nature portfolio

Peer Review File

AAV-based delivery of RNAi targeting Ataxin-2 improves survival and pathology in TDP-43 mice

Corresponding Author: Dr Defne Amado

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript from Amado and colleagues presents an AAV-based gene therapy strategy targeting the ataxin 2 gene using a miRNA. Ataxin 2 was identified as a modifier of TDP-43 toxicity in model systems and intermediate-length CAG repeat expansions in the ataxin 2 gene (ATXN2) have been identified as a genetic contributor to risk for ALS. Previous studies have found that lowering levels of ataxin 2 (either by genetic knockout or with antisense oligonucleotides (ASOs) is sufficient to extend survival and rescue phenotypes in transgenic mice engineered to express human TDP-43 at high levels. These preclinical results motivated a recent clinical trial in human ALS patients to test ataxin 2-targeting ASOs but the results of the trial showed no clinical benefit. However, data reported by the sponsor of the clinical trial (Biogen), and reviewed by the authors of this manuscript, show that the clinical trial only achieved a 21% knockdown of ataxin 2 in the CSF and taking into account that the placebo treated subjects showed a 9% lowering of ataxin 2, the net lowering due to ataxin 2 is only 12%. The authors propose that this indicates that the negative clinical trial results represent a technical failure rather than a biological one. They also correctly point out that the clinical trial was using the highest tolerable dose of ASOs, suggesting that if ataxin 2 will be pursued as a target for ALS, other strategies will be needed to achieve a more potent reduction in ataxin 2 levels.

Here the authors present such a strategy. They use a new AAV vector to deliver a miRNA against ataxin 2 and demonstrate potent knockdown of ataxin 2 levels in the spinal cord, with robust knockdown in motor neurons. Importantly, this treatment extended survival and rescued motor phenotypes. There are several important advances this paper makes compared to other similar efforts. First, they show a rescue of TDP-43 hemizygous mice (TAR4) in addition to the homozygous ones (TAR4/TAR4). Second, they demonstrate using transcriptomics the molecular changes that are rescued by ataxin 2 lowering, providing insight into the mechanism by which ataxin 2 lowering rescues TDP-43 transgenic mice.
This manuscript is very well written, and the authors are transparent about the strengths and limitations of their approach. The striking results and the timely nature of these findings will be of great interest to the field and will likely inspire efforts to translate these results into human. As a first start, the authors end their paper by showing that their approach works against human ataxin 2 in vivo and in vitro. Overall, I think this is an important paper and given the recent clinical trial results should be published without delay. I just have a few comments and suggestions for the authors to consider.

1. The authors compared their ataxin 2 AAV-miRNA treated mice to buffer treated mice. They argue that this more closely relates to what will be needed for IND-enabling studies, which is a convincing argument. However, have the authors tested other AAVs with a non-targeting miRNA as a negative control? This is not required, of course, but if the authors have such data in hand it would be informative to compare to the buffer-treated animals to see if there are any AAV or miRNA specific effects independent of ataxin 2 targeting.

2. The TAR4 hemizygous mouse results are very interesting (lowering ataxin 2 rescues phenotypes). Do the authors have transcriptomic data on these animals and their rescued littermates? Are the same types of genes and pathways altered in TAR4 mice but just at a lower level than TAR4/TAR4 mice? This analysis might be informative for readers to learn about mechanism of rescue.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
In the present manuscript, Amado and colleagues report the downregulation of Ataxin-2 using a RNAi AAV-based approach. AAV efficacy and pro-survival effect were tested in a TDP43 mouse model, a human Ataxin-2 overexpressing transgenic mouse and iPSC-derived motor neurons carrying the C9orf72 mutation. Here, they report specific targeting of the RNAi construct which reduced Ataxin-2 levels in all models included in the study. Moreover, they report amelioration of motor phenotypes in the TDP43 mouse model (in both homozygous and heterozygous genotypes) as well as increased motor neuron survival and decreased inflammation.
The authors suggests that the AAV approach is preferable to the ASO one (that was successfully tested in preclinical models but that did not produce the expected results in clinical trials) because it allows for more stable and robust decrease in Ataxin-2 levels, while being less invasive since it requires only one injection. The study is well designed and of interest for the field. However, there are some concerns that should be addressed before the manuscript can be considered for publication in Nature Communications.

