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14. ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a devastating and universally fatal neurodegenerative disease. While multiple genetic mutations have been identified as causative of ALS, the vast majority of cases remain sporadic in nature. Additionally, the precise molecular events leading to neurodegeneration in ALS and related diseases remain largely unknown. Defects in an essential cellular process which maintains communication between nuclear and cytoplasmic compartments of the cell (nucleocytoplasmic transport, NCT) have recently emerged as a prominent pathomechanism underlying ALS and other neurodegenerative diseases. The nuclear pore complex (NPC) is made up of multiple copies of ~30 individual proteins (Nups) and tightly regulates NCT. However, the mechanisms leading to alterations in NPC composition and function remain understudied. We have now identified a reproducible subset of Nups that are altered as an early event in disease pathogenesis. Moreover, we have determined that these Nups are aberrantly degraded from neuronal NPCs. We now seek to understand the mechanism by which this degradation event is initiated in ALS and related neurodegenerative diseases. In addition, we hope to evaluate the potential of this degradative pathway as a therapeutic target. This work is uniquely poised to both understand the molecular events leading to NPC injury in ALS but also identify new therapeutic targets for the treatment of devastating neurological diseases.

15. SUBJECT TERMS
ALS, nuclear pore, frontotemporal dementia

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1. Introduction

Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) comprise a spectrum of devastating and fatal neurodegenerative diseases. Over 20 genetic loci have been linked to ALS and FTD, the most common of which is an intronic GGGGCC hexanucleotide repeat expansion in the C9orf72 gene. However, about 90% of ALS cases are sporadic in nature. While deficits in nucleocytoplasmic transport (NCT) have arisen as a prominent pathomechanism underlying familial and sporadic ALS, the molecular mechanisms underlying this disruption remain largely unknown. The nuclear pore complex (NPC) is made of ~30 nucleoporins (Nups) organized into subcomplexes and arranged with eightfold rotational symmetry to regulate NCT. To date, none of the existing studies define the actual injury to the NPC itself or the mechanism by which the injury occurs. Using iPSC derived spinal neurons (iPSNs) and postmortem tissue, we have amassed substantial data suggesting that a reproducible subset of Nups are reduced from the nucleoplasm and NPCs in all C9orf72 ALS patients early in disease pathogenesis. Given that these specific Nups are not mislocalized or aggregated in the cytoplasm in iPSNs or postmortem human tissue, we have now determined that they are aberrantly degraded from the NPC. Recent work suggests that the ESCRT-III pathway plays a fundamental role in the surveillance and maintenance of properly assembled and functioning NPCs in yeast. Critically, work in these non-CNS systems suggests that recruitment of CHMP7 to the nuclear envelope initiates downstream events leading to degradation of Nups and NPCs. We hypothesized that the dysregulation of this pathway is the driving cellular process that leads to NPC injury in C9orf72 ALS patients, and potentially extend to a subset of sALS patients characterized by NPC injury. Our new data suggests that nuclear localization of CHMP7 is drastically increased in C9orf72 iPSN and postmortem C9orf72 patient nuclei. Furthermore, knock-down of CHMP7 in iPSNs completely mitigates the observed C9orf72 mediated loss of specific Nups from the nuclear envelope. These data suggest that in human neurons, aberrant activation of the ESCRT-III pathway may be a substantial contributor to disruptions in the NPC, NCT, and overall cellular survival thus highlighting the potential for CHMP7 as a therapeutic target in ALS. In this research program we are using iPSNs and a candidate-based approach to begin to understand the mechanism by which the nuclear accumulation of CHMP7 initiates aberrant Nup degradation in C9orf72 ALS/FTD. Furthermore, we will be evaluating the efficacy of CHMP7 as a therapeutic target in mitigation of nuclear pore injury in C9orf72 ALS/FTD and sALS Together, these experiments will advance our understanding of the mechanisms underlying Nup homeostasis in human neurons and in disease and evaluate new therapeutic targets for the treatment of neurodegeneration.

