein (Cell Signalling Technology, 2396; validated in Supplemental Figure 7). Followed by washes, blots were incubated with secondary antibodies as described ²³ and visualized using Odyssey Infrared Imager (LiCor, 9120). Image J was used for densitometry measurements. Data collection and analysis were not performed blind to the conditions of the experiments.

Immunoprecipitation

Immunoprecipitation (IP) analysis from cell lysates was performed as previously described ²⁶. In brief, cells were lysed with IP lysis buffer (1% Triton X100, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10% Glycerol pH 7.5, fresh protease inhibitors) and cleared through centrifugation (16000 g) for 15 min at 4°C. Protein concentration were determined using BCA assay kit (Thermo Scientific Pierce, 23227) and 500 μg - 1 mg of lysates were subjected to IP. Magnetic Dynabeads protein G (Invitrogen, 10003D) were washed and charged with 40 μg rabbit anti-FUS antibody (Genscript, lab made), 3 μg rabbit anti-Nup62 antibody (Bethyl Laboratories, A304-942A) or 3/40 μg Chrompure rabbit IgG whole molecule (Jackson ImmunoResearch, 011-000-003) for 2 hr at 4°C by rotation. Charged beads were washed and 500 μg - 1 mg of lysates were incubated with the charged beads overnight at 4°C by rotation. To test if the interactions were RNA-mediated, lysates along with beads were treated with or without 50 $\mu\text{g}/\text{mL}$ RNase A (Invitrogen, 12091021) and incubated at 37°C for additional 30 min after the overnight incubation. Supernatants were discarded and beads were washed three times thoroughly with IP buffer to eliminate unbound proteins. Beads were boiled at 95°C for 10 min in 1x SDS sample buffer (Westnet, BP-111R) to elute bound proteins. Proteins were subjected to western blotting analysis as described.

IP analysis on recombinant proteins were performed as follows. MBP-Nup62 (150 nM) was mixed with MBP-FUS (75 nM) or MBP (75 nM) and incubated at room temperature for 30min. The protein mixtures were then incubated with protein A magnetic beads (SureBeads, Biorad) pre-charged with anti-Nup62 antibody (Bethyl Laboratories, A304-942A) for 2h. After washes, proteins were eluted by heating the beads in Laemmli buffer at 95°C for 5min and analyzed by Western blot. To assess non-specific binding of FUS to the beads, similar experiment with FUS was performed in the absence of Nup62. Data collection and analysis were not performed blind to the conditions of the experiments.

Proximity ligation assay

In situ protein-protein interactions were detected using Duolink proximity ligation assay (PLA) reagents according to manufacturer's instructions. In brief, cells were fixed with 4% PFA and permeabilized with 1% Triton-X100. Following by blocking with Duolink Blocking Solution (Sigma, DUO82007) for 1 hour at 37°C, cells were incubated with primary antibodies (as described in the Immunofluorescence staining section) diluted with Duolink antibody diluent (Sigma, DUO82008) for 1 hour at room temperature. Duolink PLA secondary probes (anti-rabbit IgG PLUS, DUO92002; anti-mouse IgG MINUS, DUO92004; anti-rat IgG MINUS probes were generated using Duolink Probemaker kit DUO92101 based on manufacturer's protocol.) were applied to the cells for 1 hour at 37°C. PLA probe hybridization was performed by incubating cells with ligation mix for 30 min at 37°C. Amplification was followed by 100 min, 37°C incubation with polymerase: Texas Red oligonucleotides mix. Components of ligation and amplification were from Duolink In Situ Detection Red kit (DUO92008). Coverslips were mounted with Duolink mounting medium with DAPI (DUO82040). Data collection and analysis were not performed blind to the conditions of the experiments.

Plasmid construction

Plasmid encoding MBP-FUS WT on pTHMT backbone was obtained from Addgene (#98651). All plasmid constructs for recombinant protein expression were constructed onto this vector. Nup62, FUS M511Nfs and MBP DNA sequence were synthesized as gBlocks Gene Fragments (Integrated DNA Technologies) and assembled into vector using NEBuilder HiFi DNA Assembly (New England Biolabs).

Expression and purification of recombinant proteins

All the proteins were expressed in *E.coli* Rosetta (DE3) (Novagen). Bacterial cultures were grown at 37°C until OD=0.4, induced for protein expression with 1 mM IPTG and further grown for 16-18h at 16°C. Cells were harvested by centrifugation and stored frozen at -80°C. Only full-length FUS and Nup62 proteins were used herein.

Frozen cell pellet expressing MBP and MBP-Nup62 were thawed on ice, resuspended and sonicated in ice cold lysis buffer (20 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.6) supplemented with protease inhibitor (cOmplete, EDTA-free protease inhibitor tablet, Sigma), lysozyme and RNase A (100 µg/ml). Lysate was clarified by

centrifugation at 26,000xg for 30min, 4°C and incubated with Ni-sepharose resin (GE Healthcare) pre-equilibrated with lysis buffer for 2h at 4°C on end-to-end rotor. After washes with lysis buffer containing 40 mM imidazole, the protein was eluted in 20 mM sodium phosphate, 150 mM NaCl, 500 mM imidazole, pH 7.6. Peak fractions were pooled and further purified by size exclusion chromatography using HiLoad 16/600 Superdex 200pg (GE Healthcare) equilibrated with 20 mM Tris/Cl, 150 mM NaCl, pH 7.4 at room temperature. Fractions containing the protein of interest were pooled, concentrated using centrifugal filter (10kDa MWCO, Vivaspin) and stored at -80°C.

MBP-FUS WT and M511Nfs were purified as described in ⁵⁸. Cell lysate was prepared by sonication in ice cold lysis buffer (20 mM sodium phosphate, 1 M NaCl, 10 mM imidazole, pH 7.6 supplemented with protease inhibitor (cOmplete, EDTA-free protease inhibitor tablet, Sigma) and lysozyme. Protein was first purified using Ni-sepharose resin (GE Healthcare) pre-equilibrated with lysis buffer by batch/gravity method at room temperature. Peak fractions were pooled and further purified by size exclusion chromatography using HiLoad 16/600 Superdex 200pg (GE Healthcare) equilibrated with 20 mM sodium phosphate, 1 M NaCl, pH 7.4 at room temperature. Fractions containing pure MBP-FUS were pooled, concentrated using centrifugal filter (10 kDa MWCO, Vivaspin) and stored in 25% glycerol, 20mM sodium phosphate, 1 M NaCl, pH 7.4 at -80°C.

Fluorophore labelling of recombinant proteins

MBP-FUS WT and M511Nfs were labelled with Alexa-555 maleimide (ThermoFisher Scientific), MBP-Nup62 with Alexa-488 maleimide (ThermoFisher Scientific) and MBP with Alexa-488 TFP ester (ThermoFisher Scientific) according to manufacturer's instructions. Unreacted dye molecules were removed by two serial buffer exchanges using spin desalting columns (7k MWCO, Zeba, ThermoFisher Scientific).

***In vitro* phase separation assay**

MBP-FUS WT and M511Nfs samples were doped with $\leq 2.5\%$ of the respective Alexa-555 labelled proteins. MBP-Nup62 and MBP samples were doped with $\leq 2.7\%$ and $\leq 1\%$ respectively of the corresponding Alexa-488 labelled proteins. MBP-FUS WT (1.5 μ M) or M511Nfs (1.5 μ M) was mixed with MBP-Nup62 (1.5 μ M) and phase separation was initiated by dilution into buffer containing PEG-8000 (USB corporation). The final composition of the phase separation buffer was 20mM Tris/Cl, 158mM NaCl, 3.6% PEG-8000, 3.45% glycerol, pH 7.4. Samples were deposited on 384-well clear plate (ibidi), sealed with plastic film and shaken at 250 rpm at room temperature.

To assess the effect of RNA on FUS/Nup62 phase separation, torula yeast RNA (0.125 mg/ml) was added before addition of PEG such that mass ratio of RNA:FUS:Nup62 $\approx 1.5:1:1$. RNA stock was prepared by dissolving 10mg of torula yeast RNA (Sigma Aldrich) in 1ml of 20mM Tris/Cl, pH 7.4 on ice. After centrifugation at 13,000 rpm, 10min, 4°C and dissolved RNA was buffer exchanged into 20mM Tris/Cl, pH 7.4 using spin desalting columns (7k MWCO, Zeba, ThermoFisher Scientific). RNA concentration was estimated from absorbance at 260nm measured using Nanodrop spectrophotometer (ThermoFisher Scientific).

Images were acquired under 40X air objective with Leica DFC365 FX camera on Leica DMI 6000 inverted fluorescent microscope using AF6000 Leica Software v3.1.0 (Leica Microsystems). Images of RNA experiments were acquired using Nikon Ti E microscope with a cooled CMOS camera (Andor Zyla). Acquisition settings for each protein was same at each time point in the timecourse experiments. Brightness and contrast were adjusted equally across sample conditions in each channel to facilitate visualization of protein species. Full field of view images are shown in the figures. Images of RNA experiments and insets represent cropped and zoomed images of representative protein species. Data collection and analysis were not performed blind to the conditions of the experiments.

***Drosophila* stocks and maintenance**

Transgenic UAS-FUS WT, R518K and R521C lines were generated by the Pandey lab³⁹. The RFP-tagged FUS-WT and FUS-P525L lines were a generous gift from Dr. Jane Wu at Northwestern University. RNAi lines were obtained from Vienna *Drosophila* Research Center for Nup62 (Stock # 100588 KK), Nup54 (Stock # 103724 KK), Nup98 (Stock # 109279 KK), Nup37 (Stock # 109814 KK), Nup43 (Stock # 33645 GD), Nup153 (Stock # 107750 KK), Nup205 (Stock # 38610 GD) and Nup214 (Stock # 41964 GD). All stocks were maintained on standard dextrose media on a 12-hour light/dark cycle.

***Drosophila* assays**

To assess external eye degeneration, GMR-gal4 driver was used to selectively drive expression in fly eyes at 25°C. Quantification of external eye phenotype of 1-day old female flies was performed as previously published⁵⁹. Data collection and analysis were not performed blind to the conditions of the experiment. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications^{10,39,60,61}.

To assess nuclear membrane morphology in *Drosophila* VNC neurons, D42-gal4 driver was used to selectively drive expression in motor neurons at 25°C. Third instar larval VNC was dissected out and fixed in 4% paraformaldehyde for 20 minutes at room temperature. For immunofluorescence, fixed VNCs were blocked for 1 hour in blocking buffer (5% normal goat serum in 0.2% PBS-Triton X-100). The tissue was incubated in primary antibody overnight (1:100 Mouse anti-Lamin C, DSHB LC28.26) followed by incubation in secondary antibody for 2 hours at room temperature (1:500 Goat anti-mouse Alexa Fluor 647). This Lamin C antibody was validated previously in *Drosophila* brains⁶². The tissues were mounted on slides using Fluoroshield mounting medium (Sigma F6182). Confocal images were acquired on Nikon A1 Confocal microscope at 100X magnification. Quantification of percentage of cells with nuclear invaginations and nuclear-cytoplasmic FUS intensities were performed on ImageJ (NIH). Nuclei were considered to be positive for nuclear invagination if the nuclear membrane (Lamin C staining) visibly extended into the nuclear interior as previously described⁶². For quantification of number of P525L-RFP puncta per neuron, an ROI was assigned to each cell body and ImageJ threshold tool was used to convert puncta (>0.3µm) into pixels, followed by measurement of the number of puncta. Data collection and analysis were not performed blind to the conditions of the

experiment. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications ^{10,39}.

Survival assay:

F1 adult female flies expressing either FUS-WT, R518K and R521C driven by motor neuron driver (D42-gal4) or FUS WT-RFP and FUS P525L-RFP driven by conditional pan-neuronal driver (ElavGS-gal4) were used for survival analyses. For conditional expression, adults were grown on media containing 20mM RU486 (Mifepristone, Cayman Chemicals) to induce transgene expression starting at day-1 post-eclosion. Flies were grown at 25°C and moved to fresh media every 3-4 days. Death was recorded every other day until all flies from the experimental groups were dead. Kaplan-Meier analysis of percentage survival and graphing the survival curve were performed on Prism Graphpad. Data collection and analysis were not performed blind to the conditions of the experiment. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications ^{39,60,61}.

RNA extraction from *Drosophila* heads

Adult heads from transgenic overexpression or RNAi flies under the GMR-gal4 driver were used for RNA extraction. Fly heads were flash-frozen on dry ice and crushed using a pestle followed by lysis in TRIzol. RNA was extracted using bromo-3-chloropropane separation followed by alcohol precipitation. Contamination from genomic DNA was removed by treatment with DNaseI (Invitrogen) in accordance with manufacturer's instructions. 500ng of RNA was used for cDNA synthesis using iScript Select cDNA Synthesis kit (Biorad) followed by quantitative real-time PCR (qRT-PCR) assays performed using iQ Supermix (Bio-rad) following manufacturer's instructions. All reactions were run on StepOnePlus and cycle threshold (Ct) values were used for determining fold change differences by comparative Ct analysis as previously described ⁶³. Data collection and analysis were not performed blind to the conditions of the experiment. α -tubulin served housekeeping control for normalization of Ct values. *Drosophila* gene-specific primers and probes were designed using PrimerQuest primer design tool (Integrated DNA Technologies) and listed below:

Nup54

Forward: 5'-GAGTGAGCTGACAGAACTCAAG-3'
Reverse: 5'-CTCGGCCAGTTTCCGTTTAT-3'
Probe: 5'-CCACTGCCACAGCGAAGATACTTGA-3'

Nup62

Forward: 5'-CTTGCTGTTGTCTGCATCTC-3'
Reverse: 5'-CAGCACCAGCTTCAGGA-3'
Probe: 5'-ACATTCTCTTTCGGAACACCGGCA-3'

Nup98

Forward: 5'-GTCTTCTTCGGCAAGGAAATG-3'
Reverse: 5'-TCGTCGTCGGGATAAATGATAA-3'

Probe: 5'-ACCTCGATG/ZEN/AGATTGTCCACTTTTCGC-3'

Nup214

Forward: 5'-CCTAAGTGAGGACAAGGATGAG-3'

Reverse: 5'-GGCATAGTCTGCAGCTTCTT-3'

Probe: 5'-TGCCTTCGACACTTCTACAACGCA-3'

α -tubulin

Forward: 5'-ACCAGCCTGACCAACATG-3'

Reverse: 5'-CCTCGAAATCGTAGCTCTACAC-3'

Probe: 5'-TCACACGCGACAAGGAAAATTCACAGA-3'

Human Tissue Staining

Formalin fixed paraffin embedded tissue sections (5 μ m thick) were acquired through the Alzheimer's Disease Research Center at the Massachusetts General Hospital (Charlestown, MA) or the University of British Columbia (⁴¹, Vancouver, Canada). Available demographics information for the three de-identified cases used in the current study can be found in Supplemental Table 1. Tissues were stained and processed by multiple approaches. For the images in the main text Figure 7, the protocol described in Lee et al was used with anti-FUS (Sigma Aldrich, HPA008784), together with either a hematoxylin nuclear counterstain for immunohistochemistry or a DAPI nuclear counterstain for immunofluorescence ⁶⁴. For the images in Supplemental Figure 17, mouse anti-Nup62 (BD Bioscience, BDB610497) was diluted to 1:150 ⁴⁷. Immunohistochemistry was performed using the Dako Omnis automated immunostainer with primary incubation for 60 mins following heat-induced antigen retrieval and developed using Dako Envision Flex/HRP. Data collection and analysis were not performed blind to the conditions of the experiment

Statistical analysis

For iPSC-based experiments, at least three independent differentiations were performed for all the analyses. One-way ANOVA with multiple comparisons test was performed for analyzing more than two cell lines. Unpaired two-tailed t-test was used across for analyzing differences between the isogenic lines. For fly-based experiments, the following tests were used: Two-tailed unpaired t-test, One-way ANOVA with Tukey's multiple comparison, Kruskal-Wallis test with Dunn's multiple comparison and Log-Rank Mantel-Cox test. Error bars represent standard error of the mean (s.e.m.) for cell culture and fly data, and standard deviation for *in vitro* biochemical data. Statistical analyses were calculated using GraphPad Prism v8.1.2. The test used and resulting p-values are indicated for each analysis in the corresponding figure legend.

Randomization was not applied to the experiments herein. For *Drosophila* studies, the different experimental groups refer to independent genotypes; therefore, there was no randomization used in data sampling. Appropriate controls were always included alongside the experimental conditions.

Data availability statement: No data sets that require mandatory deposition into a public database were generated during the current study. Any data generated and/or analyzed during the current study are available from the corresponding author (DAB) on reasonable request.

Code availability statement: Code used for the FRAP analysis of recombinant proteins is provided in the Supplemental_Script_FRAP.py.