Main text:
1) Statistics and N values need to be reported following the journal guidelines, especially in the Results section and the Figure legends. Throughout the manuscript and the supplementary material exact P values, means and standard deviations, degree of freedom (where applicable), N for each condition should be included.
2) The information regarding the construct should be clarified (a schematic depicting all elements included in the construct would help); also, Supplementary figure 1 should be included in the main figures, since it helps understanding AAV efficacy. The authors should clarify how the used capsids compare to the ones published in Ranum PT et al 2023. Is the used capsid proven to target neurons in non-human primates? Considerations about this specific point should be included in the discussion because it is relevant for the translational potential of the approach.
3) The “Effect of miAtxn2 knockdown on transcriptional phenotypes” paragraph should be restructured. It should clearly state which conditions were included in this part of the study (WT, TAR4/4 mice – I guess homozygous, and miAtxn2 treated), as well as how many mice were included per condition. The authors should clarify that this is bulk sequencing, thus results include all cell types found within the spinal cord (with motor neurons being among the least abundant populations). Considerations about the potential effect of the bulk sequencing on the results obtained in the DEG analysis should be included in the Discussion paragraph. Also, more information about which genes are upregulated or downregulated should be included. The authors are currently mainly referring to differentially expressed genes.
4) Line 275-276 “in our study at 12 weeks either in numbers of DRG neurons or presence of inflammatory 275 cells in the DRGs (data not shown)”. “Data not shown” is not acceptable nowadays, so the authors should include this dataset. This is a relevant point supporting the low toxicity of the treatment.

Figures:
Fig 1B. I understand that these are normalised values but one of the +miAtxn2 is actually below 0. Why is this? Also, it is unclear from the graph and the figure legend what the black bar is representing.

Fig 1C How many animals are included in each group? The figure legends state 14/23 but it is unclear how many animals are used for each condition (treated vs untreated). It is very important that the number of mice included in the survival study is comparable or results may be skewed. What is the median survival for the treated and untreated conditions? (Mean and standard deviation should be reported here).

Fig 1D-F Which post hoc analysis was applied here?

Fig 1F Why is the WT+miAtx2 not included in all assessments?

Fig 2A-B Two-way ANOVAs should be used here since two variables are compared 1) treatment, 2) level of the spinal cord.

Fig 2A The images are out of focus.

Fig 3 Same as above regarding post hoc and reported stats.

Fig 4A Spinal cord pictures are conventionally shown with the ventral horn facing downwards. Also, why was level L6 of the spinal cord chosen here?

Fig 6A-B When showing longitudinal data a two-way ANOVA should be used.

Fig 7A The picture is not very representative of the graph presented in this panel.

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Supplementary figures:

Supp Fig 1C The PM-AAV9 picture is completely saturated and should be replaced.

Supp Fig 2A The curves should show standard deviations and means, it is not clear what is represented in the graph.

Supp Fig 2B-F The N per each condition should be clarified since it is reported 10/40. Comparable Ns should be used in each condition.

Supp Fig 4 It is unclear why this figure is included. There is no change in weight - only at 9 months and not at later timepoints. Also, this is longitudinal data and should be analysed using a two-way ANOVA.

(Remarks on code availability)
The code is present and accessible as well as the data set. Everything is well documented. I did not try to run it because I do not have time at the moment, but considering the used environment/functions it looks like a standard DEG bulk analysis performed in R. I would recommend including a READ ME file to help future users that might want to run the code and the dataset. I would also suggest to move code and data set to Zenodo to have a stable repository.

