Ataxin-2 interacts with FUS and intermediate-length polyglutamine expansions enhance FUS-related pathology in amyotrophic lateral sclerosis

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Fused in sarcoma (FUS) is mutated in both sporadic amyotrophic lateral sclerosis (ALS) and familial ALS patients. The mechanisms underlying neurodegeneration are not fully understood, but FUS redistributes from the nucleus to the cytoplasm in affected motor neurons, where it triggers endoplasmic reticulum (ER) stress. Ataxin-2 is a polyglutamine protein which normally contains 22 repeats, but expanded repeats (>34) are found in Spinocerebellar Ataxia type 2. Recently ataxin-2 with intermediate length repeats (27–33) was found to increase the risk of ALS. Here we show that ataxin-2 with an ALS-linked intermediate length repeat (Q31) is a potent modifier of FUS pathology in cellular disease models. Translocation of FUS to the cytoplasm and ER stress were significantly enhanced by co-expression of mutant FUS with ataxin-2 Q31. Ataxin-2 also co-localized with FUS in sporadic and FUS-linked familial ALS patient motor neurons, co-precipitated with FUS in ALS spinal cord lysates, and co-localized with FUS in the ER–Golgi compartments in neuronal cell lines. Fragmentation of the Golgi apparatus is linked to neurodegeneration in ALS and here we show that Golgi fragmentation is induced in cells expressing mutant FUS. Moreover, Golgi fragmentation was enhanced, and the early stages of apoptosis were triggered, when ataxin-2 Q31 was co-expressed with mutant FUS. These findings describe new cellular mechanisms linking ALS with ataxin-2 intermediate length polyQ expansions and provide further evidence linking disruption to ER–Golgi compartments and FUS pathology in ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by the degeneration and death of motor neurons. Although most ALS cases are sporadic (SALS), 10% of ALS is inherited (familial ALS, FALS). Over 30 mutations have been described in the fused in sarcoma (FUS) gene, causing ~5% of all FALS, and these cases display a classical ALS phenotype (1–3). FUS is normally located in the nucleus but in ALS, it translocates to the cytoplasm and misfolds, forming inclusions. FUS-immunoreactive inclusions are found in SALS (4,5) and in the related disorder frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTD) (6). Mutations in FUS have also been described in 1% of SALS patients (7,8).

Furthermore, FUS is the major component of nuclear polyglutamine aggregates in cellular models of Huntington’s disease and it interacts with the polyQ region of polyglutamine proteins (9,10). TDP-43 is a major protein constituent of inclusions in sporadic and familial ALS and FTD. TDP-43 and FUS are both RNA/DNA-binding proteins with striking structural and functional similarities (11,12).

Spinocerebellar Ataxia type 2 (SCA2) is a polyglutamine disorder that results from the expansion of CAG trinucleotide repeats in the ataxin-2 gene (13,14). The normal length of the polyglutamine tract is 22 or 23 residues, but in SCA2 this is...
increased up to 34 repeats. Intermediate length polyglutamine expansions in ataxin-2, greater than normal but below the threshold for SCA2 (27–33 residues), were recently identified as a significant risk factor for ALS (15). TDP-43 and ataxin-2 physically interact in an RNA-dependent manner and ataxin-2 is abnormally localized in spinal cord neurons of ALS patients (15). The biological function of ataxin-2 is not fully understood, but it has been implicated in RNA metabolism (16,17) and cellular trafficking processes (18,19). The latter function is consistent with its subcellular location in the endoplasmic reticulum (ER) (20) and Golgi apparatus (21).

Ataxin-2 is also found in stress granules which form rapidly in response to a variety of cellular insults and lead to translational repression of incorporated mRNAs (22). Similarly, TDP-43 and FUS are also found in stress granules (23–26). ER stress, leading to induction of the unfolded protein response (UPR), is now recognized to be an important pathway to cell death in ALS (27,28).

