

Correction of amyotrophic lateral sclerosis related phenotypes in induced pluripotent stem cell-derived motor neurons carrying a hexanucleotide expansion mutation in *C9orf72* by CRISPR/Cas9 genome editing using homology-directed repair

Nidaa A. Ababneh^{1,2,§,†}, Jakub Scaber^{1,§,‡}, Rowan Flynn³, Andrew Douglas^{4,5}, Paola Barbagallo¹, Ana Candalija¹, Martin R. Turner^{1,¶}, David Sims⁶, Ruxandra Dafinca^{1,#}, Sally A. Cowley^{3,#} and Kevin Talbot^{1,#,*||}

Human Molecular Genetics

Pearson Lab Journal Club

July 6, 2020.

Monika Schmidt

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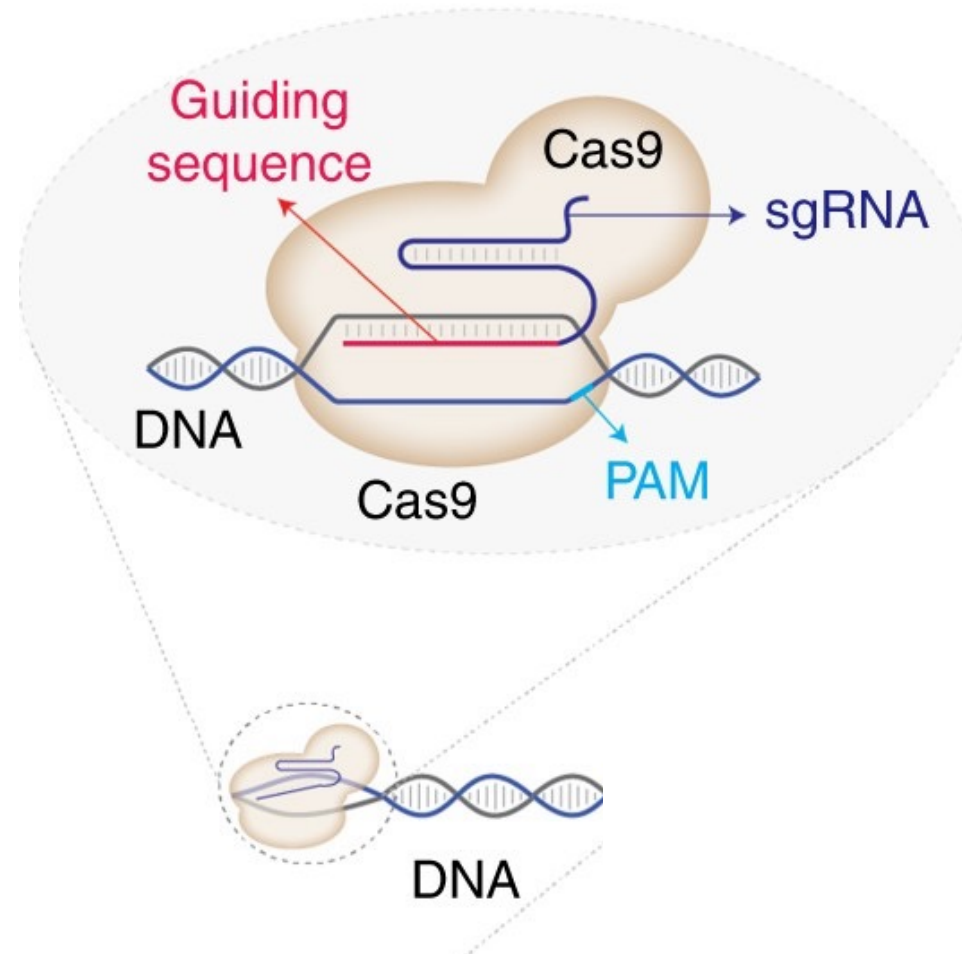
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Brief overview of CRISPR-Cas9



Adapted from Adli, 2018, *Nat Comm*.

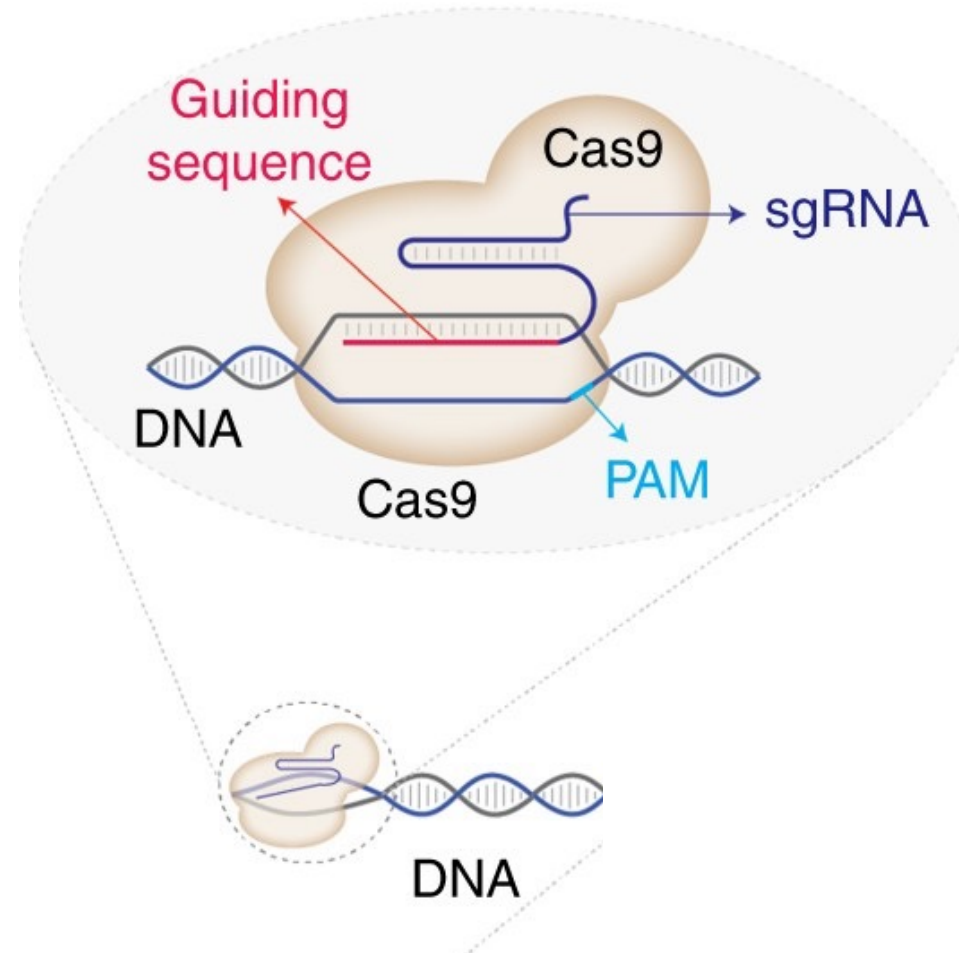
Brief overview of CRISPR-Cas9

1987 – first identified by a Japanese Team at Osaka Uni

Work through the 1990's to study the unusual repeating patterns

2002 – Rudd Jansen and team name 'CRISPR' and the accompanying 'CRISPR-associated genes' aka Cas9

2007 – Danisco identifies 'spacer' sequences that provide bacteria with viral resistance.



Adapted from Adli, 2018, *Nat Comm*.

2008 – Eugene Koonin et al., recognize that CRISPR and Cas9 work together to disable viruses

2012 – Doudna, Charpentier and team, publish CRISPR-Cas9 as a tool for cutting DNA in a test tube (Jinek *et al.*, *Science*, 2012)

2013 – multiple labs publish on how to edit genomes in human cells with CRISPR/Cas9

2016 – Alexis Komor and colleagues develop first base editor (Liu lab) using Cas9 fusion protein

Many innovative forms of Cas9!

- **Cas9:** The OG workhorse
 - isolated from *S.pyogenes*, relatively large
 - stringent PAM requirements but complimentary specifications not very stringent = off-targets
- **Cas9 variants:** recognize different PAM sequences
 - SaCas9, NmCas9, StCas9, FnCas9, CjCas9, ScCas9...
- **Cas9 nickases:** can use 2 gRNAs
- **HfCas9:** various high fidelity versions
- **xCas9:** broad range of PAM sequences
- **dCas9:** catalytically inactive, blocks transcription, used for CRISPRa/i or for fluorescent applications
- **CasX and CasY:** most compact and naturally-occurring
- **Cas12a (Cpf1):** well-suited to AT-rich regions, cuts ssDNA after binding target
- **Cas13a, 13b, 13c, 13d:** RNA editors
- **Cas14a:** does not need a PAM sequence, cuts target ssDNA, then will cut off-target ssDNA

Previous iPSC models in repeat-associated diseases

Dimos, Rodolfa, Niakan (2008) – iPSCs from **fALS** patient differentiated into MNs

Ebert, Yu, Rose (2009) – iPSC model from SMA patient

Soldner, Hockemeyer, Beard (2009) – iPSC model from PD patient

Almeida, Zhang, Copplola (2012) – iPSC model of **FTD** (progranulin-deficient FTD)

HD iPSC Consortium (2012) – iPSC model from HD patients (expansion-associated phenotypes)

Israel, Yuan, Bardy (2012) – another set of iPSC models from sAD and fAD

Juopperi, Kim, Chiang (2012) – iPSCs from HD patient differentiated into astrocyte model

Modeling key pathological features of frontotemporal dementia with *C9ORF72* repeat expansion in iPSC-derived human neurons

Sandra Almeida · Eduardo Gascon · H  l  ne Tran · Hsin Jung Chou · Tania F. Gendron · Steven DeGroot · Andrew R. Tapper · Chantal Sellier · Nicolas Charlet-Berguerand · Anna Karydas · William W. Seeley · Adam L. Boxer · Leonard Petrucelli · Bruce L. Miller · Fen-Biao Gao

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DOI: 10.1038/s41467-017-02729-0

OPEN

C9ORF72 repeat expansion causes vulnerability of motor neurons to Ca²⁺-permeable AMPA receptor-mediated excitotoxicity

Bhuvaneish T. Selvaraj^{1,2,3}, Matthew R. Livesey^{2,3,4}, Chen Zhao^{1,2,3}, Jenna M. Gregory^{2,3}, Owain T. James^{2,3,4}, Elaine M. Cleary^{1,2,3}, Amit K. Chouhan^{2,5}, Angus B. Gane^{1,2,3}, Emma M. Perkins^{2,3,4}, Owen Dando^{4,6}, Simon G. Lillico⁷, Youn-Bok Lee⁸, Agnes L. Nishimura⁸, Urjana Poreci⁹, Sai Thankamony⁹, Meryll Pray⁹, Navneet A. Vasistha^{1,3,6}, Dario Magnani^{1,2,3}, Shyamanga Borooah¹, Karen Burr^{1,2,3}, David Story^{1,2,3}, Alexander McCampbell¹⁰, Christopher E. Shaw⁸, Peter C. Kind^{4,6}, Timothy J. Aitman¹¹, C. Bruce A. Whitelaw⁷, Ian Wilmut¹, Colin Smith^{2,3}, Gareth B. Miles^{2,5}, Giles E. Hardingham^{2,4,12}, David J.A. Wyllie^{2,4,6} & Siddharthan Chandran^{1,2,3,6,12}

ORIGINAL PAPER

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Stem Cell Reports

Article



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Knocking out *C9ORF72* Exacerbates Axonal Trafficking Defects Associated with Hexanucleotide Repeat Expansion and Reduces Levels of Heat Shock Proteins

Masin Abo-Rady^{1,11}, Norman Kalmbach^{2,11}, Arun Pal³, Carina Schludi⁴, Antje Janosch⁵, Tanja Richter⁶, Petra Freitag⁷, Marc Bickle⁵, Anne-Karin Kahlert⁷, Susanne Petri², Stefan Stefanov¹, Hannes Glass^{3,8}, Selma Staeger², Walter Just⁶, Rajat Bhatnagar⁹, Dieter Edbauer⁴, Andreas Hermann^{1,3,8,10}, Florian Wegner^{2,12,*} and Jared L. Sternecker^{1,12,*}

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No protein

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Acta Neuropathologica (2020) 140:81–84
<https://doi.org/10.1007/s00401-020-02154-6>

CORRESPONDENCE



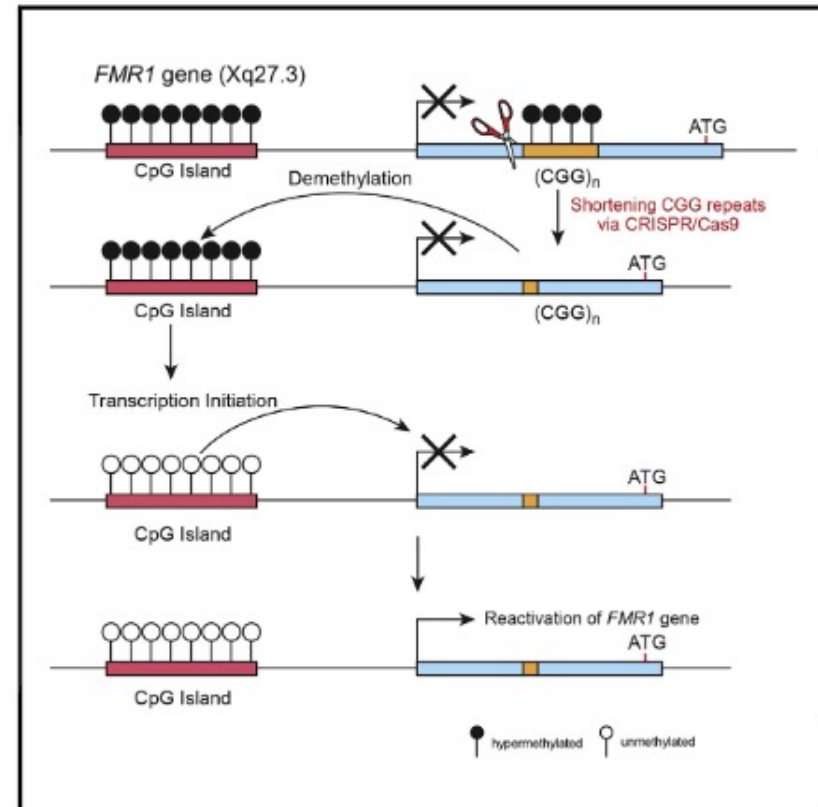
CRISPR deletion of the *C9ORF72* promoter in ALS/FTD patient motor neurons abolishes production of dipeptide repeat proteins and rescues neurodegeneration

Gopinath Krishnan¹ · Yu Zhang^{1,2} · Yuanzheng Gu³ · Mark W. Kankel³ · Fen-Biao Gao¹ · Sandra Almeida¹

Cell Reports

Reversion of *FMR1* Methylation and Silencing by Editing the Triplet Repeats in Fragile X iPSC-Derived Neurons

Graphical Abstract

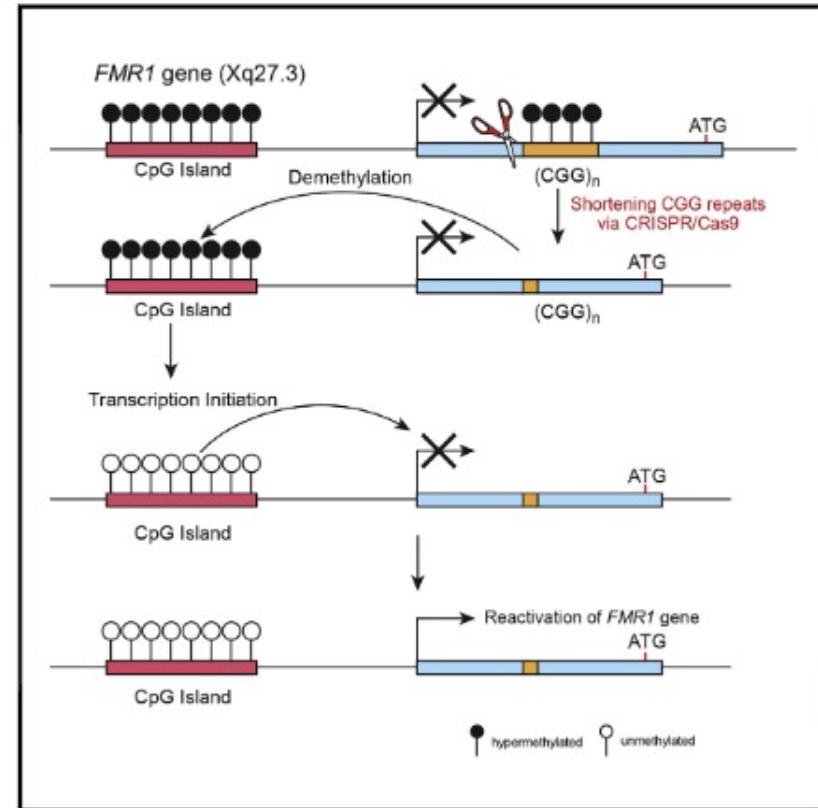


Authors

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RESEARCH ARTICLE

Reactivation of *FMR1* by CRISPR/Cas9-Mediated Deletion of the Expanded CGG-Repeat of the Fragile X Chromosome

Nina Xie^{1a}, He Gong¹, Joshua A. Suhl^{1b}, Pankaj Chopra¹, Tao Wang^{1ac}, Stephen T. Warren^{1,2*}

The Question:

Can CRISPR/Cas9 be used to correct the *C9orf72* repeat expansion? If so, does it stop/reverse disease phenotype?

Rationale:

Previous studies have only employed CRISPR/Cas9 via NHEJ to edit the repeat tract, and have not made proper isogenic controls → difficult to evaluate changes in markers disease pathology.

Principal Conclusion:

Complete correction of iPSC line derived from C9-positive ALS/FTD patient using CRISPR/Cas9 and HDR. Genetic architecture of locus preserved, and all markers of disease phenotype restored to WT levels.

A)

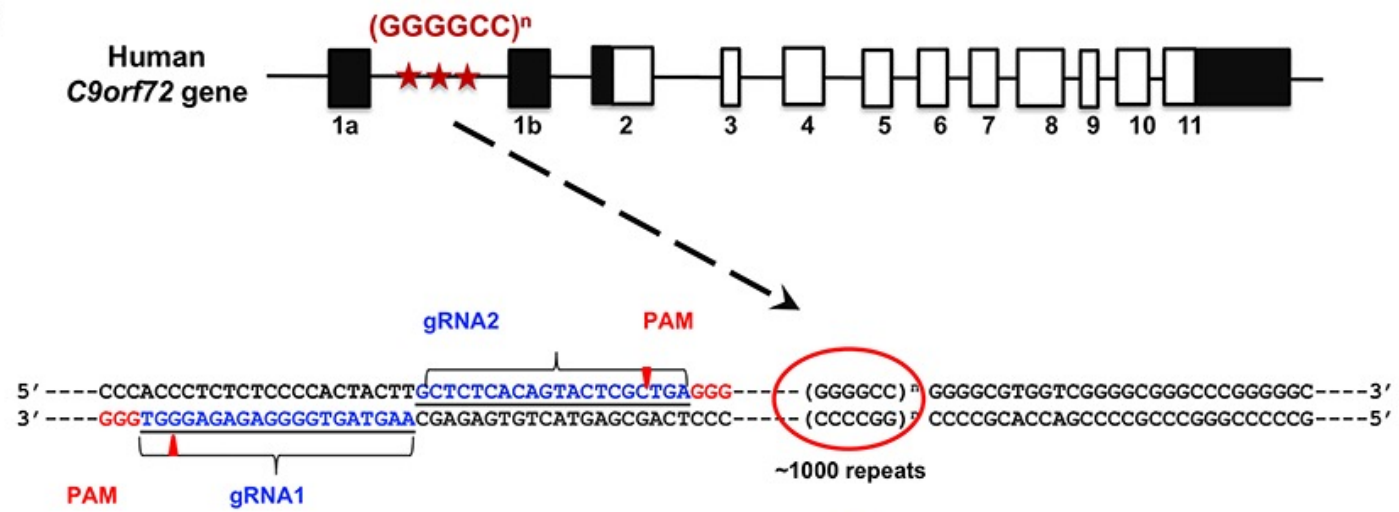


Figure 1

A)

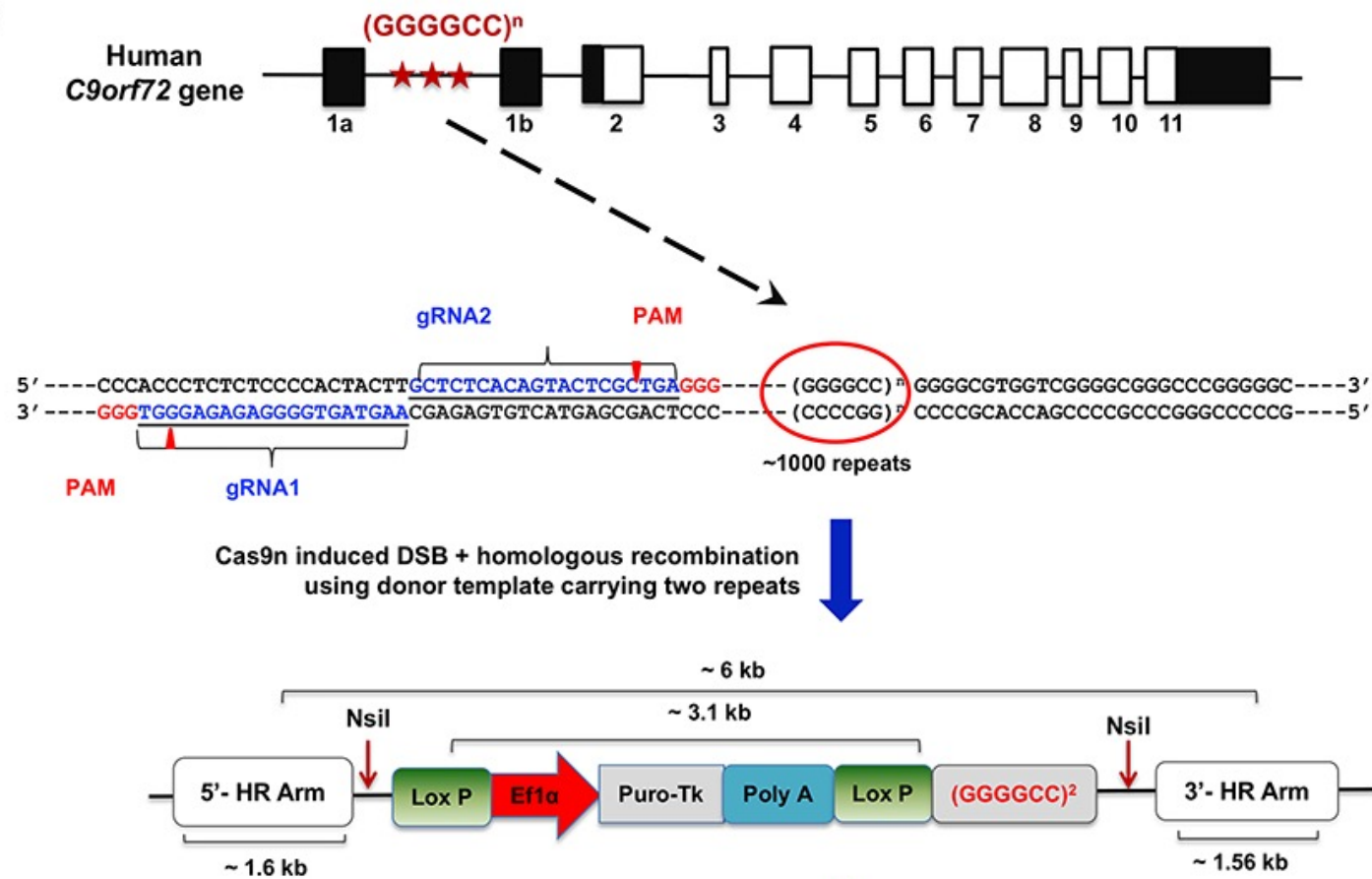


Figure 1

A)