Reviewer #3

(Remarks to the Author)
Title: AAV-based delivery of RNAi targeting Ataxin-2 improves survival, strength, and pathology in TDP-43 mice
Authors: Defne A. Amado1,2*, Ashley B. Robbins1,2, Katherine R. Whiteman2, Alicia R. Smith2, Guillem Chillon2,3, Yonghong Chen2, Joshua A. Fuller2, Nicholas A. Patty2,3, Aleksandar Izda2, Congsheng Cheng2, Shareen Nelson2, Abigail I. Dichter2, Alex Mas Monteys2, Beverly L. Davidson2,4*

Amado et. al., have developed an RNAi-based strategy to reduce Atxn2 in the TAR4/4 sporadic ALS mouse model, a strategy that is likely applicable to diseases including SCA2 and potentially additional forms of ALS characterized by TDP-43 mislocalization. The novel PM-AAV9 capsid has a potent motor neuron transduction capability using incredibly low dosage compared to AAV9 which has been used in other studies (AAV9-Cas13 moue study and AAV5-RNAi study in patients), highlighting the great translational potential of this novel capsid. In TAR4/4 mice, miAtxn2 treatment increased survival, improved behavioral phenotypes, and reduced motor neuron death compared to untreated mice. miAtxn2 treatment also convincingly reduced neuroinflammation in both the motor cortex and spinal cord and decreased phosphorylated TDP-43 levels to wild-type levels. Using RNA-seq, they found TAR4/4 ALS-associated transcriptomic signatures were rescued after Atxn2 reduction, highlighting the strong therapeutic potential of targeting Atxn2. This is a compelling story with great potential for translatability in other diseases. Below are a few minor suggestions to improve clarity.

Figure 1B: What are the levels of Atxin2 in WT mice? It would be nice to have the quantification of Atxin2 also in the WT with and without miAtxin2.
Figure 1B picture: Labels (green vs red) for Atxin2 and ChAT need to be defined in the figure legend. It would be nice to have a zoomed-in version of Figure 1B to appreciate the decrease in Atxin2 levels.
Figure 2A: CC3 and ChAT labels are missing in the picture. Are the representative images L6? You might want to separate quantification from representative pictures. In the text, the authors mention treatment with miAtxn2 improves and/or fully corrects apoptosis (CC3) in some areas. The authors may want to specify which areas are fully rescued and which are improved.
Figure 2A, 2B, 3A: Each of these figures would benefit from having the representative WT-miAtxin2 treatments shown, at least in the image panels.
Figure 4D: The authors may want to show the western blot of p-TDP43 together with Pan-TDP43 in addition to showing the quantification of p-TDP43 in Figure 4B.
Figure 5D: Are any of the gene expression changes found in Tar4/4-miAtxin2 mice also found in WT- miAtxin2 treated mice?
Figure S3: Do the gene changes found in the Mutant-miAtxin2 treated mice represent changes after normalization of the miAtxn2 effects on WT gene expression? Clarification on this point is needed.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
the authors have addressed my previous comments and the comments of the other referees and I think this excellent manuscript should be published in Nature Communications.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
In the revised manuscript, the authors have addressed all my concerns.

I do not have further comments.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
This is an important study that will be of broad interest to the scientific community. The authors have carefully addressed the critiques and I have no additional comments.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)

(Remarks on code availability)

Response to Reviewers

We thank the reviewers for their thoughtful reviews and comments, which have greatly strengthened our manuscript.

PLEASE NOTE THAT FOR LINE NUMBERING IN THIS DOCUMENT TO MATCH THE TRACKED-CHANGES MANUSCRIPT, IT MUST BE IN VIEWING (NOT REVIEWING) MODE. We also provide line numbers for the PDF/non-tracked-changes version.

Reviewer #1 (Remarks to the Author):

This manuscript from Amado and colleagues presents an AAV-based gene therapy strategy targeting the ataxin 2 gene using a miRNA. Ataxin 2 was identified as a modifier of TDP-43 toxicity in model systems and intermediate-length CAG repeat expansions in the ataxin 2 gene (ATXN2) have been identified as a genetic contributor to risk for ALS. Previous studies have found that lowering levels of ataxin 2 (either by genetic knockout or with antisense oligonucleotides (ASOs) is sufficient to extend survival and rescue phenotypes in transgenic mice engineered to express human TDP-43 at high levels. These preclinical results motivated a recent clinical trial in human ALS patients to test ataxin 2-targeting ASOs but the results of the trial showed no clinical benefit. However, data reported by the sponsor of the clinical trial (Biogen), and reviewed by the authors of this manuscript, show that the clinical trial only achieved a 21% knockdown of ataxin 2 in the CSF and taking into account that the placebo treated subjects showed a 9% lowering of ataxin 2, the net lowering due to ataxin 2 is only 12%. The authors propose that this indicates that the negative clinical trial results represent a technical failure rather than a biological one. They also correctly point out that the clinical trial was using the highest tolerable dose of ASOs, suggesting that if ataxin 2 will be pursued as a target for ALS, other strategies will be needed to achieve a more potent reduction in ataxin 2 levels.