2. Keywords: Amyotrophic lateral sclerosis, frontotemporal dementia, antisense oligonucleotide, CHMP7, ESCRT-3, nuclear pore, nucleoporin, nuclear transport, neurodegeneration, neuron, induced pluripotent stem cell

3. Accomplishments

- Major Goals:
  - **Specific Aim 1**: Investigate the contribution of CHMP7 nuclear recruitment and retention to nucleoporin alterations in C9orf72 iPSNs.
  - **Specific Aim 2**: Evaluate the ability of CHMP7 ASOs to mitigate C9orf72 ALS/FTD and sALS mediated alterations in the nuclear pore complex and nucleocytoplasmic transport in iPSNs.
- Major activities and Objectives:
  - In the last year we have focused on the optimization of the knockdown of CHMP7 by 3 different unique human targeting ASOs.
- Significant results: As shown below we now have "first pass" data that teaches us:
  - All three ASO were effective reducing human CHMP7. This effect may be dose dependent (noting that these are still early studies) (**Figure 1**)
  - ASO targeting human CHMP7 allow restoration of normal nuclear pore complex (NPC) composition of individual nucleoporins. Specifically, 3 candidate nups: nup 50, nup133 and Pom 121 were all restored. The restoration occurs even after the injury to the NPC occurs.

- CHMP7 ASO also prevent sensitivity to glutamate stress (**Figure 2**)
- Treatment with CHMP7 restores normal TDP43 function in both C9orf72 iPS cell lines as well as sporadic ALS cell lines (**Figure 3**).

In aggregate in the first year, we have tackled core question and goals in both our original Specific Aims 1 and 2.

Data Accomplishments

CHMP7 is effectively knocked down by antisense oligonucleotides (ASOs) in iPSNs (Figure 1)
To determine if ASOs targeting human CHMP7 pre-mRNA could efficiently reduce CHMP7 protein levels in iPSNs, control, C9orf72, and sALS iPSNs were subjected to treatment with 1, 5, 10 µM scrambled or CHMP7 targeting ASOs twice per week for 2 weeks. Western blot analyses confirmed a dose dependent decrease in CHMP7 protein expression in control, C9orf72, and sALS iPSNs (**Figure 1**) suggesting that ASOs are a viable tool for reduction of CHMP7 protein in non-dividing human neurons.