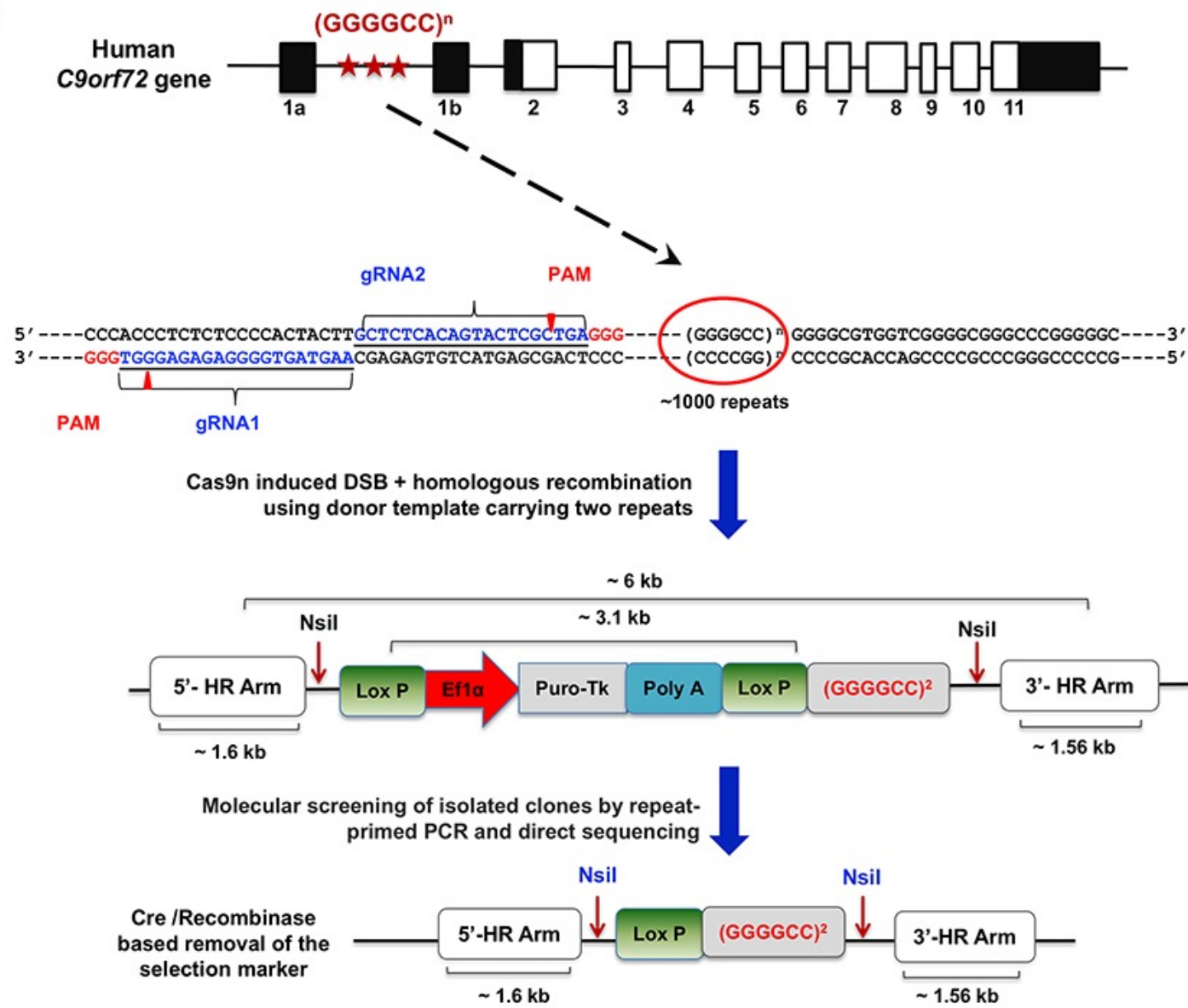


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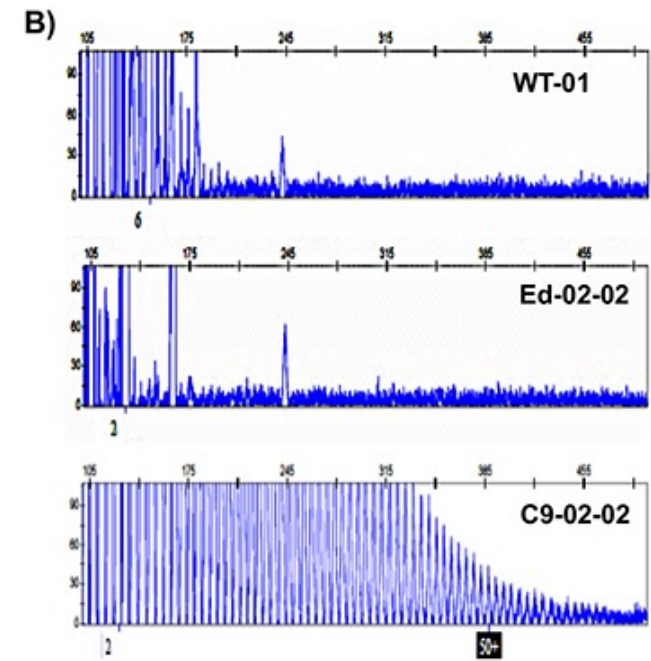
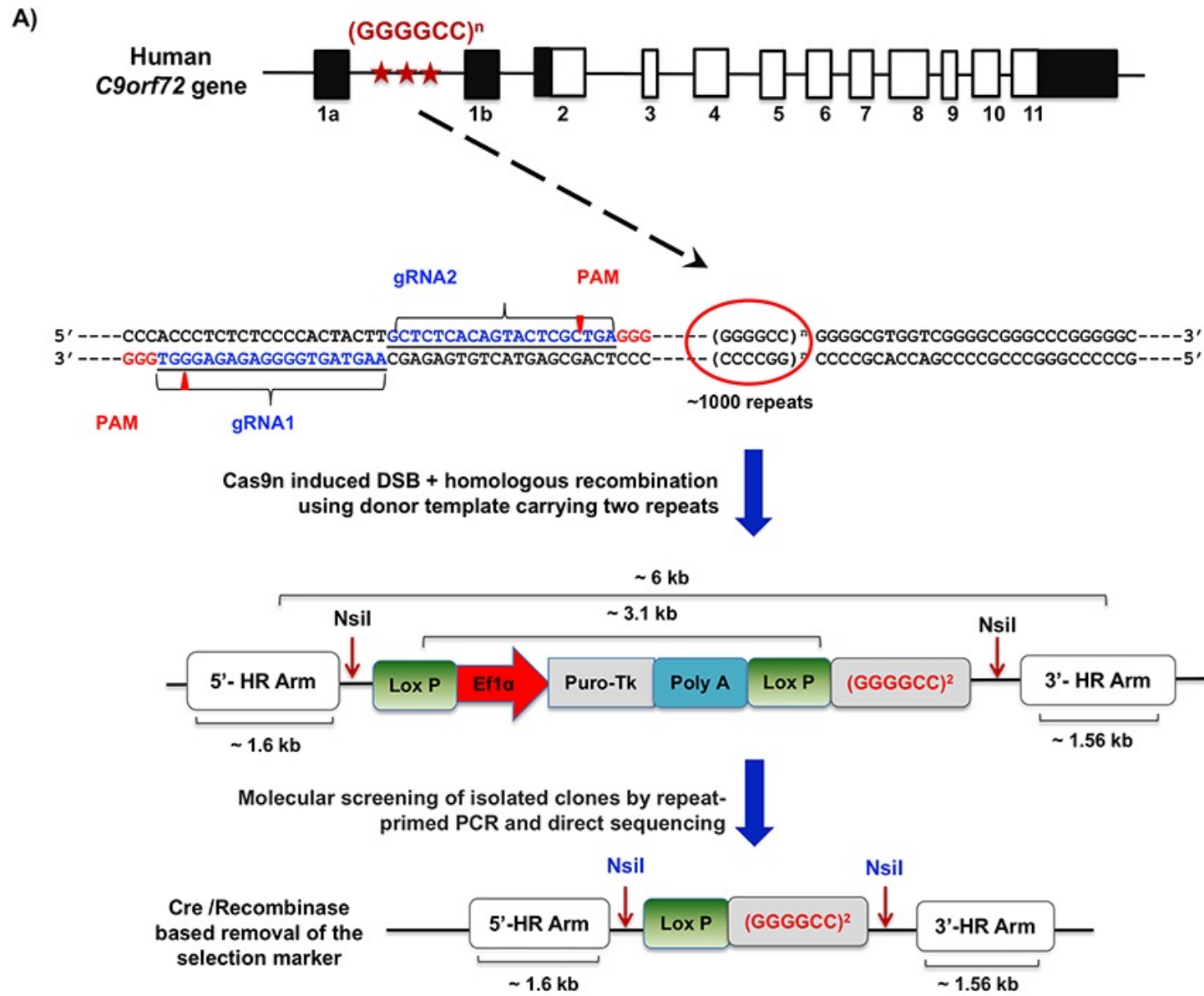
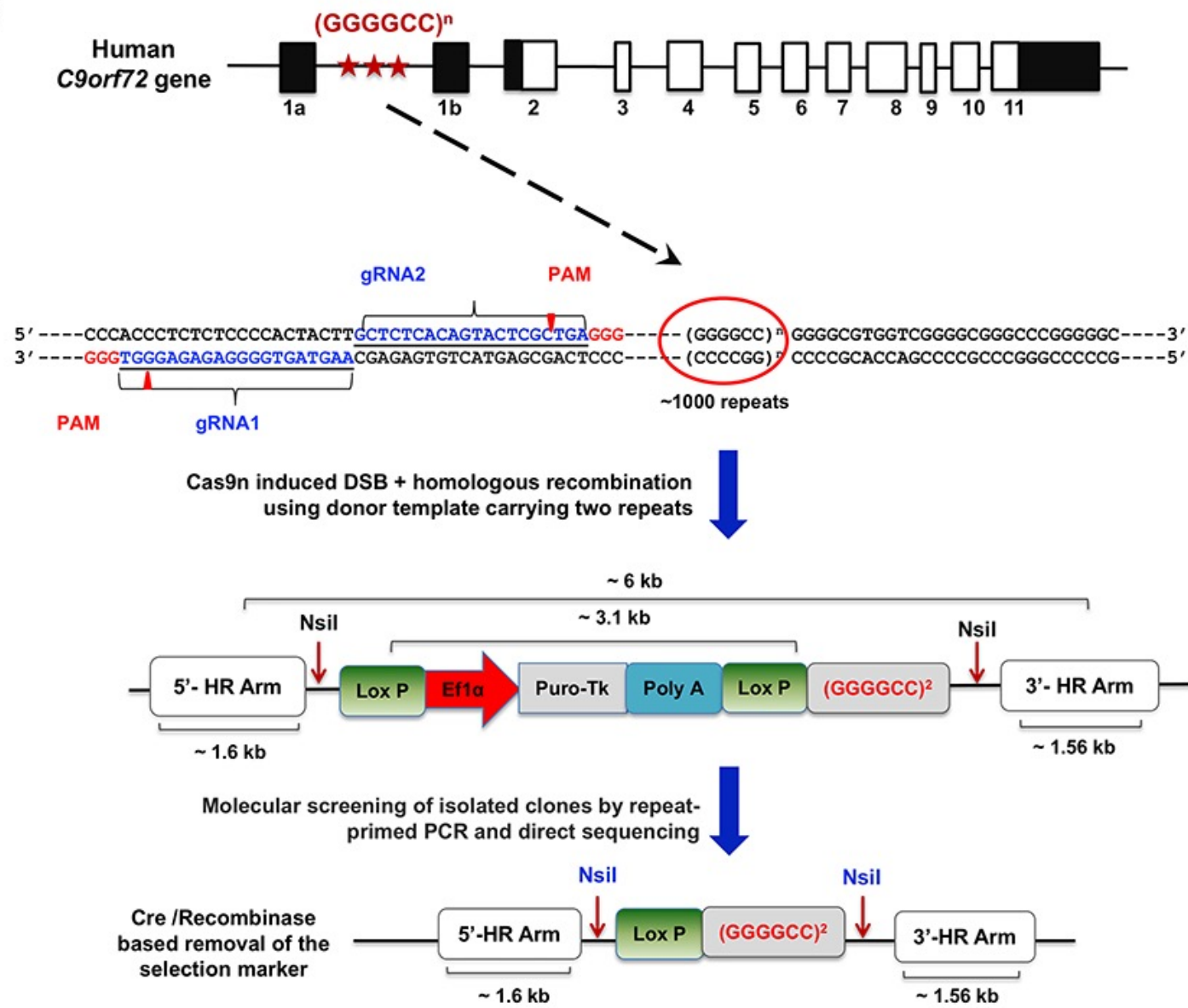
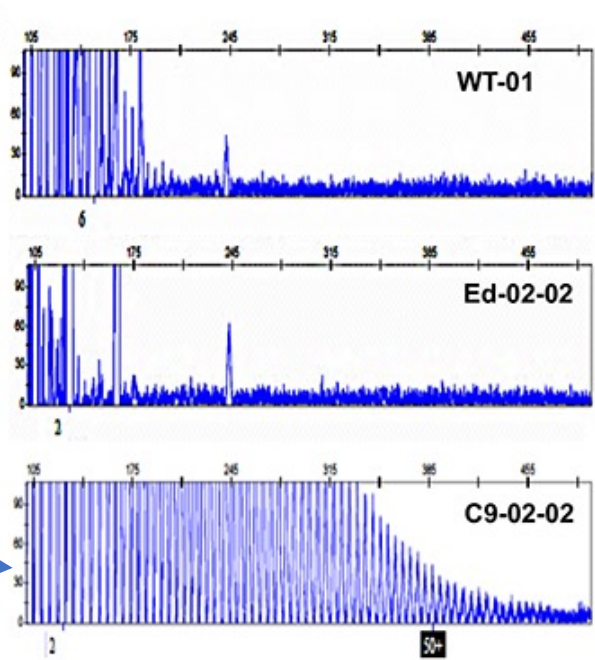


Figure 1

A)



B)



C)

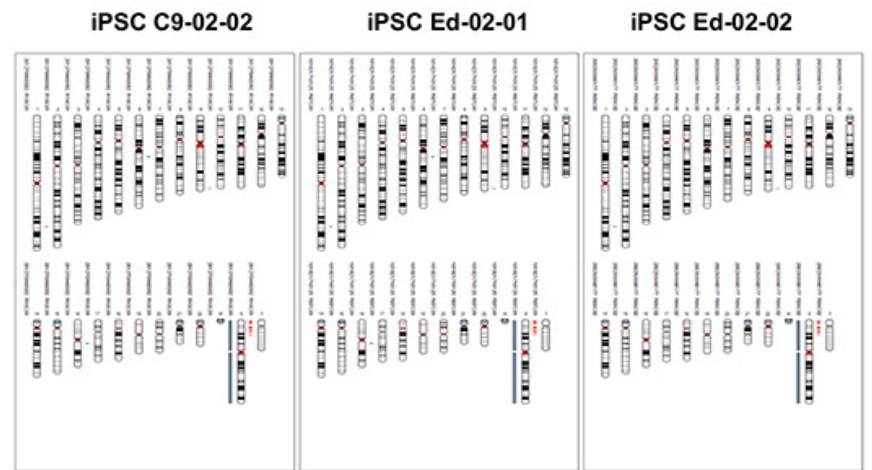


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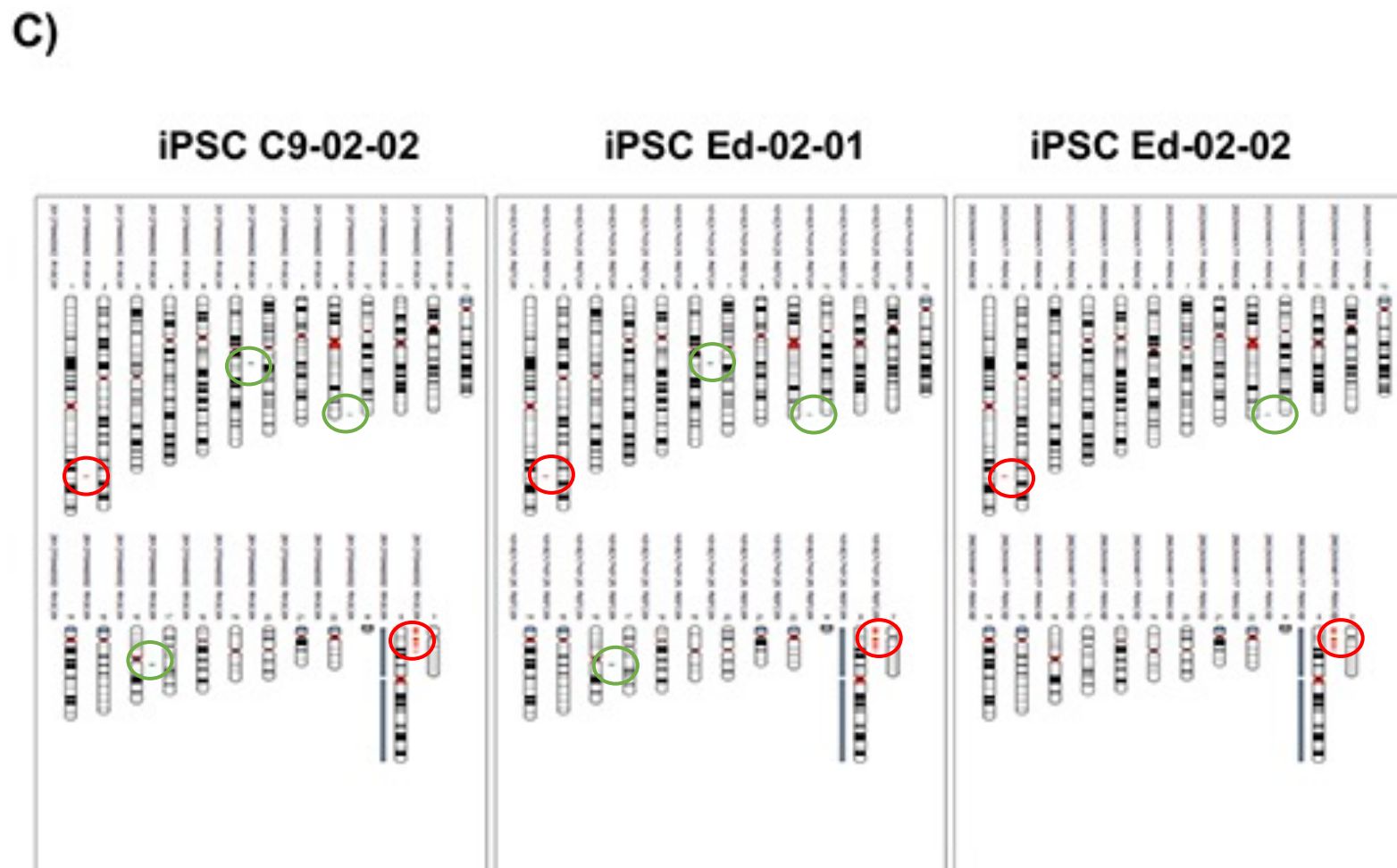
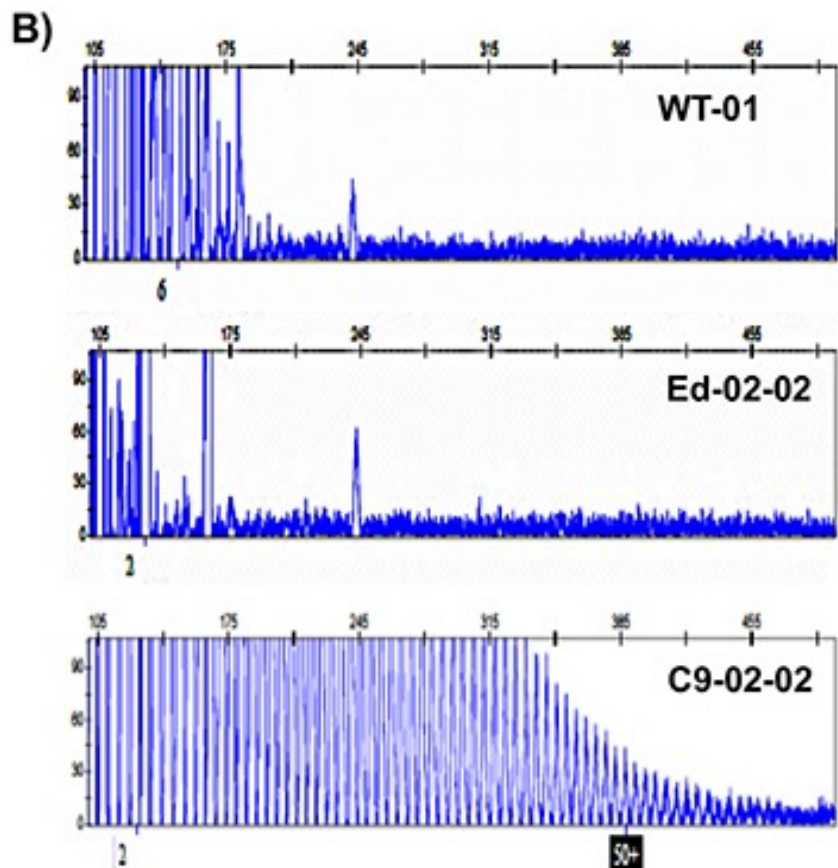
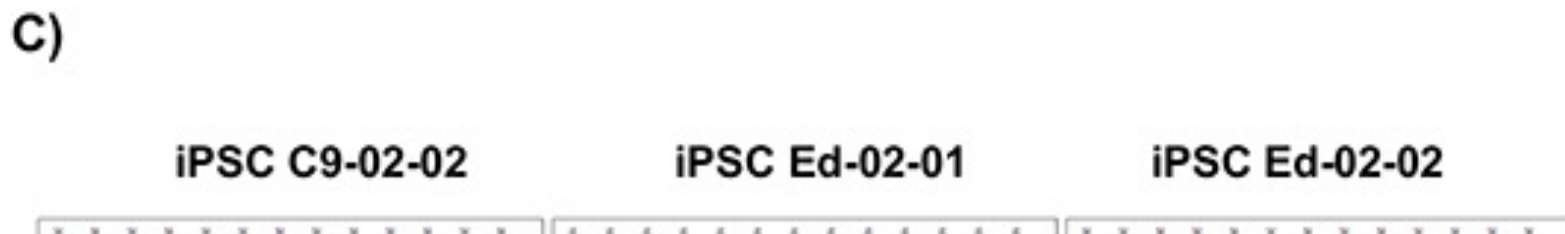