Here the authors present such a strategy. They use a new AAV vector to deliver a miRNA against ataxin 2 and demonstrate potent knockdown of ataxin 2 levels in the spinal cord, with robust knockdown in motor neurons. Importantly, this treatment extended survival and rescued motor phenotypes. There are several important advances this paper makes compared to other similar efforts. First, they show a rescue of TDP-43 hemizygous mice (TAR4) in addition to the homozygous ones (TAR4/TAR4). Second, they demonstrate using transcriptomics the molecular changes that are rescued by ataxin 2 lowering, providing insight into the mechanism by which ataxin 2 lowering rescues TDP-43 transgenic mice.
This manuscript is very well written, and the authors are transparent about the strengths and limitations of their approach. The striking results and the timely nature of these findings will be of great interest to the field and will likely inspire efforts to translate these results into human. As a first start, the authors end their paper by showing that their approach works against human ataxin 2 in vivo and in vitro. Overall, I think this is an important paper and given the recent clinical trial results should be published without delay. I just have a few comments and suggestions for the authors to consider.

We thank the reviewer for this assessment of our work.

1. The authors compared their ataxin 2 AAV-miRNA treated mice to buffer treated mice. They argue that this more closely relates to what will be needed for IND-enabling studies, which is a convincing argument. However, have the authors tested other AAVs with a non-targeting miRNA as a negative control? This is not required, of course, but if the authors have such data in hand it would be informative to compare to the buffer-treated animals to see if there are any AAV or miRNA specific effects independent of ataxin 2 targeting.

We have not performed studies with a non-targeting miRNA, as in prior studies we found that "non-targeting" miRNAs still had off-target effects. We are in the process of developing a non-ATXN2 targeting miRNA that shares the same off-target profile as miATXN2 for future studies.

2. The TAR4 hemizygous mouse results are very interesting (lowering ataxin 2 rescues phenotypes). Do the authors have transcriptomic data on these animals and their rescued littermates? Are the same types of genes and pathways altered in TAR4 mice but just at a lower level than TAR4/TAR4 mice? This analysis might be informative for readers to learn about mechanism of rescue.

We have not performed transcriptomic analyses on TAR4 hemizygous mice, which we felt was outside the scope of this study – but we agree that this would be informative for a future study. We have added language about the importance of this in the discussion (lines 596-603 in tracked-changes document, lines 333-340 in PDF).

Reviewer #2 (Remarks to the Author):

In the present manuscript, Amado and colleagues report the downregulation of Ataxin-2 using a RNAi AAV-based approach. AAV efficacy and pro-survival effect were tested in a TDP43 mouse model, a human Ataxin-2 overexpressing transgenic mouse and iPSC-derived motor neurons carrying the C9orf72 mutation. Here, they report specific targeting of the RNAi construct which reduced Ataxin-2 levels in all models included in the study. Moreover, they report amelioration of motor phenotypes in the TDP43 mouse model (in both homozygous and heterozygous genotypes) as well as increased motor neuron survival and decreased inflammation.
The authors suggests that the AAV approach is preferable to the ASO one (that was successfully tested in preclinical models but that did not produce the expected results in clinical trials) because it allows for more stable and robust decrease in Ataxin-2 levels, while being less invasive since it requires only one injection. The study is well designed and of interest for the field. However, there are some concerns that should be addressed before the manuscript can be considered for publication
in Nature Communications.

Main text:
1) Statistics and N values need to be reported following the journal guidelines, especially in the Results section and the Figure legends. Throughout the manuscript and the supplementary material exact P values, means and standard deviations, degree of freedom (where applicable), N for each condition should be included.
We have now included all statistical information, including means and SEMs, comparison method and post-hoc analyses, N, and specific p-values, in each figure legend. We have also elaborated our statistical methods in the Statistical Analysis and specific relevant sections of Methods.