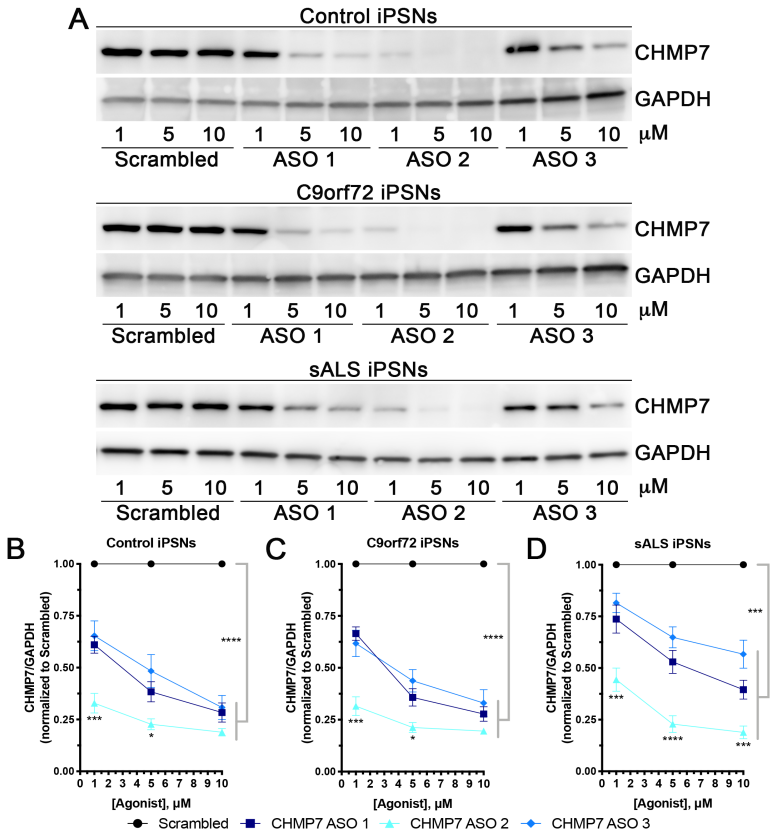

Figure 1. CHMP7 ASOs reduce CHMP7 protein in iPSNs in a dose dependent manner. (**A-D**) Western blot (**A**) and quantification (**B-D**) for CHMP7 protein in control (**A-B**), *C9orf72* (**A,C**), and sALS (**A,D**) iPSNs at day 40 of differentiation following 2 week exposure to scrambled control or CHMP7 targeting ASOs. Genotype as indicated on top, concentration and ASO as indicated on bottom. n = 6 control, 4 *C9orf72*, and 6 sALS iPSC lines. Two-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Published in.[1]

Knockdown of CHMP7 restores NPC composition, TDP-43 function, and alleviates neuronal death in response to glutamate stress in ALS iPSNs (Figure 2)
Having observed that 2 week exposure to CHMP7 ASOs can effectively reduce CHMP7 protein by ~90% (**Figure 1**), we next asked whether knockdown of CHMP7 could mitigate NPC injury cascades in iPSNs. Using SIM, we demonstrated that reduction of CHMP7 protein following the emergence of NPC injury, can restore the composition of iPSN NPCs (**Figure 2**). Moreover, one week following the restoration of NPC composition, we observed a significant improvement of TDP-43 function as evaluated by a qRT-PCR panel of mRNA target expression (**Figure2 G,H; Figure 3**) and a mitigation of neuronal death in response to exogenous glutamate stress (**Figure 2I**). Collectively, this data suggests that reduction in CHMP7 protein is not overtly toxic to human neurons and can repair reverse multiple pathologies in ALS iPSNs.