FUS mutant R521H or R521C, as previously observed (37). was mainly detected in the cytoplasm in cells expressing mutant HA-FUS also co-localized with GM130 (Fig.1C). Hence, mutant FUS

We first addressed the question of whether ataxin-2 and FUS co-localize and interact with FUS in human sporadic ALS motor neurons

The co-localization of FUS and ataxin-2 suggested that these proteins physically interact. This was examined using co-immunoprecipitation of NSC-34 cell lysates using an anti-ataxin-2 antibody. Western blotting of the resulting protein fraction revealed that both WT and mutant FUS were co-precipitated by ataxin-2, but not in control reactions with irrelevant isotype-matched control antibody, or untransfected cells (Fig. 1B).

We next asked whether mutant FUS is also localized in these compartments. We previously demonstrated that both FUS mutants R521C and R521H co-localize with ER markers in this cell line (37). As ataxin-2 is normally located in the ER and Golgi apparatus (19–21), we next asked whether mutant FUS is also localized in the Golgi. Using immunocytochemistry to Golgi marker, GM130, we found that mutant but not WT FUS Halo-tagged proteins partially co-localized with GM130 (Fig. 1C). Hence, mutant FUS associates with both ER and Golgi compartments. We examined this further using FUS proteins tagged with the small HA tag instead of the large Halo-tag (33 kDa). Immunocytochemistry for markers of the ER (KDEL) and Golgi (GM130) revealed that mutant HA-FUS also co-localized with ataxin-2 in the ER-Golgi compartments, similar to Halo-tagged proteins (Supplementary Material, Fig. S1). Hence, the size of the tag did not alter FUS or ataxin-2 localization.

RESULTS

Ataxin-2 co-localizes and interacts with FUS in human sporadic ALS motor neurons

The co-localization between FUS and ataxin-2 was then investigated in human ALS tissues. In control patients without neurological disease, ataxin-2 localized partially with FUS in a diffuse or fine-granular pattern in spinal cord motor neurons. However, in motor neurons from SALS patients or FALS patients bearing the R521C FUS mutation, ataxin-2 predominantly co-localized with FUS in cytoplasmic inclusions (Fig. 2A and B). Analysis of 50 individual motor neuron cells from SALS patients revealed FUS and ataxin-2 were co-localized in all cells examined.

Immunoprecipitations of spinal cord lysates from three SALS and two control patients followed by western blotting revealed that FUS was co-precipitated by ataxin-2 (Fig. 2C). However, more FUS was precipitated from SALS patient tissues. Again, no precipitation was observed in negative control reactions containing irrelevant, isotype-matched control antibody (N). Interestingly, in SALS patient tissues, the input control demonstrated at least three additional, lower molecular weight bands (MW ~40–60 kDa) which were not present in controls (Fig. 2C), suggesting that FUS

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is fragmented in ALS tissues. Finally, we investigated whether RNA is necessary for the interaction between FUS and ataxin-2. RNase treatment did not prevent co-precipitation between ataxin-2 and FUS, suggesting that this interaction is independent of RNA (Fig. 2D).

Intermediate polyQ expansions in ataxin-2 promote interaction with mutant FUS

We next examined whether polyQ repeat expansions in ataxin-2 modify its interaction with FUS. Hence FUS was immunoprecipitated using an ataxin-2 antibody from insoluble cell fractions. Co-expression of FUS with ataxin-2 Q22 enhanced the precipitation of both FUS mutants but not WT, suggesting that when over-expressed, ataxin-2 Q22 preferentially associates with mutant FUS rather than WT (Fig. 3A).

Moreover, over-expression of ataxin-2 Q31 strongly enhanced the precipitation of both WT and mutant FUS, particularly R521H. Hence ataxin-2 Q31 preferentially precipitates ALS-associated FUS mutants, despite a stronger interaction with WT FUS than R521H in the soluble cellular fraction in the absence of over-expression (Fig. 1). Hence, ataxin-2 with intermediate length repeat greatly increases the interaction between FUS and ataxin-2 in the insoluble fraction.