Figure 1



Conclusion:

Edited iPSC lines

- carried 2 (GGGGCC)_n repeats
- had no other significant changes to the genome (per SNP analysis)



Figure 1

A)

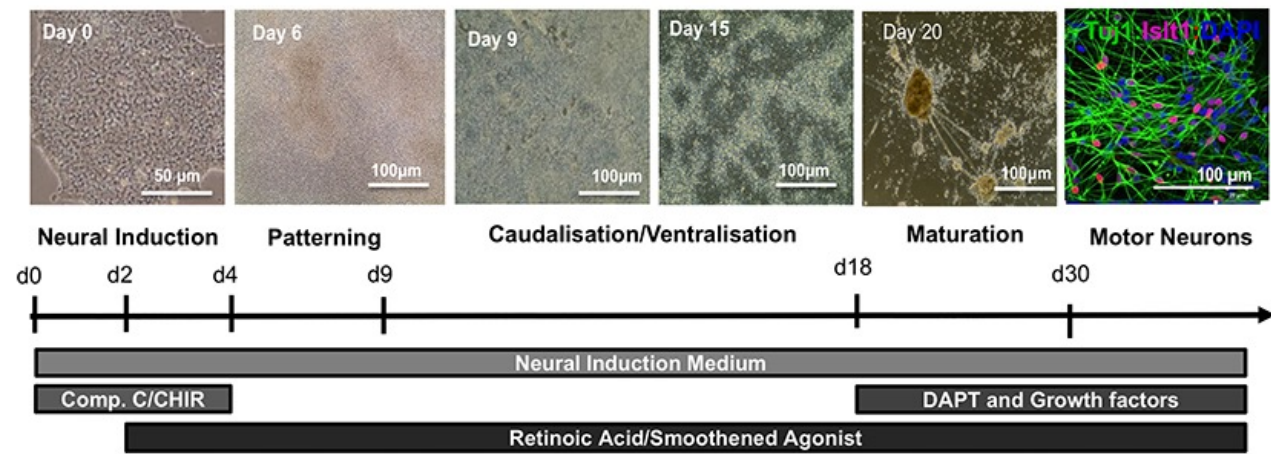


Figure 2

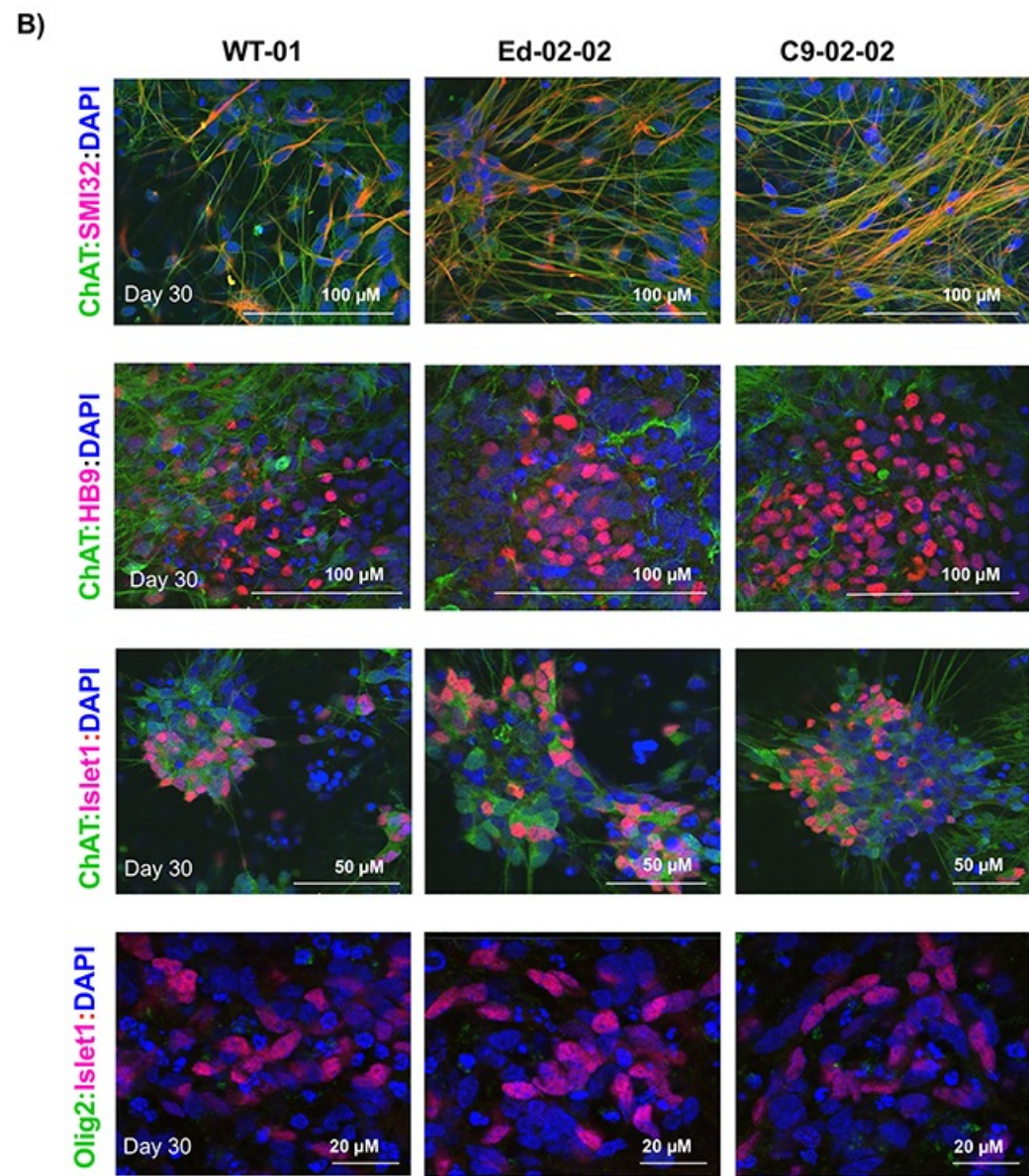
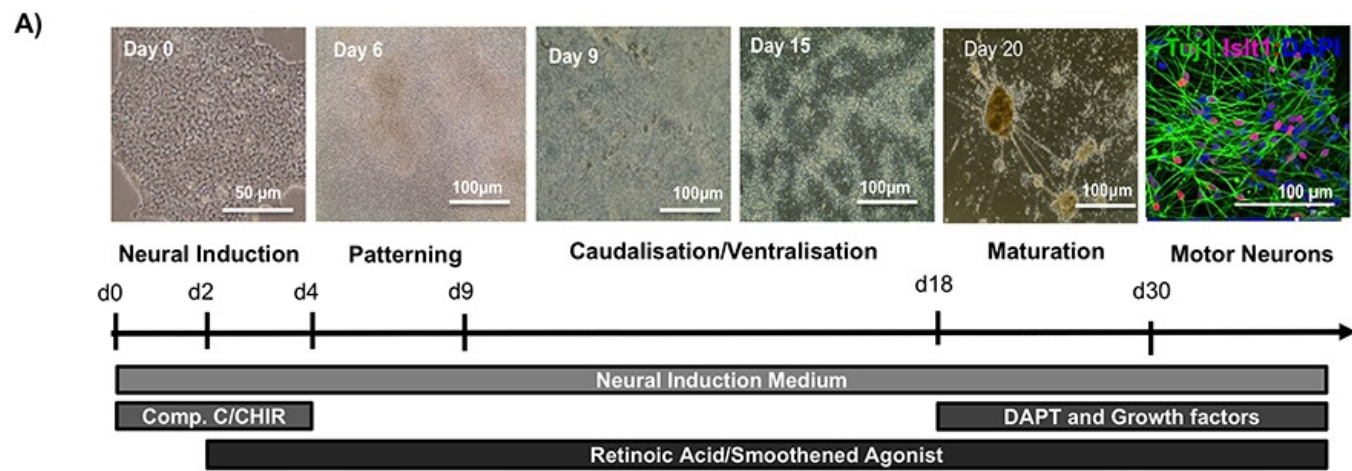


Figure 2

Modeling ALS using motor neurons derived from human induced pluripotent stem cells

S Sances^{1,12}, LI Bruijn^{2,11}, S Chandran^{3,11}, K Eggan^{4,11}, R Ho^{1,11}, J Klim^{4,11}, MR Livesey^{3,11}, E Lowry^{5,11}, JD Macklis^{6,7,8,9,11}, D Rushton^{1,11}, C Sadegh^{6,7,8,9,11}, D Sareen^{1,11}, H Wichterle^{5,11}, SC Zhang^{10,11}, and CN Svendsen¹

HB9	Early marker for MN differentiation. (Also known as MNX1.)
ISL1	Expressed by all classes of MNs early in differentiation.
OLIG2	Nuclear protein expressed in progenitor MNs and sometimes detectable in mature MNs.
SMI32	Marker of mature MNs – stains neuronal cell body via recognition of LMN-enriched neurofilament heavy chain (NFH).
ChAT	Useful for identifying any cholinergic neuron – best for mature MN identification.

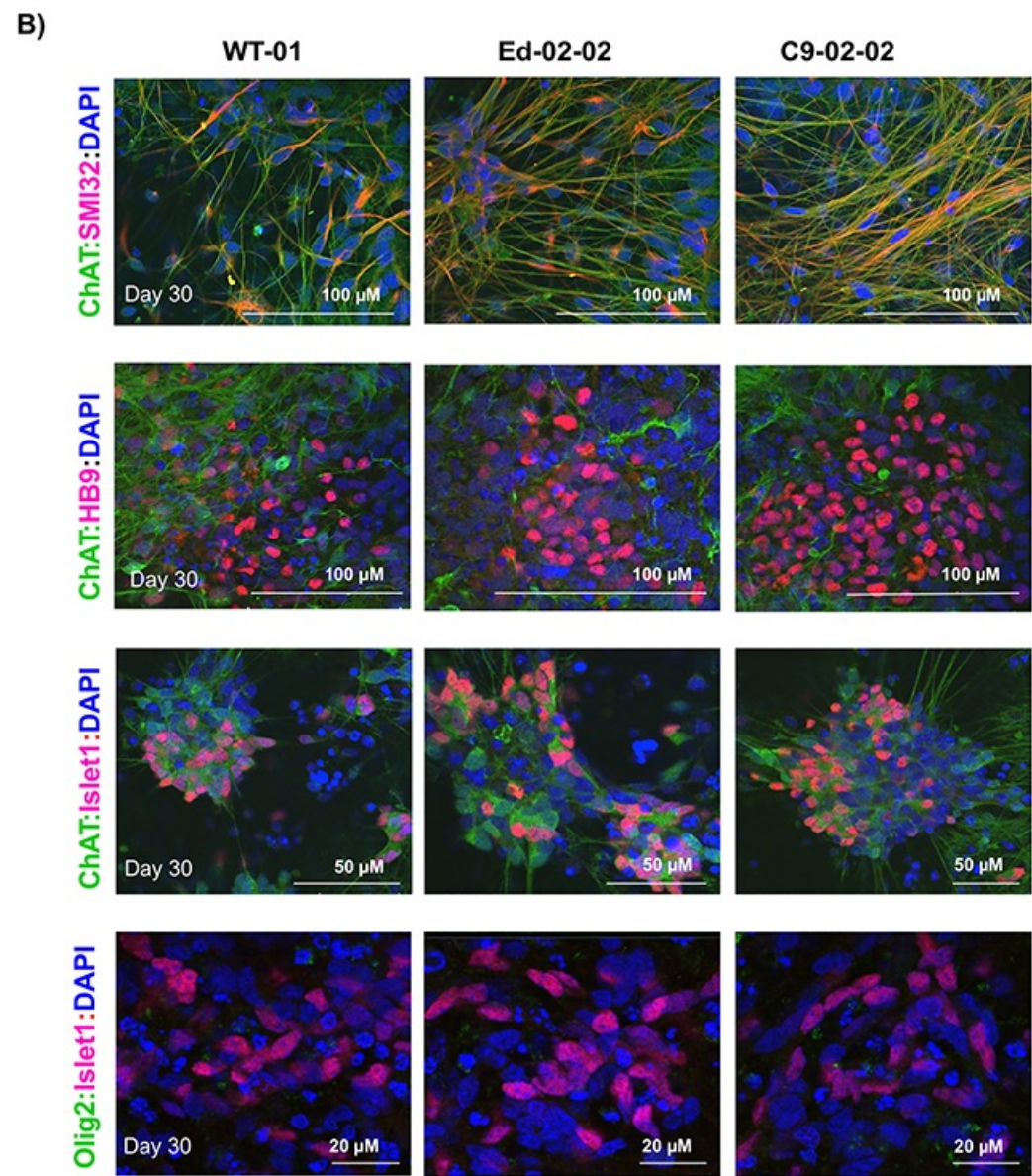
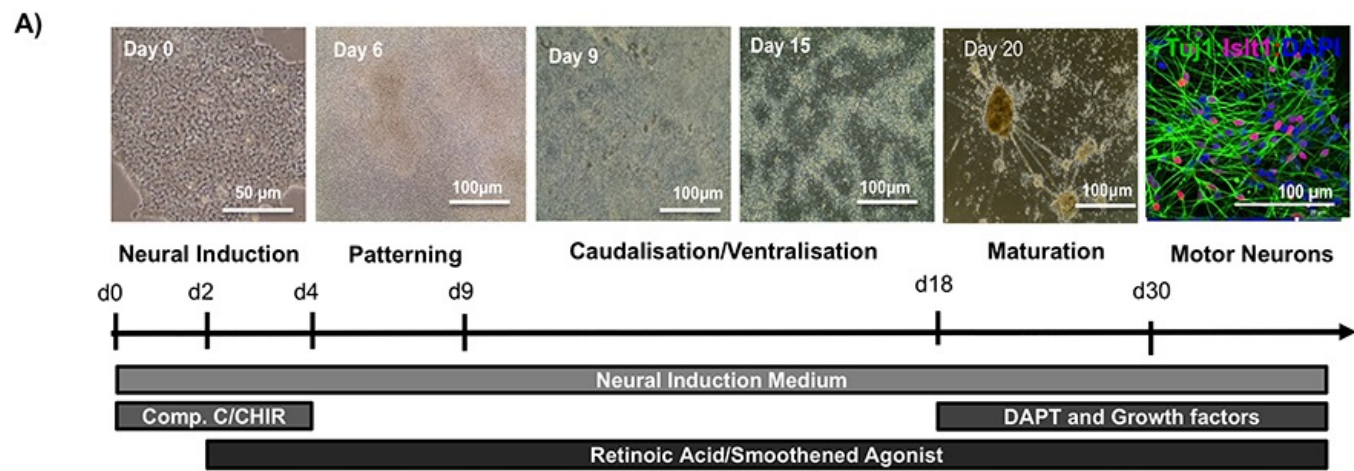


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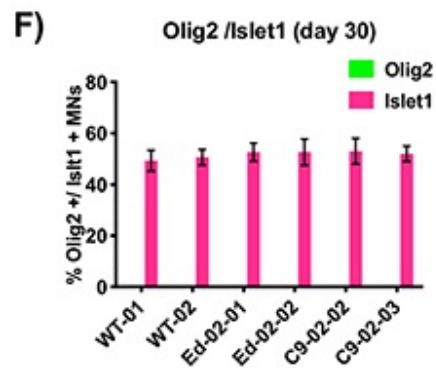
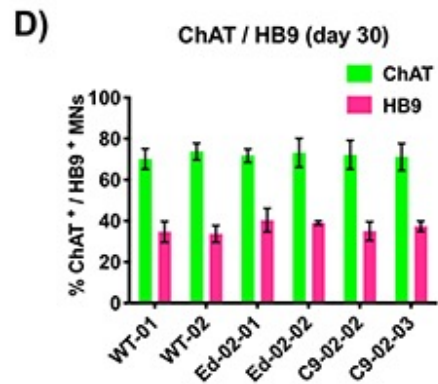
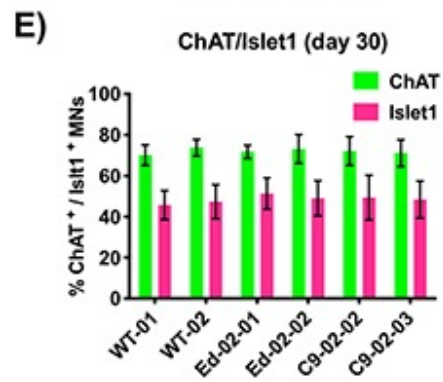
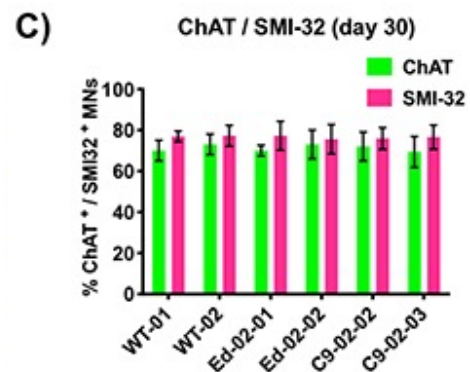
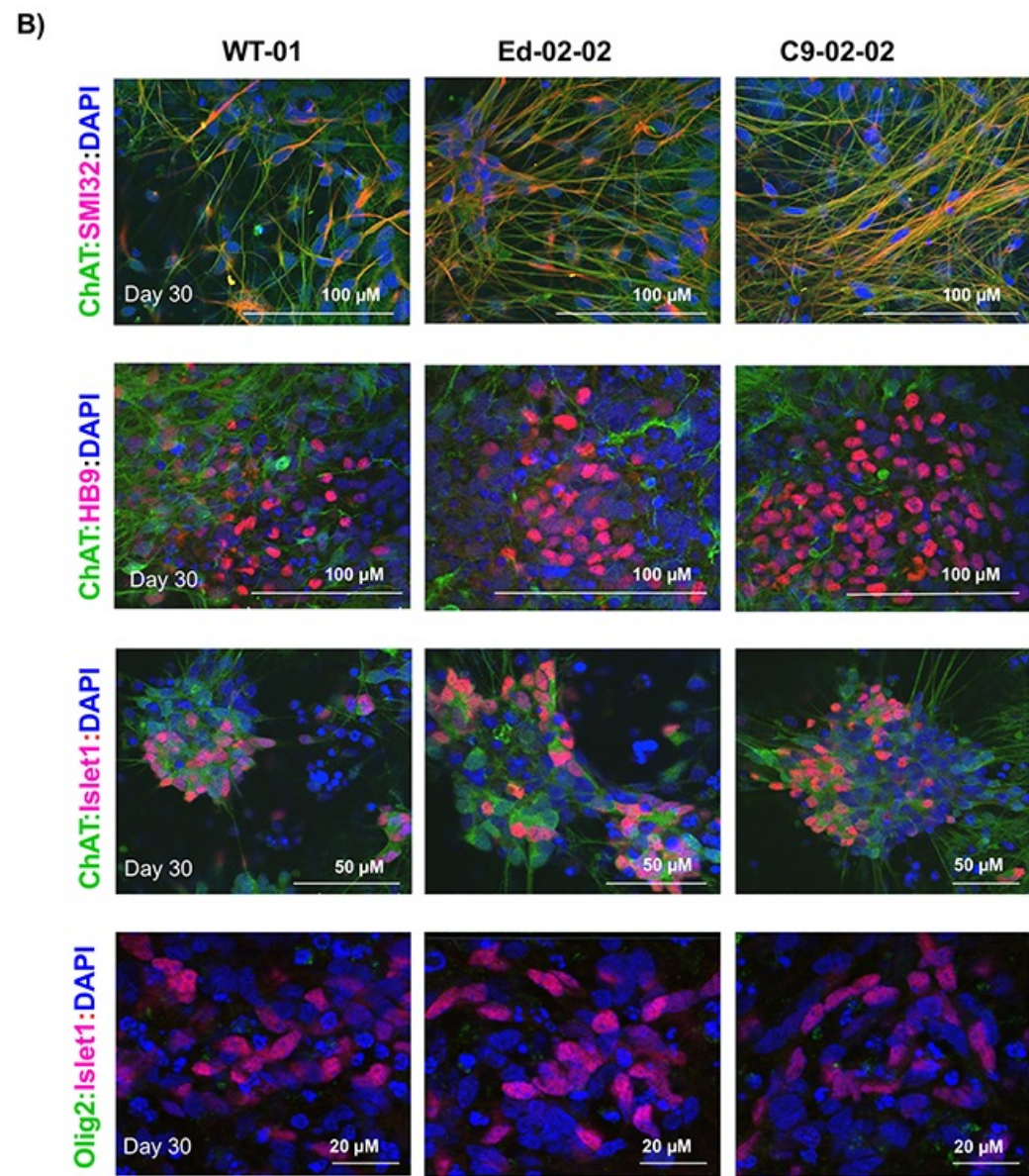
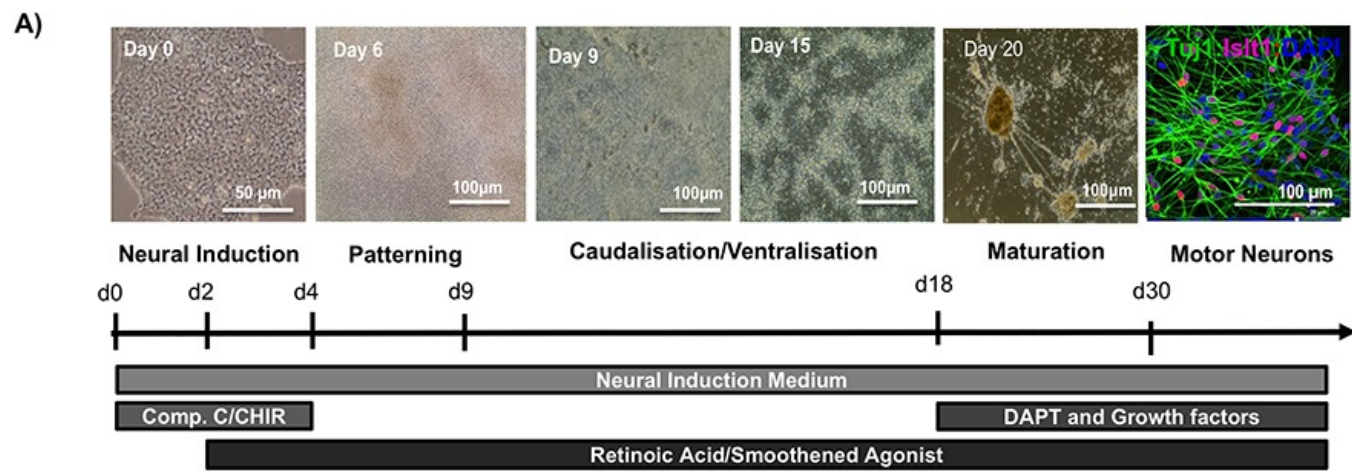
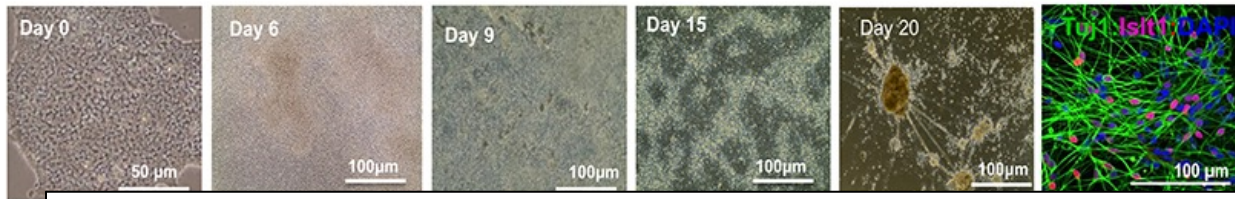
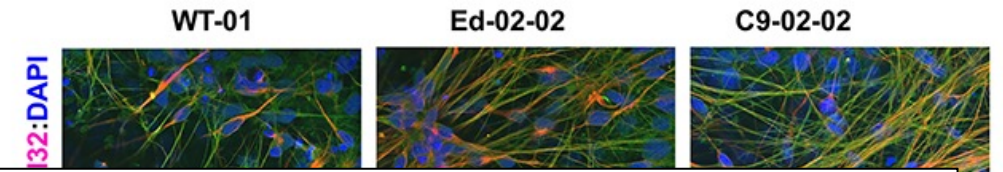