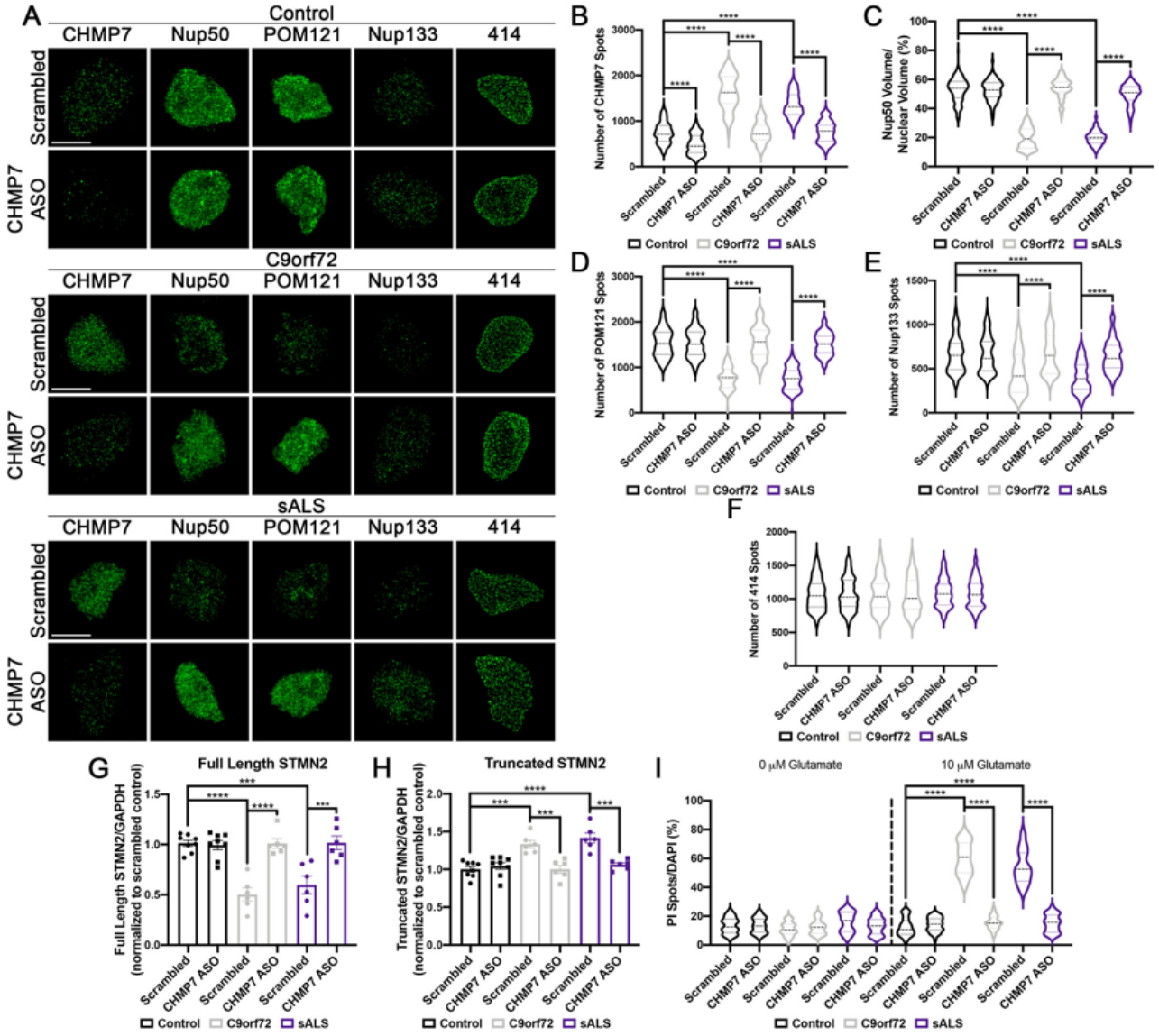

Figure 2. ASO mediated knockdown of CHMP7 restores the nuclear expression of specific Nups, mitigates TDP-43 mediated splicing defects, and reduces glutamate induced excitotoxicity in *C9orf72* and sALS iPSNs. (**A**) Maximum intensity projections from SIM imaging of nuclei isolated from control, *C9orf72*, and sALS iPSNs on day 40 of differentiation following 2-week exposure to 5 μM scrambled control ASO or CHMP7 ASO 2. Treatment as indicated on left, genotype and antibodies as indicated on top. (**B-F**) Quantification of spots and volume. n = 6 control, 4 *C9orf72*, and 8 sALS iPSC lines, 50 NeuN+ nuclei per line/treatment. Two-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. **** $p < 0.0001$. (**G-H**) qRT-PCR for full length (**G**) and truncated (**H**) *STMN2* mRNA in control, *C9orf72*, and sALS iPSNs on day 46 of differentiation following 3-week exposure to 5 μM scrambled control ASO or CHMP7 ASO 2. GAPDH was used for normalization. n = 8 control, 6 *C9orf72*, and 6 sALS iPSC lines. Two-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. *** $p < 0.001$, **** $p < 0.0001$. (**I**) Quantification of percent cell death as measured by propidium iodide (PI) incorporation following exposure to glutamate in control and *C9orf72* iPSNs following 2-week exposure to 5 μM scrambled control ASO or CHMP7 ASO 2. n = 7 control, 5 *C9orf72*, and 6 sALS iPSC lines, 5 frames per well. Two-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. **** $p < 0.0001$. Scale bar = 5 μm. Published in [1].