2) The information regarding the construct should be clarified (a schematic depicting all elements included in the construct would help); also, Supplementary figure 1 should be included in the main figures, since it helps understanding AAV efficacy. The authors should clarify how the used capsids compare to the ones published in Ranum PT et al 2023. Is the used capsid proven to target neurons in non-human primates? Considerations about this specific point should be included in the discussion because it is relevant for the translational potential of the approach.

We agree and have now made the eGFP transduction portion of Supplementary Figure 1 into a Main Figure (Figure 1). We include all information regarding the construct in Methods and have also included schematics for each relevant construct (Figure S1B, Figure 1 A and E) for greater clarity. There are no additional coding or regulatory elements in the constructs other than those depicted. The capsid used is the same as the one published in Ranum et al. 2023 which we now clarify (lines 220-222 in tracked-changes document, lines 86-88 in PDF). That paper was focused on transduction in the ear, and spinal cord and brain were not analyzed in those studies. However, although we do not have direct NHP data, our data demonstrate strong transduction of human iPSCs differentiated into lower motor neurons (Figure 8).

3) The “Effect of miAtxn2 knockdown on transcriptional phenotypes” paragraph should be restructured. It should clearly state which conditions were included in this part of the study (WT, TAR4/4 mice – I guess homozygous, and miAtxn2 treated), as well as how many mice were included per condition. The authors should clarify that this is bulk sequencing, thus results include all cell types found within the spinal cord (with motor neurons being among the least abundant populations). Considerations about the potential effect of the bulk sequencing on the results obtained in the DEG analysis should be included in the Discussion paragraph. Also, more information about which genes are upregulated or downregulated should be included. The authors are currently mainly referring to differentially expressed genes.

We agree and have now included this information in the relevant Results section (lines 351-353 in tracked-changes document, lines 173-175 in PDF), including the number of mice in each group, the fact that groups were balanced across litters such that each litter contained at least one mouse from each group, and that this is a bulk-sequencing experiment. In the Discussion we discuss the limitations of

this approach and suggest future approaches that will enable motor neuron-specific analysis (lines 596-603 in tracked-changes document, lines 333-340 in PDF). Finally, we provided more detailed information about up- and downregulated genes (lines 377-394 in tracked-changes document, lines 188-205 in PDF).

4) Line 275-276 “in our study at 12 weeks either in numbers of DRG neurons or presence of inflammatory 275 cells in the DRGs (data not shown)”. “Data not shown” is not acceptable nowadays, so the authors should include this dataset. This is a relevant point supporting the low toxicity of the treatment.

We have now included a supplemental figure showing our DRG histology and analyses, and have included corresponding information in Results (lines 243-246 in tracked-changes document, lines 100-103 in PDF) and in Methods (lines 850-854 and 879-885 in tracked-changes document, lines 578-582 and 607-613 in PDF).

Figures:
Fig 1B. I understand that these are normalised values but one of the +miAtxn2 is actually below 0. Why is this? Also, it is unclear from the graph and the figure legend what the black bar is representing.

The reason one value is below zero is the way that ATXN2 levels were computed. To remove background noise, mean fluorescence of extracellular background signal was subtracted from mean fluorescence in ChAT-positive cells. In the case of that mouse, the signal was very slightly lower in the ChAT-positive cells than in surrounding tissue. This has now been added to the relevant Methods section (lines 891-892 in tracked-changes document, lines 619-620 in PDF).

Fig 1C How many animals are included in each group? The figure legends state 14/23 but it is unclear how many animals are used for each condition (treated vs untreated). It is very important that the number of mice included in the survival study is comparable or results may be skewed. What is the median survival for the treated and untreated conditions? (Mean and standard deviation should be reported here).

Of note, Figure 1C is now Figure 2A. There were 14 untreated mutant mice and 15 treated mutant mice included in this analysis; the 23 was an error from another study and we thank the reviewer for catching this. We reported both the mean and median survival in the Results section initially but have now included this information in the figure legend as well, including standard error.