Intermediate polyQ expansions in ataxin-2 promote cytoplasmic translocation of FUS

We next examined whether intermediate length ataxin-2 polyQ expansions modify ALS-associated FUS pathology. Hence FUS was co-transfected with ataxin-2 constructs encoding the normal Q22 repeat or the ALS intermediate length repeat Q31. In cells co-expressing WT FUS with ataxin-2 Q22 or Q31, FUS remained predominantly in the nucleus and its cellular location did not change. However, in cells expressing either R521C or R521H, co-expression with ataxin-2 Q31 significantly increased redistribution of FUS to the cytoplasm ($P < 0.05$, $P < 0.01$, respectively, Fig. 3B). In contrast, co-expression with the normal repeat length (Q22) had no effect on the distribution of either FUS mutant. Hence ataxin-2 with intermediate expansion repeat enhances the translocation of mutant FUS from nucleus to the cytoplasm.

Intermediate polyQ expansions in ataxin-2 increase mutant FUS-induced ER stress

We next asked whether intermediate ataxin-2 repeats enhance ER stress in cells expressing mutant FUS. Induction of the early phases of UPR was examined using XBPI-venus linked to GFP, which detects activation of the IRE/XBP-1 pathway (44). Transfected cells were scored individually for nuclear fluorescence, which indicated splicing of XBP1 and hence IRE1 activation (Fig. 4A). There were increased percentages of cells with IRE1 activation in both mutant FUS populations as observed previously (37). Moreover, there was a significance increase in the proportion of cells with spliced XBP1 co-expressing Q31 with either R521H or R521C (2-fold, $P < 0.01$ and $P < 0.05$, respectively, Fig. 4B) compared to either mutant expressed alone, or to either mutant co-expressed with Q22. In contrast, co-expression of ataxin-2 Q22 or Q31 with WT FUS did not alter the proportion of cells with nuclear XBP1 activation, despite the interaction between ataxin-2 and WT FUS. Similarly, ataxin-2 Q22 or Q31 expressed alone did not induce ER stress. Hence, intermediate length repeats in ataxin-2 enhance the proportion of

![Figure 1](http://hmg.oxfordjournals.org/)

**Figure 1.** Ataxin-2 and mutant FUS inclusions co-localize with ER-Golgi compartments in NSC34 cells. (A) Cells were transfected for 72 h with WT or mutant FUS (R521C or R521H)-Halo-tag vectors, then fixed and stained with anti-Halo antibody (first column), anti-ataxin-2 antibody (second column) and DAPI (third column). Merge (fourth column) indicates overlays of the fluorescent images of FUS, ataxin-2 and DAPI. White arrow indicates mutant FUS proteins inclusions co-localized with ataxin-2. (B) FUS immunoprecipitated with ataxin-2 in NSC34 cell lysates, cell lysates were subjected to western immunoblotting and stained with anti-FUS antibody. (C) Cells expressing Halo-tagged FUS were fixed and stained with anti-Halo antibody (first column), anti-GM130 antibody (second column) and DAPI (third column) and merge of Halo, GM130 and DAPI (fourth column). Scale bar, 10 μm, applies to all fields.
cells expressing mutant FUS which activate IRE1 and hence induce the early phase of UPR.

The later, pro-apoptotic phases of UPR were also examined by nuclear immunoreactivity to CHOP. CHOP is a pro-apoptotic transcription factor specific for ER stress which translocates to the nucleus when activated (31). There were no significant differences in the proportion of cells with activated CHOP expressing WT FUS with either ataxin-2 Q22 or Q31 compared with WT FUS alone, despite the interactions between ataxin-2 with WT FUS. Similarly, ataxin-2 Q22 or Q31 alone did not induce activation of CHOP. However, there were increases percentages of cells expressing nuclear CHOP in both mutant FUS populations, particularly R521H (37), and this increased further when ataxin-2 Q31 was co-expressed with either mutant (2-fold, \( P < 0.05 \), \( P < 0.01 \), respectively). Hence intermediate length ataxin-2 expansions enhance both the early and late apoptotic phases of the UPR induced by ALS-mutant FUS proteins (Fig. 5A and B).

Ataxin-2 polyQ expansions do not promote recruitment to stress granules

We then asked whether intermediate length expansions in ataxin-2 alter the recruitment of FUS to stress granules or promote stress granule formation. The formation of stress granules was examined using immunocytochemistry for the marker TIAR in cells expressing FUS and ataxin2 Q22 and Q21 (Fig. 6A). We found similar percentages of cells expressing TIAR in cells co-transfected either with Q31 or Q22 compared with cells expressing FUS alone for WT or either FUS mutant (Fig. 6B). Hence, ataxin-2 Q31 does not promote the formation of stress granules or the recruitment of FUS to stress granules.