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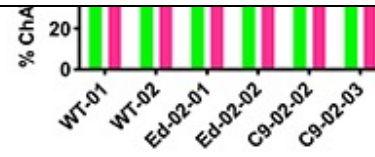
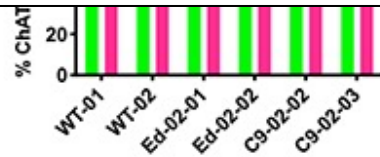
A)



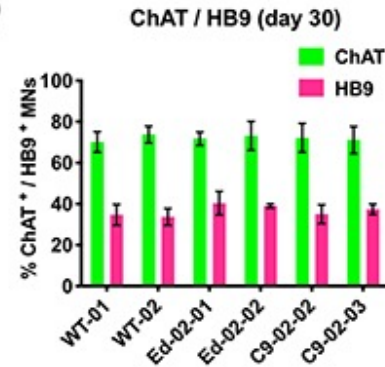
B)



Conclusion: Gene editing process had no effect on MN differentiation; similar morphology and differentiation efficiencies seen across all lines.



D)



F)

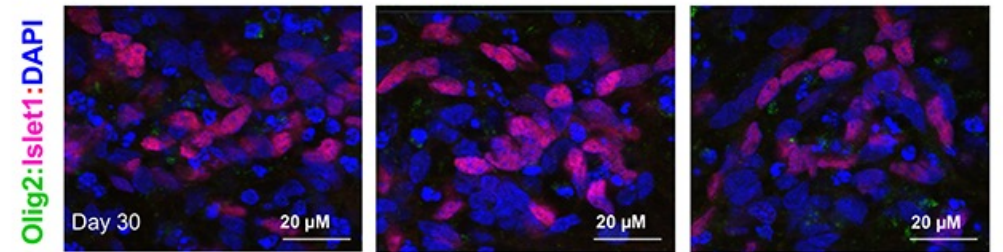
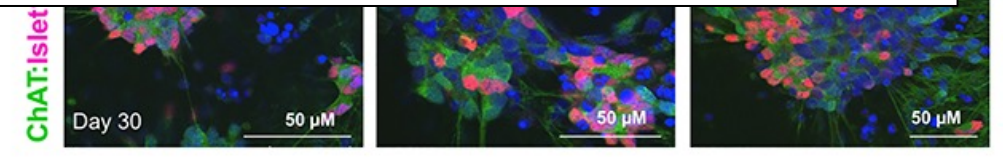
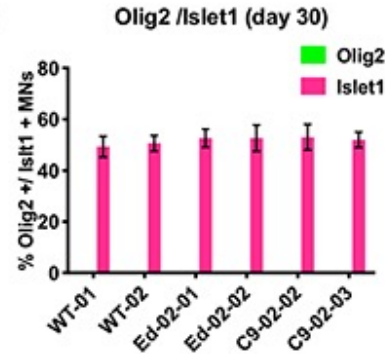


Figure 2

A

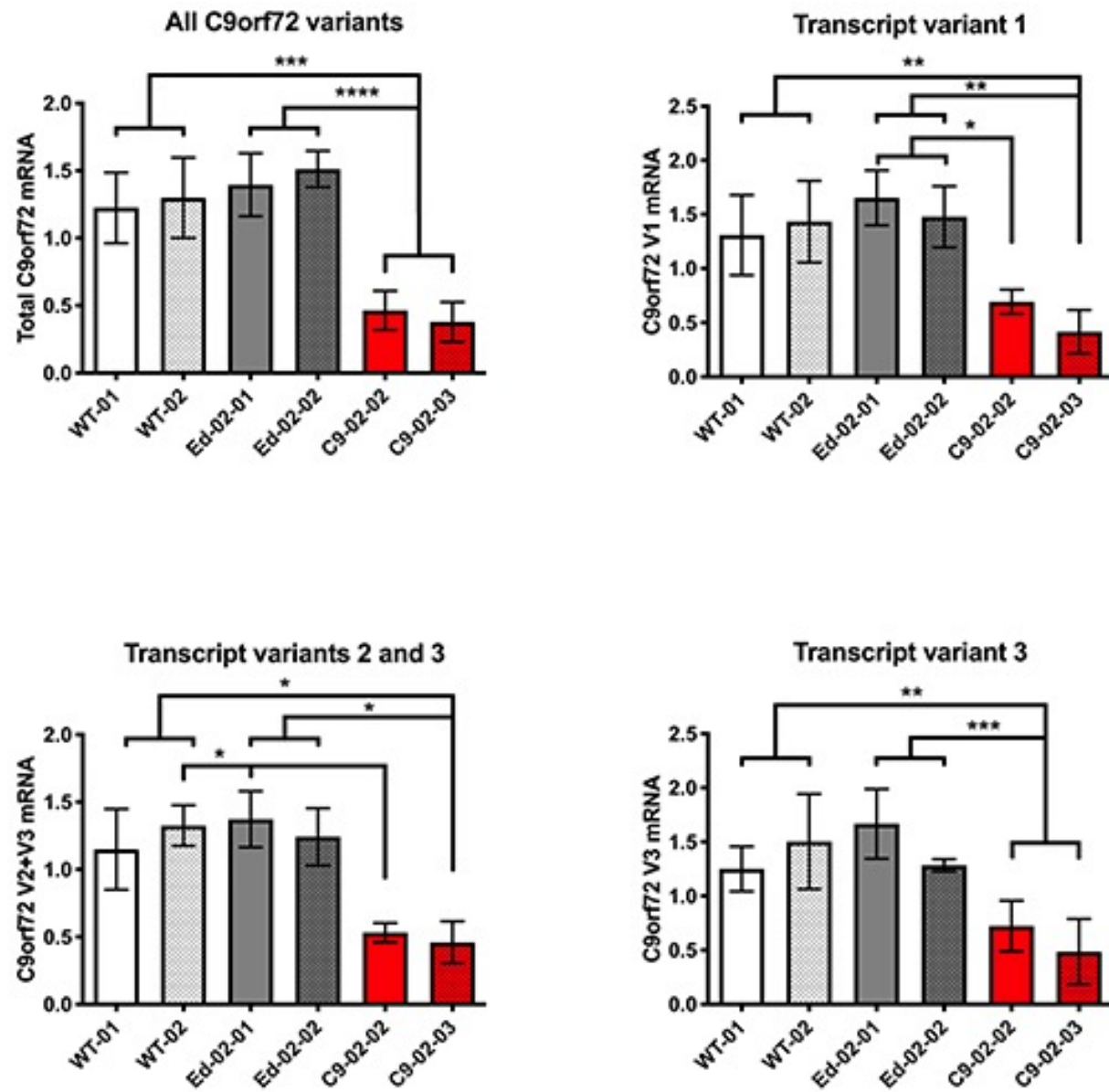


Figure 3

A

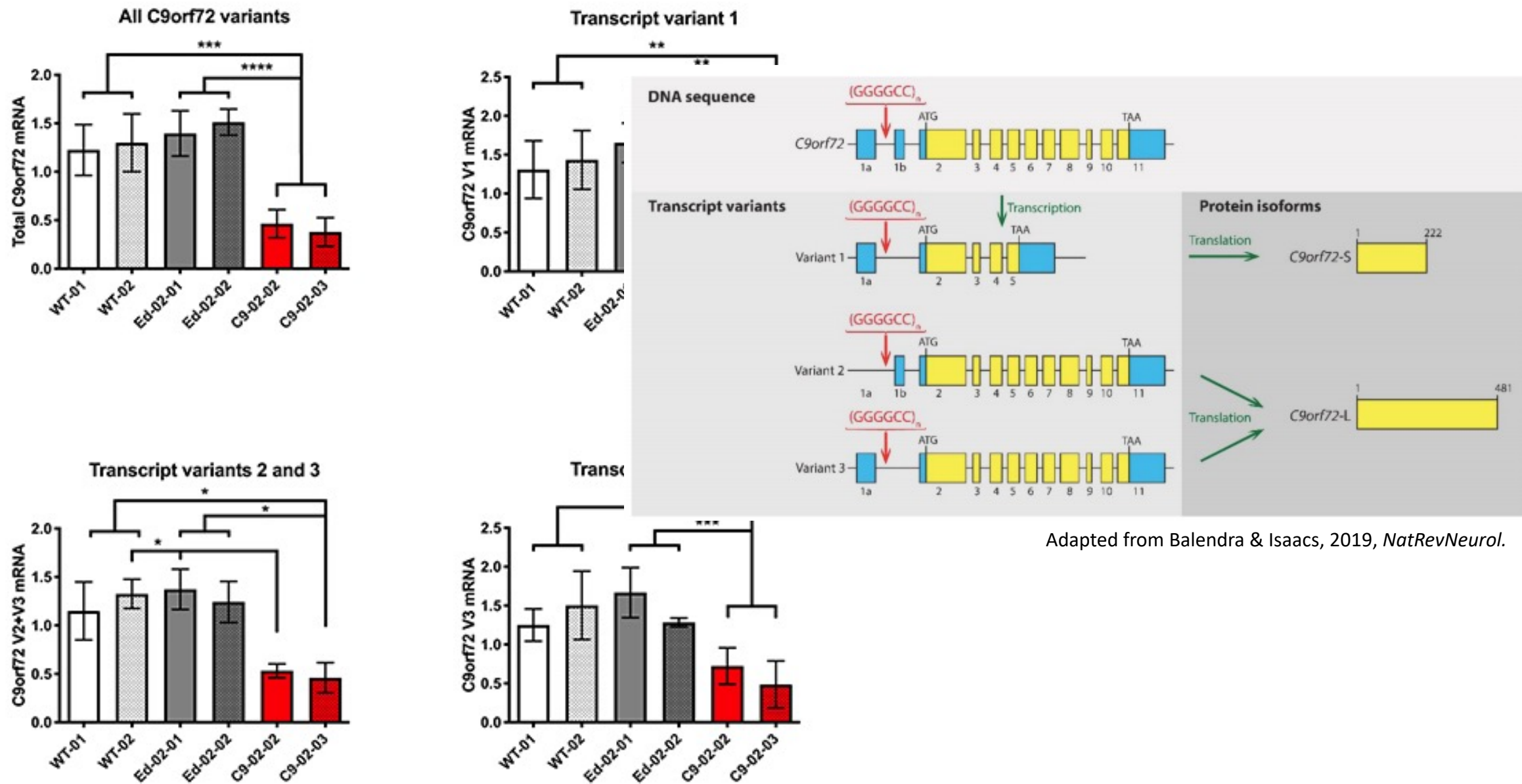
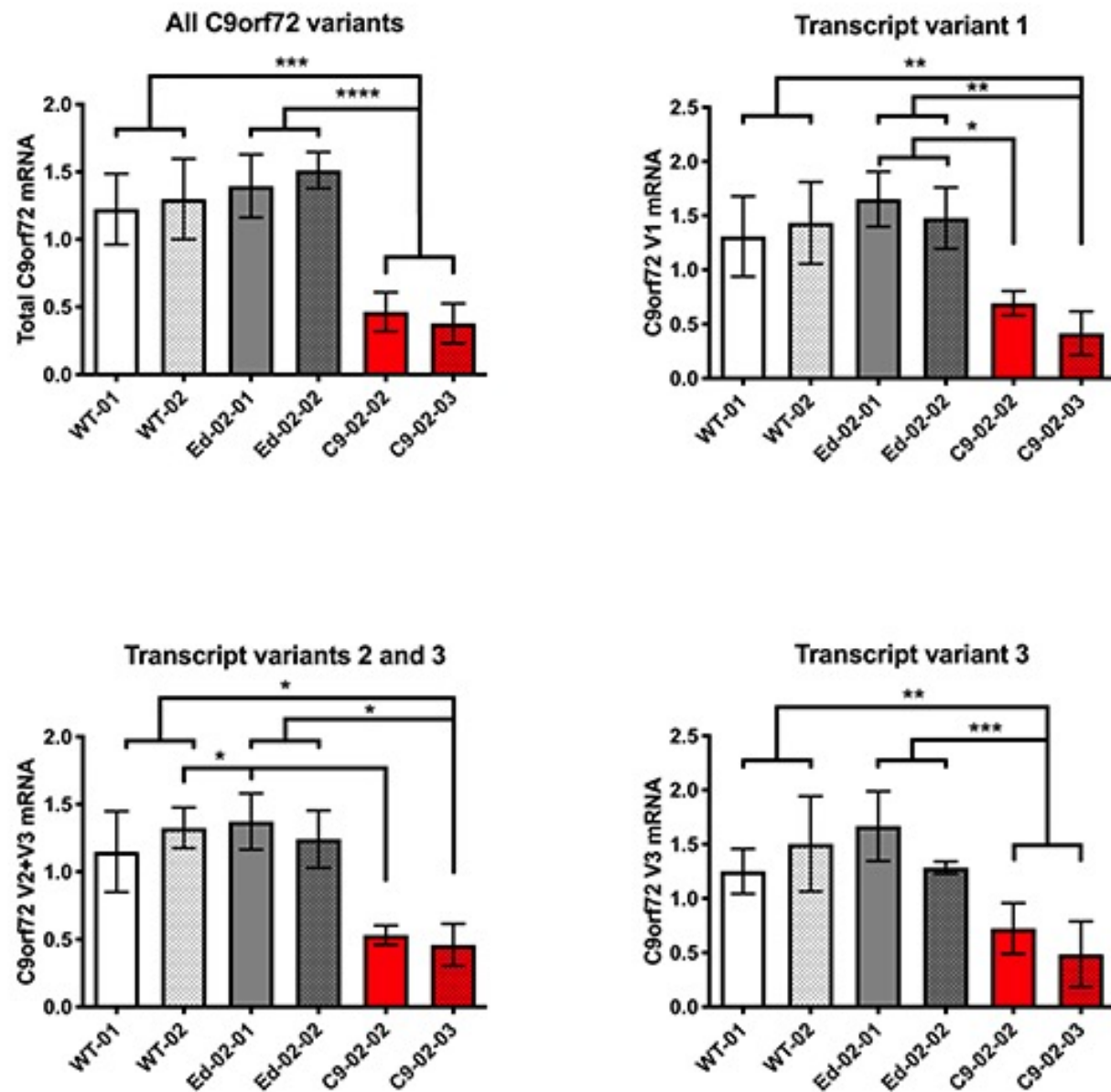


Figure 3

A



D

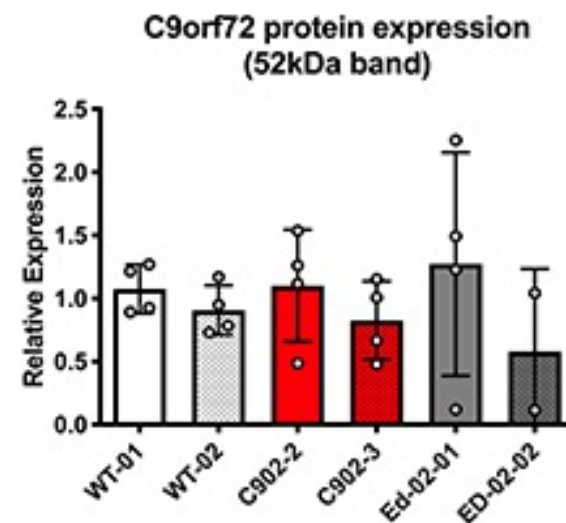
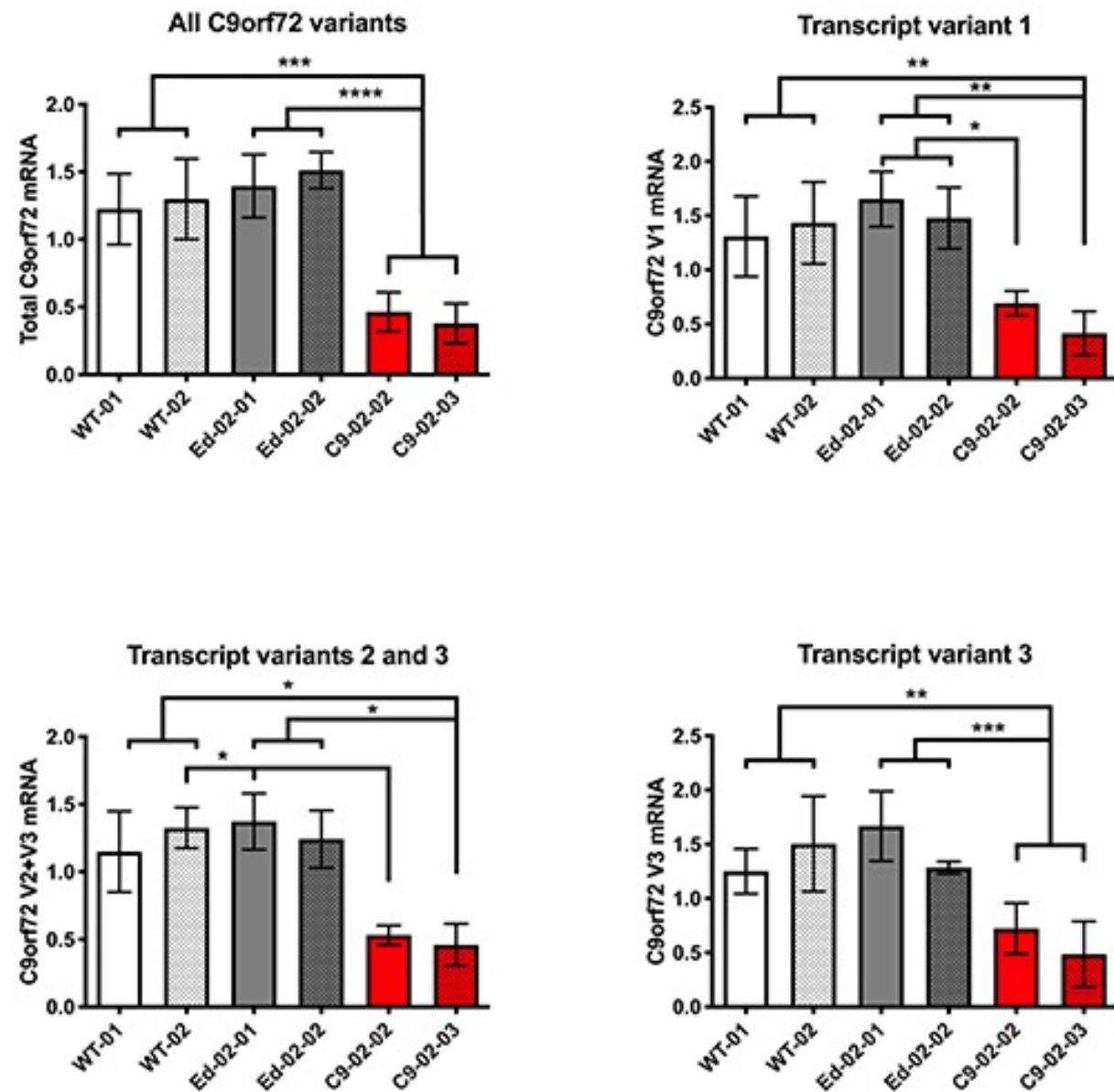
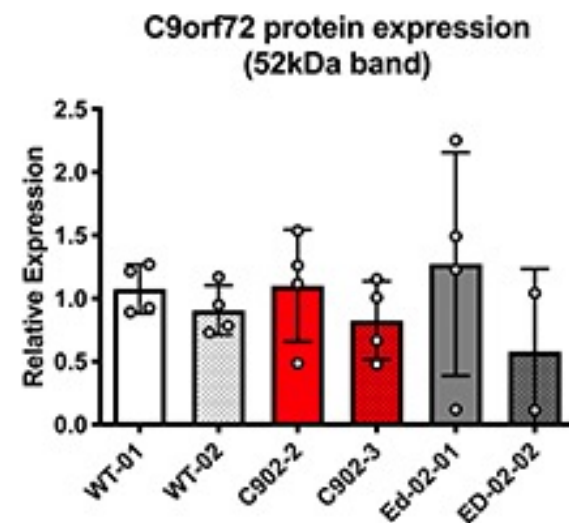
*C9orf72*-L protein

