ALS-associated mutations in FUS disrupt the axonal distribution and function of SMN


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Mutations in the RNA binding protein fused in sarcoma/translated in liposarcoma (FUS/TLS) cause amyotrophic lateral sclerosis (ALS). Although ALS-linked mutations in FUS often lead to a cytosolic mislocalization of the protein, the pathogenic mechanisms underlying these mutations remain poorly understood. To gain insight into these mechanisms, we examined the biochemical, cell biological and functional properties of mutant FUS in neurons. Expression of different FUS mutants (R521C, R521H, P525L) in neurons caused axonal defects. A protein interaction screen performed to explain these phenotypes identified numerous FUS interactors including the spinal muscular atrophy (SMA) causing protein survival motor neuron (SMN). Biochemical experiments showed that FUS and SMN interact directly and endogenously, and that this interaction can be regulated by FUS mutations. Immunostaining revealed co-localization of mutant FUS aggregates and SMN in primary neurons. This redistribution of SMN to cytosolic FUS accumulations led to a decrease in axonal SMN. Finally, cell biological experiments showed that overexpression of SMN rescued the axonal defects induced by mutant FUS, suggesting that FUS mutations cause axonal defects through SMN. This study shows that neuronal aggregates formed by mutant FUS protein may aberrantly sequester SMN and concomitantly cause a reduction of SMN levels in the axon, leading to axonal defects. These data provide a functional link between ALS-linked FUS mutations, SMN and neuronal connectivity and support the idea that different motor neuron disorders such as SMA and ALS may be caused, in part, by defects in shared molecular pathways.

INTRODUCTION

Considerable progress has been made in dissecting the genetic basis of amyotrophic lateral sclerosis (ALS), but how genetic defects cause this devastating disease remains poorly understood (1). Disease mechanisms implicated in ALS include excitotoxicity, oxidative stress, protein aggregation, impaired mitochondrial function and RNA metabolism and defective axonal transport (2). These processes frequently function in a compartmentalized manner with distinct subcellular mechanisms acting in cell bodies, axons and synapses. Intriguingly, in ALS, and other neurodegenerative disorders, the most distant cellular sites (i.e. distal axons and synapses) are often affected first or at least during initial stages of disease (3). Therefore,

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studying genetic defects in light of this selective vulnerability of axons and synapses promises to provide valuable insights into pathogenic mechanisms as well as yield therapeutic targets for early intervention.

An ever increasing number of genetic studies highlight a crucial role for RNA-related proteins such as TAR DNA binding protein (TDP-43) and fused in sarcoma/translocated in liposarcoma (FUS) in ALS pathogenesis (4–6). FUS has a predominant nuclear localization and shuttles between the nucleus and the cytoplasm (7). It functions in RNA-related processes such as splicing and mRNA transport (8). Mutations in FUS can be found in patients suffering from ALS, including juvenile and predominantly lower motor neuron forms [for an overview of juvenile ALS cases associated with FUS mutations, see (69)]. The greatest number of ALS causative FUS mutations occur in the C-terminal nuclear localization signal (NLS) of FUS leading to an aberrant, cytosolic localization of the mutant protein, both in patients and in experimental models (4,6,9,10). Both the cytosolic localization of the mutant protein and the mutations themselves contribute to the pathogenic effects of FUS mutations. Although the disease mechanisms underlying FUS mutations remain largely unexplored, mutant FUS confers cellular toxicity in yeast and induces stress granule formation in cells and zebrafish (11–13). Important questions such as the pathogenic role and composition of mutant FUS aggregates and the effect of mutations on the physiological functions of FUS remain to be addressed.

The intriguing relationship between RNA metabolism and motor neuron disease described for FUS is shared by other RNA-related proteins including the survival motor neuron (SMN) protein. Homozygous mutations or deletions of SMN cause another motor neuron disease, spinal muscular atrophy (SMA). SMN localizes to nuclear Gems and to the cytoplasm of cells and is part of the SMN complex, which functions in snRNP biogenesis and RNA splicing (14). Accordingly, models of SMA, based on severe reduction of SMN protein, show RNA splicing defects (15). In addition, however, SMN is localized to axons to regulate axonal mRNA transport and local translation (16–21). Reduced SMN levels in SMA patients and in vitro and in vivo models lead to axonal and synaptic defects (20,22), which, in part, may reflect a disruption of the axon-specific effects of SMN (20,23,24). Remarkably, both human genetic and experimental studies implicate SMN in ALS pathogenesis (25–27), suggesting overlapping disease mechanisms for ALS and SMA. However, the functional and subcellular basis of this relationship remains largely uncharacterized.