MATERIALS AND METHODS REFERENCES

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AUTHOR CONTRIBUTIONS

Y.-C.L., M.S.K., N.R., U.B.P. and D.A.B. conceptualized and designed the project. Y.-C.L., M.S.K., N.R., E.N.A, A.T.N, B.K., S.C., J.A.M, and W.C.S conducted the experiments. Y.-C.L., M.S.K., N.R., E.N.A, A.T.N, B.K., S.C., J.A.M, W.C.S, I.R.A.M, E.B.L, J.A.N, D.G, U.B.P. and D.A.B. analyzed the data. J.E.L. and N.L.F. provided critical intellectual input. R.L.-G. reprogrammed the control iPSC line designated as C1. D.A.B. wrote the paper with co-first authors and contributions from all co-authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

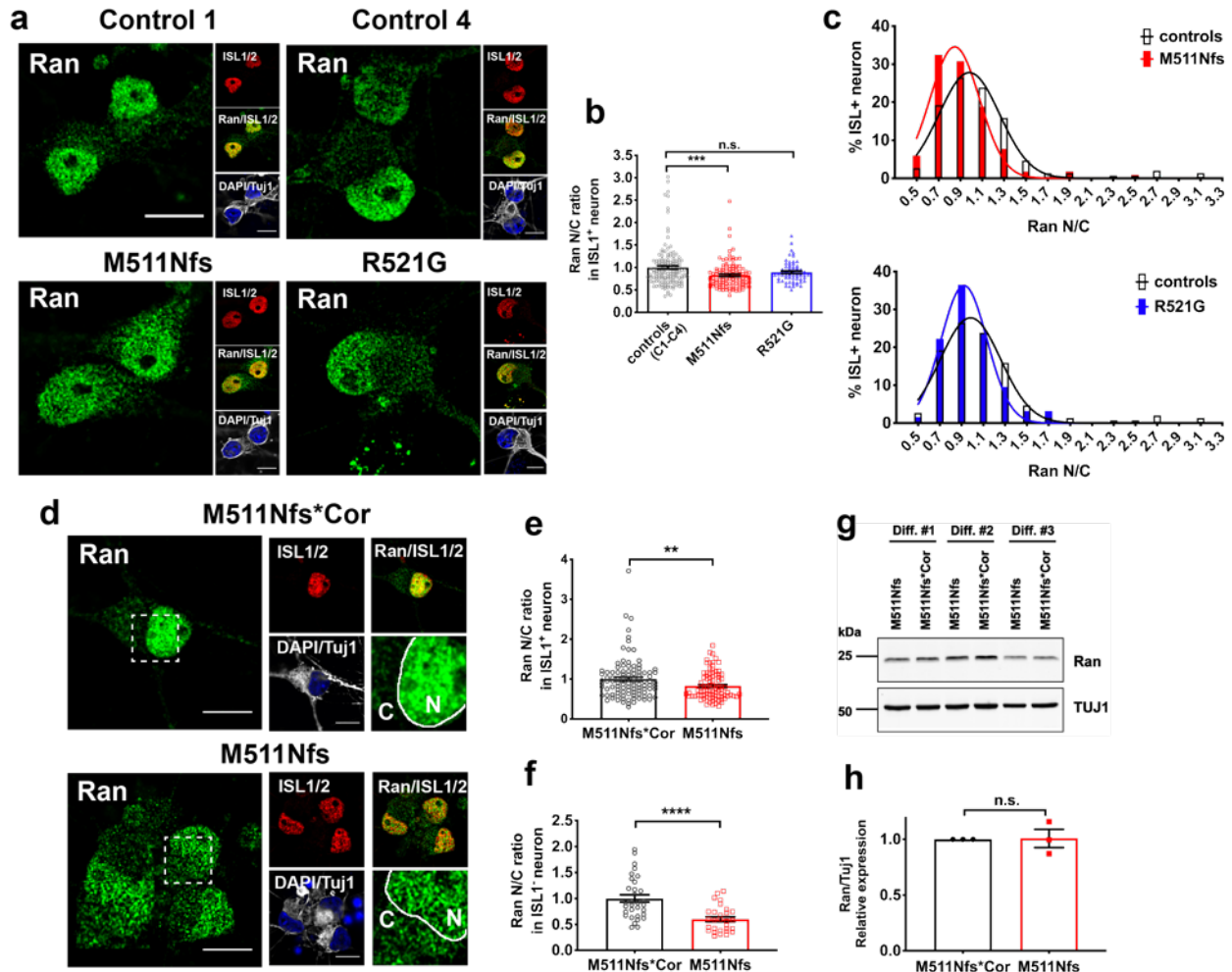


Figure 1. The Ran gradient is perturbed in motor neurons expressing ALS-linked FUS. Immunofluorescence imaging of Ran revealed enhanced cytoplasmic versus nuclear Ran localization in FUS M511Nfs iPSC-derived motor neurons compared to control and FUS R521G motor neurons. Neurons at DIV 36-42 were used and the indicated number of cells (below) were analyzed across at least three independent differentiations (each differentiation represents an experiment). **(a-c)** Four control (C1-C4) and two ALS (M511Nfs and R521G) lines were employed **(a)** Representative immunofluorescence images for neurons derived from two control and two ALS iPSC lines. Staining includes Ran (green), the motor neuron marker Islet1/2 (red), the pan-neuronal marker Tuj1 (anti-TUBB3; grey), and DAPI (blue). Scale bar= 10 μ m. The Ran N/C ratio of the chosen representative neurons: C1 (1.25 and 1.53); C4 (2.12, 0.99); M511Nfs (0.64, 0.69); R521G (1.29). **(b,c)** Quantification of Ran N/C ratio in all six lines (pooled controls, n=151; M511Nfs, n=117; R521G, n=63 cells) reveals a significant

decrease for FUS M511Nfs neurons. Each point in the bar graph represents the average across all cells for an independent experiment. Within each experiment, the ALS lines were normalized to the control lines. One-way ANOVA with Dunnett's multiple comparisons test, *** $p=0.0003$, n.s. $p=0.0967$. $F(2, 328)=7.632$. Error bars represent s.e.m. **(c)** Frequency distribution graphs of Ran N/C ratio for FUS M511Nfs (left) and R521G (right) neurons compared to controls. **(d-f)** Similar analysis as A-D using FUS M511Nfs motor neurons compared to the isogenic, corrected control (M511Nfs*Cor). **(d)** Immunofluorescence staining as in (a). Scale bar= 10 μm . The Ran N/C ratio of the chosen representative neurons: M511Nfs*Cor (1.19); M511Nfs (0.55, 0.58, 0.91). Abbreviation within the zoom insets: N (nucleus), C (cytoplasm). **(e)** Quantification of the Ran N/C ratio as in (b). Ran N/C is significantly lower in M511Nfs versus M511Nfs*Cor neurons (M511Nfs*Cor, $n=103$; M511Nfs, $n=90$ cells). Unpaired two-tailed t-test, ** $p=0.0063$. $t=2.762$, $df=191$. **(f)** Quantification of the Ran N/C ratio in ISL1/2 negative M511Nfs and M511Nfs*Cor neurons (non-motor neurons). Ran N/C is significantly lower in M511Nfs versus M511Nfs*Cor neurons (M511Nfs*Cor, $n=32$; M511Nfs, $n=29$ cells). Unpaired two-tailed t-test, **** $p<0.0001$. $t=4.594$, $df=59$. **(g)** A Western blot analysis of total Ran protein levels in neurons derived from the indicated line; data from three independent differentiations are shown. Tuj1 was used as loading control. Source data are provided as a Source Data file. **(h)** Quantification of (g). Unpaired two-tailed t-test, n.s.= not significant. $t=0.09654$, $df=4$. For all quantifications, data distribution was assumed to be normal but this was not formally tested.

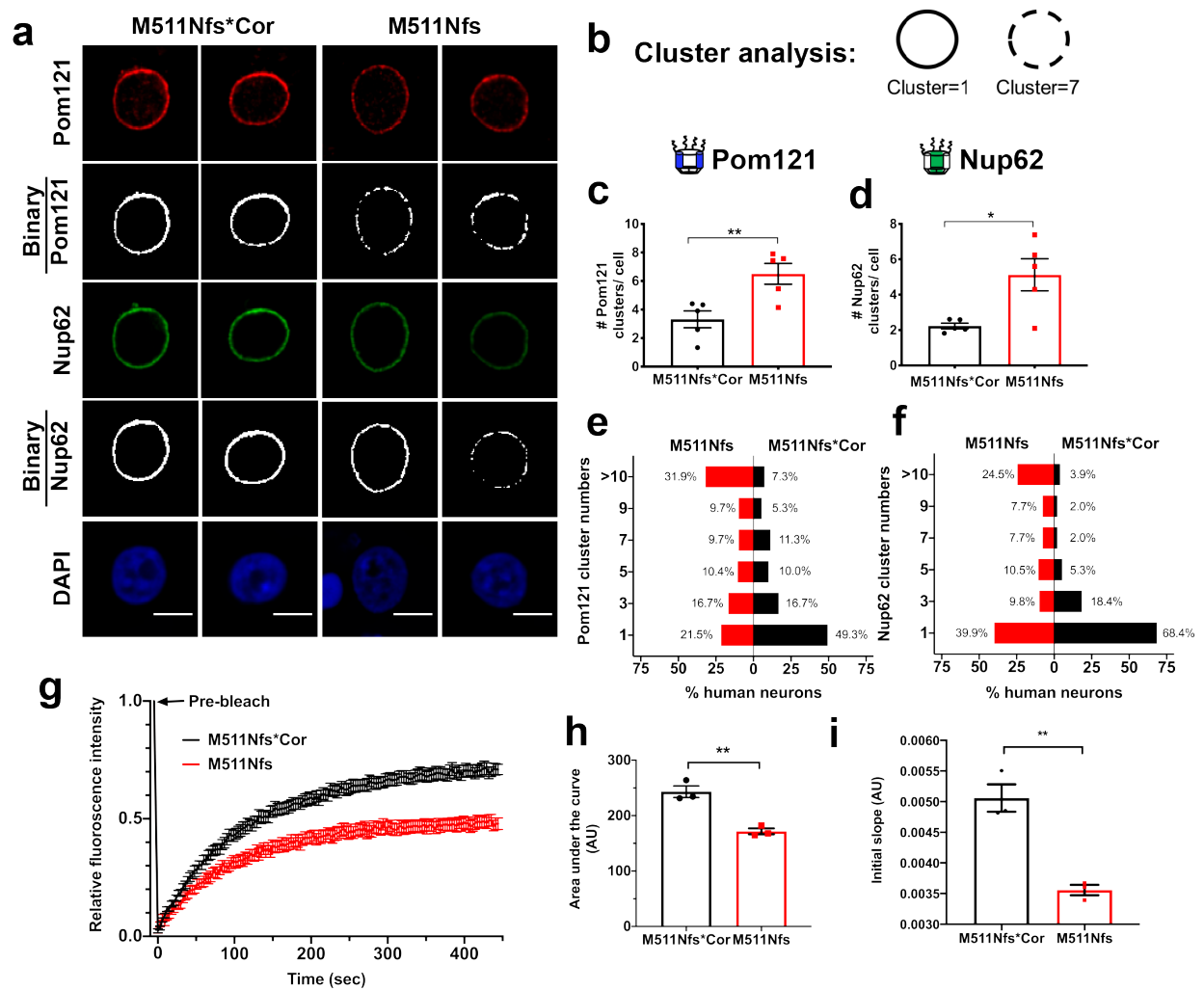


Figure 2. Diminished nuclear pore signal and nucleocytoplasmic transport in ALS-FUS human neurons. (a) Immunofluorescence images of Pom121 (red) and Nup62 (green), representative of $n=5$ experiments, reveals weaker signals in FUS M511Nfs nuclei compared to nuclei from the isogenic control neurons. Binary images (white) derived from the fluorescence images are shown. DAPI (blue). Scale bar = 5 μ m. (b) An illustration for the cluster analysis used to quantify the continuity of Pom121 and Nup62 staining around the perimeter of the nucleus. (c-f) Results of the cluster analysis for Pom121 (scaffold Nup; c, e) and Nup62 (central channel Nup; d, f) for $n=152$ M511Nfs*Cor and $n=144$ M511Nfs nuclei that were analyzed across five independent differentiations (each differentiation represents an experiment) from neurons at DIV 36-45. (c, d): significantly higher cluster numbers in FUS M511Nfs versus M511Nfs*Cor nuclei through analysis of Pom121 (Unpaired two-tailed t-test, $**p=0.0095$, $t=3.391$, $df=8$) and Nup62 (Unpaired two-tailed t-test, $p=0.0137$, $t=3.144$, $df=8$; error bars represent s.e.m.). (e, f): results of the cluster analysis presented in a population pyramid format show the cluster number for every neuron analyzed. Each point in a bar graph represents the average across all cells in that experiment, normalized to M511Nfs*Cor. (g-i) Results of the FRAP experiment with the NLS-tdTomato-NES shuttling reporter in FUS M511Nfs*Cor neurons (black; $n=29$ cells) and M511Nfs neurons (red; $n=36$ cells) across

three independent differentiations. Neurons at DIV 56-60 were used for FRAP experiments. **(g)** FRAP recovery curves. **(h)** The area under the recovery curve was significantly lower in FUS M511Nfs neurons compared to M511Nfs*Cor neurons (Unpaired two-tailed t-test, $**p=0.0036$, $t=6.112$, $df=4$; error bars represent s.e.m.). Each point in the bar graph represents an independent differentiation. **(i)** The initial slopes of the FRAP recovery curves (comprising the first 50 seconds from panel **g**) were determined using a non-linear regression analysis and found to be significantly slower in FUS M511Nfs neurons compared to M511Nfs*Cor neurons (Unpaired two-tailed t-test, $**p=0.0033$, $t=6.271$, $df=4$; error bars represent s.e.m.). For all quantifications, data distribution was assumed to be normal but this was not formally tested.

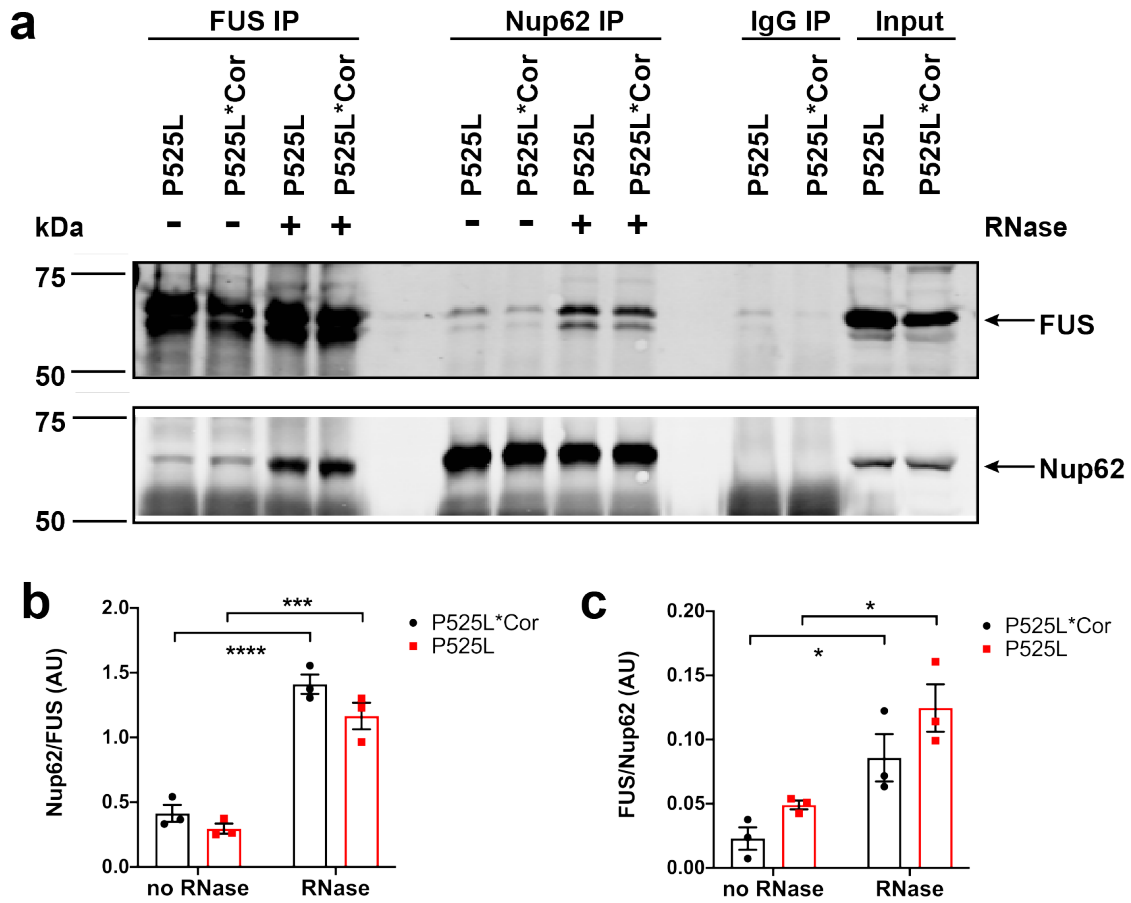


Figure 3. Wild-type and mutant FUS interact with Nup62 in iPSCs. (a) Left: FUS IP. Nup62 was pulled down with wild-type (P525L*Cor) and mutant (P525L) FUS. Middle: Nup62 IP. Both wild-type and mutant FUS were pulled down with Nup62. In both IPs, interactions of FUS and Nup62 were retained and significantly enhanced upon RNase treatment, suggesting the interactions were not mediated through RNA. Right: IgG and input controls for IP. Source data are provided as a Source Data file. (b) Quantification for FUS IP. Densitometry analysis of FUS and Nup62 signals in the Western blot shown in (a). Two-way ANOVA with Tukey's multiple comparisons test. P525L*Cor: **** $p < 0.0001$; P525L: *** $p = 0.0002$. $F(1, 8) = 0.7543$. (c) Quantification for Nup62 IP, as described for (b). Two-way ANOVA with Tukey's multiple comparisons test. P525L*Cor: * $p = 0.0497$; P525L: * $p = 0.0204$. $F(1, 8) = 0.2072$. Only significant comparisons were denoted. (b,c) Each point in the bar graph represents a biological replicate ($n = 3$). Error bars represent s.e.m. Data distribution was assumed to be normal but this was not formally tested.