Figure 3

A



D

*C9orf72*-L protein

E

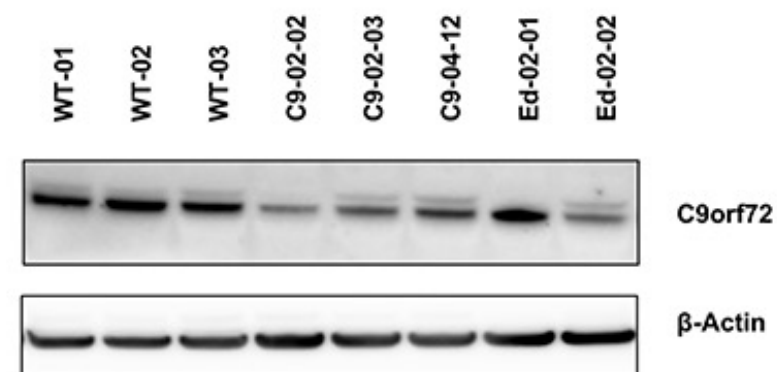
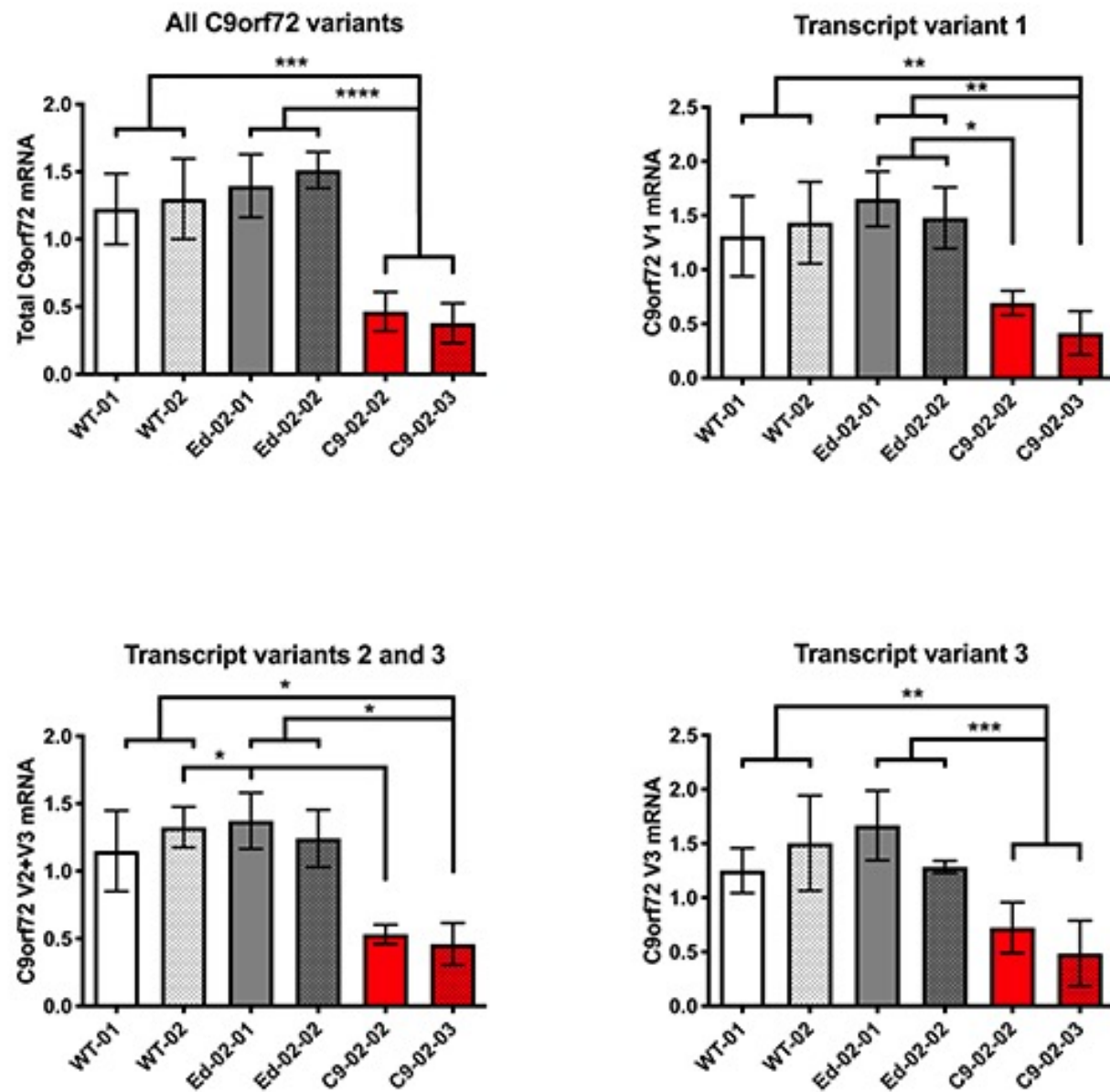
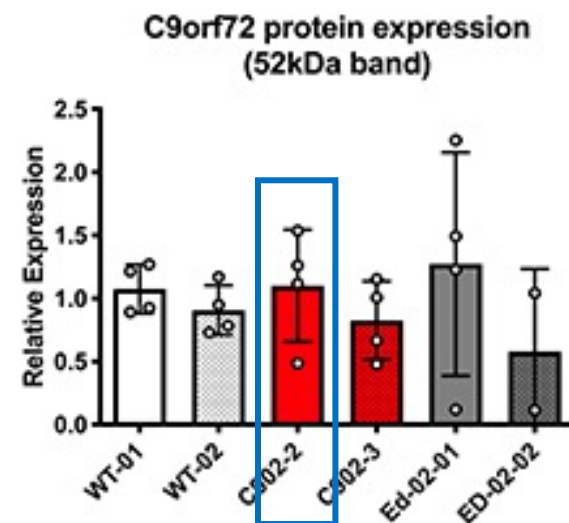


Figure 3

A



D

*C9orf72*-L protein

E

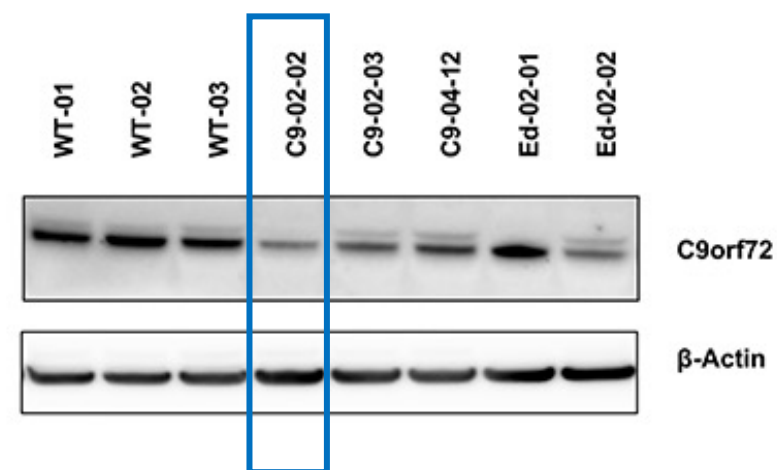
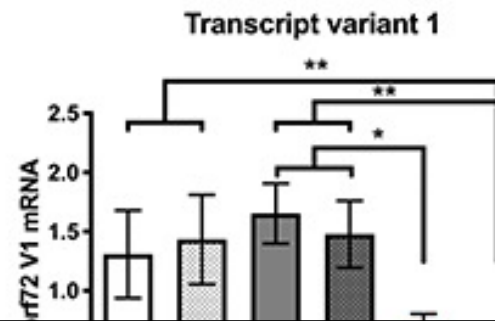
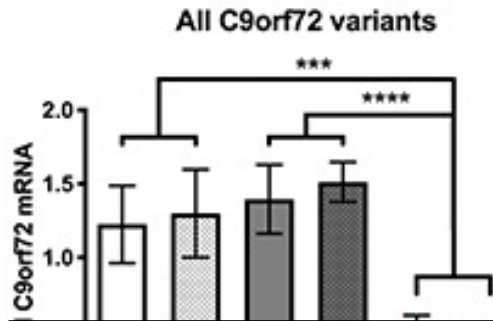


Figure 3

A



D



Conclusion:

Patient iPSCs have decreased levels of transcription of V1 and V2, while the edited and WT lines have similar levels of transcription.

Protein expression is reported similar across iPSCs regardless of expansion.

WT-01
WT-02
Ed-02-01
Ed-02-02
C9-02-02
C9-02-03

WT-01
WT-02
Ed-02-01
Ed-02-02
C9-02-02
C9-02-03

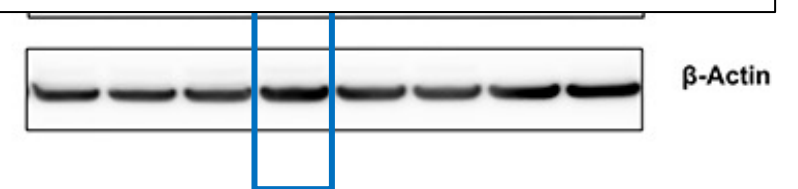


Figure 3

B

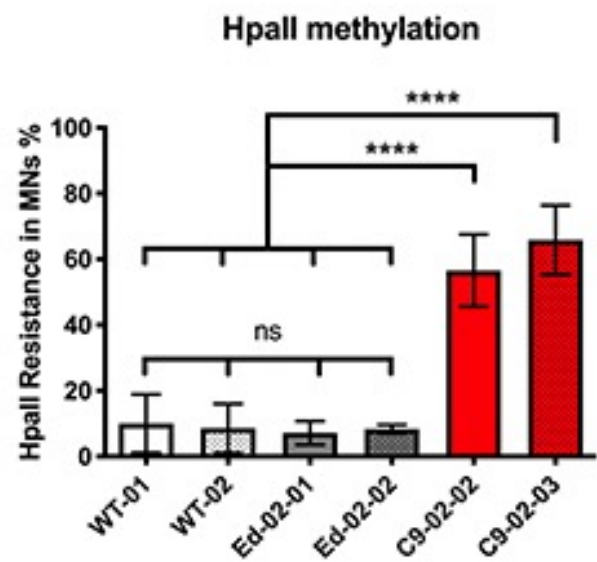
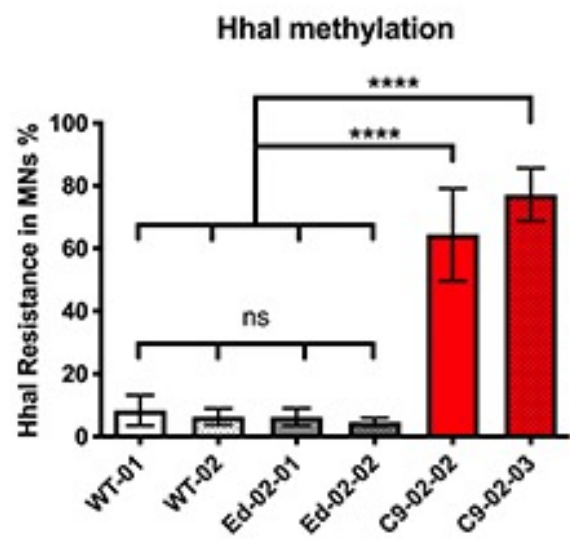
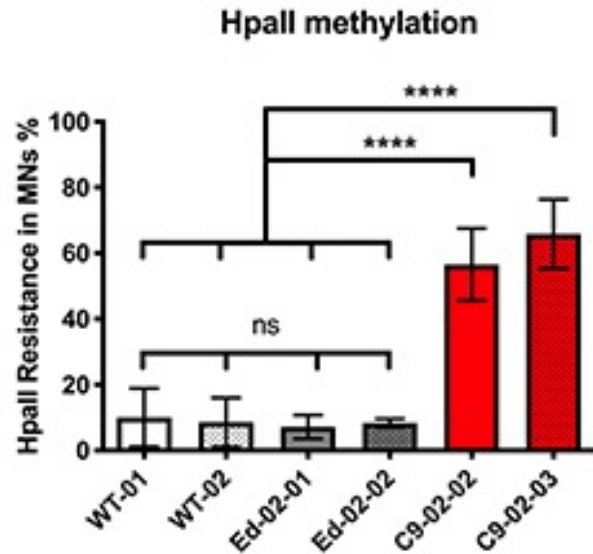
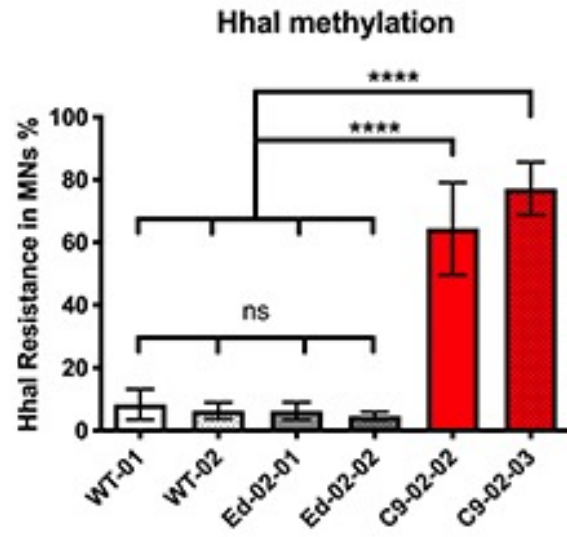
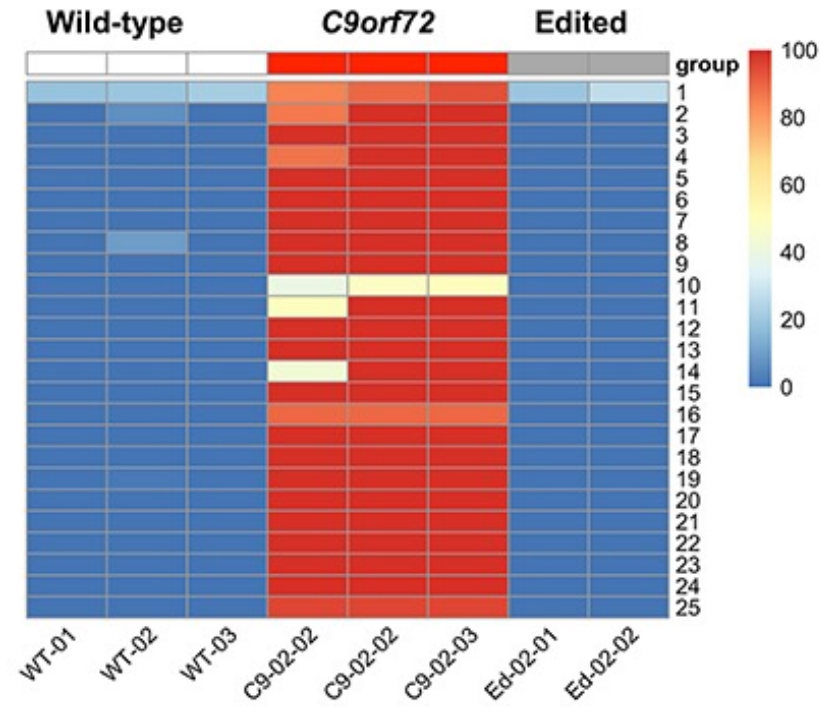


Figure 3

B



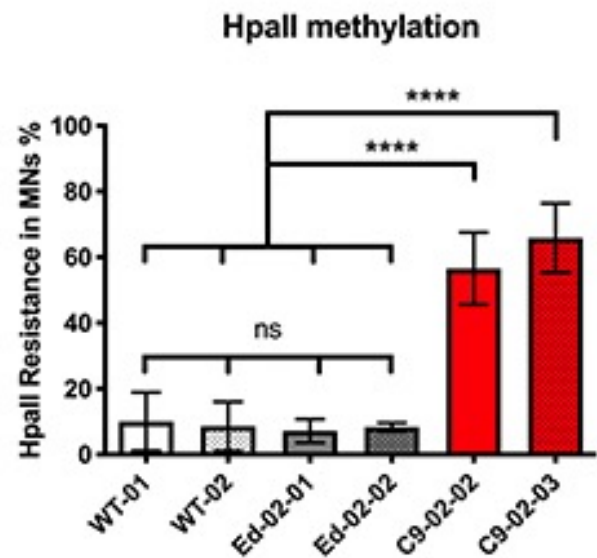
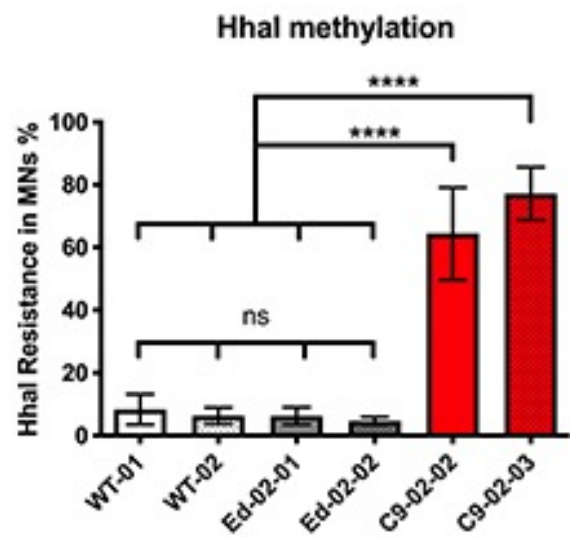
C



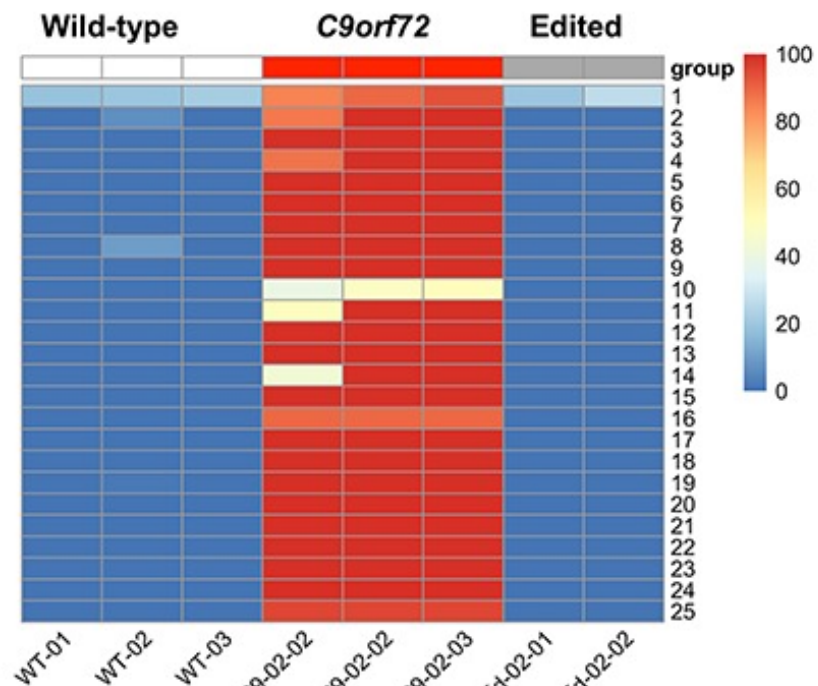
Bisulfite sequencing
at all 26 CpG sites in
the 5' region
upstream of the
(GGGGCC)_n repeat

Figure 3

B



C



Bisulfite sequencing
at all 26 CpG sites in
the 5' region
upstream of the
(GGGGCC)_n repeat

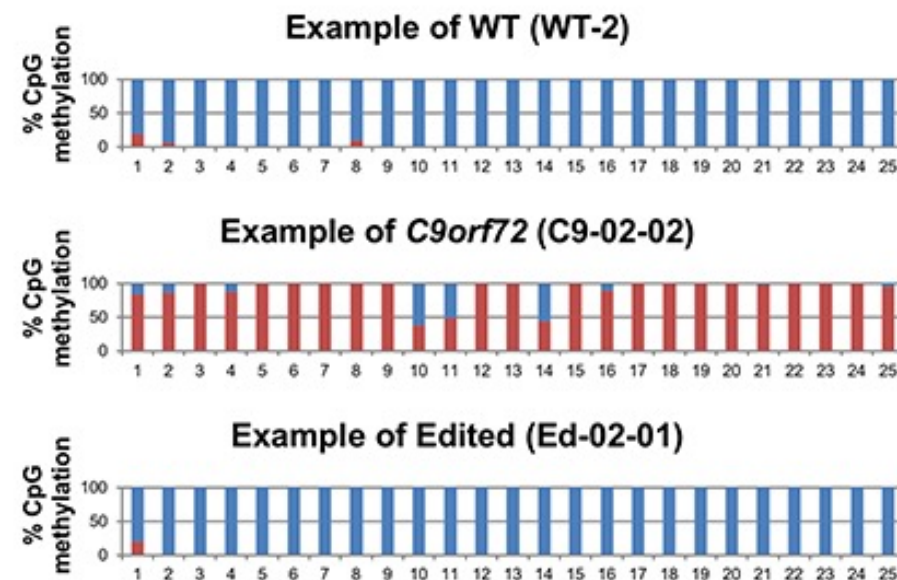
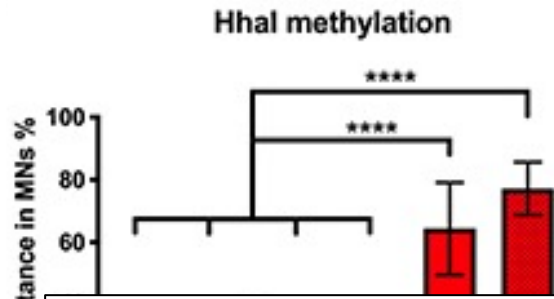
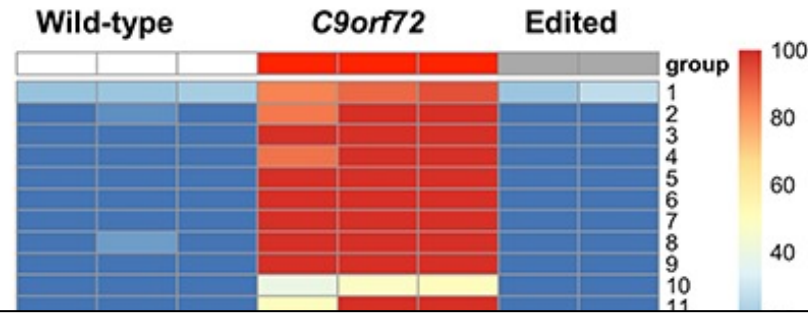


Figure 3

B



C



Bisulfite sequencing at all 25 CpG sites in

Conclusion:

CpG island 5' of repeat is highly methylated in the patient iPSMNs.