Mutant FUS induces fragmentation of the Golgi apparatus, and this is enhanced by ataxin-2 Q31

Fragmentation of the neuronal Golgi apparatus is a consistent feature of ALS (38,39) and the co-location of mutant FUS with GM130 led us to ask whether mutant FUS also induces Golgi fragmentation. Hence, we examined the morphology of the Golgi apparatus in cells expressing FUS using immunocytochemistry. In untransfected cells or cells expressing WT FUS, the Golgi apparatus mostly localized to a compact, perinuclear ribbon. In contrast, the Golgi apparatus fragmented into punctate structures dispersed throughout the cytoplasm in 25–40% of cells expressing mutant FUS (Fig. 7A). Quantification revealed significantly more cells expressing FUS R521C mutation (Fig. 7B). Hence ALS-associated mutant FUS induce fragmentation of the Golgi apparatus.
Next, we investigated whether ataxin-2 intermediate length expansions enhance Golgi fragmentation in cells. Ataxin-2 Q31 co-expression with either R521H or R521C significantly enhanced the proportion of cells with Golgi fragmentation compared with either FUS mutant alone (~3-fold \( P < 0.001 \) and 2-fold, respectively \( P < 0.05 \)). Importantly, expression of ataxin-2 Q22 or Q31 alone did not induce Golgi fragmentation, in contrast to ataxin-2 with the longer polyglutamine repeats (Q58 or Q104) characteristic of SCA (21). Similarly, ataxin-2 Q22 did not induce Golgi fragmentation in any cell population, and Q31 did not induce Golgi fragmentation in WT FUS expressing cells, despite the observed ataxin-2-WT FUS co-precipitation (Fig. 1B). Hence intermediate length expansions in ataxin-2 enhance fragmentation of the Golgi apparatus in cells expressing mutant FUS (Fig. 7C).

**Figure 3.** Ataxin-2 Q31 enhances FUS cytoplasmic translocation and interaction with mutant FUS in NSC34 cells. (A) Cells were co-transfected with mutant FUS (R521C or R521H)-Halo-tag and ataxin-2 Q22 or Q31 vectors for 72 h. Cells were then lysed at 72 h post-transfection and the pellet fractions were co-immunoprecipitated using an anti-ataxin-2 antibody followed by immunoblotting using anti-FUS antibody. Input control shows similar levels of FUS in each sample. Blot was reprobed with β-actin as a loading control. More mutant FUS is immunoprecipitated with ataxin-2 when Q31 is co-expressed with FUS. (B) Cells were co-transfected with mutant FUS (R521C or R521H)-Halo-tag and ataxin-2 Q22 or Q31 vectors for 72 h, then fixed and stained with anti-Halo antibody. Cells were then scored individually as to whether FUS was expressed in the nucleus or cytoplasm. For each of three replicate experiments, 50 cells were scored for each population. Data are represented as mean ± SEM; *\( P < 0.05 \), **\( P < 0.01 \) by one-way ANOVA with Tukey’s post-hoc test.

**Figure 4.** Ataxin-2 poly Q31 enhances XBP-1 splicing in NSC-34 cells. Cells were co-transfected with mutant FUS (R521C or R521H)-Halo-tag, ataxin-2 poly Q22 and Q31 and GFP XBP1-venus vectors for 72 h (A) Cells were fixed and immunostained with Halo-tag antibody (first column), and DAPI (third column). XBP1 splicing is shown as GFP fluorescence (second column). Merge (fourth column) indicates overlays of the fluorescent images of Halo-tag, XBP-1 and DAPI. Scale bar, 10 μm, applied to all fields. (B) Quantification of FUS-transfected cells using XBP1-venus as a marker for IRE1 activation and hence ER stress. Cells expressing both FUS and XBP1-venus together were scored. Cells expressing FUS in both the nucleus and cytoplasm were counted as nuclear. For each of three replicate experiments, 50 cells were scored for each population. Data are represented as mean ± SEM; *\( P < 0.05 \), **\( P < 0.01 \) by one-way ANOVA with Tukey’s post-hoc test.
Intermediate polyQ expansions in ataxin-2 induce early apoptosis in mutant-FUS expressing cells