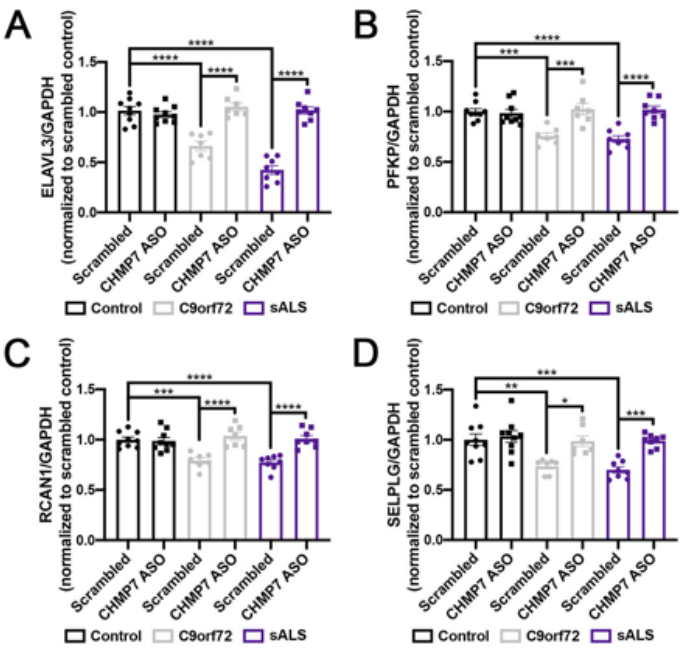

Figure 3 ASO mediated knockdown of CHMP7 restores the levels of additional mRNAs affected by TDP-43 loss of function. (**A-D**) qRT-PCR for *ELAVL3* (**A**), *PFKP* (**B**), *RCAN1* (**C**), and *SELPLG* (**D**) mRNA in control, *C9orf72*, and sALS iPSNs on day 46 of differentiation following 3 week exposure to 5 μM scrambled control ASO or CHMP7 ASO 2. GAPDH was used for normalization. n = 8 control, 6 *C9orf72*, and 6 sALS iPSC lines. Two-way ANOVA with Tukey's multiple comparison test was used to calculate statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

- Note, a more detailed discussion of data in Figure 2 and 3 also found in our recent publication (Coyne et al, Sci Transl Med, 2021). [1]
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4. Professional development: Nothing to report
5. Results dissemination: Data from Fig 1,2,3 recently published, [1]
6. Plans:
    - Aim 1. In the next year we will more fully explore, and importantly repeat dose and time response curves.
    - Aim 2. The mitigation of TDP43 dysfunction is critically important and in year 2 we intend on expanding multiple other TDP43 misplicing RNA products after ASO treatment along with the tie dependence of recovery of normal TDP43 function in sporadic ALS iPS neuronal cell lines. We will assay expression of additional mRNAs and cryptic exon inclusion products including AARS1, MAST1, MGAT1, SYT7, CAMK2B, ATG4B, AGRN, SEPTIN11, CELF5, KCNQ2, PTPRN2, SEMA6D, SET6D[2]. These studies will provide preclinical validation that disease associated biomarkers respond to CHMP7 ASO treatment.
7. Impact
    - Impact on the development of the principal discipline(s) of the project: Nothing to Report
    - Impact on other disciplines: Nothing to Report
    - Impact on technology transfer: Noting to report
    - Impact on Society beyond science: Nothing to report

8. Changes/Problems

- Changes in approach: Nothing to Report
- Anticipated Problems: Nothing to Report
- Change in expenditures: Nothing to Report
- Changes in Human subjects: Nothing to Report
- Changes in use and care of vertebrate animals: Nothing to Report
- Changes in biohazards: Nothing to Report

9. Products: Noting to report

10. Participants & Other Collaborating Organizations: The current study reflects a collaboration with Ionis Pharmaceuticals who have been collaborating to provide optimized CHMP7 ASO. (See Figure 1)

11. Special Reporting Requirements: Noting to report

12. REFERENCES

1. Coyne, A.N*., et al.* Nuclear accumulation of CHMP7 initiates nuclear pore complex injury and subsequent TDP-43 dysfunction in sporadic and familial ALS. *Sci Transl Med* **13**(2021)
2. Brown, A.L*., et al.* TDP-43 loss and ALS-risk SNPs drive mis-splicing and depletion of UNC13A. *Nature* **603**, 131-137 (2022).PMC8891020 described in this work has been protected in the patent PCT/EP2021/084908 and UK patent 2117758.9 (patent applicant, UCL Business Ltd and NIH; status pending), in which A.-L.B., O.G.W., M.J.K., S.E.H., M.E.W. and P.F. are named as inventors. The other authors declare no competing interests.