DATA NOTE

REVISED The identification of high-performing antibodies for RNA-binding protein FUS for use in Western Blot, immunoprecipitation, and immunofluorescence [version 2; peer review: 2 approved]

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Abstract

RNA-binding protein Fused-in Sarcoma (FUS) plays an essential role in various cellular processes. Mutations in the C-terminal domain region, where the nuclear localization signal (NLS) is located, causes the redistribution of FUS from the nucleus to the cytoplasm. In neurons, neurotoxic aggregates are formed as a result, contributing to neurogenerative diseases. Well-characterized anti-FUS antibodies would enable the reproducibility of FUS research, thereby benefiting the scientific community.

In this study, we characterized ten FUS commercial antibodies for Western Blot, immunoprecipitation, and immunofluorescence using a standardized experimental protocol based on comparing read-outs in knockout cell lines and isogenic parental controls.

We identified many high-performing antibodies and encourage readers to use this report as a guide to select the most appropriate antibody for their specific needs.

Keywords

Uniprot ID P35637, FUS, RNA-binding protein FUS, antibody characterization, antibody validation, Western Blot, immunoprecipitation, immunofluorescence

Open Peer Review

Approval Status ✓ ✓

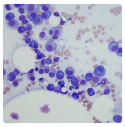
	1	2
version 2 (revision) 26 Jun 2023	✓ view	
version 1 06 Apr 2023	? view	✓ view

1. **Ryota Hikiami**, Shiga University, Hikone, Japan

2. **Ralph H. Kehlenbach**, Georg-August-Universitat Gottingen, Göttingen, Germany

Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the YCharOS (Antibody Characterization through Open Science) gateway.



This article is included in the Cell & Molecular Biology gateway.

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Author roles: Alshalfie W: Investigation, Methodology; **Fotouhi M**: Investigation; **Ayoubi R**: Investigation, Methodology, Visualization, Writing – Review & Editing; **You Z**: Investigation; **Southern K**: Writing – Original Draft Preparation, Writing – Review & Editing; **McPherson PS**: Conceptualization, Funding Acquisition, Resources, Supervision; **Laflamme C**: Conceptualization, Data Curation, Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Validation, Writing – Review & Editing;

Competing interests: For this project, the laboratory of Peter McPherson developed partnerships with high-quality antibody manufacturers and knockout cell line providers. The partners provide antibodies and knockout cell lines to the McPherson laboratory at no cost. These partners include: - Abcam- ABclonal -Aviva Systems Biology -Bio Techne -Cell Signalling Technology -Developmental Studies Hybridoma Bank -GeneTex – Horizon Discovery – Proteintech – Synaptic Systems -Thermo Fisher Scientific.

Grant information: This work was supported in part by the ALS-Reproducible Antibody Platform (ALS-RAP). ALS-RAP is a private-public partnership created by the ALS Association (USA), the Motor Neurone Disease Association (UK), and the ALS Society of Canada. The grant was from a Canadian Institutes of Health Research Foundation Grant (FDN154305) and by the Government of Canada through Genome Canada, Genome Quebec and Ontario Genomics (OGI-210). The Structural Genomics Consortium is a registered charity (no. 1097737) that receives funds from Bayer AG, Boehringer Ingelheim, Bristol-Myers Squibb, Genentech, Genome Canada through Ontario Genomics Institute (grant no. OGI-196), the EU and EFPIA through the Innovative Medicines Initiative 2 Joint Undertaking (EUbOPEN grant no. 875510), Janssen, Merck KGaA (also known as EMD in Canada and the United States), Pfizer and Takeda. RA and WA were supported by a Mitacs fellowship.

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REVISED **Amendments from Version 1**

To the introduction, we included frontotemporal local degeneration (FTLD) to the list of neurodegenerative diseases that the FUS gene may contribute to.