The activation of CHOP in cells co-expressing mutant FUS with ataxin-2 suggested that apoptosis was triggered in these cells. We examined this further using immunocytochemistry to Bcl-2-associated X protein (Bax). Bax becomes recruited to mitochondria where it is activated (45) during the initial stages of mitochondrial apoptosis (Fig. 8A). In the absence of ataxin-2, there was no difference in the proportion of cells with Bax recruitment between WT FUS and mutant FUS populations, consistent with previous observations that FUS expressed in cell culture does not induce toxicity (46,47). Similarly, the expression of ataxin-2 Q22 or Q31 alone did not induce recruitment of Bax to mitochondria and hence apoptosis, unlike ataxin-2 Q34 in SCA (21).

However, co-expression of ataxin-2 Q31 with R521H significantly increased the proportion of cells in which Bax was recruited to mitochondria (Fig. 8B), suggesting that early mitochondrial apoptosis was underway in these cells. There was a slight increase in the proportion of cells with Bax recruitment in cells expressing Q31 with R521C, but this did not reach statistical significance. Again Q22 had no effect on mutant FUS expressing cells, and similarly, Q31 and Q22 had no effect in WT FUS expressing cells. Hence, Q31 enhances the toxicity of FUS mutant R521H. We could not detect induction of the later stages of apoptosis in any cell population using propidium iodide staining, nuclear condensation using DAPI staining or cytochrome c release assessed by immunoblotting.
DISCUSSION

In this study, we show that intermediate length polyQ expansions in ataxin-2 (27–33 residues), greater than the normal 22–23 repeats but below the threshold for SCA2 (>34), contribute to the pathology associated with FUS in ALS. We demonstrate that in a motor neuron cell line, ataxin-2-Q31 enhances both translocation of FUS from the nucleus to the cytoplasm and ER stress induced by mutant FUS. Furthermore, we show that ALS FUS mutants induce fragmentation of the Golgi apparatus which is enhanced by ataxin-2 polyQ31 in NSC34 cells. Cells were co-transfected for 72 h with FUS-Halo-tag (mutants R521C or R521H) and ataxin-2 Q22 or Q31 vectors. (A) Cells were fixed and immunostained with Halo-tag antibody (first column), Golgi marker GM130 antibody (second column) and DAPI (third column). Merge (fourth column) indicates overlays of the fluorescent images of Halo-tag, GM130 and DAPI. White arrows indicate intact Golgi in WT FUS cells and fragmented Golgi in mutant FUS expressing cells. Scale bar, 10 μm, applied to all fields. (B) Mutant FUS induces Golgi fragmentation in comparison with untransfected NSC34 cells. Cells were examined individually for fragmented Golgi, identified by immunocytochemistry using anti-GM130 antibodies. For each of three replicate experiments, 50 cells were scored for each population. Data are represented as mean ± SEM; **P < 0.01, *P < 0.05 mutant versus WT, by analysis of variance (ANOVA) with Tukey’s post-hoc test. (C) Quantification also reveals ataxin-2 enhances Golgi fragmentation in NSC-34 cells. Cells were examined individually for fragmented Golgi, identified by immunocytochemistry using anti-GM130 antibodies. For each of the three replicate experiments, 50 cells were scored for each population. Data are represented as mean ± SEM; *P < 0.05, **P < 0.01 by one-way ANOVA with Tukey’s post-hoc test.
induced by mutant FUS (37), the increased risk of ALS associated with intermediate FUS, and elucidates a new molecular mechanism to explain though we could not detect later markers of apoptosis. 