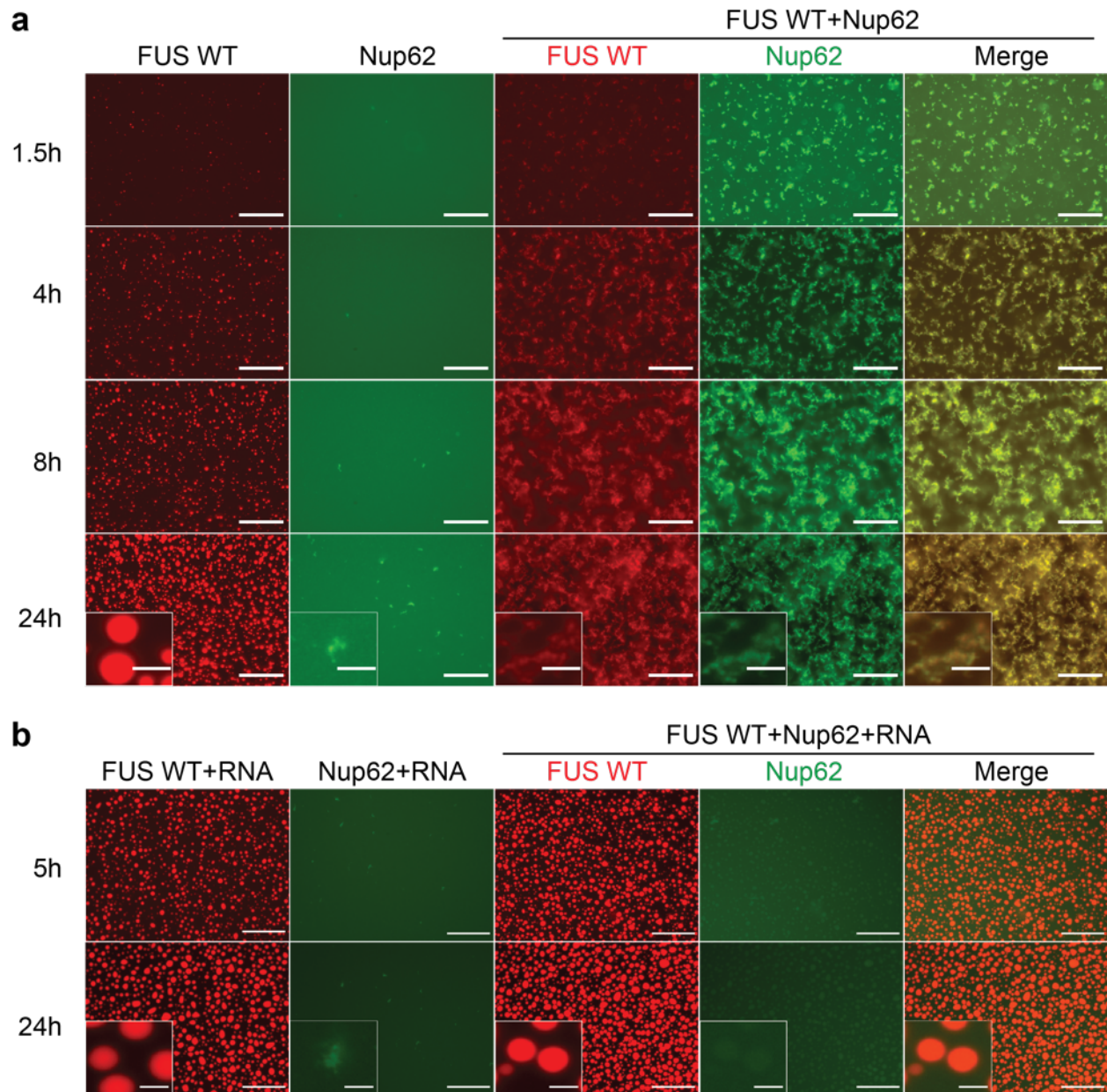


Figure 4. Nup62 and FUS alter the phase separation properties of each other in a manner that is modulated by RNA *in vitro*. Fluorescence images showing phase separation of 1.5 μ M MBP-FUS WT (red) doped with $\leq 2.5\%$ Alexa555-labelled MBP-FUS WT and 1.5 μ M MBP-Nup62 (green) doped with $\leq 2.7\%$ Alexa488-labelled MBP-Nup62. Full-length FUS and Nup62 proteins were used herein. **(a)** MBP-FUS forms dynamic spherical droplets (Supplemental Video 1). Co-mixing MBP-FUS with MBP-Nup62 promotes the formation of FUS/Nup62-containing amorphous assemblies that appear less dynamic (Supplemental Video 2). **(b)** Co-mixing as in (a) in the presence of RNA (mass ratio of RNA:FUS:Nup62 $\approx 1.5:1:1$) prevents the formation of amorphous assemblies, giving way to spherical droplets MBP-FUS with some MBP-Nup62 co-localization. Images were taken at the indicated timepoints after initiation of phase

separation by 3.6% PEG. Results are representative of n=4 independent experiments.
Scale bar= 50 μm (inset = 5 μm).

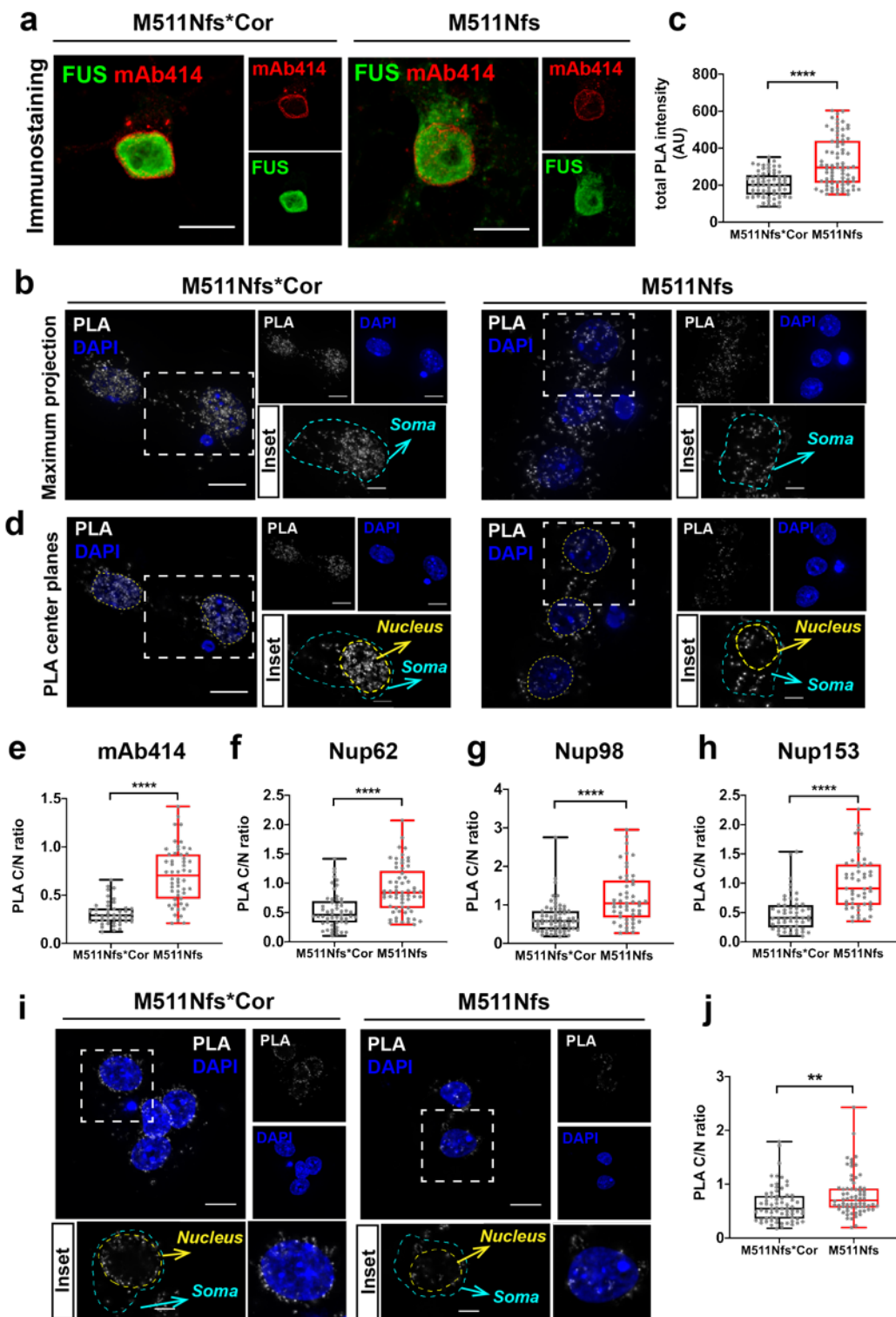


Figure 5. Mutant FUS interacts with F/G Nup in the cytoplasm of human neurons. (a) Immunofluorescence images of FUS (green) and F/G Nup marker mAb414 (red) in human neurons are representative of n=3 experiments. Tuj1 (grey). DAPI (blue). Scale

bar= 10 μ m. **(b)** Proximity ligation assay (PLA) using the FUS and mAb414 antibodies in (a). **(b)** Maximum intensity projections of the all z-planes for cell soma (delineated with blue dotted line). **(c)** Quantification of (b). A significant increase of total PLA signal in FUS M511Nfs neurons compared to control neurons; **** $p < 0.0001$. $t = 6.630$, $df = 141$. **(d)** Maximum intensity projections of the center ten optical planes, allowing for quantification of nuclear (delineated with yellow dotted line surrounding DAPI) versus cytoplasmic PLA signals detected in between the blue and yellow lines. PLA signal at the nuclear membrane is counted as “nuclear” here. **(e)** Quantification of (d). PLA C/N ratio revealed significantly higher cytoplasmic PLA signal in FUS M511Nfs neurons compared to control neurons; **** $p < 0.0001$. $t = 8.384$, $df = 97$. The PLA C/N ratio of the chosen representative neurons: M511Nfs*Cor (0.33, 0.30); M511Nfs (1.05, 1.00, 1.42). **(f-h)** Similar PLA C/N analyses were performed with FUS and Nup62 (f), Nup98 (g), Nup153 (h) antibodies. **** $p < 0.0001$. (f) $t = 5.240$, $df = 112$. (g) $t = 5.142$, $df = 114$. (h) $t = 6.797$, $df = 102$. **(i)** PLA analysis with mAb414 and Nup62 antibodies show a loss of nuclear Nup PLA signal (including the nuclear membrane) and an increase of cytoplasmic Nup PLA signal in FUS M511Nfs neurons compared to the control. PLA signal (grey); DAPI (Blue). The PLA C/N ratio of the chosen representative neurons: M511Nfs*Cor (0.49, 0.27, 0.18); M511Nfs (0.71, 1.49). **(j)** Quantification of (i). M511Nfs*Cor, $n = 69$; M511Nfs, $n = 68$ cells; ** $p = 0.0018$. $t = 2.832$, $df = 135$. All the images were chosen to be representative of the mean quantified value. Scale bar= 10 μ m. **(c,e,f,h,j)** Box and whiskers plot showing all cells analyzed from three independent differentiations. Plots indicate the 25th (above) and 75th (below) percentiles, with a line within showing the median; whiskers above and below the box indicate the maximum and minimum values. Unpaired two-tailed t-test was used for all comparisons. Data distribution was assumed to be normal but this was not formally tested.

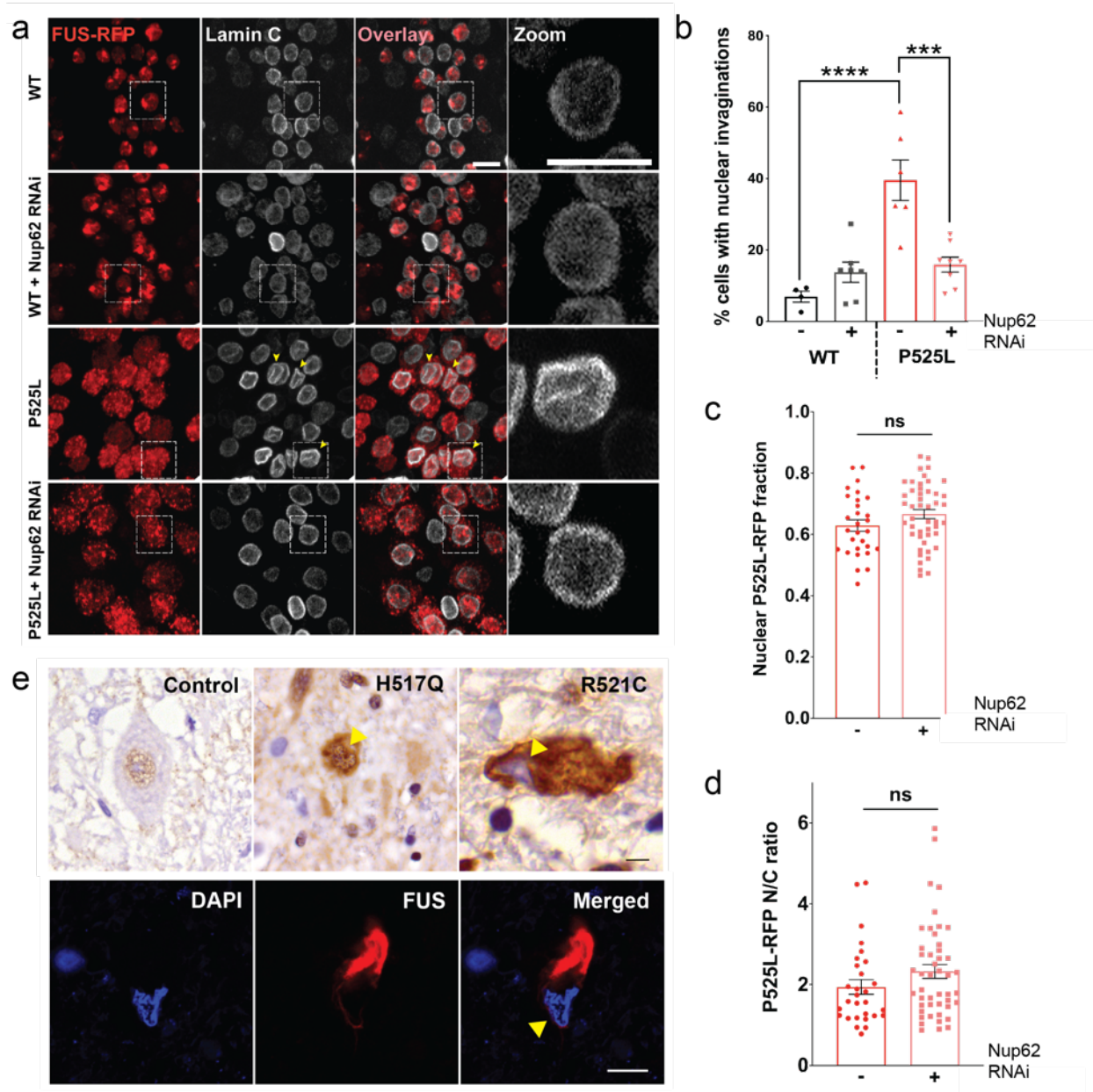


Figure 6. Nuclear abnormalities associated with ALS-linked FUS are rescued by knock-down of Nup62 *in vivo* (a) Immunofluorescence imaging of larval ventral nerve chord in *Drosophila* expressing FUS WT-RFP (red, top) and ALS mutant FUS P525L-RFP (red, bottom), with and without Nup62 knockdown. Lamin C (grey) staining was used to mark the nuclear membrane. Nuclear membrane invaginations, which appear as wrinkled membranes, were observed in P525L-expressing neurons (denoted by yellow arrowheads). Zoom images show normal nuclear membrane morphologies for all conditions except P525L. Scale bar= 10µm. (b) Quantification of percentage of neurons that have nuclear membrane defects in all four groups (Each point in the bar graph

represents an animal. FUS WT, n=4; FUS WT + Nup62 RNAi, n=7; FUS P525L, n=6; FUS P525L + Nup62 RNAi n=8) reveals a significant increase in percentage of neurons with nuclear morphology defects in P525L-expressing neurons compared to WT. Upon Nup62 knockdown, there was a significant decrease in percentage of P525L-expressing neurons with nuclear morphology defects. Each point represents data from one larva. One-way ANOVA with Tukey's multiple comparisons test was used for analysis (F-value=14.94, df=3). Only significant comparisons were denoted. WT-RFP vs P525L-RFP ****p-value<0.0001; P525L-RFP vs P525L-RFP + Nup62 RNAi ***p-value=0.0003. Error bars indicate s.e.m. **(c)** Quantification of nuclear FUS-P525L intensities and **(d)** nuclear-cytoplasmic (N/C) ratio of FUS-P525L intensities with and without Nup62 knockdown (P525L-RFP, n=29; P525L + Nup62 RNAi, n=47) indicates no difference in either nuclear levels (p-value=0.128) **(c)**, or nuclear-cytoplasmic FUS distribution (p-value=0.145) **(d)** in P525L-expressing neurons upon Nup62 knockdown. Each point in (c) and (d) represent individual neurons. Unpaired two-tailed Student's t-test was used for analysis (For [c]: t=1.539, df = 74; and for [d]: t=1.473, df = 74). Error bars indicate s.e.m. **(b-d)** Data distribution was assumed to be normal but this was not formally tested. **(e)** Post-mortem human spinal cord from two ALS-FUS cases (H517Q and R521C) compared to a neurologically normal control demonstrate abnormally misshapen nuclei (i.e., deviating from a round shape and/or containing membrane invaginations), which are denoted by yellow arrow-heads. Images are representative of FUS inclusion-bearing neurons within n=1 staining experiments. Tissues were stained with anti-FUS antibody and a nuclear dye, revealing cytoplasmic mislocalization of ALS-FUS. Top panel: immunohistochemistry. Bottom panel: confocal immunofluorescence images of a neuron from the FUS R521C case with FUS accumulation (red) proximal to the nucleus (blue). Scale bar= 10µm.