Any further responses from the reviewers can be found at the end of the article

Introduction

Fused-in Sarcoma (FUS) encodes a DNA/RNA-binding protein involved in numerous cellular processes including transcriptional regulation, RNA splicing, RNA transport and DNA repair.[1] Predominantly localized in the nucleus, FUS can shuttle between the nucleus and cytoplasm.[2] The *FUS* transcript is reported to have multiple domains including an N-terminal Gln-Gly-Ser-Tyr -rich region, an RNA-recognition motif, Arg-Gly-Gly repeat regions, a zinc finger motif and a highly conserved C-terminal NLS.[3–5]

Variants in the *FUS* gene have been identified as potential causative factors for amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and frontotemporal lobar degeneration (FTLD).[6–9] FUS related mutations found in familial ALS/FTD patients are clustered in the C-terminal NLS, causing FUS to be mislocalized and accumulate as aggregates in the cytoplasm of neurons, initiating a pathway that contributes to neurodegeneration.[6,7] FUS function is reduced when aggregates form, but it is not yet known whether this initiates the pathogenic process or if the aggregates are pathogenic.[10] Mechanistic studies would be greatly facilitated with the availability of high-quality antibodies.

Here, we compared the performance of a range of commercially-available antibodies for RNA-binding protein FUS and validated several antibodies for Western Blot, immunoprecipitation and immunofluorescence, enabling biochemical and cellular assessment of FUS properties and function.

Table 1. Summary of the cell lines used.

Institution	Catalog number	RRID (Cellosaurus)	Cell line	Genotype
ATCC	CCL-2	CVCL_0030	HeLa	WT
Montreal Neurological Institute	-	CVCL_A8VH	HeLa	*FUS* KO

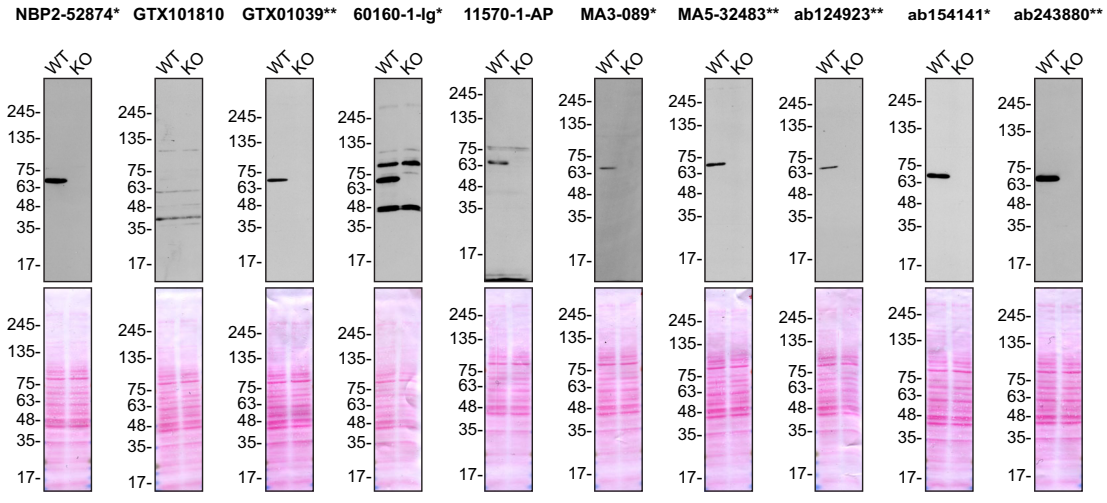

Figure 1. FUS antibody screening by Western Blot. Lysates of HeLa (WT and *FUS* KO) were prepared and 30 µg of protein were processed for Western Blot with the indicated FUS antibodies. The Ponceau stained transfers of each blot are presented to show equal loading of WT and KO lysates and protein transfer efficiency from the acrylamide gels to the nitrocellulose membrane. Antibody dilutions were chosen according to the recommendations of the antibody supplier. An exception was given for antibody GTX101810, which was titrated to 1/3000, as the signal was too weak when following the supplier's recommendation. Antibody dilution used: NBP2-52874* at 1/1000; GTX101810 at 1/3000; GTX01039* at 1/1000; 60160-1-Ig* at 1/10000; 11570-1-AP at 1/4000; MA3-089* at 1/2000; MA5-32483** at 1/1000, ab124923** at 1/5000; ab154141* at 1/1000; ab243880** at 1/1000. Predicted band size: 53 kDa. Observed specific band size: ~70 kDa. *Monoclonal antibody; **Recombinant antibody.