Figure 8. Bax is recruited to mitochondria, indicating induction of early apoptosis in cells expressing mutant FUS and ataxin-2 poly Q31. (A) NSC-34 cells were transfected with mutant FUS (R521C or R521H)-Halo-tag vectors as indicated. Cells were fixed and immunostained with Halo-tag antibody (first column), Bax antibody (second column) and DAPI (third column). White arrow shows Bax recruitment to mitochondria and depletion from the nucleus. Scale bar 10 μm. (B) Quantification of Bax recruitment in (A) demonstrates that ataxin-2 Q31 increased Bax recruitment to mitochondria in mutant FUS R521H expressing cells but not in other populations. For each of the three replicate experiments, 100 cells were scored for each population. Data are represented as mean ± SEM; *P < 0.05, ***P < 0.01 by one-way ANOVA with Tukey’s post-hoc test. 

of the Golgi apparatus, and this is further enhanced by the presence of ataxin-2 Q31. We also demonstrate that early mitochondrial apoptosis, mediated by Bax and CHOP, was underway in cells expressing mutant FUS and ataxin-2, although we could not detect later markers of apoptosis. Hence this study provides new clues into the pathology of FUS, and elucidates a new molecular mechanism to explain the increased risk of ALS associated with intermediate length polyQ expansions (15,43,48).

The cellular mechanisms underlying FUS-mediated neurodegeneration in ALS remain unclear. We previously demonstrated that ER stress is induced by mutant FUS (37), including up-regulation of CHOP which mediates the transition from the pro-survival to pro-apoptotic phase of UPR (28,32). Fragmentation of the Golgi apparatus is one of the earliest changes in ALS pathology (49), and here we show that mutant FUS also induces Golgi fragmentation. Hence together these data show that mutant FUS induces considerable dysfunction to the ER–Golgi compartments in ALS. ER–Golgi abnormalities have also been described in association with other proteins linked to ALS, including TDP-43, SOD1 and VAPB (50–54), pointing to common pathogenic pathways in ALS linked to the ER. Our study also shows that ataxin-2 can modulate these pathways, consistent with its normal ER–Golgi location. However, mutant FUS may also have indirect effects in ALS. A recent study showed that mutant FUS R521C induces mutant-like misfolding in SOD1 (55), suggesting overlap between the pathogenic mechanisms triggered by TDP-43, FUS and SOD1. Mutant SOD1 induces ER stress (33,56,57) and Golgi fragmentation (38,49), hence, mutant FUS may also induce misfolding of SOD1, which subsequently perturbs the ER–Golgi via this mechanism.

This study also shows that ataxin-2 associates preferentially with pathological forms of FUS. Ataxin-2 Q31 precipitated more FUS in ALS patients compared to controls and more mutant FUS than WT FUS in cell lines. Similarly, ataxin-2 Q22 also precipitated more mutant FUS than WT, but significantly more FUS was precipitated with ataxin-2 Q31. Hence these data suggest a preferential interaction between pathological forms of ataxin-2 Q31 and mutant FUS. As we examined insoluble cell lysates, it is possible that the interaction between ataxin-2 Q31 and FUS may increase the insolubility of FUS, therefore promoting aggregation. However, it should be noted that the interaction between ataxin-2 and FUS may not be the only reason for the observed effects of ataxin-2 on FUS ER–Golgi pathology. A proteomic screen to identify other ataxin-2 interacting proteins which could modify FUS pathology may therefore be warranted.

We also found evidence of FUS protein fragments (40–60 kDa) in ALS patient spinal cords but not in controls. The significance of these fragments remains unclear, but they suggest impairment of protein degradation processes which is well documented in ALS. A recent study in yeast demonstrated that a 422 residue (~46 kDa) region in FUS was necessary for toxicity and aggregation, suggesting that fragmented FUS as well as the intact protein can modulate toxicity (46). However, only full-length FUS protein immunoprecipitated with ataxin-2 in patient spinal cord lysates, suggesting that the ataxin-2 binding region was not present in these fragments.