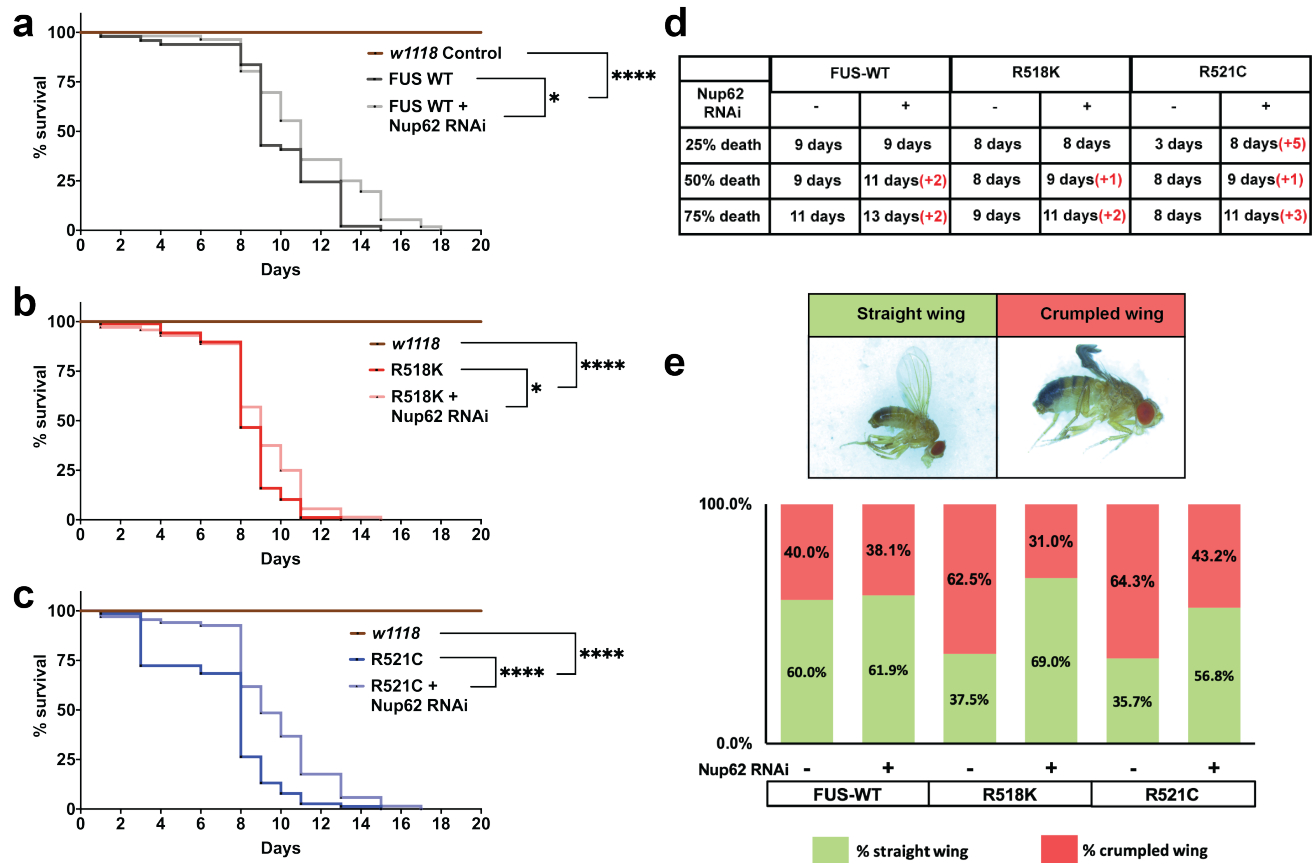
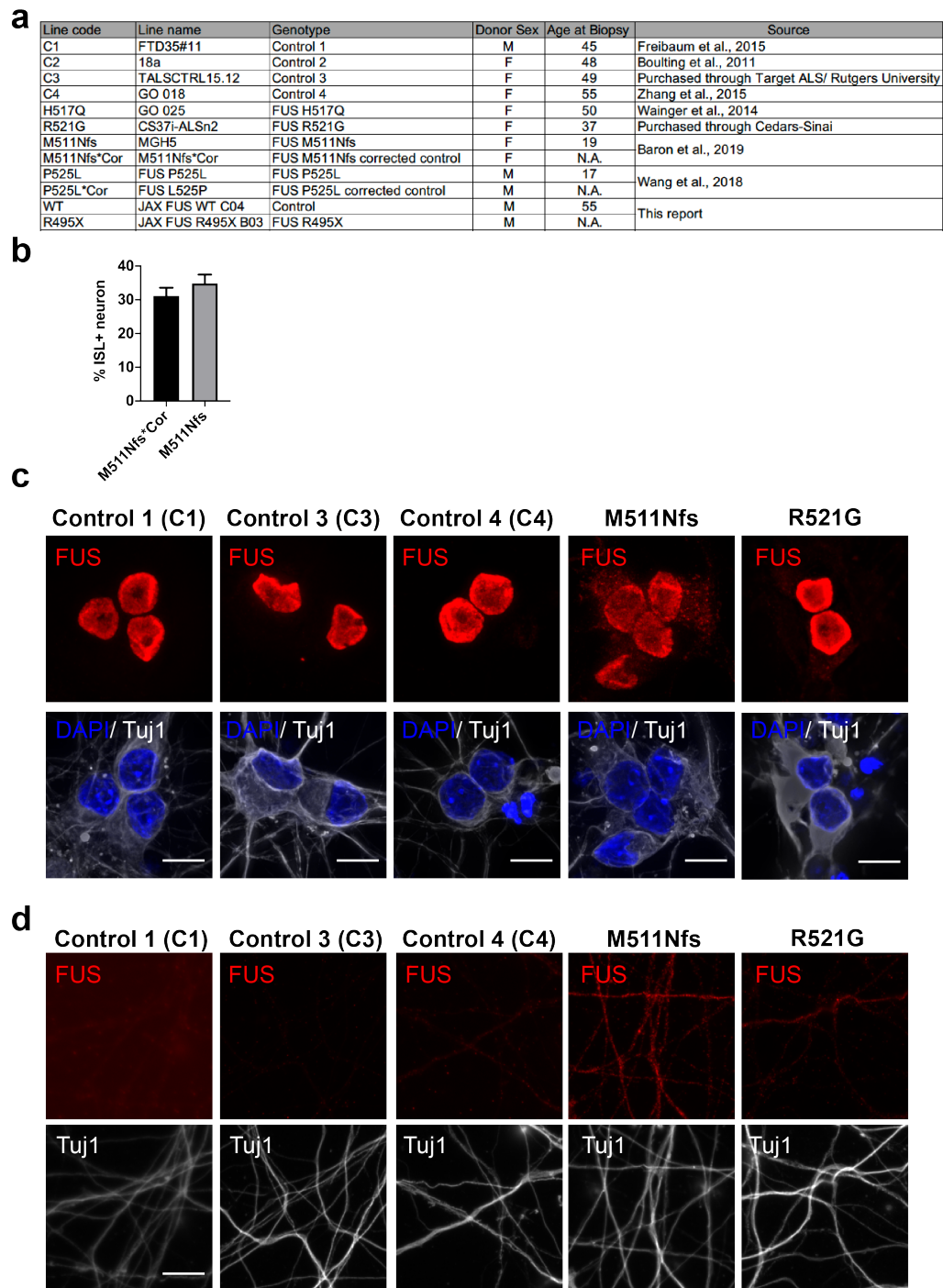


Figure 7. Modulation of Nup expression rescues FUS-induced toxicity *in vivo*.

(a-c) Kaplan-Meier survival curves of flies expressing (a) FUS WT, (b) R518K mutant and (c) R521C mutants with and without Nup62 RNAi in adult motor neurons (D42-gal4). Non-transgenic (*w1118*) flies were used as controls. Log-Rank (Mantel-Cox) statistical test was used for analysis of survival curves. ****p-value<0.0001; FUS-WT vs FUS-WT + Nup62 RNAi p-value=0.0397; R518K vs R518K + Nup62 RNAi p-value=0.0128. Data distribution was assumed to be normal but this was not formally tested. (d) Tabular representation of number of days it took for 25%, 50% and 75% death respectively for each genotype. The number of days that Nup62 siRNA confers for survival for each genotype are shown in red. (e) Percentage of flies expressing FUS WT, R518K and R521C in motor neurons through development, with and without Nup62 RNAi, knockdown that eclosed/hatched with normal straight wings (green) or abnormal crumpled wings (red) (*w1118* Control n = 99; FUS WT n = 49; FUS WT + Nup62 RNAi n = 56; R518K n=88; R518K + Nup62 RNAi n = 72; R521C n = 76; R521C + Nup62 RNAi n = 68).

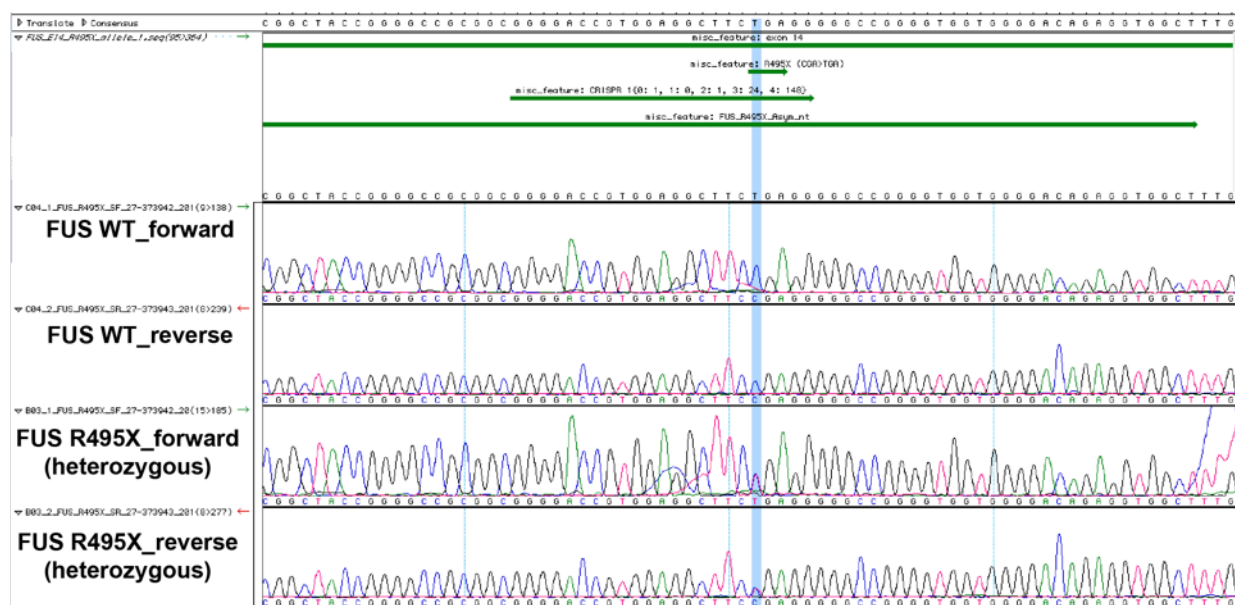


Supplemental Figure 1. iPSC lines and immunofluorescence images of FUS localization. (a) Table of iPSC lines used in this report ^{10,23,28,65-67}. (b) Percentages of motor neurons yield from n=3 independent differentiations of FUS M511Nfs isogenic lines. Error bars represent s.e.m. (c) Immunofluorescence images of FUS in iPSC-derived

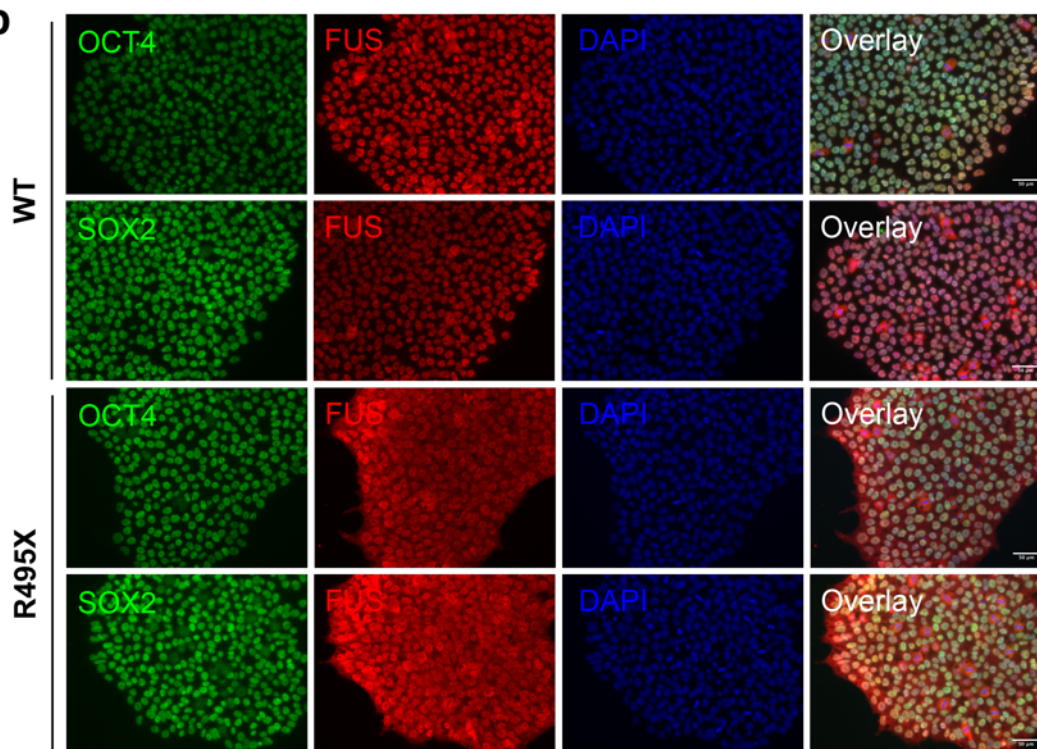
neurons DAPI (blue), Tuj1 (grey). **(d)** FUS staining in the neurites of iPSC-derived neurons. Scale bar= 10 μ m. **(c-d)** Images are representative of at least n=3 experiments.