Fig 1D-F Which post hoc analysis was applied here?

Holm-Sidak post-hoc analysis was applied, now specified in this and all figure legends.

Fig 1F Why is the WT+miAtx2 not included in all assessments?

WT + miAtxn2 is not included because we noted early on that there were no phenotypes at P17/P18 in this group and subsequently only studied those mice longitudinally as depicted in Figure 7. As noted in the figure, even at 21 months of age, miAtxn2-treated wildtype mice had no phenotype.

Fig 2A-B Two-way ANOVAs should be used here since two variables are compared 1) treatment, 2) level of the spinal cord.

We have changed our statistical analysis to two-way ANOVA split by mouse and followed by Holm-Sidak post-hoc and have included this information in the figure legend.

Fig 2A The images are out of focus.

We have replaced these with clearer images.

Fig 3 Same as above regarding post hoc and reported stats.

This is now addressed as above.

Fig 4A Spinal cord pictures are conventionally shown with the ventral horn facing downwards. Also, why was level L6 of the spinal cord chosen here?

We have corrected the orientation of the spinal cord images shown. Level L6 was chosen because this was a region where ChAT+ cell loss and rescue with miAtxn2 treatment were noted (Fig. 3B). We wished to know whether motor neuron cell loss was accompanied by gliosis. We have clarified this rationale in the Results section (lines 323-324 in tracked-changes document, lines 156-157 in PDF).

Fig 6A-B When showing longitudinal data a two-way ANOVA should be used.

We have re-analyzed our longitudinal data from Figure 7 with two-way ANOVA and Holm-Sidak post-hoc, and added this information in the figure legend.

Fig 7A The picture is not very representative of the graph presented in this panel.

We apologize that this was confusing. The image shown represents the transduction pattern in the cerebellum after delivery of PM-AAV9.eGFP. The purpose is to demonstrate that our vector reaches Purkinje cells effectively. The graph represents the reduction of mutant human ATXN2 in the Purkinje cells after delivery of PM-AAV9.miAtxn2. Figure 7A, which is now Figure 8A, has been split into two sub-figures and language clarified in the figure legend.

Fig 7C The picture should show treated vs untreated cultures.

We agree and now show treated vs untreated cultures.

Supplementary figures:

Supp Fig 1C The PM-AAV9 picture is completely saturated and should be replaced.

The inset picture, now separately labeled as Figure 1C, is the unsaturated version of PM-AAV9. The reason we chose to show this data the way that we do is to enable comparison of transduction at the same exposure, which is critical for demonstrating the degree to which transduction is increased using PM-AAV9 over AAV9. When imaged at lower exposure, AAV9 and AAV-PHP.eB transduction are no longer visible. This is the reason we included the inset picture, at a more appropriate exposure, which we have now labeled as a separate sub-figure and added clarifying language in the figure legend.

Supp Fig 2A The curves should show standard deviations and means, it is not clear what is represented in the graph.

We have replaced this depiction of our phenotyping with a more quantifiable dataset, as we had a large number of mice that were phenotyped at both P12 and P18 for more robust statistical analysis.

Supp Fig 2B-F The N per each condition should be clarified since it is reported 10/40. Comparable Ns should be used in each condition.

By chance of litter composition, we had more untreated WT mice available for analysis than other groups. To make our N per condition a more comparable size, we now exclude from analysis litters in which no TAR4/4 homozygous mice were born. This results in more comparable group sizes.

Supp Fig 4 It is unclear why this figure is included. There is no change in weight - only at 9 months and not at later timepoints. Also, this is longitudinal data and should be analysed using a two-way ANOVA.

We have eliminated this figure.

Reviewer #2 (Remarks on code availability):

The code is present and accessible as well as the data set. Everything is well documented. I did not try to run it because I do not have time at the moment, but considering the used environment/functions it looks like a standard DEG bulk analysis performed in R. I would recommend including a READ ME file to help future users that might want to run the code and the dataset. I would also suggest to move code and data set to Zenodo to have a stable repository.

Data will be made available on GeoQuery and code will be available on GitHub. A README file and detailed instructions to run existing code will be made available.