Here, we report that ALS-associated FUS mutants cause axonal defects in primary neurons. A protein interaction screen performed to explain these phenotypes identifies numerous FUS interactors including SMN. SMN directly and endogenously interacts with FUS, and this interaction can be regulated by FUS mutations. Furthermore, mutant FUS aggregates co-localize with SMN in primary neurons. The redistribution of SMN to cytosolic FUS aggregates leads to a decrease in axonal SMN. Strikingly, expression of FUS mutants and reducing SMN levels in neurons elicits similar axonal phenotypes. Overexpression of full-length (FL) SMN or a C-terminal SMN fragment of SMN, known to mediate SMN axonal functions (28), rescues the axonal defects induced by mutant FUS, providing a functional link between mutant FUS and SMN. These observations suggest that FUS mutations may indirectly, through aggregate formation and sequestering of SMN, affect neuronal connectivity, thereby providing a functional link between ALS and SMA.

RESULTS

ALS-associated mutations in FUS lead to axonal defects

FUS has a predominant nuclear localization, but shuttles between the nucleus and the cytosol (7). Mutations in FUS associated with ALS often lead to a cytosolic mislocalization of the mutated protein (4,6,9,10). This mislocalization may perturb the normal, physiological roles of FUS and its interacting proteins, for example in RNA-related processes or in the development and plasticity of axons and synaptic contacts. Given the fact that axonal changes are among the first pathological hallmarks of ALS, we assessed the effect of mutations in FUS on axonal morphology. FUS aggregates have been reported in spinal motor neurons and cortical neurons (29–31). Here, we used cortical neurons as a model. Primary cortical neurons isolated from E14.5 mouse embryos were cultured and transfected with GFP (control, Fig. 1A), wild-type FUS (GFP-FUS–WT, Fig. 1B) or a mutant of FUS associated with ALS, R521C (GFP-FUS-R521C, Fig. 1C). After 2 days in culture, axon length, branching and growth cone area were quantified as hallmarks of axon growth and morphology. A moderate decrease in axon length was observed in neurons transfected with FUS–WT (9% decrease, \( P = 0.051 \)) or FUS-R521C (11% decrease, \( P = 0.01 \)) when compared with GFP control neurons. In contrast, the number of primary axon branches was strongly decreased in neurons transfected with FUS-R521C when compared with GFP and FUS–WT transfected neurons. While the average number of primary branches was similar for the FUS (3.9 branches) and FUS–WT (3.7 branches) conditions, it was significantly lower in FUS-R521C neurons (2.8, \( P < 0.001 \)) (Fig. 1G). Finally, a pronounced decrease in axonal growth cone area was detected in primary neurons transfected with FUS-R521C (74% of GFP, \( P < 0.05 \)) (Fig. 1H). The growth cone area of primary neurons expressing FUS-WT was similar to control (109% of GFP control, \( P = 0.39 \)) (Fig. 1H). Together, these findings show that FUS mutations lead to changes in the regulation of axon complexity and morphology.

Identification of the FUS interactome

To identify cellular processes through which FUS influences axon morphology and which may be perturbed by FUS mutations, we used a biotin–streptavidin-based purification system to identify FUS-interacting proteins (32). Neuronal NSC34 cells were co-transfected with FUS-WT and the bacterial biotin ligase BirA, allowing for highly specific pull-down of biotinylated FUS and its interacting partners using streptavidin beads (Fig. 2A). Mass spectrometry analysis of the isolated bioGFP–FUS protein complexes revealed 222 proteins that were present in bioGFP–FUS complexes, but not in control bioGFP complexes. FUS was found to form potential complexes with heterogeneous ribonucleoproteins (hnRNPs) and numerous RNA binding proteins, such as the splicing factors YBX1 and
Gene ontology (GO) analysis of all genes showed a strong enrichment for categories that further reflect previously reported functions of FUS. These include RNA splicing [88 proteins, Bonferroni-adjusted $P$-value (adj-p) $= 1.46 \times 10^{-21}$], mRNA processing (90 proteins, adj-p $= 1.53 \times 10^{-98}$) and spliceosomal complex (60 proteins, adj-p $= 1.24 \times 10^{-80}$) (Supplementary Material, Table S1). Notably, the pull-down experiment also led to the identification of several interactors with known roles in motor neuron disease, including TDP-43, ATXN2-paralog ATXN2L and SMN. In addition to SMN, other components of the SMN complex were identified, including Gem-associated protein 2 (GEMIN2), GEMIN5 and SMN-domain containing-1 (SMNDC1) (Table 1). Importantly, several of these interactions have recently been shown by others (26,36,37), providing support for the specificity of the FUS pull-down experiment. To further confirm the mass spectrometry results, the interaction of several binding partners of FUS was tested in neuronal cells.