65. Almad, A. A. *et al.* Connexin 43 in astrocytes contributes to motor neuron toxicity in amyotrophic lateral sclerosis. *Glia* **64**, 1154-1169 (2016).
66. Wainger, B. J. *et al.* Intrinsic Membrane Hyperexcitability of Amyotrophic Lateral Sclerosis Patient-Derived Motor Neurons. *Cell Rep* **7**, 1–11 (2014).
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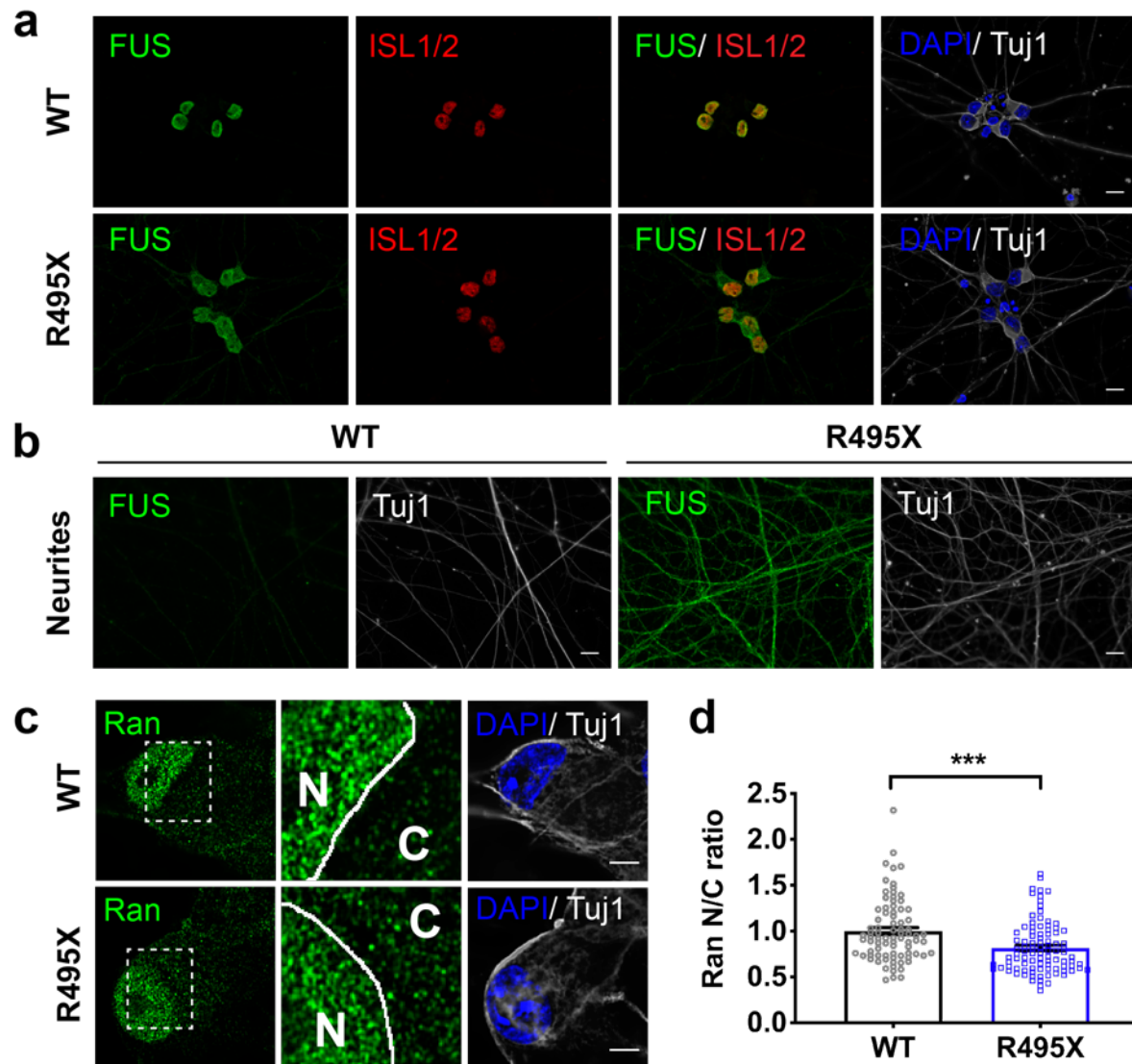
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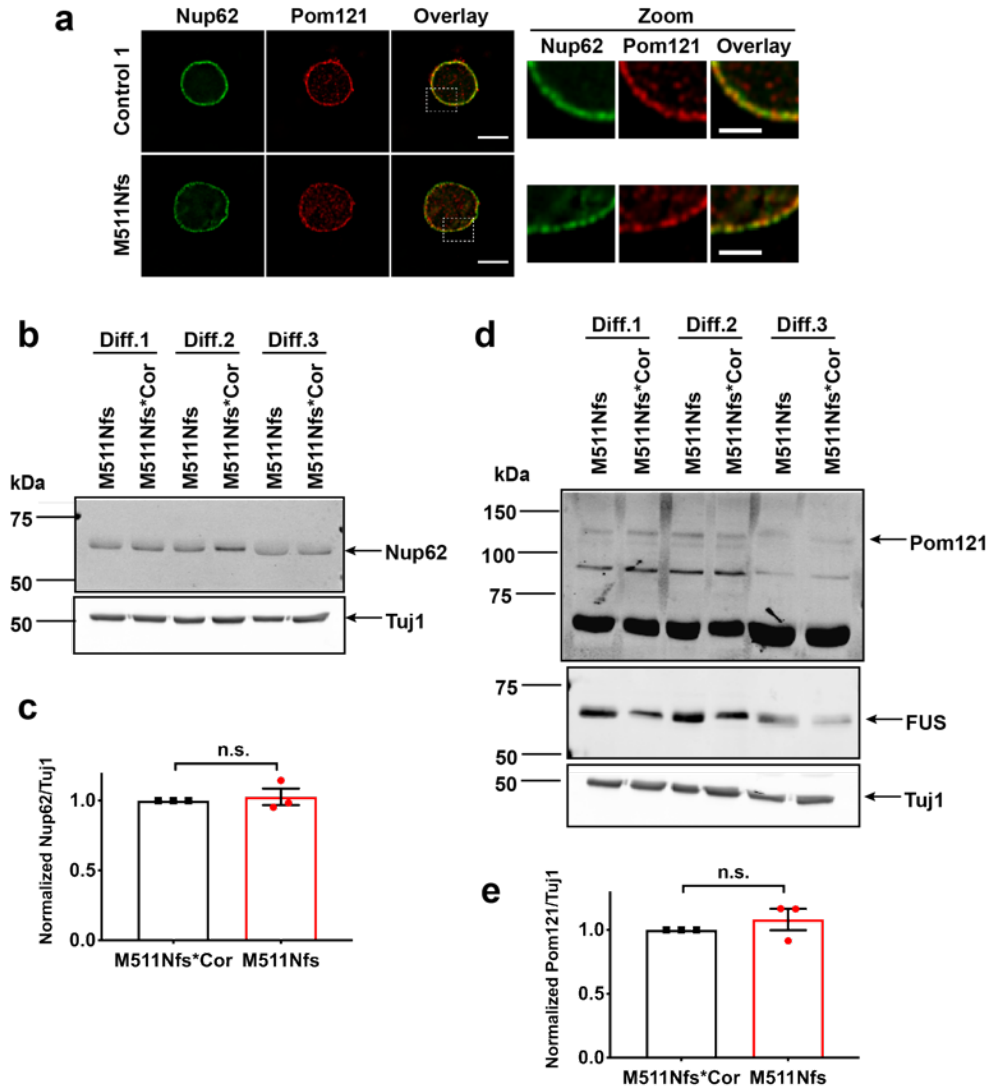
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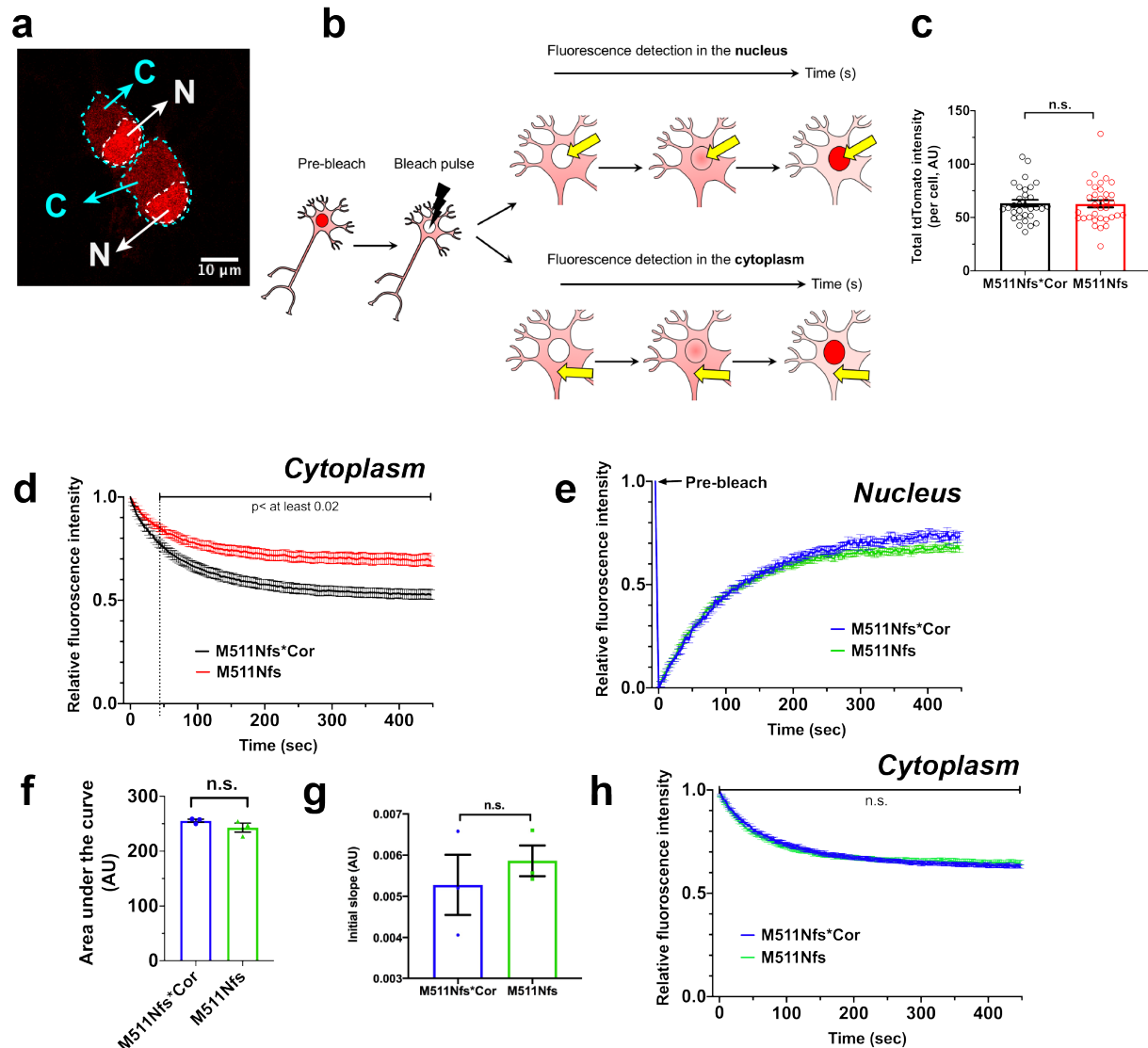
Supplemental Figure 2. Generation of FUS R495X isogenic induced pluripotent stem cells. (a) Results of Sanger sequencing after gene-editing to introduce the FUS R495X mutation. **(b)** Immunofluorescence image of pluripotency markers OCT4 and SOX2 (green) and FUS (red) in iPSCs, representative of n=1 experiments. DAPI (blue). Scale bar= 50 μ m.



Supplemental Figure 3. Differentiation and assessment of the Ran gradient in isogenic FUS R495X iPSC lines. (a) Immunofluorescence image of FUS in iPSC-derived neurons. ISL1/2 (red), DAPI (blue), Tuj1 (grey). (b) FUS staining in the neurites of iPSC-derived neurons. Scale bar= 10 μ m. (a,b) Images are representative of n=1 experiments. (c) Immunofluorescence image of Ran (green), DAPI (blue), Tuj1 (grey), representative of n=3 experiments. The Ran N/C ratio of the chosen representative neurons: WT (1.25); R495X (0.87). Abbreviation within the zoom insets: N (nucleus), C (cytoplasm). Neurons at DIV 56 (8 weeks) were used for this analysis. Scale bar= 10 μ m. (d) Quantification of the Ran N/C ratio. Ran N/C is significantly lower in R495X versus WT neurons (WT, n=82; R495X, n=95 cells; from n=3 independent biological replicates). Unpaired two-tailed t-test, ***p=0.0001, t=3.935, df=175. Error bars represent s.e.m. Data distribution was assumed to be normal but this was not formally tested.

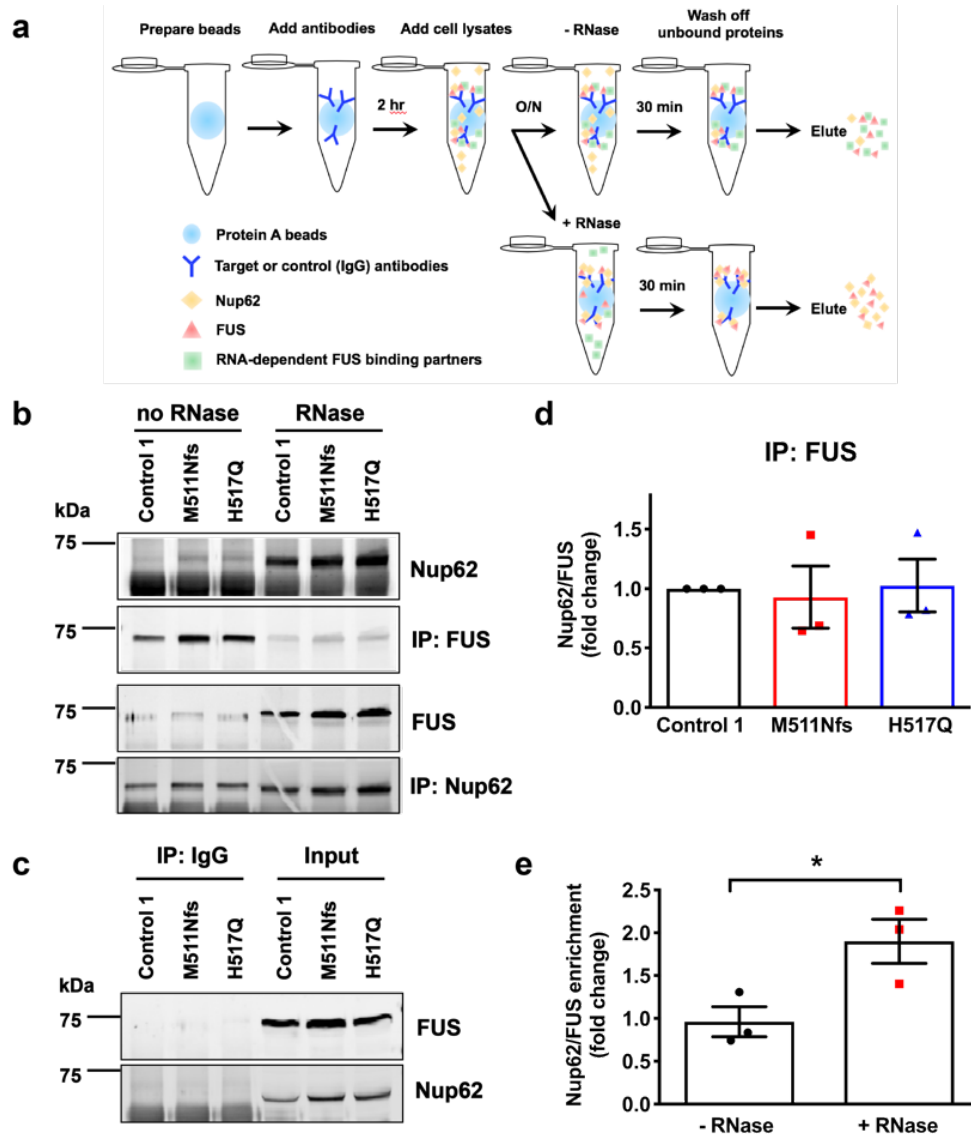


Supplemental Figure 4. A Western analysis demonstrates similar levels of nuclear pore proteins Pom121 and Nup62 in mutant and control human neurons. (a) Immunofluorescence images of Nup62 (green) and Pom121 (red), representative of n=5 experiments. Scale bar= 5 μ m. Zoom scale bar= 2 μ m. (b) Western blots probed with anti-Nup62, anti-FUS and anti-Tuj1 (TUBB3) antibodies. Source data are provided as a Source Data file. (c) Quantification of (b) revealed similar levels of Nup62 in lysates derived from mutant and control neurons. $t=0.4338$, $df=4$. (d) Western blots probed with anti-Pom121, anti-FUS and Tuj1 antibodies. Source data are provided as a Source Datafile. (e) Quantification of (d) revealed similar levels of Pom121 in lysates derived from mutant and control neurons. $t=0.9636$, $df=4$. Tuj1 was used as a loading control. Expression level in FUS M511Nfs neurons was normalized to M511Nfs*Cor neurons within each experiment. Blots showing all three biological replicates. Diff.= differentiation. (c,e) Unpaired two-tailed t-test, n.s.= not significant. Error bars represent s.e.m. Data distribution was assumed to be normal but this was not formally tested.

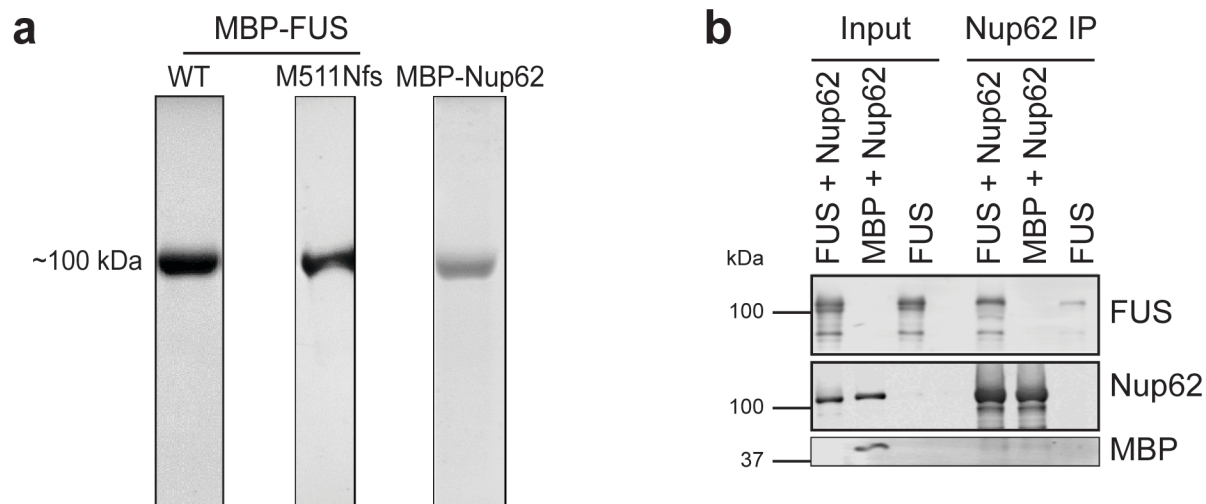


Supplemental Figure 5. Additional live-cell fluorescence analysis of the NLS-tdTomato-NES shuttling reporter in ALS-FUS human neurons. (a) Representative image of the transduced NLS-tdTomato-NES shuttling reporter (red) in human neurons. The fluorescence signal for this reporter construct is diffuse and predominately localized to the nucleus. Scale bar= 10 μ m. N=nucleus, C=cytoplasm. (b) Illustration for FRAP analysis. Top panel: Fluorescence detection in the nucleus monitors the import of unbleached tdTomato (red) from the cytoplasm. Nuclear red fluorescence increases over time as a function of tdTomato (red) import. Since there are fewer fluorescent molecules in the cytoplasm to begin with, the fluorescence never fully recovers in the nucleus on the time-scale of this experiment. Bottom panel: Fluorescence detection in the cytoplasm measures bleached tdTomato (non-fluorescent or “white”) exported from the nucleus. The red fluorescence in the cytoplasm decreases as the red signal becomes diluted with bleached molecules exported from the nucleus. Yellow arrows indicate the area of the cell where the fluorescence is detected as a function of time. (c) The total reporter expression level/ transduction efficiency was similar in FUS M511Nfs and control. M511Nfs*Cor: n=29 cells; M511Nfs: n=36 cells, examined from n=3 independent