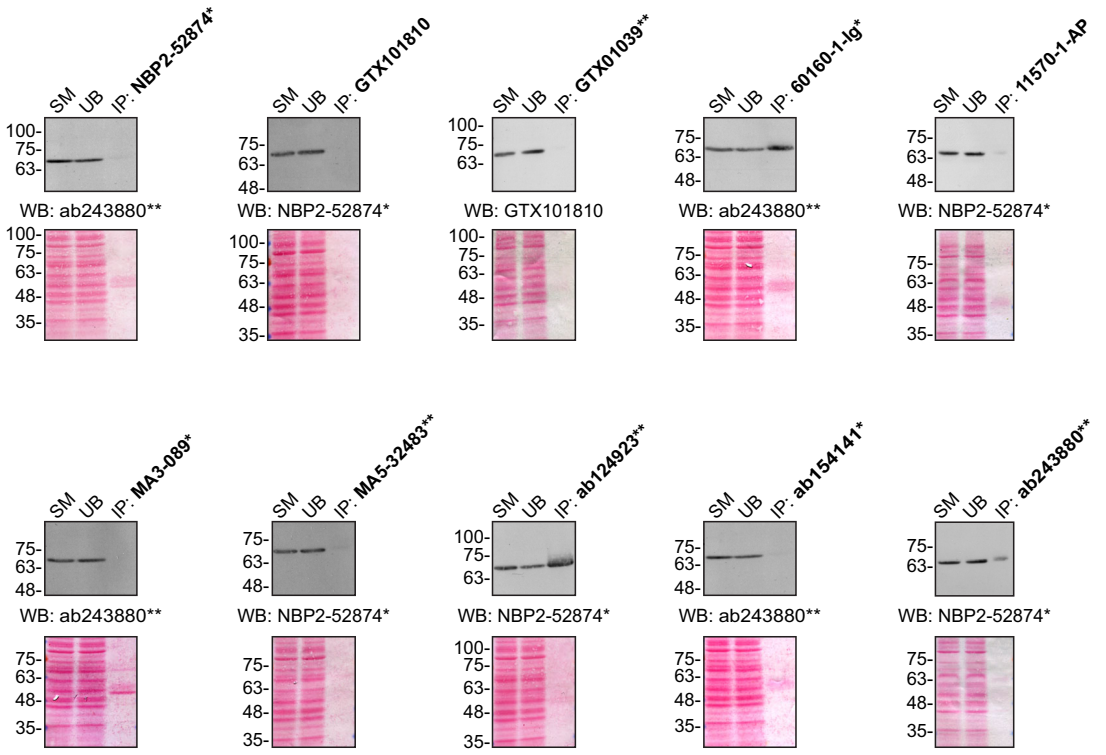

Figure 2. FUS antibody screening by immunoprecipitation. HeLa lysates were prepared, and IP was performed using 1.0 µg of the indicated FUS antibodies pre-coupled to protein G or protein A Sepharose beads. Samples were washed and processed for Western Blot with the indicated FUS antibody. For Western Blot, NBP2-52874* and ab243880** were used at a dilution of 1/2000. The Ponceau stained transfers of each blot are shown for similar reasons as in Figure 1. SM=10% starting material; UB=10% unbound fraction; IP=immunoprecipitated. *Monoclonal antibody; **Recombinant antibody.

Results and discussion

Our standard protocol involves comparing readouts from wild-type (WT) and knockout (KO) cells.[11–15] To identify a cell line that expresses adequate levels of FUS protein to provide sufficient signal to noise, we examined public proteomics databases, namely PaxDB[16] and DepMap.[17] HeLa was identified as a suitable cell line and thus HeLa was modified with CRISPR/Cas9 to knockout the corresponding *FUS* gene (Table 1).

For Western Blot experiments, we resolved proteins from WT and *FUS* KO cell extracts and probed them side-by-side with all antibodies in parallel[12–15] (Figure 1).

For immunoprecipitation experiments, we used the antibodies to immunopurify FUS from HeLa cell extracts. The performance of each antibody was evaluated by detecting the FUS protein in extracts, in the immunodepleted extracts and in the immunoprecipitates[12–15] (Figure 2).

For immunofluorescence, as described previously, antibodies were screened using a mosaic strategy.[18] In brief, we plated WT and KO cells together in the same well and imaged both cell types in the same field of view to reduce staining, imaging and image analysis bias (Figure 3).