FUS interacts with SMN in neurons

The pull-down experiment revealed that FUS associates with SMN, which is significant as SMN knockdown induces axonal and growth cone phenotypes resembling those observed following overexpression of mutant FUS (Fig. 1) (38). This raises the possibility that FUS and SMN are functionally linked. Deletions or mutations of SMN are the cause of the motor neuron disease SMA (39). Next to a well-described role in the biogenesis of snRNPs and RNA splicing (40), SMN has an important function in the development and plasticity of axons and synapses. SMN regulates the axonal transport of mRNAs and their translation...
Figure 2. FUS is able to directly interact with SMN through its C-terminal domains. (A) Streptavidin pull-down assays were performed with lysates of NSC-34 cells co-expressing bio-GFP or bio-GFP-FUS together with BirA. Proteins bound to streptavidin beads were analyzed by western blotting using anti-FUS and anti-GFP antibodies (two left panels). The same samples were separated on a gradient gel followed by silver staining (right panel). Arrows, bioGFP or bioGFP-FUS; arrowheads, proteins enriched in bioGFP-FUS pull-down. (B) Lysates of NSC-34 cells were immunoprecipitated with anti-Myc or IgG (control), anti-SMN or anti-FUS antibodies. The immunoprecipitates were analyzed with the indicated antibodies. (C) Mouse brain lysate was immunoprecipitated with anti-FUS, anti-SMN or IgG (control). The precipitates were analyzed with the indicated antibodies. (D) Purified FUS and SMN proteins were mixed and immunoprecipitated with IgG (control) or anti-FUS antibodies. Immunoprecipitates were analyzed with the indicated antibodies. (E) Schematic representation of FL FUS and truncation and deletion mutants of FUS used in this study. QGSY, glutamine, glycine, serine, tyrosine rich domain; G rich, glycine rich; NES, nuclear export signal; NLS, nuclear localization signal; RGG, arginine glycine rich; RRM, RNA recognition motif; ZnF, Zinc finger. (F) Lysates of NSC-34 cells transfected with the indicated FUS constructs were precipitated with streptavidin beads following which the precipitates were probed with the indicated antibodies.
Table 1. Summary of FUS-interacting proteins identified in this study

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Summary of FUS-interacting proteins identified in this study. Shown are putative FUS-associated proteins with a Mascot score of >60 and multiple different peptide hits in the streptavidin pull-down from bioGFP-FUS expressing NSC-34 cells. The list is corrected for common contaminants and background proteins, which were identified in a control bioGFP pull-down. Proteins are categorized according to their known functions. Proteins involved in motor neuron disease are indicated in bold.

...and a region enriched for glycines (D1-165) and a region enriched for glycines (Δ165-276), bound to SMN. In contrast, both C-terminal truncation mutants (containing the RGG-rich regions and RNA-recognition motifs of FUS), Δ285-372 and Δ360-501, were unable to bind SMN (Fig. 2F). Importantly, all mutants were localized in the nucleus, the endogenous site of FUS expression (Supplementary Material, Fig. S2). Together these data support a direct and endogenous interaction between FUS and SMN in the brain. Although the specific binding sequence mediating the interaction between FUS and SMN remains to be determined, our data indicate that this interaction requires the C-terminal region of FUS (containing the RGG-rich regions and RNA-recognition motifs).

ALS-associated mutations in FUS increase its interaction with SMN

Next, we studied the interaction of SMN with FUS carrying ALS-linked mutations. Since mutations in TDP-43, another RNA-related protein implicated in ALS, are known to affect protein binding (37), we examined the binding of SMN to several FUS mutants, including R521C, R521H and P525L. These mutations are well characterized, and the resulting mutant FUS proteins localize extensively in the cytosol (10). Interestingly, the interaction between FUS and SMN was significantly enhanced by the R521C mutation (177% of FUS–WT interaction), confirming the interaction of FUS and SMN in endogenous protein complexes in neuronal cells (Fig. 2B, lower panel). Next, immunoprecipitation of FUS and SMN was performed from mouse brain tissue, in order to assess whether FUS and SMN complexes occur in the brain in vivo. Indeed, when FUS was precipitated SMN could be detected, and vice versa (Fig. 2C). Importantly, FUS and SMN are known to associate with many RNAs and other RNA binding proteins. Thus, their interaction may be indirect and mediated by other proteins or RNAs. However, immunoprecipitation of recombinant FUS and SMN proteins revealed a direct interaction in the absence of any other proteins, RNA molecules or cellular components (Fig. 2D). Finally, to clarify which region(s) of FUS mediates its interaction with SMN, various bioGFP-FUS deletion mutants were generated and expressed in neuronal cells (Fig. 2E). FL FUS and truncation mutants lacking the N-terminal half of the FUS protein, containing a region enriched for glutamine, glycine, serine and tyrosine residues (QGSY region) (Δ1-165) and a region enriched for glycines (Δ165-276), bound to SMN. In contrast, both C-terminal...
performed with two other, novel interactors of FUS, SYNCRIP and GEMIN2. The interaction of SYNCRIP and GEMIN2 with all three FUS mutants was similar to control (Fig. 3B). Thus, the interaction of FUS and SMN can be modulated by specific ALS-linked mutations in FUS.