FUS is the major component of nuclear polyQ aggregates in Huntington’s disease and it is also found in the inclusions in other polyglutamine diseases (9). The interaction between FUS and ataxin-2 could imply that all polyQ proteins play a role in ALS and other neurodegenerative diseases. However, a recent study demonstrated that other polyQ disease proteins, including ataxin-1,-3, -6 and -7, are not associated with ALS (58). Hence, the effects of ataxin-2 on FUS must be specific to the biological function of ataxin-2 rather than the presence of the expanded polyQ repeat. The normal physiological function of ataxin-2 is unclear, but it has been implicated both in RNA-processing and in the regulation of intracellular trafficking (19,59). We show that the interaction between FUS and ataxin-2 is independent of RNA in contrast to the interaction between ataxin-2 and TDP-43 (15). Both FUS and ataxin-2 also co-localized with the ER and Golgi apparatus in our study and expression of FUS did not change the normal subcellular location of ataxin-2. Rather this suggests that ataxin-2 promotes the localization of FUS in the ER and Golgi apparatus. Hence it is possible that ataxin-2 Q31 enhances FUS pathology via a
mechanism related to cellular trafficking rather than RNA function. Consistent with this notion, disruption to cellular trafficking triggers ER stress and Golgi fragmentation, which were both enhanced by ataxin-2 in our study (60).

Similarly, the ER–Golgi localization of ataxin-2 places this protein apart from other members of the polyQ family (21), suggesting that the effects of ataxin-2 Q31 on FUS involve ER–Golgi specific functions. These effects were evident for both FUS mutants but were greater for R521H than R521C. We did not detect apoptosis in cells expressing mutant FUS alone consistent with previous studies (37,46). However, ataxin-2 enhances CHOP activation and induces Bax recruitment to mitochondria, suggesting that ataxin-2 triggers mitochondrial apoptosis in cells expressing R521H. The cells appear to be in the early stages of apoptosis, as we found no evidence of induction of the later stages, suggesting that apoptosis was stalled or slowed in these cells. Hence, additional stresses may be required to induce cell death. However, while both ataxin-2 and FUS are components of stress granules, co-expression of Q31 did not enhance their formation. Hence stress granule formation appears to be unrelated to ER stress and Golgi fragmentation in FUS pathology.

SCA2 presents motor neuron characteristics that resemble ALS in some cases, and this study and others suggest further overlap between ALS, FTD and SCA2 (61–63). Here, we also show that the effects of ataxin-2 Q31 on FUS pathology are distinct from both the normal length repeat (Q22) and the expanded repeats (Q > 35) found in SCA2. While ataxin-2 Q > 35 induces fragmentation of the Golgi apparatus and apoptosis (21), ataxin-2 Q31 does not induce either process in NSC-34 cells. Ataxin-2 Q < 35 repeats also lose their co-localization with the Golgi (21), whereas in our study, Golgi localization was retained in cells co-expressing ataxin-2 Q31. Similarly, ataxin-2 Q31 had little effect on WT FUS, despite the co-precipitation of these proteins. The effects of Q31 on FUS are not simply due to over-expression of ataxin-2 because the normal length repeat Q22 did not affect any of the parameters we examined. Hence the effects of the intermediate repeat are specific to mutant FUS and the intermediate length polyQ expansion.

In summary, ataxin-2 with an intermediate-length polyglutamine expansion, Q31, interacts with FUS and enhances ALS-mutant cellular FUS pathology. These findings provide new molecular and cellular mechanisms to explain why intermediate-length polyglutamine expansions in ataxin-2 are associated with an increased risk of ALS (15,43,64). The identification of pathological interactions between ataxin-2 Q31 and FUS also provides novel targets for future therapeutic intervention in ALS.

MATERIALS AND METHODS

Constructs

The FUS-Halo constructs used in this study were as previously described (37). WT FUS, R521C and P525L constructs tagged with HA were obtained from Dr Dorothee Dorman, Ludwig Maximilians University München. For detection of ER stress, the XBP-1-venus reporter was used (65). Ataxin-2 constructs Q22 and Q31 were kindly provided by Dr Aaron Gitler, University of Pennsylvania.

Table 1. Patient clinical information

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M, male; F, female; Age, age at death; PMI, post-mortem interval.

Human tissue samples

Human lumbar spinal cord segments (L3–L5) from three sporadic patients who died of ALS were provided by the Victorian Brain Bank Network (Table 1): the clinical diagnosis was confirmed at post-mortem. Immunohistochemistry was performed on paraffin-embedded spinal cord tissues from individual IV-5 from family ALS53, carrying the FUS R521C mutation (38). Control samples were obtained from two individuals without evidence of neurological or psychiatric disease. Studies were approved by La Trobe University Human Ethics Committee.