After editing, the methylation signal appears to be reversed/lost.

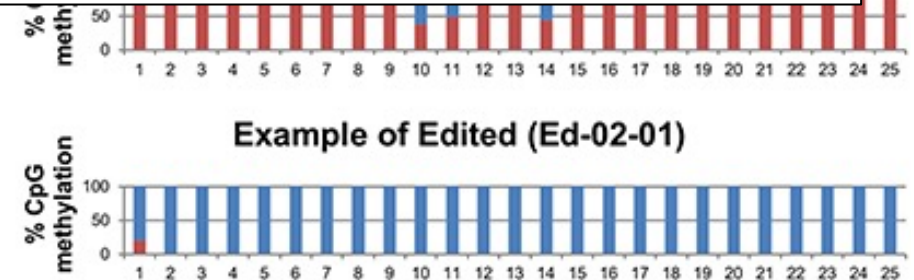
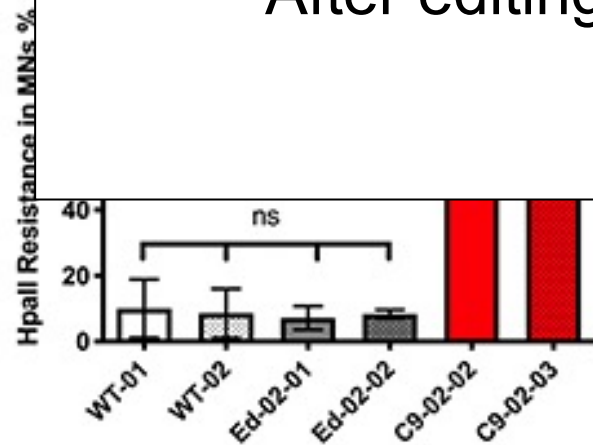


Figure 3

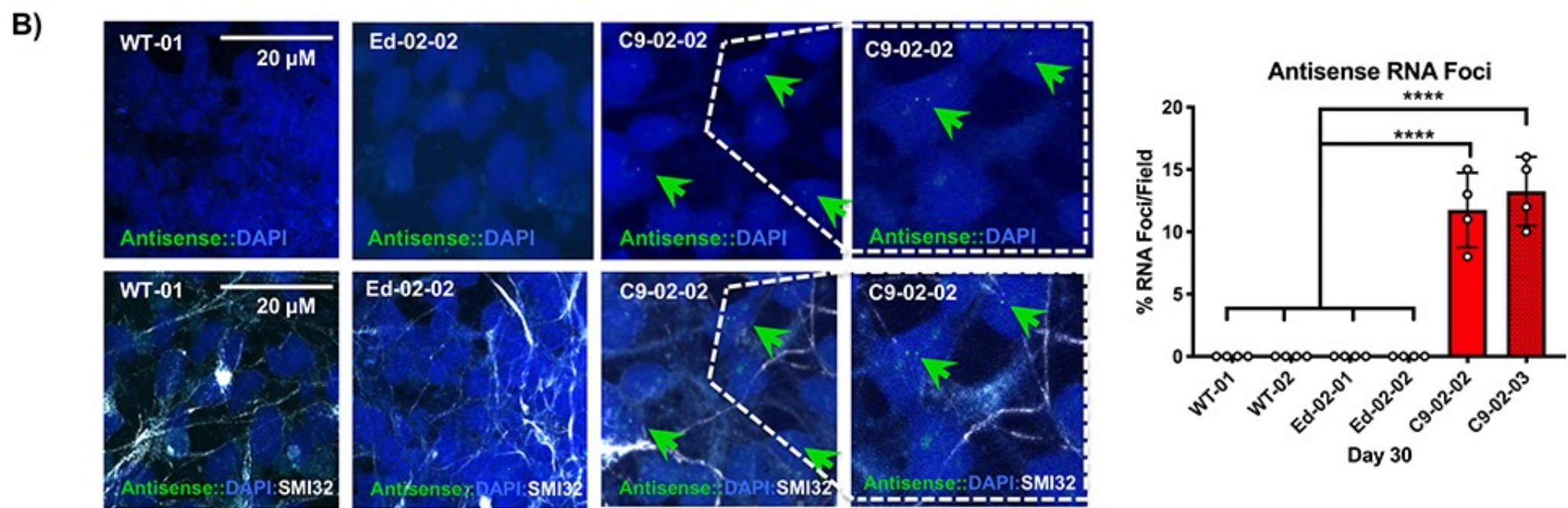
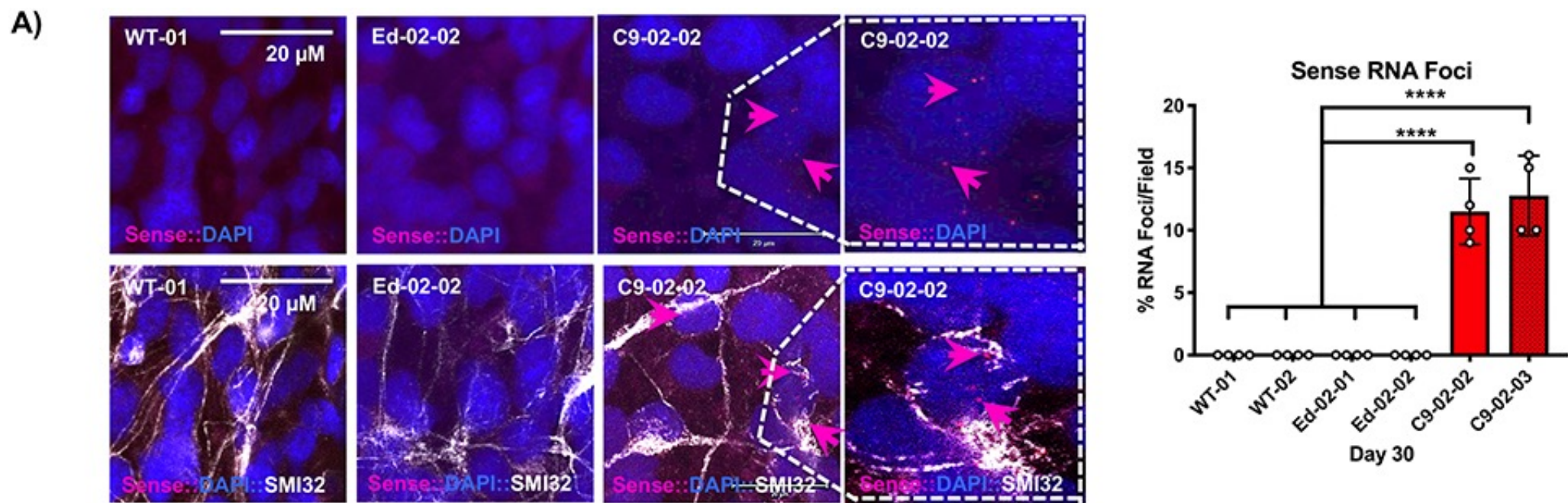
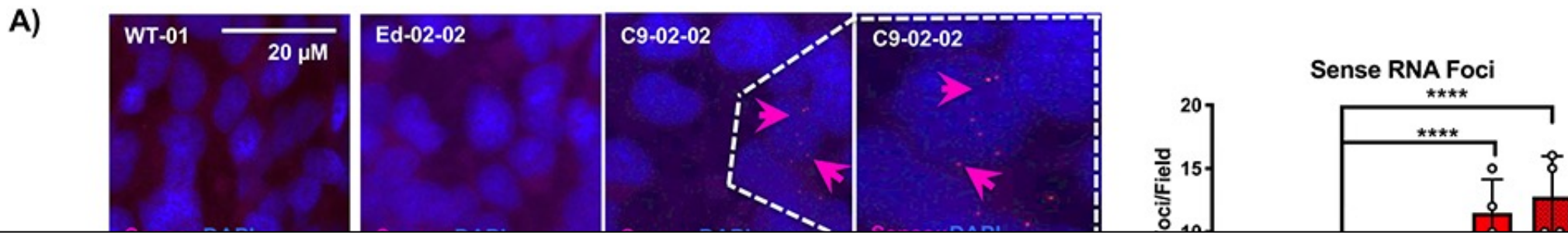


Figure 4



Conclusion:

Sense and antisense RNA foci are present at significantly higher levels in the patient iPSCMNs.

RNA foci are absent in the WT and edited iPSCMNs.

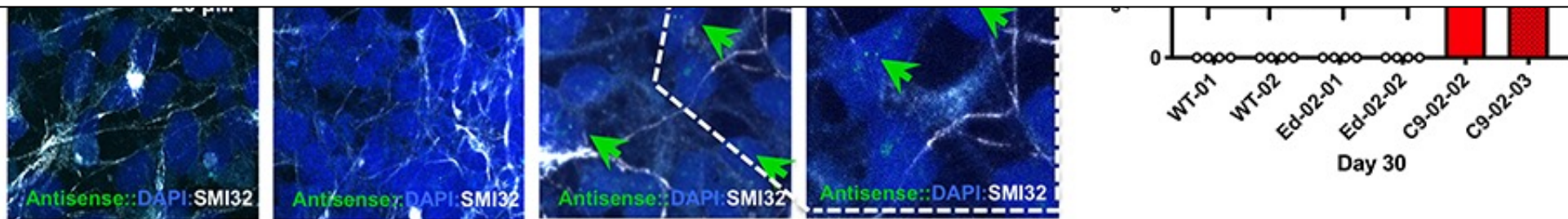


Figure 4

c)

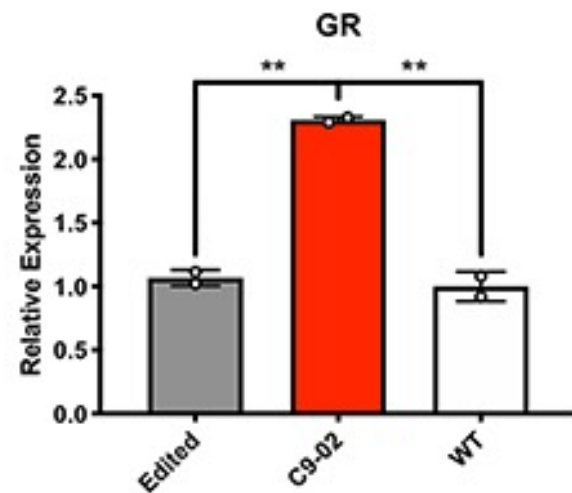
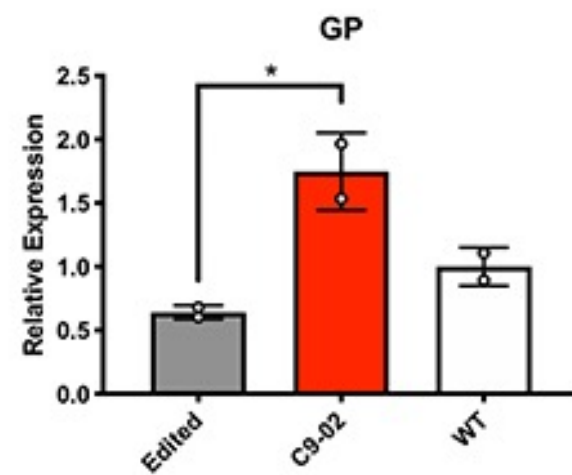
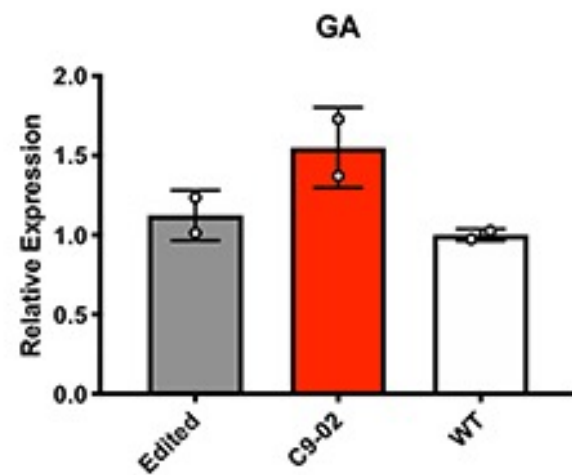
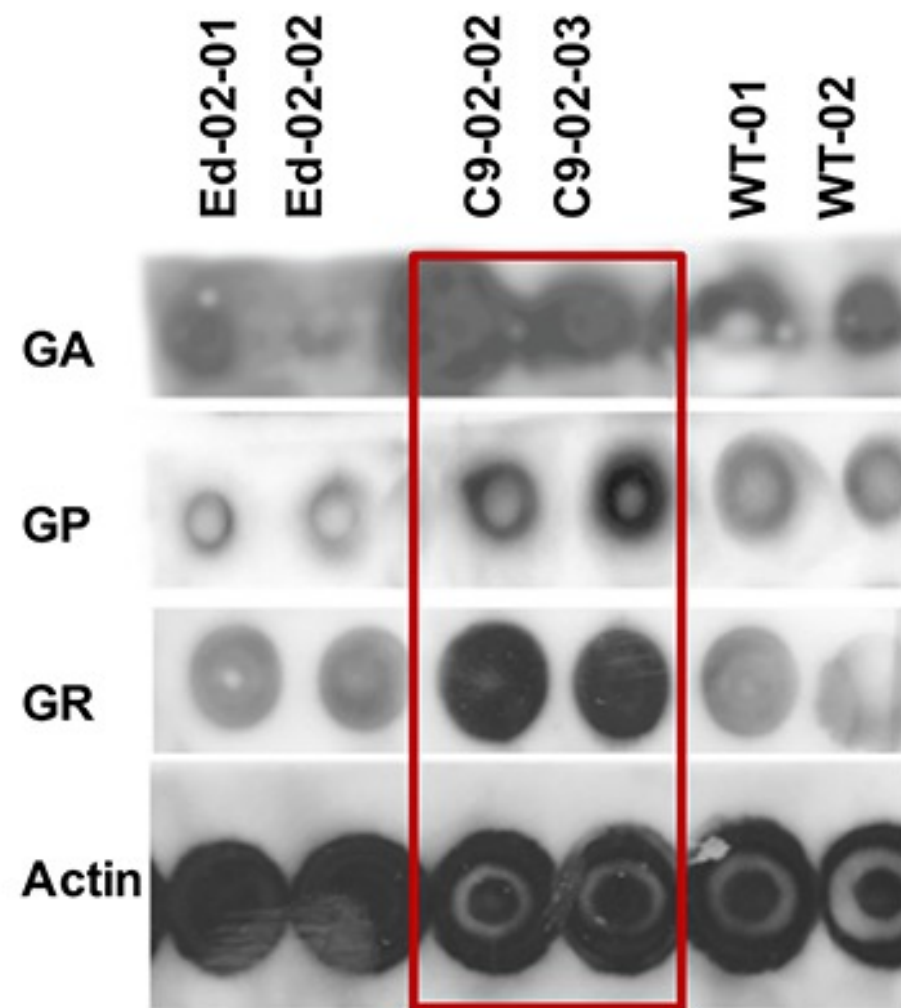


Figure 4

c)

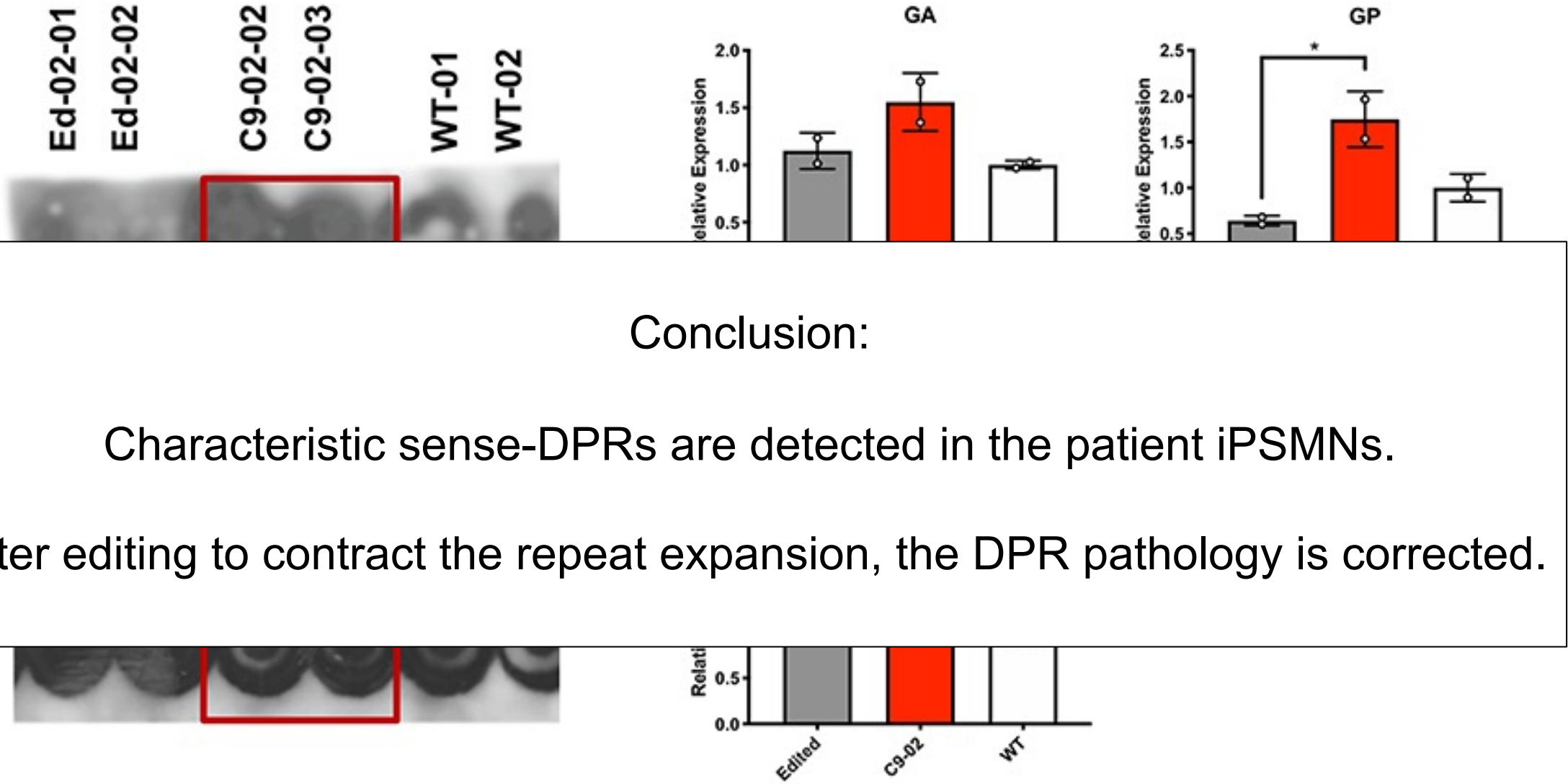
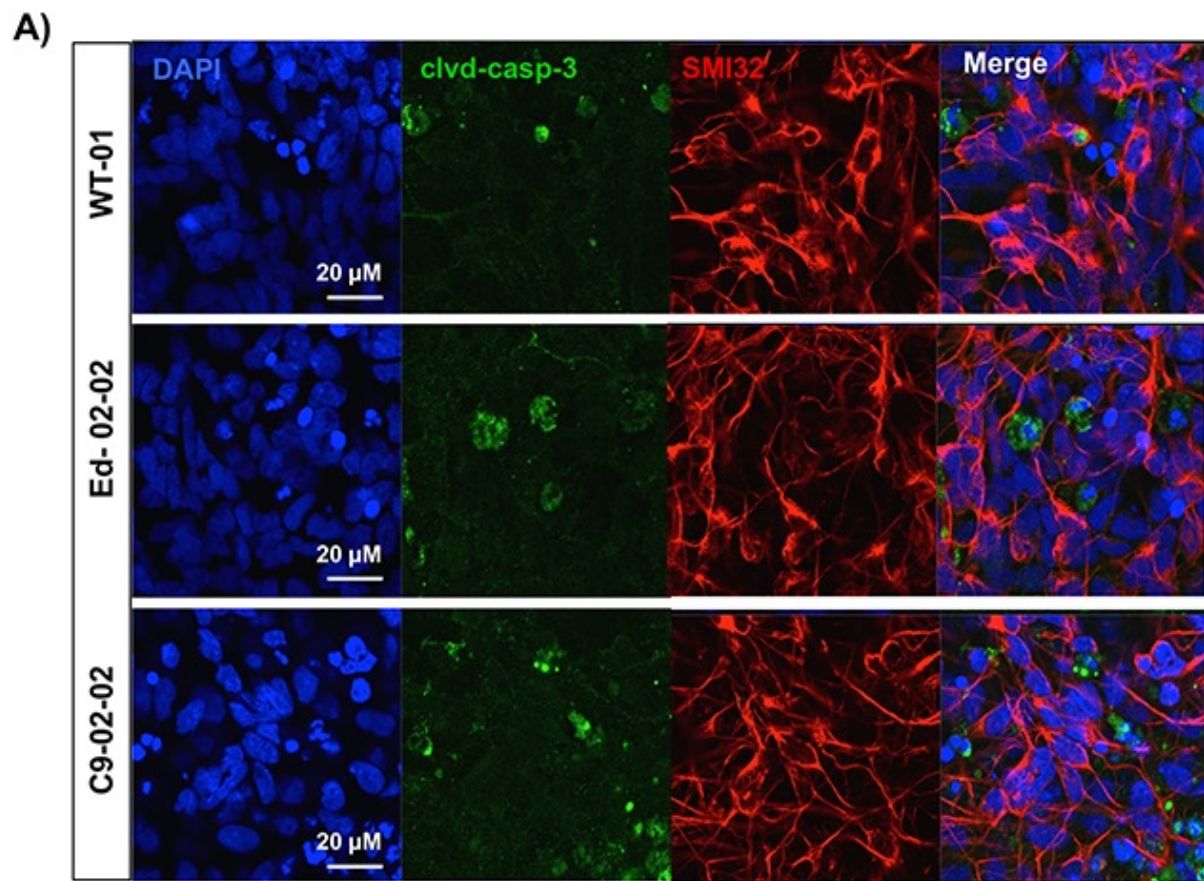


Figure 4



Cleaved caspase-3:

Implicated as an “effector” caspase associated with initiation of apoptotic signaling pathway.