**FUS mutations disrupt the localization of SMN in neurons**

Many of the ALS-linked mutations reported in FUS occur in its C-terminal NLS causing mislocalization of the protein to the cytoplasm (10). Because of the biochemical interaction between SMN and mutant FUS (Fig. 3A and B), it is possible that FUS mutant proteins sequester SMN in the cytoplasm. Therefore, we wanted to investigate whether FUS mutations affect the distribution pattern of SMN in neurons. The distribution of SMN and its co-localization with (mutant) FUS was analyzed in primary neurons overexpressing FUS-WT, FUS-R521C, FUS-R521H or FUS-P525L. In neurons overexpressing FUS–WT, FUS labeling was predominantly nuclear while SMN showed a diffuse cytosolic distribution and sparse nuclear

**Figure 3.** The R521C mutation in FUS increases its interaction with SMN. (A) Interaction of SMN with WT and R521C-, R521H- and P525L-mutant FUS. Lysates of NSC-34 cells transfected with bioGFP, bioGFP-FUS or bioGFP-FUS containing the different mutants were subjected to precipitation with streptavidin beads (IP) followed by immunoblotting (IB) against SMN, SYNCRIP and GEMIN2. (B) Quantification of three independent co-immunoprecipitation experiments as shown in (A). Data are normalized to GFP and shown as a percentage of the expression of WT bioGFP-FUS. Shown are means ± SEM. *P < 0.05.
labeling (Fig. 4A–D). Although the expression of FUS and SMN only partially overlaps, as visualized by immunohistochemistry, both proteins are known to shuttle between the nucleus and the cytoplasm. Therefore, at any moment in time part of the pool of total SMN and FUS proteins is located in the same cellular compartment (7, 41). In neurons that expressed FUS-R521C, the cytosolic distribution of SMN was significantly perturbed. Rather than displaying a diffuse, homogenous distribution pattern, a large amount of SMN protein was confined to cytoplasmic accumulations which also contained mutant FUS (Fig. 4E–H). A similar altered distribution was found for FUS mutants R521H or P525L, although the amount of FUS aggregation differed between the different mutants (Fig. 4E–P). Quantification of these experiments revealed a significant increase in the cytosolic co-localization of mutant FUS and SMN as compared with FUS-WT (Fig. 4Q, P < 0.001). To provide biochemical support for these findings, NSC34 cells were transfected with GFP, FUS-WT and FUS-R521C followed by the isolation of cytosolic and nuclear protein fractions. Whereas the nuclear interaction between FUS and SMN was unaffected by the mutation, the association between FUS-R521C and SMN in the cytosolic fraction was drastically increased (Fig. 4R). The increase in cytosolic FUS-SMN association in the absence of a decreased nuclear FUS-SMN interaction may indicate that FUS mutations stabilize the association with SMN, as has been shown for TDP-43–FUS interactions (37). These data show that the cytosolic mislocalization of mutant FUS leads to a redistribution of SMN, i.e. co-localization with mutant FUS-containing areas and reduced levels in other regions of the cell.

**ALS-associated mutations in FUS disturb the axonal localization of SMN**

The increase in cytosolic FUS–SMN interactions and changes in the normal cell body distribution of SMN in the presence of mutant FUS aggregates suggest that mutant FUS may sequester SMN away from its normal sites of action, including the axon. To study the effect of FUS mutations on axonal SMN, primary neurons were transfected with GFP control, FUS-WT and different mutants (FUS-R521C, -R521H, -P525L) and stained for SMN (Fig. 5A–C). To assess axonal SMN, the ratio of SMN staining intensity between the distal and proximal half of the axon was determined. For GFP and FUS-WT, the ratio between these axon segments was 0.94 and 1.16 (not significant), indicating a relatively even distribution of SMN throughout the entire axon. In contrast, this ratio was significantly decreased for all three mutants investigated; R521C (0.71, P < 0.01), R521H (0.84, P < 0.05) and P525L (0.60, P < 0.01) (Fig. 5D). This shows that expression of FUS mutants leads to a reduction of SMN in the distal axon.