Immunocytochemistry

NSC-34 cells grown on coverslips were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Cells were permeabilized in 0.1% Triton X-100 in PBS for 2 min, blocked for 30 min with 1% BSA in PBS, and incubated with primary anti-ataxin-2 (1:100), anti-FUS (1:100), (ab23439), anti-CHOP (SC-7351) (1:50), anti TIAR (1:1000), BD transduction, 610352), anti-Halo antibodies (1:200, BD transduction, 610352), anti-Halo antibodies (1:200, G928A, Promega), anti- KDEL (1:100, Stressgen SPA-827) for 16 h at 4°C. Secondary AlexaFluor-594 conjugated antibodies (1:200, Molecular Probes) were then incubated for 1 h at room temperature, and cells were counter-stained with DAPI and mounted. Images were acquired using an Olympus inverted confocal laser-scanning...
microscope or an Olympus inverted fluorescence microscope. To examine fragmentation of the Golgi apparatus, cells were stained with anti-GM130 antibody (BD transduction 610823 (1:50). The number of cells containing fragmented Golgi per 100 transfected cells was calculated using fluorescent microscopy. For SG quantitation, five random fields were analyzed. Cells bearing two or more TIAR-positive and FUS-positive SGs greater than ≈1 μm in diameter, were counted as containing SGs.

**Immunohistochemistry**

Paraffin sections (14 μm thick) were treated as previously described (37). Post-mortem spinal cords were blocked with normal goat serum for 30 min and then stained with anti-FUS (1:50) and anti-ataxin-2 (1:100) antibodies for 48 h. After three washes with PBS, Alexa Fluor secondary antibodies were used and images were acquired using confocal microscopy.

**Protein extraction**

Cells were lysed in Tris–NaCl (TN) buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) with 0.1% (v/v) NP-40, 0.1% (w/v) SDS, and 1% (v/v) protease inhibitor mixture (Sigma) for 10 min on ice. Cellular lysates were clarified by centrifugation at 16g for 10 min. Proteins were quantified using the BCA assay kit (Pierce).

**Immunoblotting**

Protein samples (20 μg) were electrophoresed through 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (Millipore), then blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline (TBS), pH 8.0 for 1 h. Primary antibodies were added for 16 h at 4°C; FUS (1:1000 Abcam, ab23439) or ataxin-2 (1:500, SC-18477). Membranes were incubated for 1 h at room temperature with secondary antibodies (1:4000, HRP-conjugated donkey anti-sheep, goat anti-rabbit or goat anti-mouse antibodies, Chemicon), and detected with enhanced chemiluminescence reagents (Roche).

**Immunoprecipitation**

One hundred micrograms of either human spinal cord lysates or transfected cell lysates were incubated with anti-ataxin-2 antibody, and 30 μL of 50% (w/v) protein A-Sepharose CL-4B (Amersham Biosciences) in Tris buffer [50 mM Tris–HCl, pH 7.5, 0.02% (w/v) NaNO₃] on a rotating wheel overnight at 4°C. Samples were centrifuged for 1 min at 8000 g. Immunoprecipitates were liberated by boiling in 2% (w/v) SDS loading buffer and then immunoblotted using anti-FUS or ataxin-2 antibodies. Controls were used to determine the specificity of the interaction: Tris buffer only or irrelevant antibody, anti-Flag (F2555) (Sigma) as the precipitating antibodies. For RNAase treatment, ataxin-2 samples were treated with 5 μL (100 mg/ml) RNAse for 1 h at room temperature on a rotating wheel, then the beads were washed and immunoprecipitated as described above. The activity of the RNAase enzyme was tested by incubating extracted RNA with enzyme for 1 h, and digestion was confirmed by 1% agarose gel electrophoresis.

**Statistical analysis**

Comparisons were performed as indicated by one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test unless otherwise stated, from three independent experiments. P < 0.05 was considered significant. Data are presented as mean ± standard error of the mean.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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