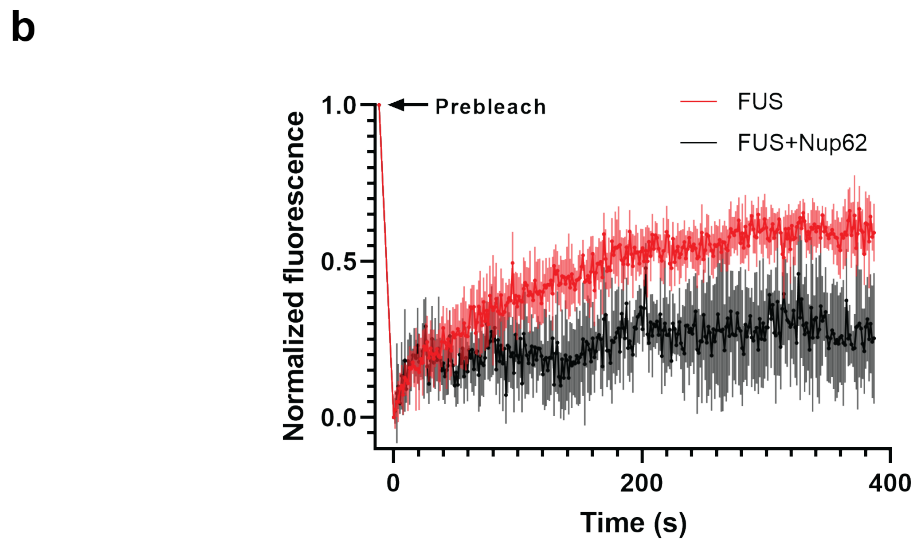
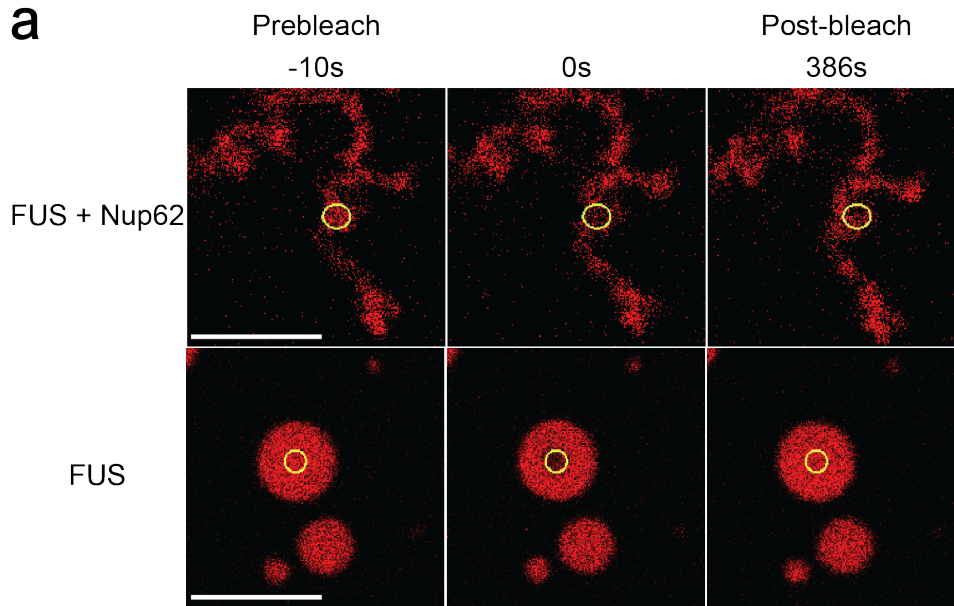
differentiations. Unpaired two-tailed t-test, n.s.= not significant. $t=0.1524$, $df=62$. Error bars represent s.e.m. **(d)** Corresponding to Figure 2i. Reporter intensity curves generated from the cytoplasm of FUS M511Nfs and M511Nfs*Cor neurons (DIV 56-60), consistent with a disruption of nuclear export in M511Nfs neurons. Curves are significantly different at time points between 48s and 450s by two-way ANOVA. Errors represent s.e.m. **(e-h)** Analysis of 'young' neurons. **(e)** The nuclear fluorescence recovery curves for the FRAP experiment with the NLS-tdTomato-NES shuttling reporter in 'young' (DIV 18) FUS M511Nfs*Cor neurons (blue curve; $n=61$ cells) and M511Nfs neurons (green curve; $n=78$ cells) across three independent differentiations. **(f)** The area under the recovery curve was not significantly different between FUS M511Nfs neurons ($n=78$) and M511Nfs*Cor neurons ($n=61$) (Unpaired two-tailed t-test, n.s.= not significant. $p=0.2227$, $t=1.442$, $df=4$). Each point in the bar graph represents an independent differentiation. Error bars represent s.e.m. **(g)** The initial slopes of the FRAP recovery curves (comprising the first 50 seconds from panel **e**) were determined using a non-linear regression analysis and found to be similar between FUS M511Nfs ($n=78$) and M511Nfs*Cor ($n=61$) neurons (Unpaired two-tailed t-test, $p=0.05142$, $t=0.7148$, $df=4$). **(h)** Reporter intensity curves generated from the cytoplasm of 'young' FUS M511Nfs and M511Nfs*Cor neurons are not significantly different (two-way ANOVA), consistent with similar nuclear export efficiencies in both. Errors represent s.e.m. For all quantifications, data distribution was assumed to be normal but this was not formally tested.



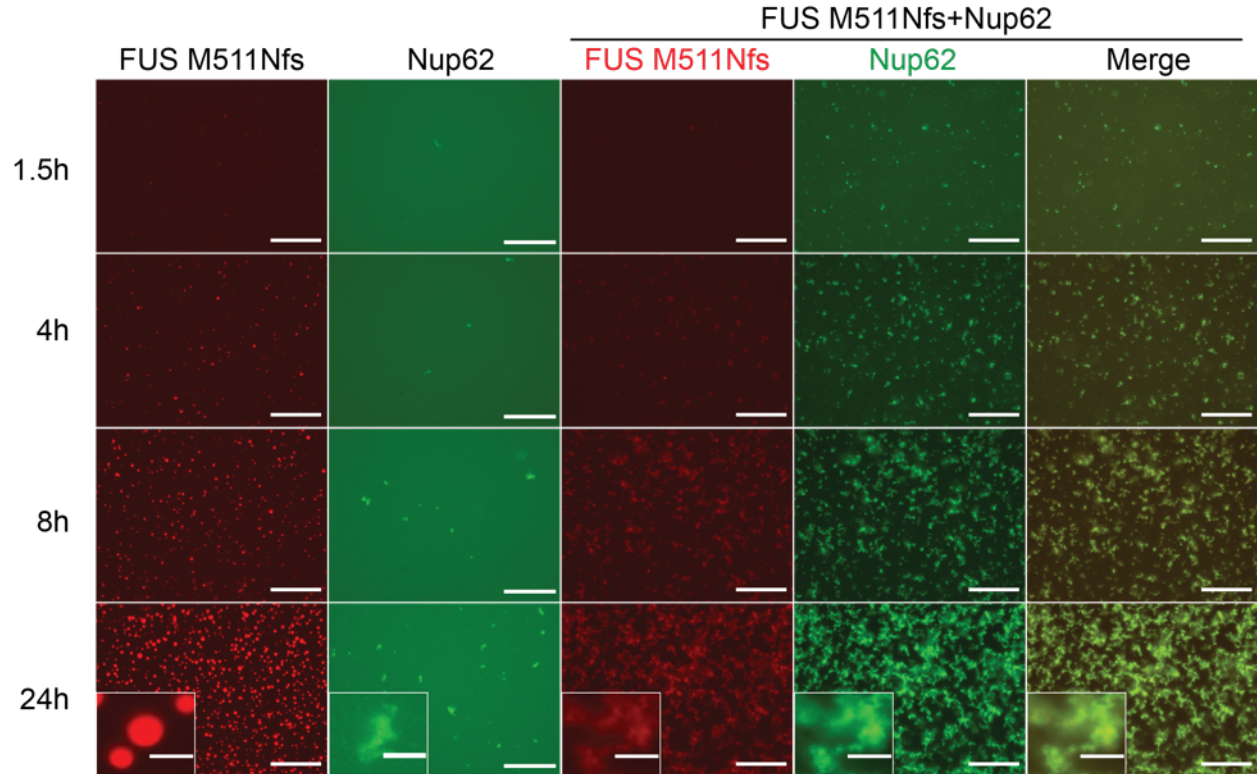
Supplemental Figure 6. Additional analyses of FUS/Nup62 binding in iPSCs. (a) Schematic co-immunoprecipitation (IP) experimental design. (b) Top 2 blots: FUS IP. Nup62 was pulled down with control and mutant (M511Nfs and H517Q) FUS. Bottom 2 blots: Nup62 IP. Both wild-type and mutant FUS were pulled down with Nup62. In both IPs, interactions of FUS and Nup62 were retained and significantly enhanced upon RNase treatment, suggesting the interactions were not mediated through RNA. Source data are provided as a Source Data file. (c) IgG and input controls for IP. Source data are provided as a Source Data file. (d) Quantification for FUS IP. Densitometry analysis of FUS and Nup62 signals in the Western blot shown in (b). One-way ANOVA with Tukey's multiple comparisons test. Control v.s. M511Nfs: $p=0.8047$. Control v.s. H517Q: $p=0.9316$. $F(2, 6)=0.06531$. (e) Quantification for FUS IP with and without RNase treatment. Unpaired two-tailed t-test. $*p=0.0391$. $t=3.021$, $df=4$. Each point in the bar graph represents a biological replicate. Error bars represent s.e.m. (d,e) Data distribution was assumed to be normal but this was not formally tested.



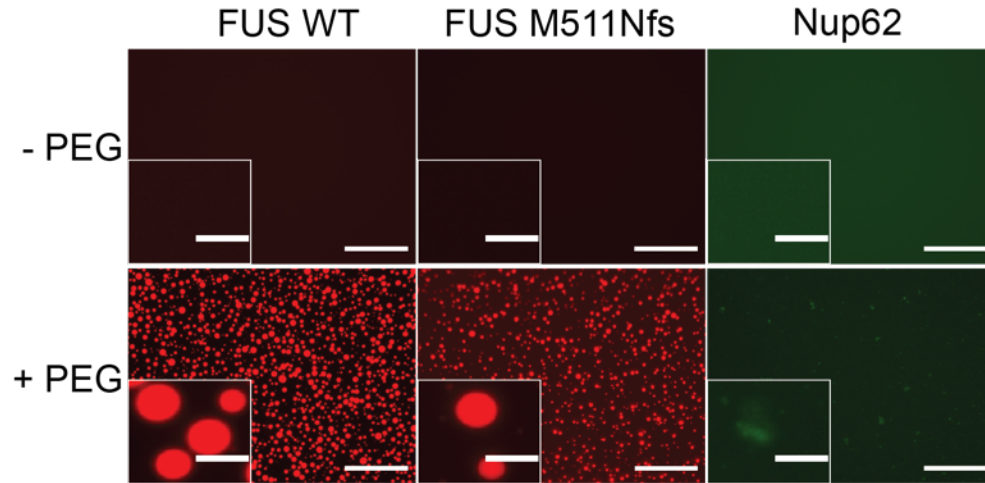
Supplemental Figure 7. Characterization of recombinant proteins and binding of FUS with Nup62. (a) Coomassie blue staining of purified MBP-FUS WT, MBP-FUS M511Nfs and MBP-Nup62. Results are representative of n=3 (MBP-FUS WT), n=1 (MBP-FUS M511Nfs) and n=3 (MBP-Nup62) independent purifications. Source data are provided as a Source Data file. (b) Recombinant MBP-FUS binds to MBP-Nup62: Western blot of co-IP binding assay showing that MBP-FUS co-elutes when MBP-Nup62 is pulled down with an anti-Nup62 antibody from a mixture containing MBP-FUS and MBP-Nup62. This interaction is not mediated by the MBP tag as MBP protein in the absence of MBP-FUS fails to co-elute with MBP-Nup62. Results are representative of n=2 independent experiments. Source data are provided as a Source Data file. These experiments serve to validate the following antibodies: rabbit anti-Nup62 antibody (Bethyl Laboratories, A304-942A), which was used to charge the beads and to probe for nup62 in the Western blot; mouse anti-FUS (Santa Cruz, SC-373698), which was used to probe for FUS in the Western blot; and mouse anti-maltose binding protein (Cell Signalling Technology, 2396), which was used to probe for MBP in the Western blot.



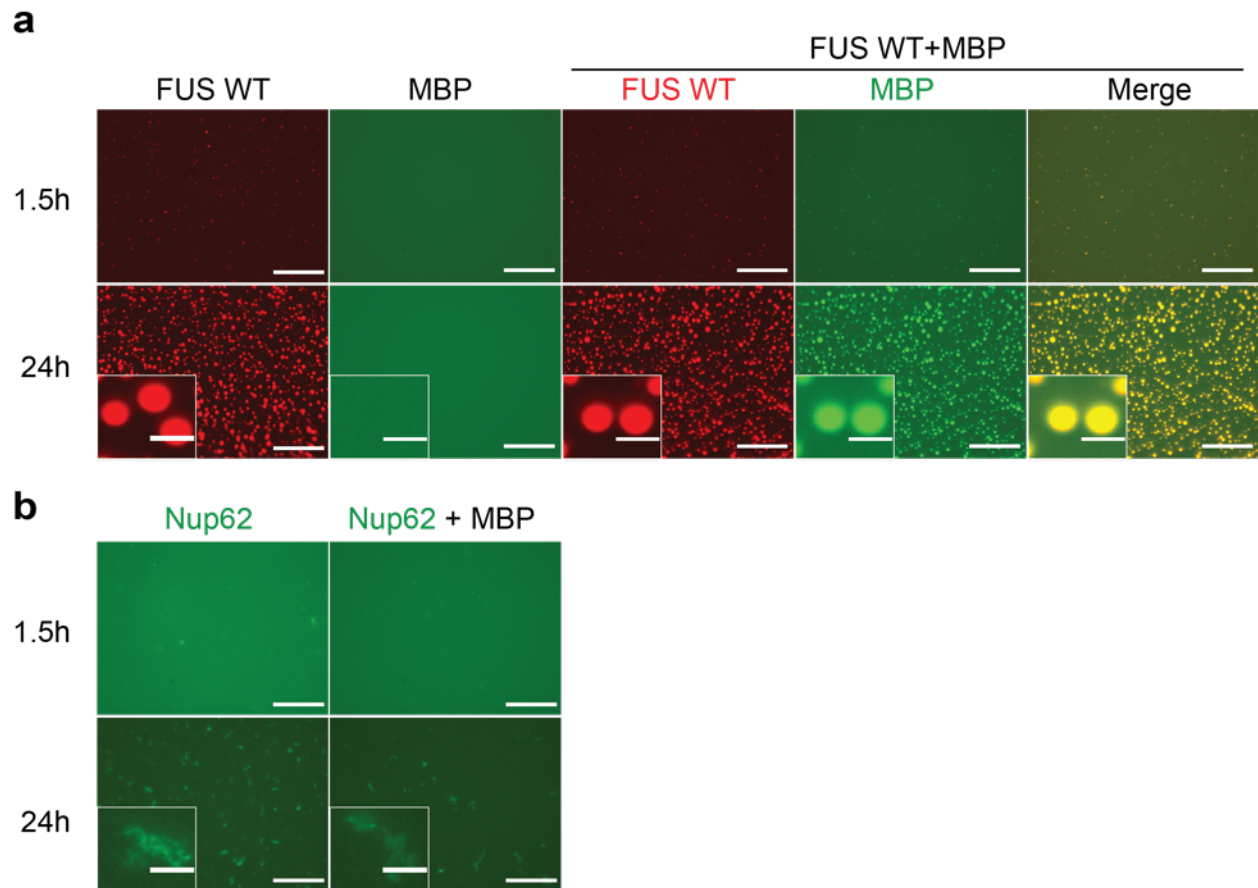
Supplemental Figure 8: FRAP measurements of recombinant FUS/Nup proteins. (a) Time lapse fluorescence images of FRAP experiment. MBP-FUS/Nup62 assemblies (top panel) and MBP-FUS droplets formed without addition of Nup62 (bottom panel) were photobleached and the fluorescence recovery was monitored. Image taken before bleaching (pre-bleach), immediately after bleaching (post-bleach: 0s) and endpoint (post-bleach: 386s) are shown. Yellow circle outlines the bleach region. Supplemental Video 1 and 2 correspond to these FRAP measurements. Results are representative of $n=5$ and $n=6$ FUS and FUS/Nup62 assemblies respectively. Scalebar = $5\mu\text{m}$. **(b)** Fluorescence recovery curves of FRAP experiment on FUS droplets (red curve, $n=6$ droplets) and FUS/Nup62 aggregates (black curve, $n=5$ regions). Error bars represent standard deviation. The custom code for this analysis has been provided as supplemental material (code_frap_invitro.py).