**Overexpression of SMN rescues mutant FUS-induced axonal defects**

SMN is normally transported into the axon, mediates the transport and local translation of mRNAs, such as β-actin, and controls axon morphology (38). Knockdown of SMN induces axonal defects, such as a decrease in growth cone area, reminiscent of what is observed following expression of FUS mutants.
Furthermore, our data show that mutant FUS cytosolic aggregates may sequester SMN in the cell body leading to reduced axonal SMN levels (Fig. 4A–Q and Fig. 5A–D). To test this potential functional link between FUS mutations and reduced axonal SMN, we asked whether the reduction in growth cone area in mutant FUS neurons could be rescued by expression of exogenous SMN. Primary neurons were co-transfected (1) with TdTomato and GFP (control), FUS-WT or FUS-R521C or (2) with SMN tagged with red fluorescent protein (RFP-SMN) and FUS-R521C (Fig. 6A). As reported in Figure 1, quantification of the growth cone area revealed that FUS-R521C induced a marked decrease in growth cone area (Fig. 6A and B, growth cone area 55% of GFP control, \( \text{P} < 0.001 \)). Interestingly, in neurons co-expressing RFP-SMN and FUS-R521C, growth cone area was completely restored to control levels (Fig. 6A and B, growth cone area 100% of GFP control). Similarly, the branching phenotype induced by mutant FUS (Fig. 1) could be restored by exogenous SMN (Fig. 6C and D). Expression of FUS-R521C led to a decrease in the number of primary branches per neuron (1.5 branches, \( \text{P} < 0.001 \)). However, following co-expression of FUS-R521C and RFP-SMN primary branch number was comparable with GFP and FUS-WT conditions (GFP 4.7, WT 4.2 and FUS-R521C + RFP-SMN 4.6 branches respectively, not significant) (Fig. 6C). Overexpression of RFP-SMN or RFP-SMN and FUS-WT, did not affect growth cone size or axon branching (Supplementary Material, Fig. S4).

The C-terminal part of SMN has been shown to be required for the axonal localization of the SMN protein and is sufficient to rescue defects in axon growth and branching following SMN knockdown in PC12 cells and in zebrafish (23,28). To provide further support for an axonal SMN deficiency induced by FUS mutations, we asked whether the C-terminal part of SMN could rescue mutant FUS-induced growth cone area defects. GFP-tagged SMN deletion constructs were transfected into primary neurons in combination with FUS-R521C tagged with

Figure 5. Redistribution of SMN into mutant FUS aggregates leads to decreased axonal SMN expression. (A–C) Primary cortical neurons were transfected with constructs for GFP or FUS-WT, -R521C, -R521H or -P525L and stained for SMN (red, left panels of A–C) and GFP (green, middle panels of A–C). GFP, FUS-WT and -R521C are shown as representative examples. The right panels in A–C show traces of cell body (red), axon (blue) and branches (green) based on phase contrast images of the left and middle panels. (D) SMN staining intensity was quantified and the ratio between signals in the proximal and distal half (as depicted in A and C) of the axon were calculated from at least three independent experiments. * \( \text{P} < 0.05 \), n.s, not significant.
mCherry (mCherry-FUS-R521C) (Fig. 6E). Expression of mCherry-FUS-R521C alone induced a decrease in growth cone size (Fig. 6F, 68.5% of control growth cone area, $P < 0.05$), as shown in Figures 1 and 6B. This decrease could be restored to control levels by co-transfection with a SMN deletion construct lacking the N-terminal but not C-terminal domain (C- and N-SMN, respectively) (Fig. 6F, N-SMN: 68.5% of GFP control, $P < 0.05$; C-SMN: 89.1% of control, not significant). Thus restoring axonal SMN expression is sufficient to rescue axonal defects caused by ALS-associated FUS mutants.

**DISCUSSION**

Work in the past several years has firmly established that mutations in FUS are the primary cause of familial ALS linked to chromosome 16 (ALS6). Recent studies show that mutant FUS triggers stress granule formation and loss of nuclear GEMs, is toxic when expressed in yeast or *Drosophila*, and induces locomotion and branching defects in zebrafish (10,11,13,26,27,42–44). However, the molecular defects induced by these mutations leading to neuron degeneration remain poorly understood. Here,
we show that neuronal aggregates formed by mutant FUS protein appear to sequester the SMA-causing protein SMN and consecutively cause a reduction of SMN levels in the distal axon and growth cone, leading to axonal defects. Restoring SMN expression levels is sufficient to rescue these phenotypes, providing a functional link between ALS-linked FUS mutations, SMN and neuronal connectivity. Together these observations support the idea that different motor neuron disorders such as SMA and ALS may be caused, in part, by defects in shared molecular pathways.