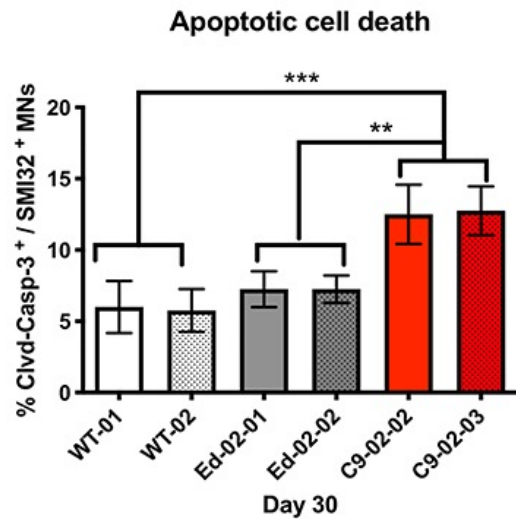


Figure 5

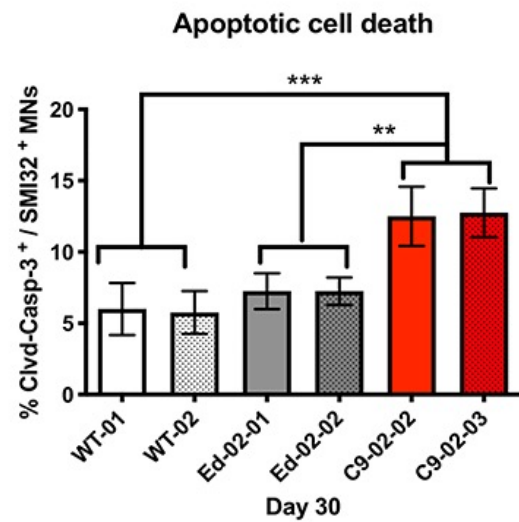
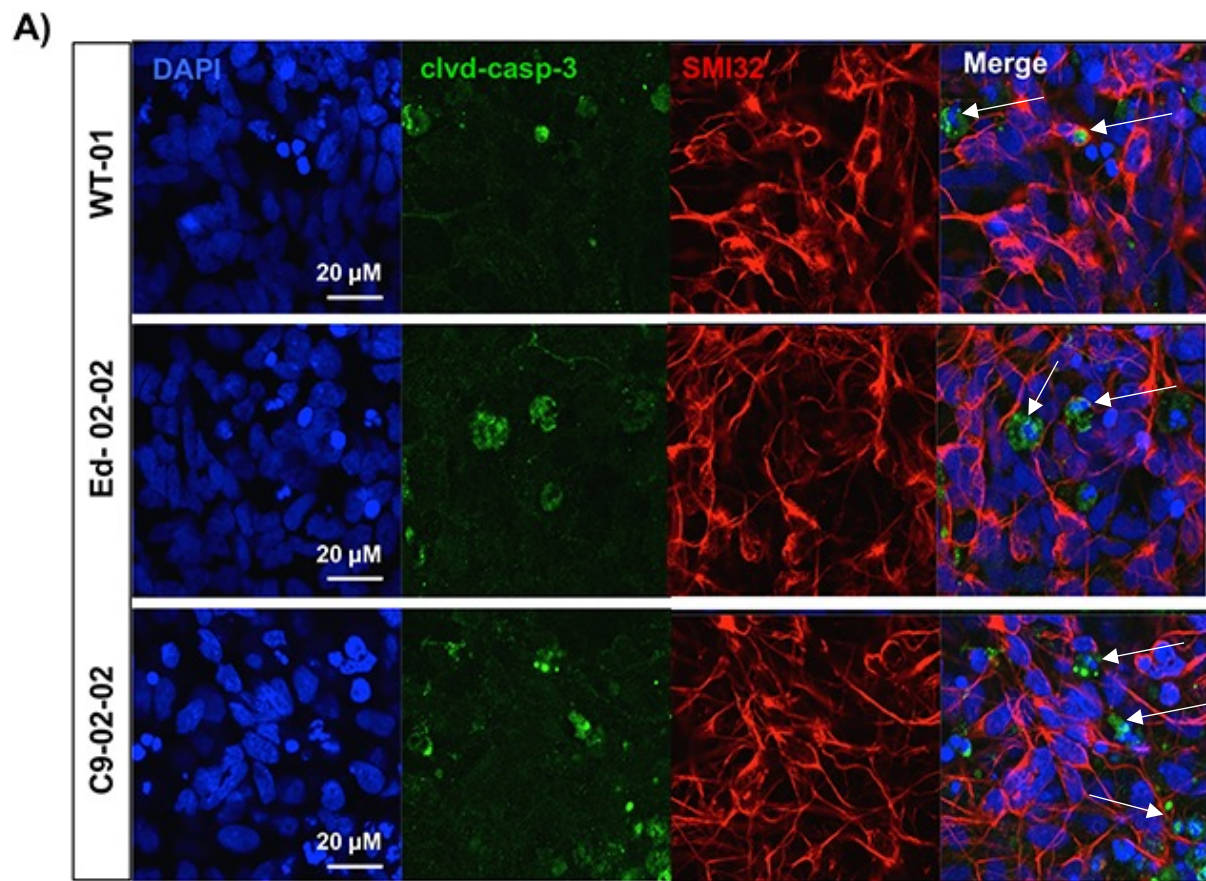


Figure 5

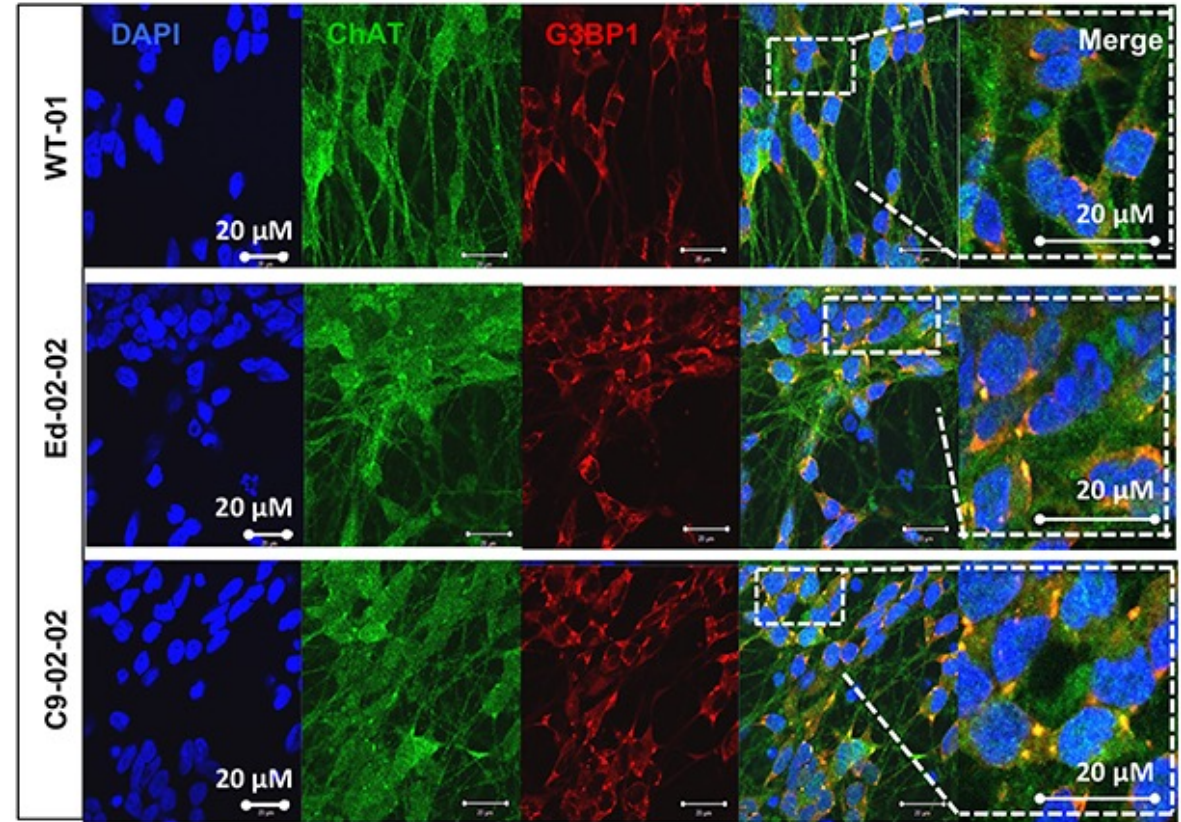
Ras GTPase-activating protein-binding protein 1 (G3BP1):

Has DNA & RNA/RNA, RNA/DNA unwinding capabilities.

→ Can initiate stress granule formation.

→ Labelled G3BP1 is a marker for stress granules.

B)



Stress Granules - G3BP1
(0.5mM Sodium Arsenite)

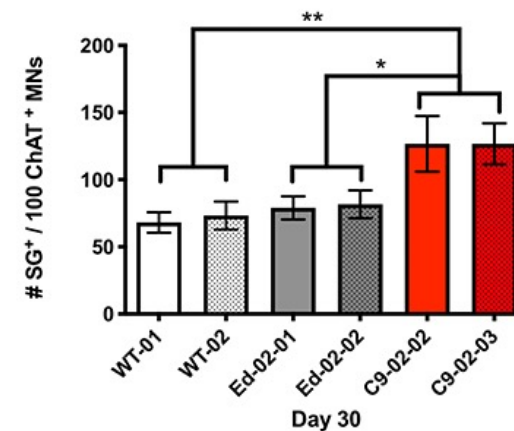


Figure 5

Ras GTPase-activating protein-binding protein 1 (G3BP1):

Has DNA & RNA/RNA, RNA/DNA unwinding capabilities.

→ Can initiate stress granule formation.

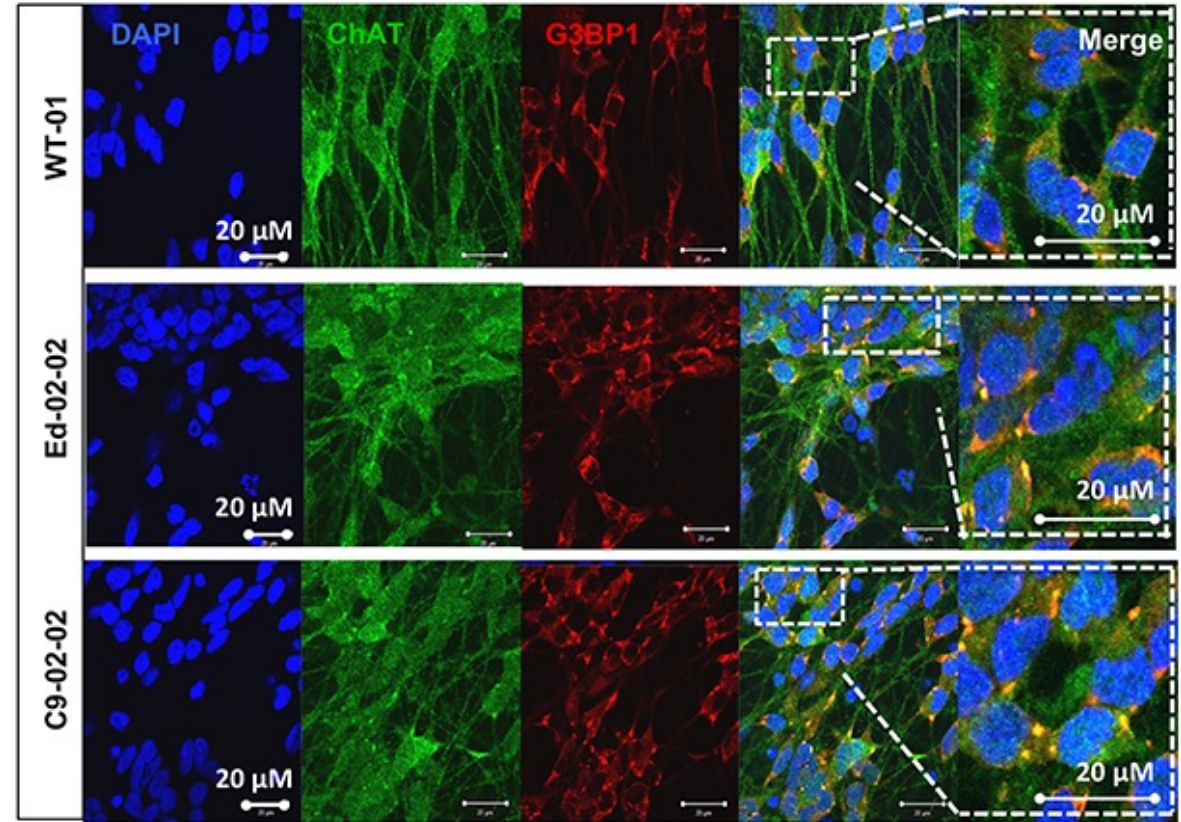
→ Labelled G3BP1 is a marker for stress granules.

Poly(A)-binding protein (dPABP):

→ Detects RNA granules, which are linked to neurodegenerative diseases

(RNA granules & RNA-binding proteins regulate RNA biogenesis; there is a relationship between stress granules and neuronal RNA granules.)

B)



Stress Granules - G3BP1
(0.5mM Sodium Arsenite)

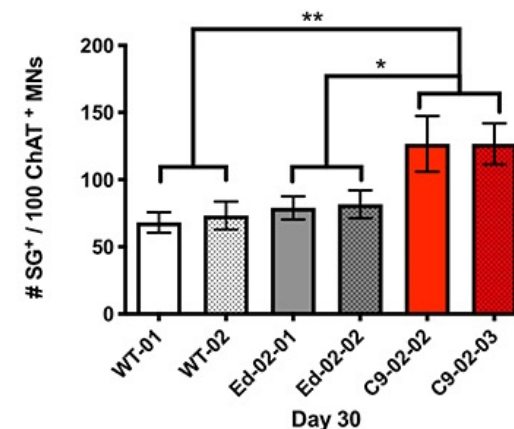
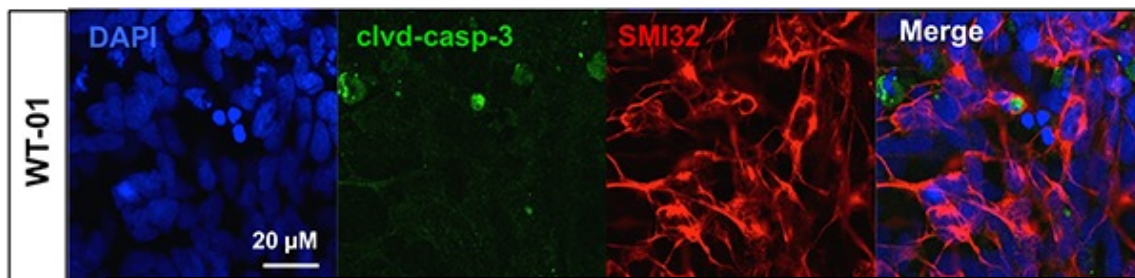
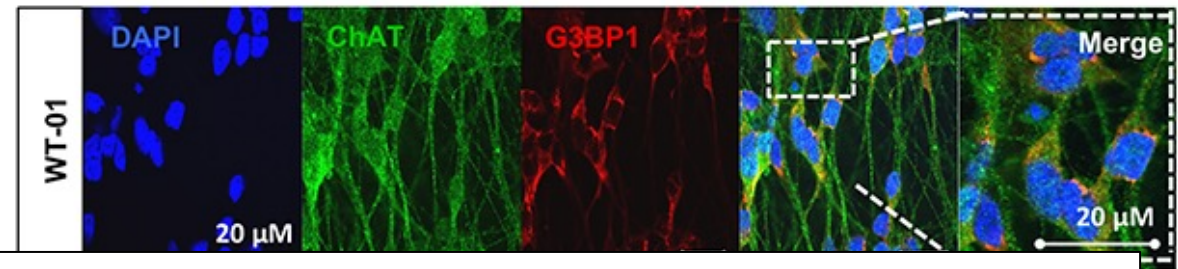


Figure 5

A)



B)



Conclusion:

Common indicators of cell stress/death are significantly higher in the expansion-carrying iPSCMNs.

Edited cell lines have these markers decreased to near-WT levels.



Figure 5

A) Principal Component Analysis
after batch correction

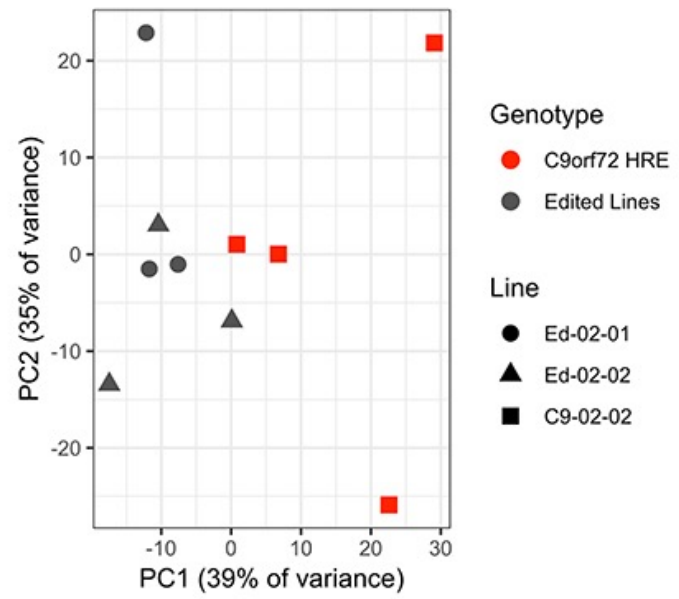
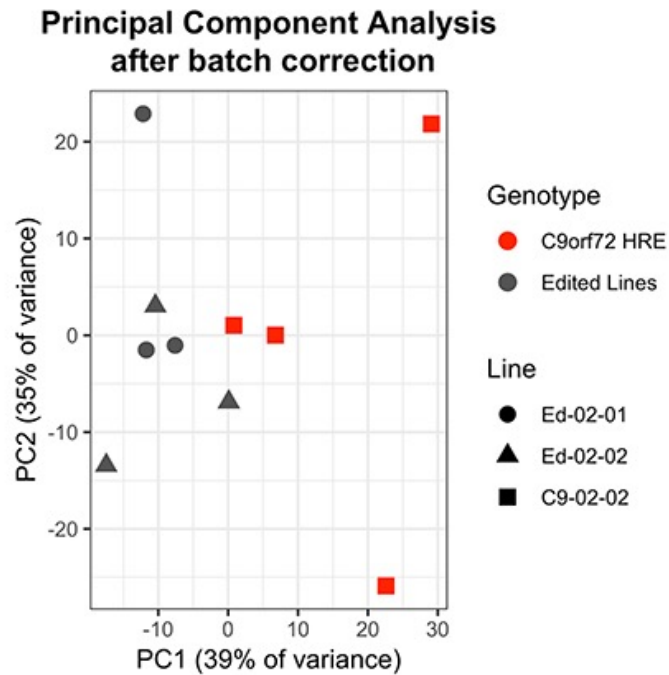


Figure 6

A)



POINTS OF SIGNIFICANCE

Principal component analysis

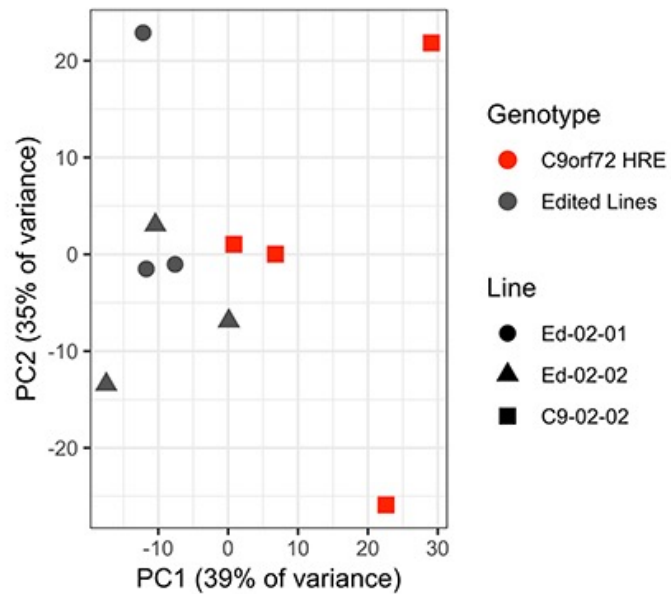
PCA helps you interpret your data, but it will not always find the important patterns.