In conclusion, we have screened FUS commercial antibodies by Western Blot, immunoprecipitation and immunofluorescence and identified several high-quality antibodies under our standardized experimental conditions. The underlying data can be found on Zenodo.[19,20]

Methods

Antibodies

All FUS antibodies are listed in Table 2, together with their corresponding Research Resource Identifiers, or RRID, to ensure the antibodies are cited properly.[21] Peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies are from

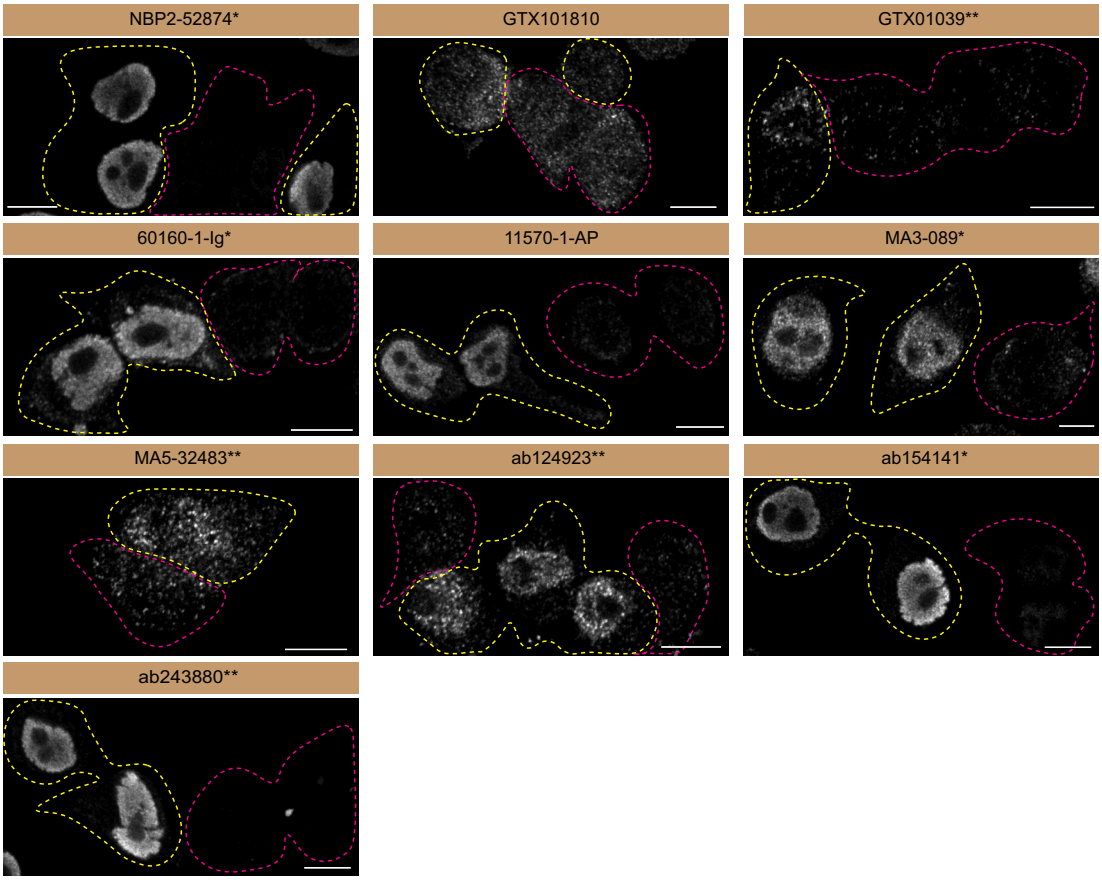

Figure 3. FUS antibody screening by immunofluorescence. HeLa WT and *FUS* KO cells were labelled with a green or a far-red fluorescent dye, respectively. WT and KO cells were mixed and plated to a 1:1 ratio on coverslips. Cells were stained with the indicated FUS antibodies and with the corresponding Alexa-fluor 555 coupled secondary antibody. Acquisition of the green (identification of WT cells), red (antibody staining) and far-red (identification of KO cells) channels was performed. Representative images of the red (grayscale) channels are shown. WT and KO cells are outlined with yellow and magenta dashed line, respectively. Antibody dilutions were chosen according to the recommendations of the antibody supplier. Exceptions were given for antibodies GTX101810, GTX01039*, 60160-1-Ig*, 11570-1-AP, MA5-32483** and ab124923**, which were titrated as the signals were too weak when following the supplier's recommendations. Antibody dilution used: NBP2-52874* at 1/1000; GTX101810 at 1/700; GTX01039* at 1/1000; 60160-1-Ig* at 1/2000; 11570-1-AP at 1/1000; MA3-089* at 1/1000; MA5-32483** at 1/1000; ab124923** at 1/1000; ab154141* at 1/1000; ab243880** at 1/500. Bars=10 µm. *Monoclonal antibody; **Recombinant antibody.