**SMN is sequestered into cytosolic FUS aggregates**

To dissect the biochemical pathways in which FUS functions, we performed an unbiased protein interaction screen using FUS as a bait (Table 1). In line with previous work, several proteins associated with motor neuron disorders were identified, i.e. TDP-43, ATXN2 and SMN. FUS–TDP-43, interactions were previously discovered in proteomics-based analyses of the TDP-43 interactome (37,45), while extended polyQ-repeat lengths in ATXN2 were found to potently modify FUS-associated cellular toxicity (36). Very recent work in non-neuronal cells revealed that FUS binds SMN, is required for nuclear GEM formation, and that FUS mutations lead to a loss of GEMs (26,27). Our study confirms and extends these findings by revealing (i) direct, endogenous interactions between FUS and SMN in neurons (Fig. 2); (ii) a perturbed subcellular distribution SMN due to cytosolic aggregation of mutant FUS in primary neurons (Figs 4 and 5) and (iii) mutant FUS-induced axonal defects remarkably similar to changes in axon morphology reported following SMN deletion or knockdown and that can be rescued by expression of exogenous SMN (Figs 1 and 6). Interestingly, knockdown of FUS leads to growth cone enlargement, supporting the view that mutations in FUS cause gain-of-function rather than loss-of-function effects (46).

Others and we report that the C-terminal RRM and RGG domains of FUS are required for SMN interaction (Fig. 2E–F) (26,27). Despite the fact that this region of FUS mediates RNA binding (7), FUS and SMN can associate in an RNA-independent manner (Fig. 2D) (26). It has therefore been proposed that RNAs may not mediate but rather modulate this interaction (26). Vice versa, SMN binds FUS via residues in its tudor domain, mutations of which cause SMA and reduce binding to Sm proteins (27). The interactions between FUS and SMN through domains important for RNA binding and processing further implicate defects in RNA-related processes in ALS.

ALS-linked mutations have been shown to affect protein–protein interactions. For example, FUS interacts more strongly with mutant when compared with WT TDP-43 following overexpression of TDP-43 in HeLa cells (37). Furthermore, a recent paper examining the interaction of FUS and ATXN2 detected a particularly strong association of ATXN2 and FUS-R521C (36). We therefore tested the impact of different mutations in FUS on its interaction with SMN. Of the different mutants tested, only FUS-R521C displayed an increased interaction with SMN. In contrast, binding of two other, novel FUS interactors SYNCRIP and GEMIN2 was unchanged. Although, cysteine residues can mediate strong biochemical interactions with specific proteins (47), it remains unclear why interaction with SMN but not SYNCRIP or GEMIN2 is enhanced by the R521C mutation. Furthermore, the functional consequences of the increased binding remain to be determined. One possibility is that this increased association leads to enhanced sequestering of SMN in the cytoplasm. However, this will be challenging to determine as different FUS mutations by themselves can cause differing degrees of cytosolic mislocalization, often in a cell type- and cellular context-dependent manner (9,10,48).

Cellular aggregation of proteins or RNAs is a hallmark of many neurodegenerative disorders. The precise role or consequence of aggregate formation remains incompletely understood but aggregates may sequester mutant proteins and their interactors away from their normal site of action. For example, ALS associated with intronic repeat expansions in C9ORF72 is proposed to be caused by the formation of nuclear RNA foci that contain mutant C9ORF72 transcripts which tritrate crucial RNA binding proteins (49). Similarly, mutant TDP-43 is thought to mediate its toxic effects by sequestering RNAs and RNA-binding proteins into cytosolic aggregates. Interestingly, this toxicity can be suppressed through knockdown of the RNA lariat debranching enzyme Db1. In the absence of Db1 enzymatic activity, intronic lariats accumulate in the cytoplasm and act as decoys to sequester TDP-43, preventing it from interfering with essential cellular RNAs and RNA-binding proteins (50,51). It is tempting to speculate that mutant FUS induces similar gain-of-function effects by trapping its binding partners. First, using biochemical approaches and neuron cultures, we observed an increased cytosolic co-localization of SMN and FUS mutant proteins (Fig. 4). Second, FUS mutations induced decreased SMN levels in nuclear GEMs (26,27) and the distal axon (Fig. 5). Together, these observations support the idea that mutant FUS aggregates capture SMN in the cytoplasm reducing its levels at other subcellular sites. However, as FUS has several known binding partners and is part of axonal RNA granules (52), it is likely that mutant FUS aggregates also contain other proteins. Several components of axonal RNA granules were found in our FUS interaction screen, including ELAV-live proteins, elongation–initiation factor (eIF) proteins and polyA/ rC-binding proteins (Table 1), and future studies are needed to address whether these interactors are present in mutant FUS aggregates.