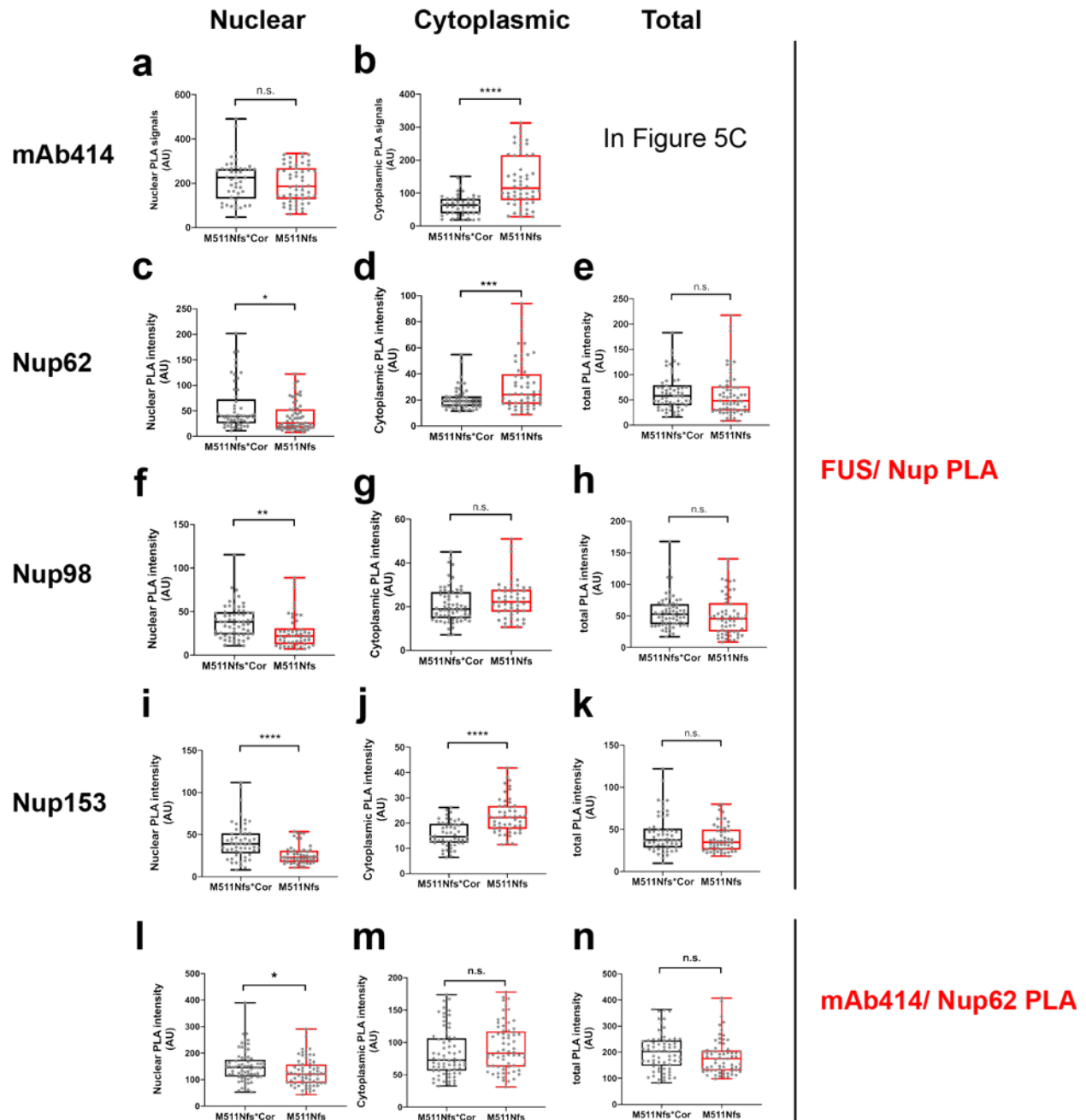
Supplemental Figure 9. Nup62 alters FUS M511Nfs phase separation in a similar manner to FUS WT. Fluorescence images showing phase separation of 1.5 μ M MBP-FUS M511Nfs (red) doped with 2.5% Alexa555-labelled MBP-FUS M511Nfs. MBP-FUS droplet formation is altered by co-incubation with 1.5 μ M MBP-Nup62 (green; doped with 2.7% Alexa488 labelled MBP-Nup62). Images were taken at the indicated timepoints after initiation of phase separation by 3.6% PEG. Results are representative of n=4 independent experiments. Scale bar= 50 μ m (inset = 5 μ m).



Supplemental Figure 10. FUS and Nup62 assemblies form only in the presence of PEG. Fluorescence images showing absence of FUS or Nup62 assemblies when PEG is not added (top). FUS and Nup62 assemblies are formed only upon addition of 3.6% PEG (bottom). MBP-FUS WT and -M511Nfs (1.5 μ M) are doped with 2.5% Alexa555-labelled FUS WT or -M511Nfs and MBP-Nup62 (1.5 μ M) is doped with 2.7% Alexa488 labelled MBP-Nup62. Images were taken 24h after addition of PEG. Results are representative of n=4 independent experiments. Scale bar= 50 μ m (inset = 5 μ m).

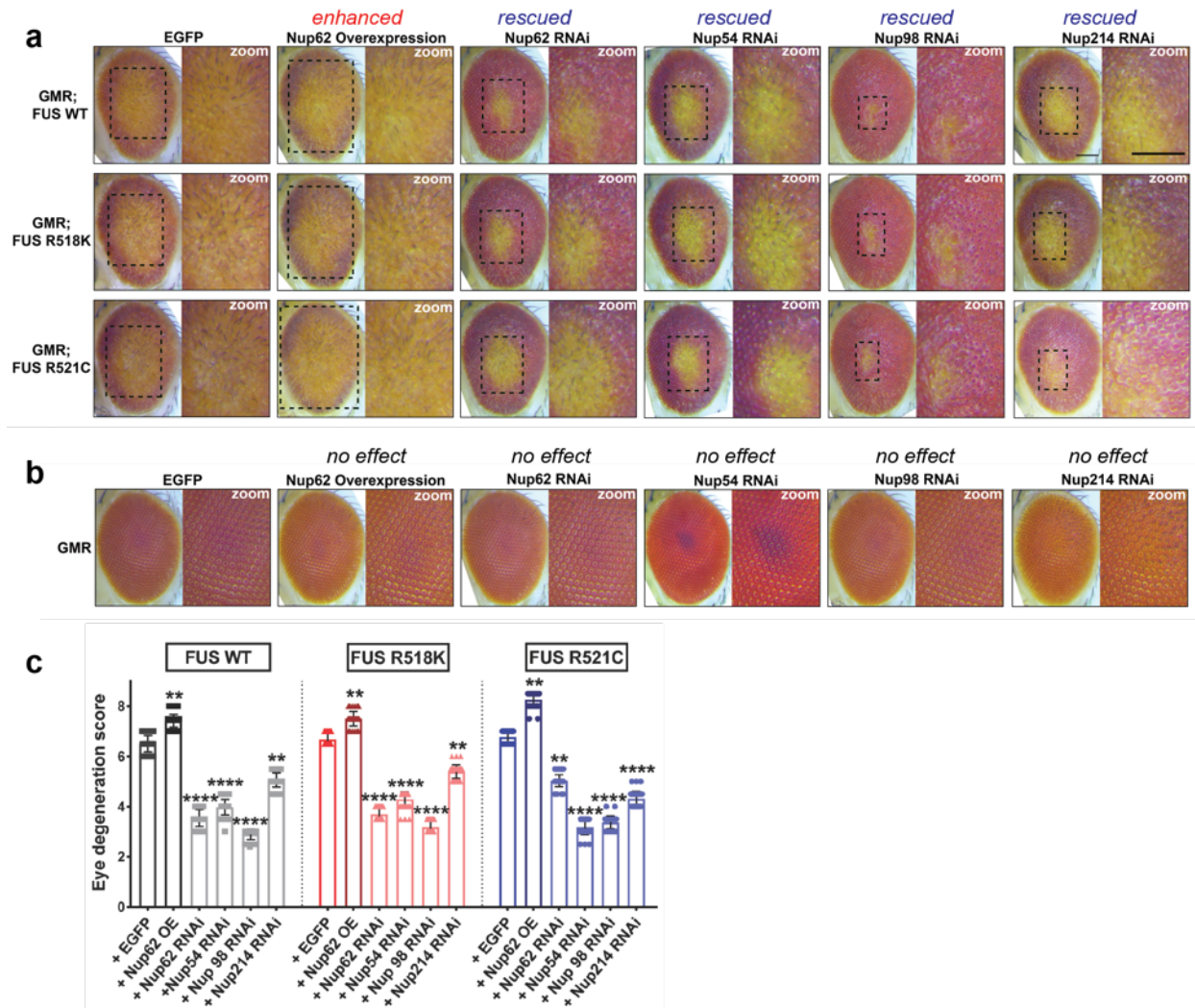


Supplemental Figure 11. MBP does not alter FUS or Nup62 phase separation. (a) Fluorescence images showing phase separation of 1.5 μ M MBP-FUS WT (red) doped with 2.5% Alexa555-labelled MBP-FUS WT. MBP-FUS droplet formation is not altered by co-incubation with 1.5 μ M MBP-MBP (green; doped with 1% Alexa488 labelled MBP), although MBP is detected within FUS droplets. (b) Fluorescence images showing amorphous assemblies of 1.5 μ M MBP-Nup62 (green) doped with 2.7% Alexa488 labelled MBP-Nup62. Formation of MBP-Nup62 amorphous assemblies is not enhanced upon co-incubation with 1.5 μ M MBP (unlabeled). Images were taken at the indicated timepoints after initiation of phase separation by 3.6% PEG. Results are representative of n=4 (for panel A) and n=2 (for panel B) independent experiments. Scale bar= 50 μ m (inset = 5 μ m).



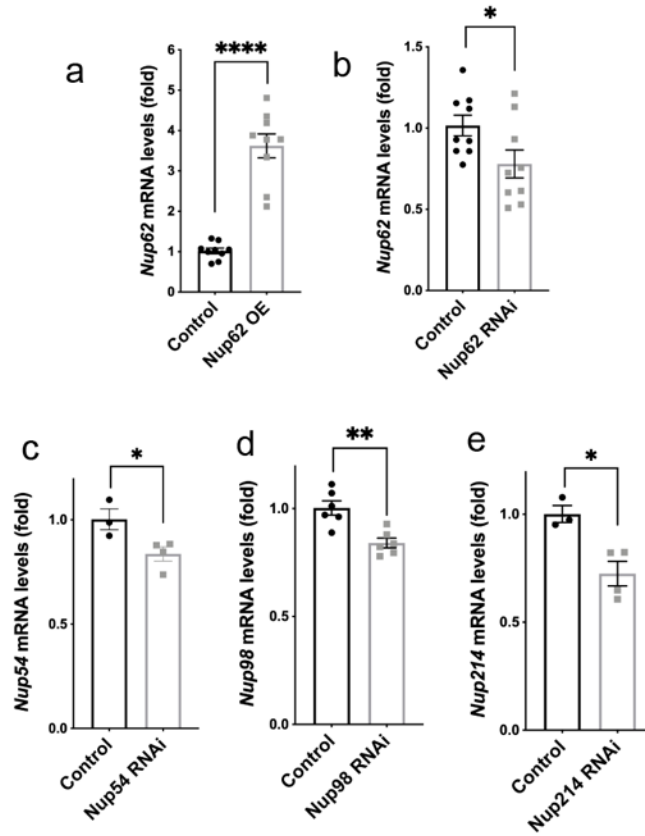
Supplemental Figure 12. Additional PLA data analyses corresponding to Figure 5. (a-k) FUS/Nup PLA. (a, b) FUS/mAb414 PLA. Similar nuclear (a) and a significantly higher cytoplasmic (b) PLA intensity in FUS M511Nfs neurons compared to control neurons. M511Nfs*Cor, n=45; M511Nfs, n=54 cells. n.s.= not significant, ****p<0.0001. (c-e) FUS/Nup62 PLA. A significantly lower nuclear (c), higher cytoplasmic (d) and similar total (e) PLA intensity in FUS M511Nfs neurons compared to control neurons. *p=0.0117, ***p=0.0008; n.s.= not significant. (c) t=2.562, df=112. (d) t=3.455, df=104. M511Nfs*Cor, n=53; M511Nfs, n=53 cells (e) t=0.5965, df=121. M511Nfs*Cor, n=58; M511Nfs, n=65 cells. (f-h) FUS/Nup98 PLA. A significantly lower nuclear (f), similar cytoplasmic (g) and total (h) PLA intensity in FUS M511Nfs neurons compared to control neurons. **p=0.0014,

n.s.= not significant. (f) $t=3.278$, $df=114$. (g) $t=1.498$, $df=114$. M511Nfs*Cor, $n=66$; M511Nfs, $n=50$ cells. (h) $t=0.9271$, $df=123$. M511Nfs*Cor, $n=65$; M511Nfs, $n=60$ cells. (i-k) FUS/Nup153 PLA. A significantly lower nuclear (i), higher cytoplasmic (j) and similar total (k) PLA intensity in FUS M511Nfs neurons compared to control neurons. **** $p<0.0001$, n.s.= not significant. (i) $t=4.576$, $df=102$. (j) $t=6.190$, $df=102$. M511Nfs*Cor, $n=53$; M511Nfs, $n=51$ cells. (k) $t=1.502$, $df=106$. M511Nfs*Cor, $n=55$; M511Nfs, $n=53$ cells. (l-n) mAb414/Nup62 PLA. A significantly lower (l), similar cytoplasmic (m) and total (n) PLA intensity in between FUS M511Nfs and control neurons. * $p=0.0171$, n.s.= not significant. (l) $t=2.415$, $df=135$. (m) $t=1.417$, $df=135$. (n) $t=1.517$, $df=135$. M511Nfs*Cor, $n=69$; M511Nfs, $n=68$ cells. (a-n) Box and whiskers plot showing all cells analyzed from three independent differentiations. The box plots indicate the 25th (above) and 75th (below) percentiles, with a line within showing the median; whiskers above and below the box indicate the maximum and minimum values. Unpaired two-tailed t-test. For all quantifications, data distribution was assumed to be normal but this was not formally tested.

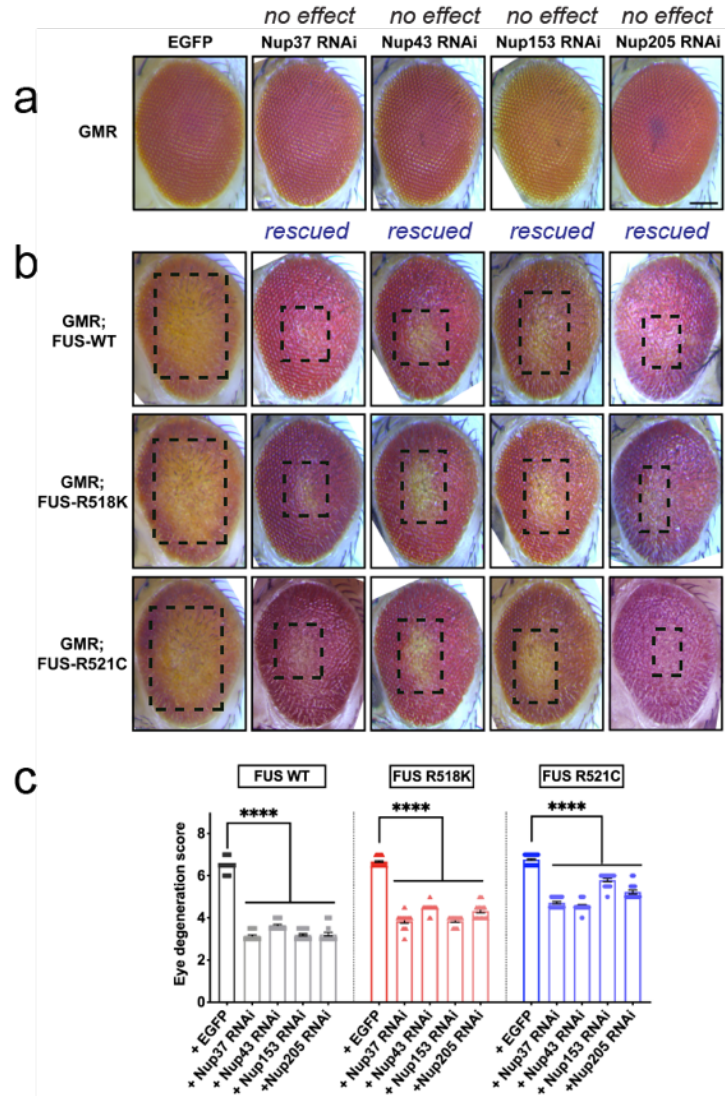


Supplemental Figure 13. Genetic interaction between FUS and nucleoporins *in vivo*. (a) Representative images of fly eyes showing that modulation of nucleoporins in FUS WT and mutant backgrounds modifies the external eye degeneration caused by FUS expression, indicated by areas of depigmentation and ommatidial fusion. Black dashed boxes highlight areas of marked eye degeneration; the size of the box corresponds to the area of degeneration. Zoom panels are used to further emphasize the extent of eye degeneration. Overexpression of Nup62 (Nup62 OE) enhances external eye degeneration in FUS-expressing flies. Knockdown of Nup62, Nup54, Nup98 and Nup214 rescues the external eye degeneration in FUS-expressing flies. Scale bar= 100µm. (b) Representative images of eye phenotypes of flies overexpressing Nup62 or expressing RNAi for Nup62, Nup54, Nup98, and Nup214 indicates no external eye degeneration caused by modulation of Nup expression. Zoom images show intact and healthy ommatidia. (c) Quantification of eye degeneration (from b) indicates statistically significant difference in eye phenotypes upon modulation of nucleoporins in FUS WT, FUS R518K and FUS R521C backgrounds (n=48 per group). Each point represents one adult fly, Kruskal-Wallis test with Dunn's multiple comparisons test was used for analysis. ****p-value<0.0001; FUS-WT + EGFP vs FUS-WT + Nup62 OE p-value=0.0046; FUS-

WT + EGFP vs FUS-WT + Nup214 RNAi p-value=0.0019; FUS-R518K + EGFP vs FUS-R518K + Nup62 OE p-value=0.0052; FUS-R518K + EGFP vs FUS-R518K + Nup214 RNAi p-value=0.0017; FUS-R521C + EGFP vs FUS-R521C + Nup62 OE p-value=0.0026; FUS-R521C + EGFP vs FUS-R521C + Nup62 RNAi p-value=0.0014. Normality was tested using the Kolmogorov-Smirnov test on Prism Graphpad and the data was determined to not have normal distribution. Error bars indicate s.e.m.