Reviewer #3 (Remarks to the Author):

Title: AAV-based delivery of RNAi targeting Ataxin-2 improves survival, strength, and pathology in TDP-43 mice
Authors: Defne A. Amado1,2*, Ashley B. Robbins1,2, Katherine R. Whiteman2, Alicia R. Smith2, Guillem Chillon2,3, Yonghong Chen2, Joshua A. Fuller2, Nicholas A. Patty2,3, Aleksandar Izda2, Congsheng Cheng2, Shareen Nelson2, Abigail I. Dichter2, Alex Mas Monteys2, Beverly L. Davidson2,4*

Amado et. al., have developed an RNAi-based strategy to reduce Atxn2 in the TAR4/4 sporadic ALS mouse model, a strategy that is likely applicable to diseases including SCA2 and potentially additional forms of ALS characterized by TDP-43 mislocalization. The novel PM-AAV9 capsid has a potent motor neuron transduction capability using incredibly low dosage compared to AAV9 which has been used in other studies (AAV9-Cas13 moue study and AAV5-RNAi study in patients), highlighting the great translational potential of this novel capsid. In TAR4/4 mice, miAtxn2 treatment increased survival, improved behavioral phenotypes, and reduced motor neuron death compared to untreated mice. miAtxn2 treatment also convincingly reduced neuroinflammation in both the motor cortex and spinal cord and decreased phosphorylated TDP-43 levels to wild-type levels. Using RNA-seq, they found TAR4/4 ALS-associated transcriptomic signatures were rescued after Atxn2 reduction, highlighting the strong therapeutic potential of targeting Atxn2. This is a compelling story with great potential for translatability in other diseases. Below are a few minor suggestions to improve clarity.

We thank the reviewer for these comments.

Figure 1B: What are the levels of Atxin2 in WT mice? It would be nice to have the quantification of Atxin2 also in the WT with and without miAtxin2.

This data is now included in our Revised Figure 1 and referenced in Results (lines 274-277 in tracked-changes document, lines 121-124 in PDF). Interestingly, Ataxin-2 levels are actually downregulated in TAR4/4 mice compared to wildtype mice, and are further downregulated by miAtxn2.

Figure 1B picture: Labels (green vs red) for Atxin2 and ChAT need to be defined in the figure legend. It would be nice to have a zoomed-in version of Figure 1B to appreciate the decrease in Atxin2 levels.

We include these labels in Revised Figure 1 and include zoomed-in images.

Figure 2A: CC3 and ChAT labels are missing in the picture. Are the representative images L6? You might want to separate quantification from representative pictures. In the text, the authors mention treatment with miAtxn2 improves and/or fully corrects

apoptosis (CC3) in some areas. The authors may want to specify which areas are fully rescued and which are improved.

We agree and have now clarified which areas are fully rescued and have added clarifying labels to the image. We also separated quantification from pictures as suggested.

Figure 2A, 2B, 3A: Each of these figures would benefit from having the representative WT-miAtxin2 treatments shown, at least in the image panels.

We unfortunately do not have this data, as we did not analyze miAtxn2-treated wildtype mice histologically.

Figure 4D: The authors may want to show the western blot of p-TDP43 together with Pan-TDP43 in addition to showing the quantification of p-TDP43 in Figure 4B.

We did attempt this originally but found that pulling out p-TDP43, which aggregates, required urea extraction which interfered with running an SDS-based gel. This in turn caused highly variable yields between experiments, which coupled with a low p-TDP43 amount (as a fraction of total protein) led to unreliable quantification. For this reason, we quantified p-TDP43 by IF.

Figure 5D: Are any of the gene expression changes found in Tar4/4-miAtxin2 mice also found in WT- miAtxin2 treated mice?

We did not include wildtype mice treated with miAtxn2 in this dataset. We agree this would be of value in future studies.

Figure S3: Do the gene changes found in the Mutant-miAtxin2 treated mice represent changes after normalization of the miAtxn2 effects on WT gene expression? Clarification on this point is needed.

We did not include wildtype mice treated with miAtxn2 in this dataset, as this study was designed primarily to assess the efficacy of a translational therapeutic. We agree that this would be of value in future studies.