**Mutations in FUS induce SMN-dependent axonal defects**

Here, we show that expression of FUS carrying ALS-linked mutations induces axonal phenotypes resembling those observed in models of SMA, i.e. SMN knockdown or depletion (53). This together with a decrease in SMN at the distal axon and the ability of SMN overexpression to restore these defects suggests that changes in the function and/or localization of SMN contribute to the pathological effects of mutant FUS in axons. These mutant FUS-induced axonal changes are intriguing as accumulating evidence identifies synapses and distal axonal segments as early and important subcellular sites of pathology in ALS and SMA (3). For example, in both ALS and SMA mouse models pathological changes in the distal axon and at the neuromuscular junction (NMJ) occur well before degeneration of motor neuron cell bodies and onset of clinical symptoms (22,54). In addition, in post-mortem material of an ALS patient who died unexpectedly shortly after disease onset, muscle denervation was detected in the absence of signs of
degeneration in the spinal cord (54). It is therefore tempting to speculate that the pathogenic mechanisms of FUS mutations include early changes in neuronal connectivity, as has for example been shown for SOD1 (54).

In neurons, SMN localizes in the nucleus and the cytoplasm and exerts different functions, such as in snRNP biogenesis and axonal mRNP transport (38). Similar to expression of mutant, but not WT, FUS, diminished expression of SMN reduces axonal complexity and growth cone size (20,24,28,55). These SMN-associated effects are caused by the impaired axonal transport and local translation of SMN binding mRNAs, such as β-actin, and by altered biochemical control of the growth cone cytoskeleton (16–18,20,56). The remarkably similarities between axonal defects caused by mutant FUS and SMN knockdown suggest that mutant FUS may impair the non-canonical, axonal functions of SMN. This is supported by our observation that the C-terminal part of SMN, known to be required for its localization to axons (57,58), is sufficient to restore growth cone defects induced by mutant FUS (Fig. 5E and F). However, since mutant FUS reduces SMN expression both in nuclear GEMs and distal axons it is important to note that SMN-dependent splicing has recently been shown to contribute motor circuit development and function in Drosophila (59,60). Therefore, further studies are needed to establish whether changes in canonical or non-canonical SMN functions underlie the axonal effects of mutant FUS.

**FUS and SMN link the motor neuron diseases ALS and SMA**

Our study together with recent work examining nuclear GEMs in relation to (mutant) FUS (26,27) indicates that the motor neuron diseases ALS and SMA are functionally related. SMA is caused by SMN deficiency and characterized by a loss of nuclear GEMs and axonal defects. By analogy, ALS-linked FUS mutations induce a local depletion of SMN and SMN-dependent defects in GEMs and axons. This link between SMA (SMN) and ALS is also apparent at the genetic and clinical levels. An SMN1 copy number variation is associated with an increased risk of developing ALS (25,61). SMA affects lower motor neurons and in FUS-linked ALS there seems to be predominance to a lower motor neuron phenotype (6,62). Furthermore, SMA is an early-onset disorder and a large number of juvenile ALS cases are associated with mutations in FUS, including cases with age at onset as early as 11 years (63–69). In addition to recent work providing links between ALS and frontotemporal dementia (49,70), spinocerebellar ataxia type 2 (SCA2) (71) and inclusion body myopathy with Paget’s disease and frontotemporal dementia (IBMPFD) (72), our findings strengthen the need for further research into shared mechanisms between neurodegenerative diseases. Overlap between disease mechanisms may provide new opportunities in finding common approaches for the treatment of neurodegenerative disease. For example, raising SMN levels or signaling in the presence of mutant FUS restores defects in GEM number (26) and axon morphology (Fig. 6). This supports the possibility that strategies developed to increase SMN levels in SMA patients may also be used for ALS. It is possible that such drug candidates can also be applied to combat ALS cases with different genetic causes, as mutations in TDP-43 (73) and SOD1 (74,75) may also cause SMN-dependent phenotypes.

**MATERIALS AND METHODS**

**Antibodies and constructs**

Rabbit polyclonal anti-FUS antibody was purchased from Bethyl Labs (A300-302A). Mouse monoclonal anti-SMN (610647) and anti-ataxin-2 (61378) antibodies were from BD-Transduction. Rabbit polyclonal anti-RHA (ab26271) and anti-GFP (ab290) antibodies and monoclonal anti-Gemin2 (ab6084) were from Abcam. Polyclonal rabbit-anti-SYNCRIP was from Aviva Systems Biology (ARP0640). Human FUS cDNA (obtained from Origene) was subcloned C-terminal to the GFP sequence of a modified pEGFP-C1 vector containing a biotin tag sequence N-terminal to the GFP sequence (76). In addition, FUS cDNA was cloned into a mCherry-vector (described previously (77)), a gift from dr. Rob Willemse (Erasmus MC, Rotterdam, The Netherlands). ALS-associated mutations were introduced into bioGFP-FUS and mCherry-FUS using the QuickChange Site-directed Mutagenesis kit (Stratagene). pCS2-RFP-SMN was as described previously (78) and a gift from Dr Christine Beattie (Ohio State University, Columbus, OH, USA). Deletion constructs for SMN (1-239 and 235-294) fused to GFP were as described previously (28) and a gift from Dr Peter Claus (Hannover University, Hannover, Germany).