Thermo Fisher Scientific (cat. number 65-6120 and 62-6520). Alexa-555-conjugated goat anti-rabbit and anti-mouse secondary antibodies are from Thermo Fisher Scientific (cat. number A21429 and A21424).

CRISPR/Cas9 genome editing

The HeLa *FUS* KO clone was generated with low passage cells using an open-access protocol available on Zenodo.org: https://zenodo.org/record/3875777#.ZA-Rxi-96Rv. Two guide RNAs were used to introduce a STOP codon in the *FUS* gene (sequence guide 1: AGGGAGUCACAAAAGCCACC, sequence guide 2: GGUACGGUGGUGUUGAUGUC).

Cell culture

Both HeLa WT and *FUS* KO cell lines used are listed in Table 1, together with their corresponding RRID, to ensure the cell lines are cited properly.[22] Cells were cultured in DMEM high-glucose (GE Healthcare cat. number SH30081.01) containing 10% fetal bovine serum (Wisent, cat. number 080450), 2 mM L-glutamate (Wisent cat. number 609065), 100 IU penicillin and 100 µg/mL streptomycin (Wisent cat. number 450201).

Table 2. Summary of the FUS antibodies tested.

Company	Catalog number	Lot number	RRID (Antibody Registry)	Clonality	Clone ID	Host	Concentration (μg/μL)	Vendors recommended applications
Bio Techne	NBP2-52874*	MAB-03520	AB_2885157	monoclonal	CL0190	mouse	1.00	Wb, IF
GeneTex	GTX101810	40366	AB_2036972	polyclonal	-	rabbit	0.70	Wb
GeneTex	GTX01039*	822100287	AB_2888934	monoclonal	JJ09-31	rabbit	1.00	Wb, IF
Proteintech	60160-1-Ig*	10017695	AB_10666169	monoclonal	3A10B5	mouse	2.36	Wb, IP, IF
Proteintech	11570-1-AP	00086256	AB_2247082	polyclonal	-	rabbit	0.90	Wb, IP, IF
Thermo Fisher Scientific	MA3-089*	VB301448	AB_2633334	monoclonal	1FU-1D2	mouse	not provided	Wb, IF
Thermo Fisher Scientific	MA5-32483**	VL3152611	AB_2809760	recombinant-mono	JJ09-31	rabbit	1.00	Wb, IF
Abcam	ab124923**	GR85761-9	AB_10972861	recombinant-mono	EPR5812	rabbit	0.15	Wb, IF
Abcam	ab154141*	GR3368481-1	AB_2885092	monoclonal	CL0190	mouse	1.00	Wb, IF
Abcam	ab243880**	GR3376392-2	AB_2885123	recombinant-mono	BLR023E	rabbit	not provided	Wb, IP, IF

Wb=Western Blot; IF=immunofluorescence; IP=immunoprecipitation.
*Monoclonal antibody.
**Recombinant antibody.

Antibody screening by Western Blot

Western Blots were performed as described in our standard operating procedure.[23] HeLa WT and *FUS* KO were collected in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1.0 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1× protease inhibitor cocktail mix (MilliporeSigma, cat. number P8340). Lysates were sonicated briefly and incubated for 30 min on ice. Lysates were spun at ~110,000 × g for 15 min at 4°C and equal protein aliquots of the supernatants were analyzed by SDS-PAGE and Western Blot. BLUelf prestained protein ladder from GeneDireX (cat. number PM008-0500) was used.

Western Blots were performed with large 5-16% polyacrylamide gels and transferred on nitrocellulose membranes. Proteins on the blots were visualized with Ponceau S staining (Thermo Fisher Scientific, cat. number BP103-10) which is scanned to show together with individual Western Blot. Blots were blocked with 5% milk for 1 hr, and antibodies were incubated overnight at 4°C with 5% bovine serum albumin (BSA) (Wisent, cat. number 800-095) in TBS with 0.1% Tween 20 (TBST) (Cell Signaling Technology, cat. number 9997). Following three washes with TBST, the peroxidase conjugated secondary antibody was incubated at a dilution of ~0.2 µg/mL in TBST with 5% milk for 1 hr at room temperature followed by three washes with TBST. Membranes were incubated with Pierce ECL (Thermo Fisher Scientific, cat. number 32106) prior to detection with the HyBlot CL autoradiography films (Denville, cat. number 1159T41).