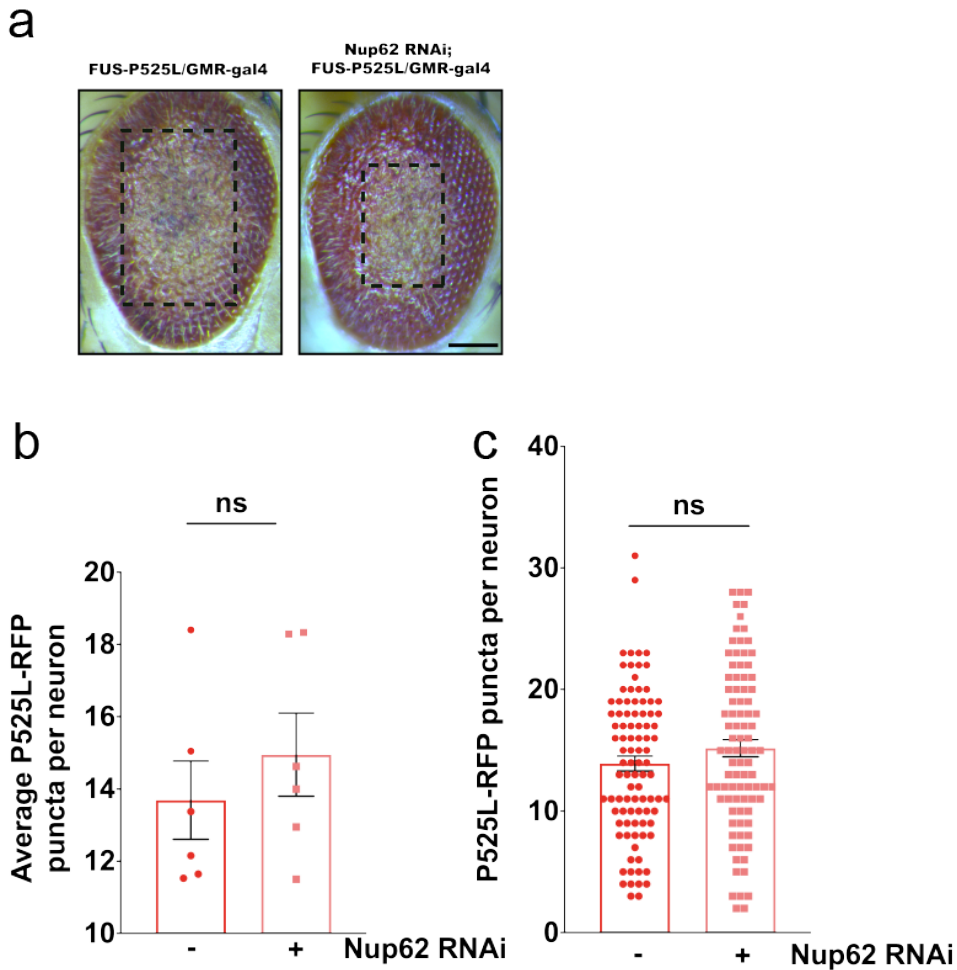


Supplemental Figure 14. Nups mRNA levels in flies measured in fly heads after overexpression or knock-down. Quantification plots of fold-change differences in mRNA levels shows (a) statistically significant overexpression (OE) of transgenic *Nup62* (fold change = 3.6; Control, n=9; Nup62 OE, n=9), and statistically significant RNAi-mediated knockdown of (b) *Nup62* (fold change = 0.77; Control, n=9; Nup62 RNAi, n=9), (c) *Nup54* (fold change = 0.85; Control, n=3; Nup54 RNAi, n=4), (d) *Nup98* (fold change = 0.84; Control, n=6; Nup98 RNAi, n=6) and (e) *Nup214* (fold change = 0.72; Control, n=4; Nup214 RNAi, n=4) compared to *w1118* Control. (a-e) Each point represents a technical replicate. Unpaired Two-tailed Student's t-test was used for analysis. (For [a]: $t=8.542$, $df = 16$; for [b]: $t=2.211$, $df = 16$; for [c]: $t=2.85$, $df = 5$; for [d]: $t=4.05$, $df = 10$; for [e]: $t=3.689$, $df = 5$) ****p-value<0.0001; Control vs Nup62 RNAi p-value=0.0420; Control vs Nup54 RNAi p-value=0.0358; Control vs Nup98 RNAi p-value=0.0023; Control vs Nup214 RNAi p-value=0.0142. Data distribution was assumed to be normal but this was not formally tested. Error bars indicate s.e.m.

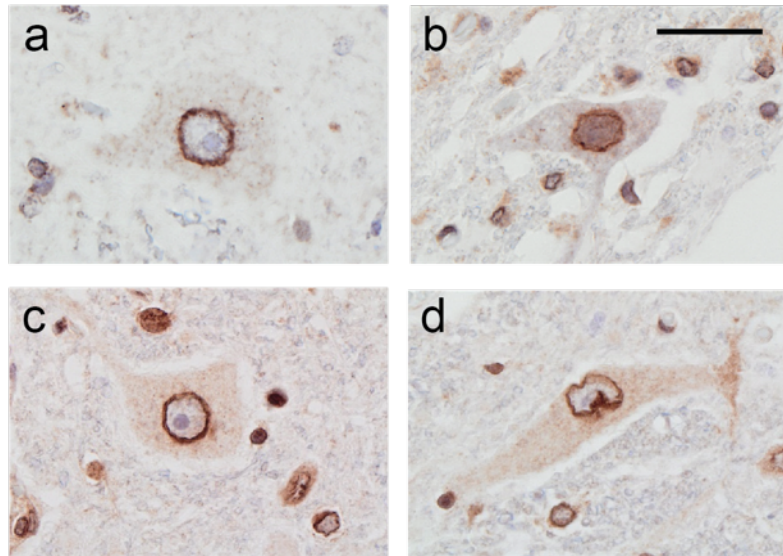


Supplemental Figure 15. RNAi-mediated knockdown of Nup37, Nup43, Nup153 and Nup205 results ameliorates FUS-induced eye degeneration. (a) Representative images of eye phenotypes of flies expressing RNAi for Nup37, Nup43, Nup153, and Nup205 indicates no external eye degeneration. (b) Representative images of fly eyes showing that external eye degeneration, indicated by areas of depigmentation and ommatidial fusion, caused by expression of FUS WT and mutants (R518K and R521C) are suppressed by Nup27, Nup43, Nup153 and Nup205 RNAi. Black dashed boxes highlight areas of marked eye degeneration; the size of the box corresponds to the area of degeneration. Scale bar= 100µm. (c) Quantification of eye degeneration (from b) indicates statistically significant difference in eye phenotypes upon modulation of nucleoporins in FUS WT, FUS R518K and FUS R521C backgrounds (FUS-WT+EGFP, n=48; FUS-R518K+EGFP, n=48; FUS-R521C+EGFP, n=48; FUS-WT+Nup37/Nup43/Nup153/Nup205 RNAi, n=20; FUS-R518K+Nup37/Nup43/Nup153/Nup205 RNAi, n=20; FUS-R521C+Nup37/Nup43/Nup153/Nup205 RNAi, n=20). Each point represents one adult fly, Kruskal-Wallis test with Dunn's multiple comparisons test was used for analysis. ****p-

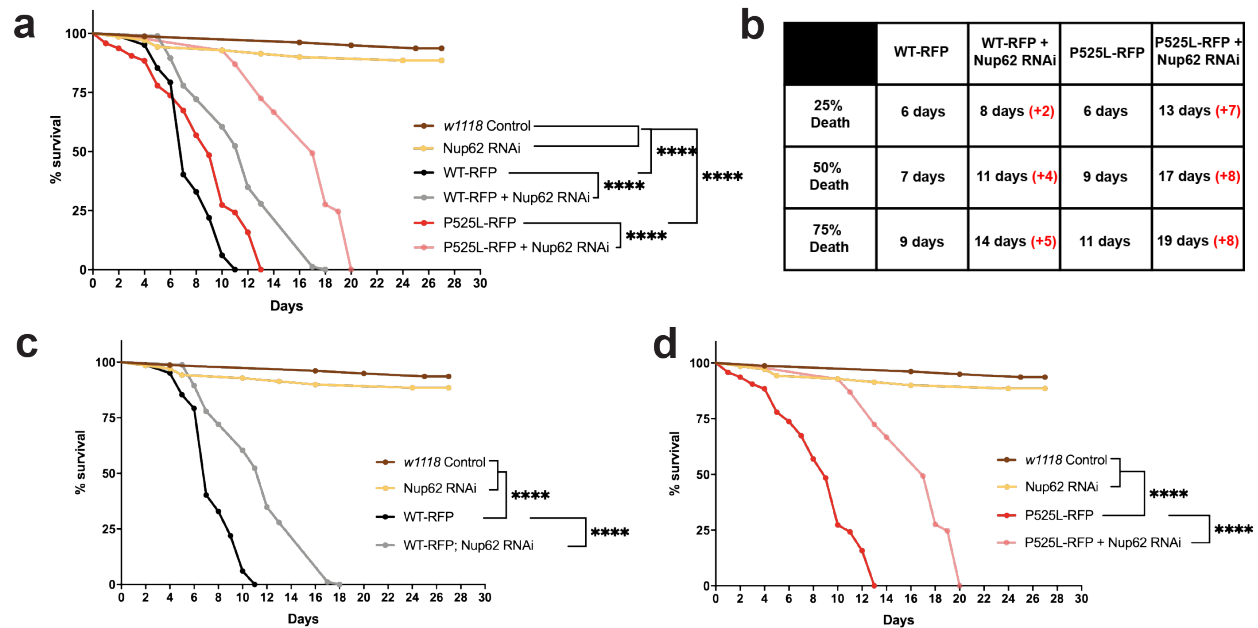
value<0.0001. Normality was tested using the Kolmogorov-Smirnov test on Prism Graphpad and the data was determined to not have normal distribution. Error bars indicate s.e.m.



Supplemental Figure 16. Phenotypes in flies due to expression of FUS P525L. (a) Representative images of fly eyes showing that external eye degeneration, indicated by areas of depigmentation and ommatidial fusion, caused by expression of FUS P525L mutant alone (left) and suppressed eye degeneration upon co-expression with Nup62 RNAi (right). Black dashed boxes highlight areas of marked eye degeneration; the size of the box corresponds to the area of degeneration. Scale bar= 100 μ m (b-c) Quantification of P525L-RFP puncta in larval ventral nerve cord neurons shows no significant differences in (b) average number of puncta per neuron (p-value=0.4447) or (c) the number of puncta in each individual neuron pooled across all larva with or without Nup62 knockdown (p-value=0.1768) (n = 6 larvae per genotype; P525L-RFP n = 94 neurons; P525L+Nup62 RNAi n = 90 neurons). Each point represents data from one larva in (b), and one neuron in (c). (b) Data distribution was assumed to be normal but this was not formally tested. (c) Normality was tested using the Kolmogorov-Smirnov test on Prism Graphpad and the data was determined to have normal distribution. (b,c) Two-tailed Student's T-test used for analysis (For [b]: $t=0.7956$, $df = 10$; for [c]: $t=1.356$, $df = 182$). ns = not significant. Error bars indicate s.e.m.

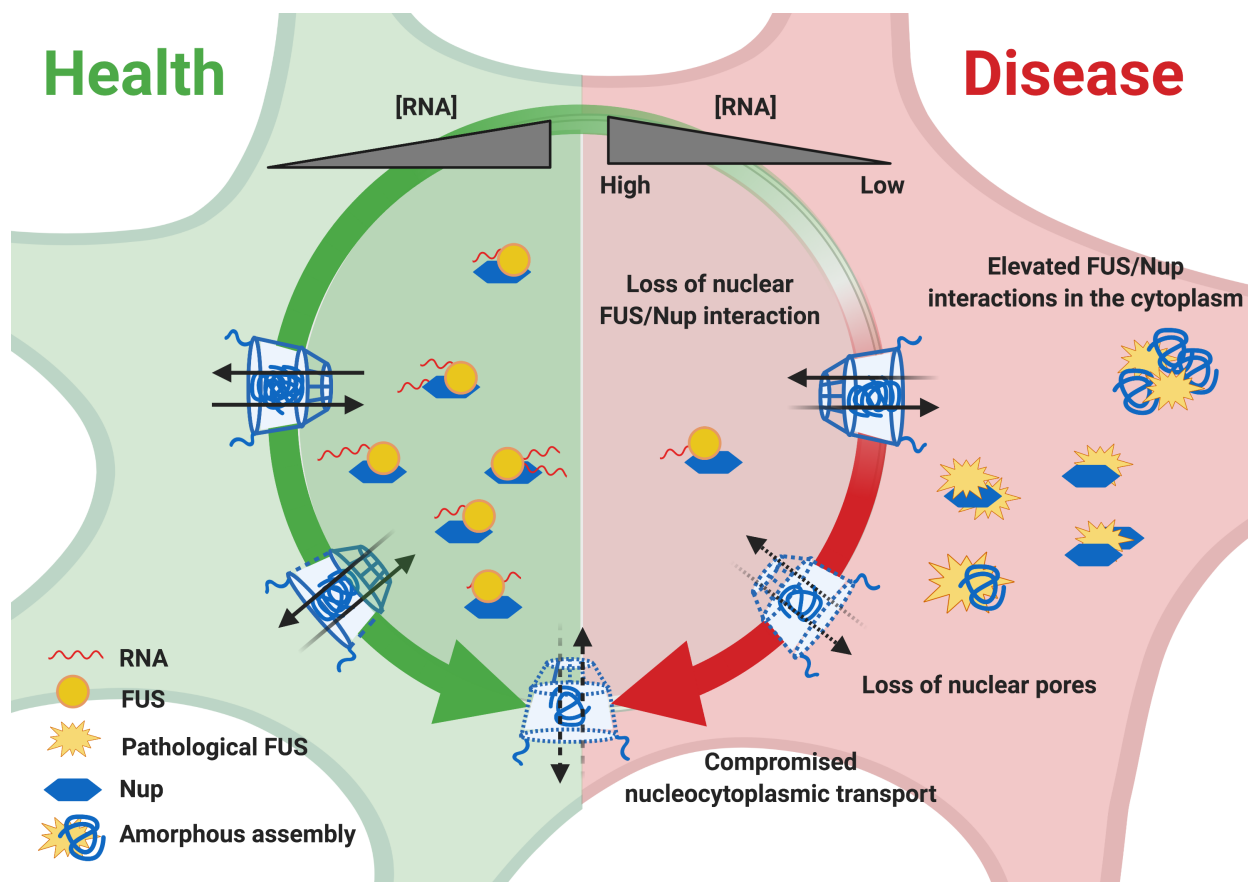


Supplemental Figure 17. Nup62 immunohistochemistry of human post-mortem spinal cord tissue. In the ALS-FUS R521G case (a and b) some lower motor neurons had normal nuclear morphology and strong Nup62 staining of the nuclear membrane (a) but many showed either abnormal morphology or Nup62 staining that was not confined to the nuclear membrane (b). In the control sections (c and d), most of the neurons had normal appearing Nup62 signal (c) but there were also some with abnormal morphology (d). (**a-d**) Images are representative of n=1 staining experiments. Scale bar = 50 μ m.



Supplemental Figure 18. Nup62 RNAi extends survival in RFP-FUS expressing flies.

(a) Kaplan-Meier survival curves of flies expressing FUS WT-RFP and P525L-RFP with and without Nup62 RNAi in adult neurons. Non-transgenic (*w1118*) and flies expressing Nup62 RNAi alone were used as controls. **(b)** Tabular representation of number of days it took for 25%, 50% and 75% death respectively for each genotype (*w1118* Control *n* = 79; Nup62 RNAi *n* = 70; WT-RFP *n* = 82; WT-RFP + Nup62 RNAi *n* = 86; P525L-RFP *n* = 95; P525L-RFP + Nup62 RNAi *n* = 69). The number of days that Nup62 siRNA confers for survival for each genotype are shown in red. **(c-d)** The same data in (a) is separated into graphs for **(c)** FUS WT-RFP and **(d)** P525L-RFP with and without Nup62 RNAi in adult neurons. Controls include *w1118* and flies expressing Nup62 RNAi. Log-Rank (Mantel-Cox) statistical test was used for analysis of survival, *****p* < 0.0001. For all quantifications, data distribution was assumed to be normal but this was not formally tested.



Supplemental Figure 19. Proposed model for how aberrant mutant FUS/Nup62 interactions contribute to nucleocytoplasmic transport decline. The efficiency of nucleocytoplasmic transport declines with age (left) and may be accelerated in neurodegenerative disorders (right). Mutations in the nuclear localization sequence of FUS cause the protein to mislocalize and accumulate in the cytoplasm (right). Our data uncovered direct interactions between FUS and nucleoporins, including Nup62. In control neurons, FUS/Nup interactions are enriched within the nucleus (left). Conversely, in ALS-FUS neurons (right), FUS/Nup interactions and Nup/Nup interactions are enriched within the cytoplasm, where relatively low levels of RNA could favor aberrant FUS/Nup interactions. Consistent with this model, recombinant FUS and Nup62 proteins alter the phase separation properties of each other, where FUS transitions from a liquid droplet into a less dynamic amorphous assembly under conditions of low RNA concentration. Over time, the accumulation and stabilization of cytoplasmic mutant FUS/Nup complexes in neurons is expected to reduce Nup occupancy at the nuclear membrane, thereby reducing NCT capacity and cell health. Decreasing Nup expression rescues toxic FUS phenotypes in flies, including abnormal nuclear membrane morphology in mutant FUS neurons. Collectively, our results indicate the mislocalization of mutant FUS to the cytoplasm within neurons, rather than a physicochemical property of the FUS protein itself, facilitates aberrant interaction with Nups.

Case ID	Tissue	Disease	Gene / Mutation	Age of Onset	Survival (trach/death) months	Age at death	Site Onset	Sex	Race	Tissue Source
MGH-FUS	Lumbar cord	ALS	FUS-H517Q	42	110	52	lower and upper limb	F	Cape Verdean	Massachusetts General Hospital
UBC-FUS	Cervical cord	ALS	FUS - R521C	62	42	~65	lower and upper limb	F	N.A.	Rademakers et al, 2010; University of British Columbia
control 1	Cervical cord	none; neurologically normal	none	N/A	N/A	66	N/A	F	non- hispanic caucasian	University of British Columbia

N/A= not applicable

N.A.=information not available

Supplemental Table 1. Demographics information for post-mortem human tissues used in this study