**Animal use and primary neuron dissociation and culture**

All animal care and use was done in accordance with institutional regulations. Mice (C57BL/6) were purchased from Charles River. To generate dissociated cortical neuron cultures, the cortex was dissected from E14.5 C57BL/6 mouse embryos. Timed-pregnant mice were sacrificed using cervical dislocation. Embryonic day 0.5 (E0.5) was set to be the morning on which a vaginal plug was detected. Subsequently, dissociated neuron cultures were obtained by triturating the cortical fragments in DMEM/F12 medium containing 12.5 mM glucose, 1× pen/strep, 10% FCS and DNaseI (Roche). Cells were seeded on sterilized coverslips coated with poly-ν-lysine (Sigma, 100 μg/μl) and laminin (Life Technologies, 40 μg/μl). Neurons were cultured in Neurobasal medium (Gibco), with 1× l-glut, Pen/Strep and B-27 serum-free supplement (Gibco). Neurons were transfected after 24 h using Lipofectamine 2000 (Life Technologies). 48 h after transfection, cells were fixed in 4% paraformaldehyde (PFA) or 4% PFA with 10% sucrose to study growth cone morphology. In order to obtain comparable levels of transgene expression, the total amount of plasmid DNA in each transfection was kept constant throughout experiments.

**Immunocytochemistry**

Primary cortical neurons were fixed with 4% PFA for 15 min at RT, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at RT, blocked in PBS containing 2.5% bovine serum albumin (BSA), and incubated with primary antibodies in BSA at RT for 1 h. After several washes in PBS, cells were incubated with a mixture of the appropriate Alexa Fluor-labeled secondary antibodies (Life Technologies) for 1 h at RT. Then, cells were washed, counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma), washed extensively with PBS and mounted in Mowiol (Sigma). For growth cone area measurements, neurons were stained for F-actin...
using phallloidin-conjugated with Alexa Fluor 350 or 594 (Life Technologies).

**Cell culture and transfection**

NSC-34 cells (mouse neuroblastoma and motor neuron hybrid, CELLution Biosystems Inc.) were grown in DMEM high-glucose medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Lonza), 1× L-glutamine (L-glu, PAA) and 1× penicillin/streptavidin (pen/strep, PAA). Cells were transfected using X-tremeGENE HP (Roche), according to the manufacturer’s instructions.

**Biotin–streptavidin pull down of FUS and interacting proteins**

Immunoprecipitation of biotin-tagged proteins was performed as described previously (76). In brief, paramagnetic streptavidin beads (Dynabeads M-280, Life Technologies) were blocked in blocking buffer (20 mM Tris–HCl, pH 8, 150 mM KCl, 20% glycerol) with 200 ng/μl purified chicken serum albumin (Sigma-Aldrich). NSC-34 cells expressing bioGFP, bioGFP-FUS or bioGFP-FUS deletion constructs were lysed (lysis buffer: 20 mM Tris–HCl, pH 8, 150 mM KCl, 1% Triton X-100, 0.2 μg/μl, phosphatase inhibitor cocktail (Sigma), complete protease inhibitor cocktail (Roche)), and lysates were centrifuged. The resulting supernatants were incubated with X-tremeGENE HP (Roche), according to the manufacturer’s instructions.

**Mass spectrometry and bioinformatics analysis**

Mass-spectrometry analysis of FUS interacting proteins was done as described previously (79). The Mascot-score cut-off value for a positive protein hit was set to 60. GFP-control mass-spectrometry data, as required.

**Image analysis and statistics**

Imaging of primary neurons was done using an Olympus Fluoview FV1000 confocal microscope. All transfected neurons on a coverslip were imaged. For morphological analysis, neurons were selected on the basis of (i) a clear neuronal morphology, i.e. clearly discernable and intact axons and dendrites, and (ii) nuclear integrity. All functional experiments in cortical neurons are based on three independent cell preparations, containing at least 20 neurons per experimental condition per cell preparation. All analyses were performed in ImageJ. Axon length, branching and staining intensity were quantified using the Neurol J plugin. Quantification of co-localization was done using the Intensity Correlation Analysis plugin. All statistical analyses were done in the R (http://www.r-project.org). Morphological changes in cortical neurons were analyzed using a mixed linear effects model using the nlme package in R. Other statistical analyses were done using an ANOVA followed by Student’s t-test or Tukey’s post-hoc test for non-parametric data, as required.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.
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