Antibody screening by immunoprecipitation

Immunoprecipitation was performed as described in our standard operating procedure.[24] Antibody-bead conjugates were prepared by adding 1.0 µg of antibody to 500 µL of phosphate-buffered saline (PBS) (Wisent, cat. number 311-010-CL) with 0,01% triton X-100 (Thermo Fisher Scientific, cat. number BP151-500) in a 1.5 mL microcentrifuge tube, together with 30 µL of protein A- (for rabbit antibodies) or protein G- (for mouse antibodies) Sepharose beads. Tubes were rocked overnight at 4°C followed by two washes to remove unbound antibodies.

HeLa WT were collected in HEPES buffer (20 mM HEPES, 100 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, pH 7.4) supplemented with protease inhibitor. Lysates were rocked 30 min at 4°C and spun at 110,000 × g for 15 min at 4°C. One mL aliquots at 1.0 mg/mL of lysate were incubated with an antibody-bead conjugate for ~2 hours at 4°C. The unbound fractions were collected, and beads were subsequently washed three times with 1.0 mL of HEPES lysis buffer and processed for SDS-PAGE and Western Blot on a 5-16% polyacrylamide gels.

Antibody screening by immunofluorescence

Immunofluorescence was performed as described in our standard operating procedure.[12–15,18] HeLa WT and *FUS* KO were labelled with a green and a far-red fluorescence dye, respectively. The fluorescent dyes used are from Thermo Fisher Scientific (cat. number C2925 and C34565). WT and KO cells were plated on glass coverslips as a mosaic and incubated for 24 hrs in a cell culture incubator at 37°C, 5% CO_2. Cells were fixed in 4% paraformaldehyde (PFA) (Beantown chemical, cat. number 140770-10ml) in PBS for 15 min at room temperature and then washed 3 times with PBS. Cells were permeabilized in PBS with 0,1% Triton X-100 for 10 min at room temperature and blocked with PBS with 5% BSA, 5% goat serum (Gibco, cat. number 16210-064) and 0.01% Triton X-100 for 30 min at room temperature. Cells were incubated with IF buffer (PBS, 5% BSA, 0.01% Triton X-100) containing the primary FUS antibodies overnight at 4°C. Cells were then washed 3 × 10 min with IF buffer and incubated with corresponding Alexa Fluor 555-conjugated secondary antibodies in IF buffer at a dilution of 1.0 µg/mL for 1 hr at room temperature with DAPI. Cells were washed 3 × 10 min with IF buffer and once with PBS. Coverslips were mounted on a microscopic slide using fluorescence mounting media (DAKO).

Imaging was performed using a Zeiss LSM 880 laser scanning confocal microscope equipped with a Plan-Apo 40× oil objective (NA = 1.40). Analysis was done using the Zen navigation software (Zeiss). All cell images represent a single focal plane. Figures were assembled with Adobe Photoshop (version 24.1.2) to adjust contrast then assembled with Adobe Illustrator (version 27.3.1).

Data availability

Underlying data

Zenodo: Antibody Characterization Report for RNA-binding protein FUS, https://doi.org/10.5281/zenodo.5259944.[19]

Zenodo: Dataset for the RNA-binding protein FUS antibody screening study, https://doi.org/10.5281/zenodo.7764130.[20]

Acknowledgments

We would like to thank the NeuroSGC/YCharOS/EDDU collaborative group for their important contribution to the creation of an open scientific ecosystem of antibody manufacturers and knockout cell line suppliers, for the development of community-agreed protocols, and for their shared ideas, resources and collaboration. Members of the group can be found below.

NeuroSGC/YCharOS/EDDU collaborative group: Riham Ayoubi, Thomas M. Durcan, Aled M. Edwards, Carl Laflamme, Peter S. McPherson, Chetan Raina, Kathleen Southern and Zhipeng You.

An earlier version of this of this article can be found on Zenodo (doi: https://doi.org/10.5281/zenodo.5259945)

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 26 June 2023

https://doi.org/10.5256/f1000research.151937.r181569

✔ **Ryota Hikiami**
Department of Therapeutics for protein misfolding diseases, Shiga University, Hikone, Shiga Prefecture, Japan

Thank you for considering my comments and suggestions on your manuscript. I am pleased to say that the majority of the concerns I raised have been adequately addressed.

I have only a minor suggestion.

- Introduction (P3, L7 'familial ALS/FTD'): Let me write again, as my writing does not seem to be coming across well. If literature reference 9 (Van Langenhove et al., 2010[1]) includes "familial" ALS/FTD with a 'family history', please disregard it. However, if reference 9 pertains to "sporadic" ALS/FTD without a family history, please include references such as "Frameshift and novel mutations in FUS in familial amyotrophic lateral sclerosis and ALS/dementia." as literature on "familial" ALS/FTD.

Thank you once again for your diligent cooperation throughout this process. I truly appreciate your responsiveness and your commitment to addressing the comments and suggestions.

References

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Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular biology, neuroscience

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 23 June 2023

https://doi.org/10.5256/f1000research.146199.r178227

Ralph H. Kehlenbach
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In their manuscript „The identification of high-performing antibodies for RNA-binding protein FUS for use in Western Blot, immunoprecipitation, and immunofluorescence", Alshalfie and coauthors perform a thorough comparison of commercial antibodies against FUS, a protein that has gained a lot of interest in the field of neurodegenerative diseases and also in general molecular cell biology. Since many antibodies do not perform as good as they are advertised, such a comparison is highly welcome. The approach (also using knockout cell lines as control) is solid and the results are clear and very well documented. I only have one suggestion: in Table 2, the authors should include one or several column(s) (next to "vendors recommended application") with comments reflecting the results of the study (e.g. ab243880 seems to work well in WB, in IP and in IF).

Is the rationale for creating the dataset(s) clearly described?
Yes

Are the protocols appropriate and is the work technically sound?
Yes

Are sufficient details of methods and materials provided to allow replication by others?
Yes

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 15 June 2023

https://doi.org/10.5256/f1000research.146199.r177190

?

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Dear Laflamme C and co-authors,

Thank you for allowing me to review your manuscript titled "The identification of high-performing antibodies for RNA binding protein FUS for use in Western Blot, immunoprecipitation, and immunofluorescence." Your study investigates the utility of commercially available antibodies targeting FUS, one of the causative genes of ALS, through WB, IP, and IF techniques. The experimental findings presented by the authors are expected to be valuable and beneficial for FUS researchers. While the overall methodology and results are sound, I propose minor revisions.

1. Introduction (P3, L6-):
Unlike ALS, FTD with the *FUS* gene mutation is rare. Does the attached literature include cases of familial FTLD? If not, I recommend adding relevant references or modifying the text accordingly.

2. Figure 3 (P5):
The mosaic arrangement of WT and KO cells in the image is clear and visually appealing. However, the image coverage appears to be somewhat limited. I suggest expanding the image coverage or adding additional images to enhance the readers' understanding.

I hope these suggestions are helpful in improving the quality and impact of your manuscript. Thank you again for considering my feedback, and I look forward to seeing the revised version.

Best regards,
Ryota Hikiami

Is the rationale for creating the dataset(s) clearly described?
Yes

Are the protocols appropriate and is the work technically sound?
Yes

Are sufficient details of methods and materials provided to allow replication by others?
Yes

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular biology, neuroscience

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Jun 2023

Kathleen Southern

Dear Ryota Hikiami,

Thank you for your thorough review of this Data Note which analyses the performance of commercial antibodies for RNA binding protein FUS, a potential causative gene in many neurodegenerative diseases.

To answer your first point of feedback, literature reference 9, which refers to the publication by Van Langenhove et al., performs a mutational analysis of *FUS* in patients with cases of FTLD. To further elucidate this fact, we will be submitting a revised version with modified text to include FTLD in the list of neurodegenerative diseases potentially caused by *FUS* gene variants.

As for the second point, we always include a dataset in our reports, which includes all underlying raw data for the experiments performed (reference 20). This increases the transparency and reproducibility of our work. It also allows viewers to have a better understanding of the results and see what wasn't included in the figures. As for immunofluorescence, the dataset includes czi files of each antibody under the microscope. We hope this provides the additional image coverage you were looking for.

Thank you again for your suggestions!

Competing Interests: No competing interests were disclosed.