Jake Lever, Martin Krzywinski & Naomi Altman

“High-dimensional data are very common in biology and arise when **multiple features**, such as expression of many genes, **are measured for each sample**. This type of data presents several challenges that PCA mitigates: computational expense and an increased error rate due to multiple test correction when testing each feature for association with an outcome. **PCA is an unsupervised learning method and is similar to clustering** — it **finds patterns without reference to prior knowledge about whether the samples come from different treatment groups or have phenotypic differences.**”

Figure 6

A) Principal Component Analysis after batch correction



B)

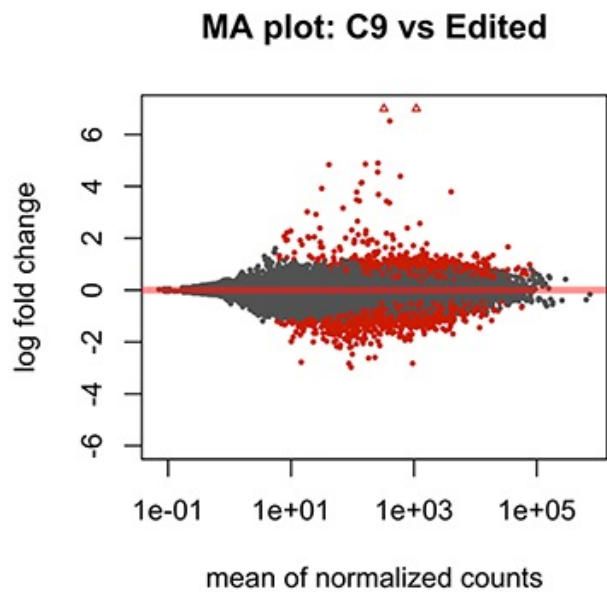


Figure 6

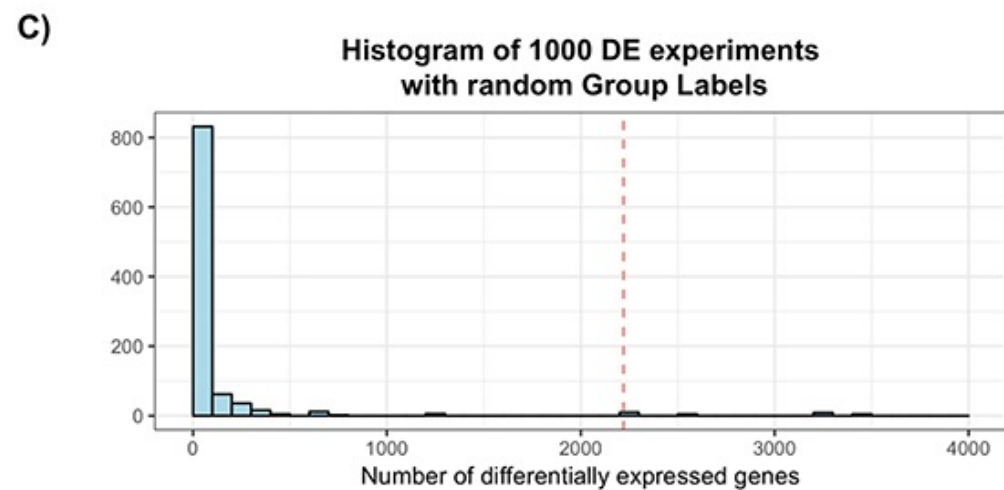
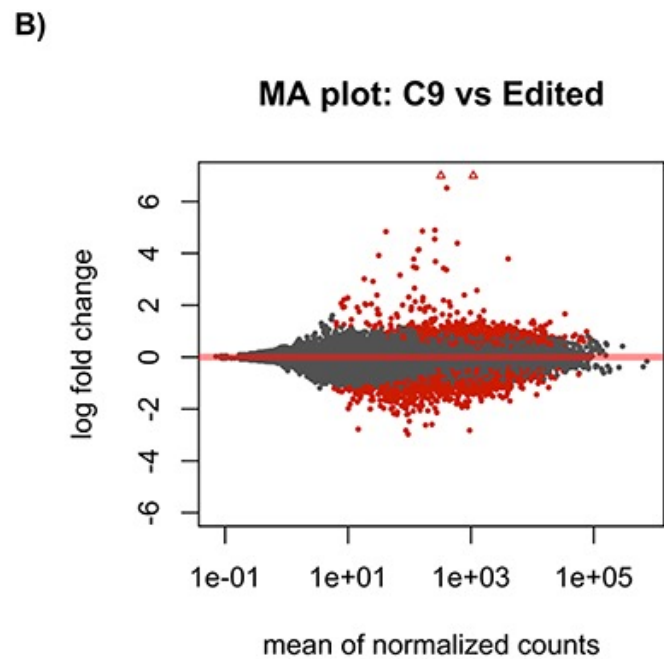
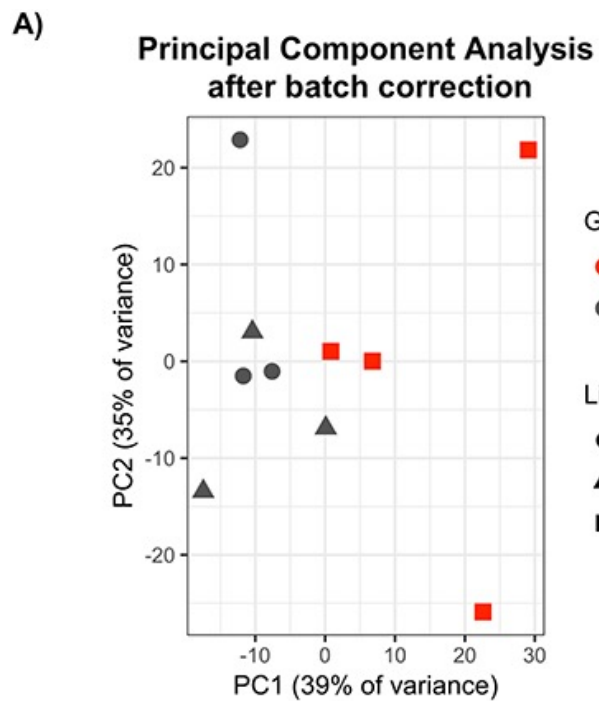


Figure 6

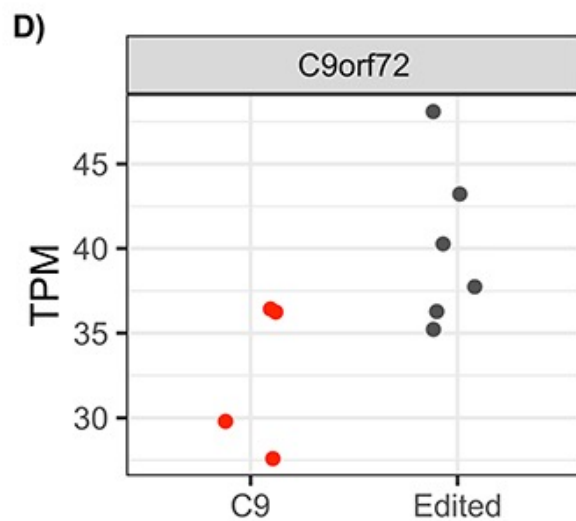
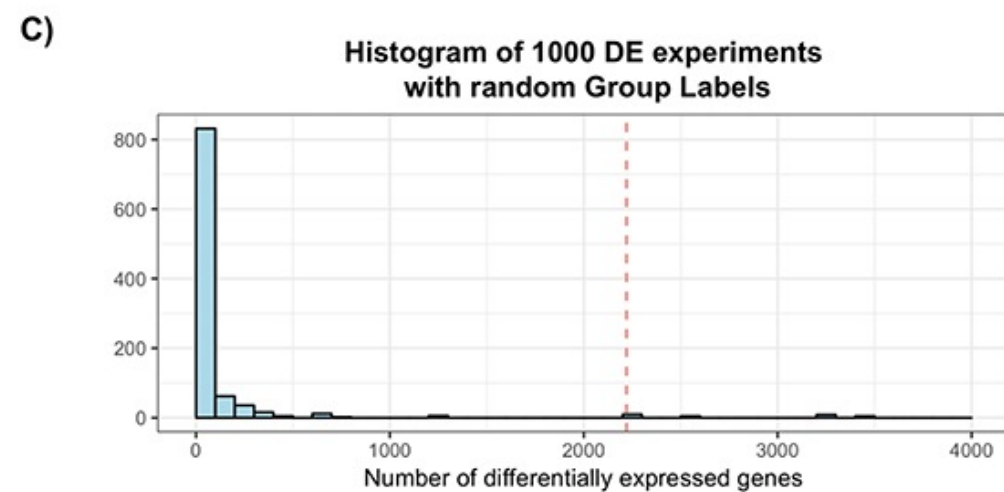
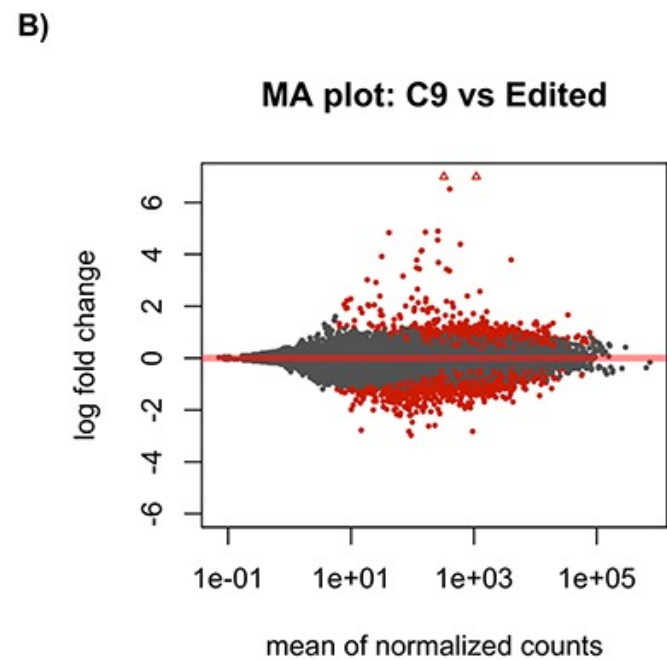
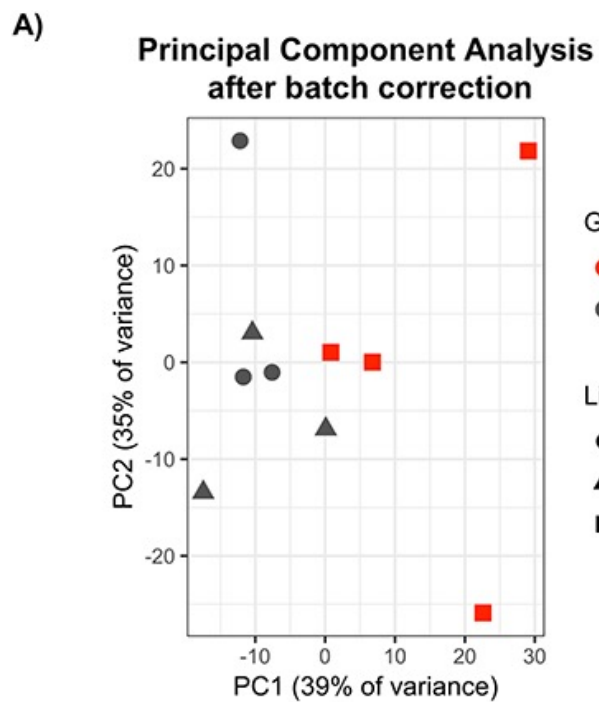


Figure 6

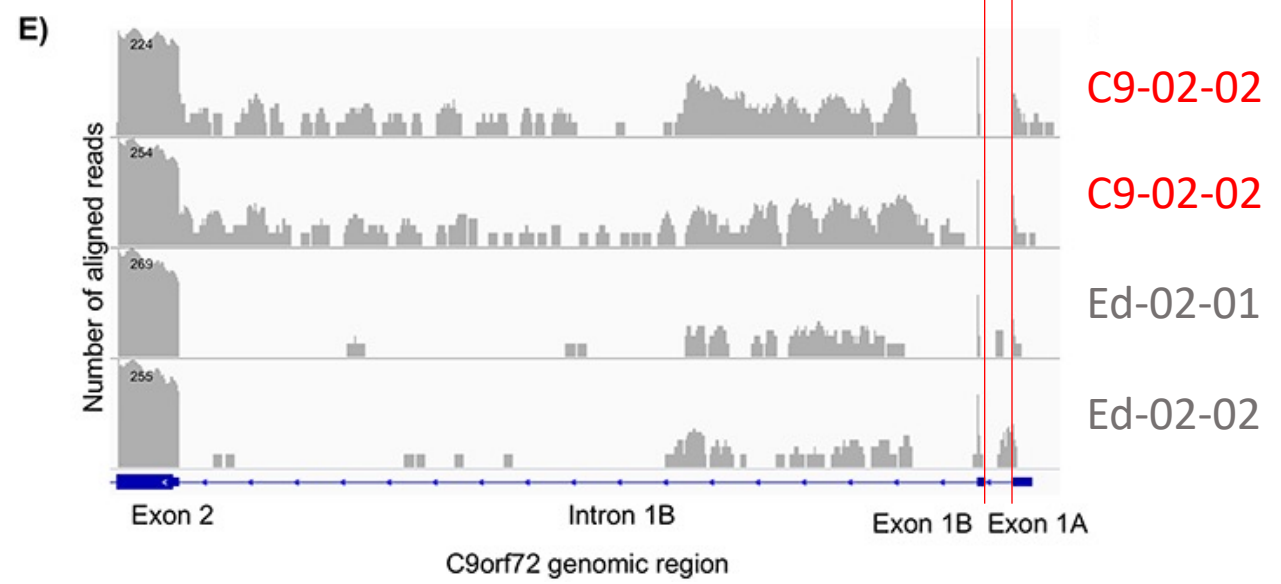
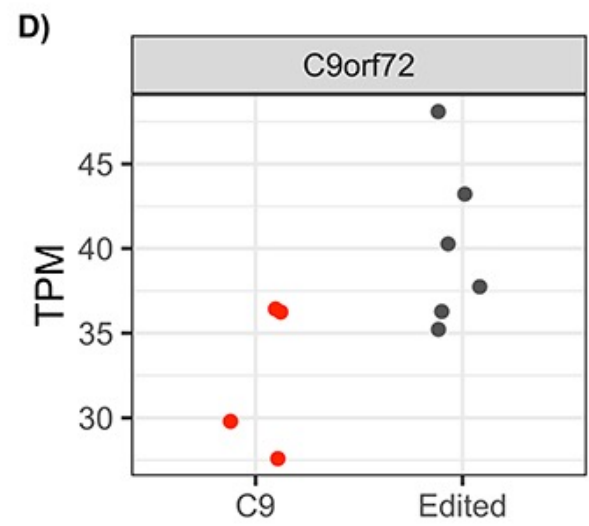
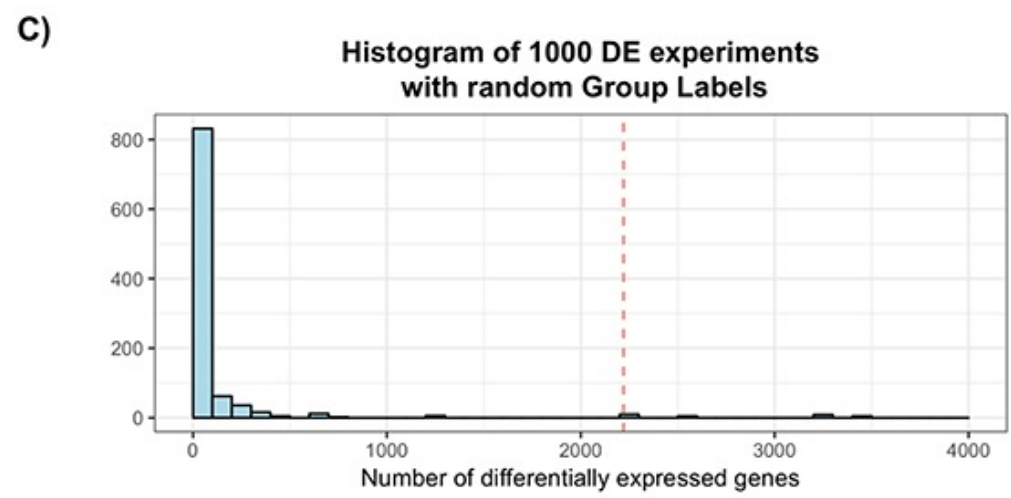
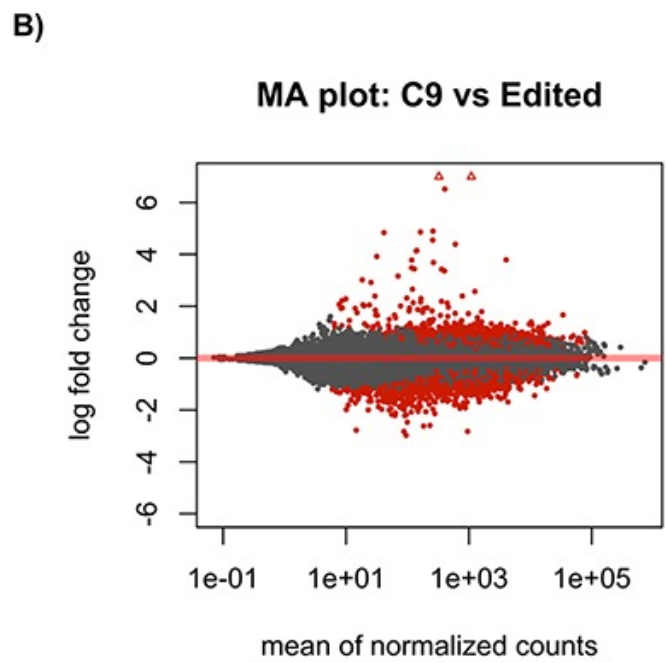
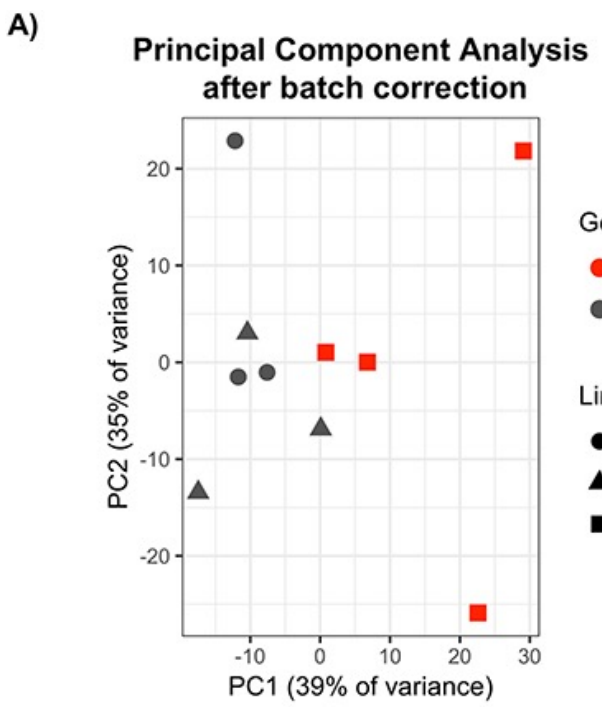


Figure 6

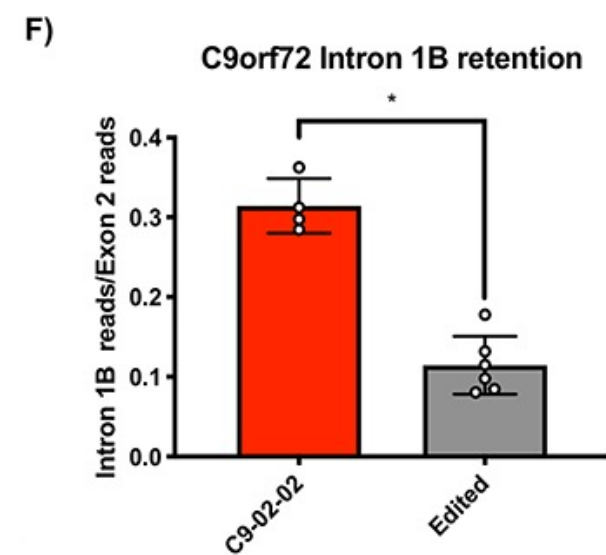
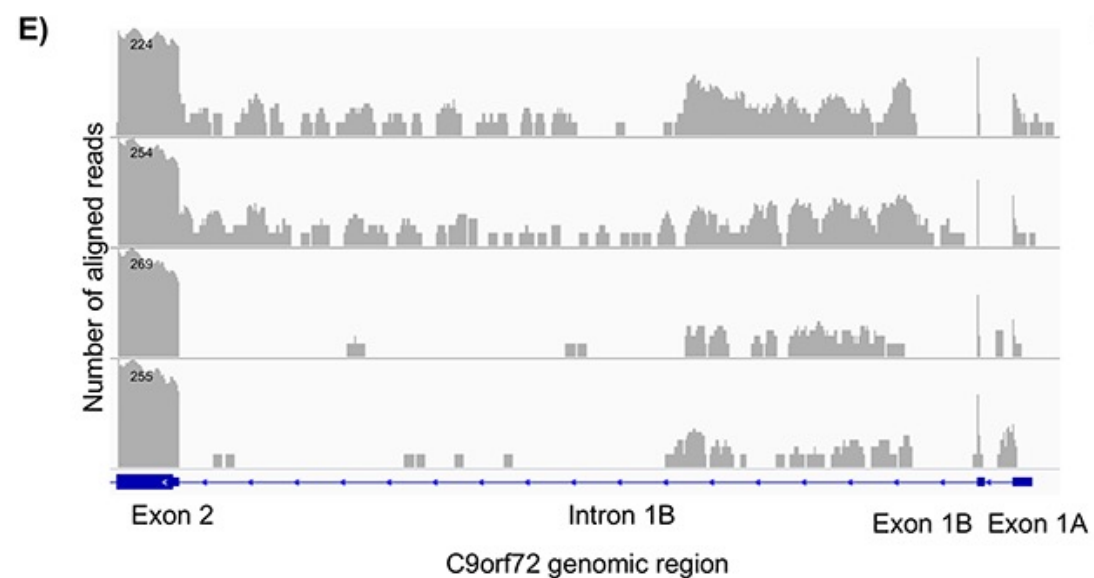
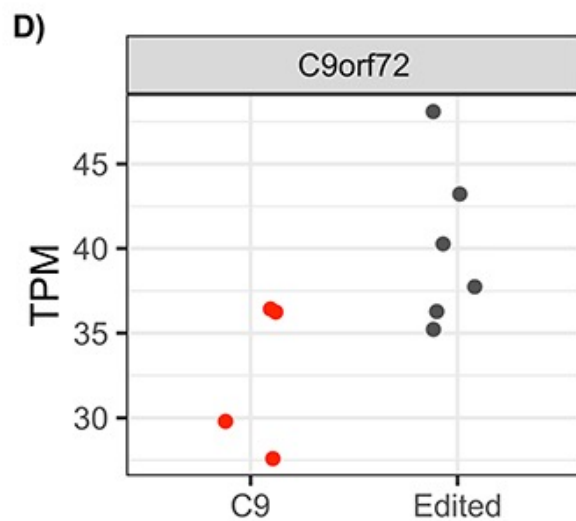
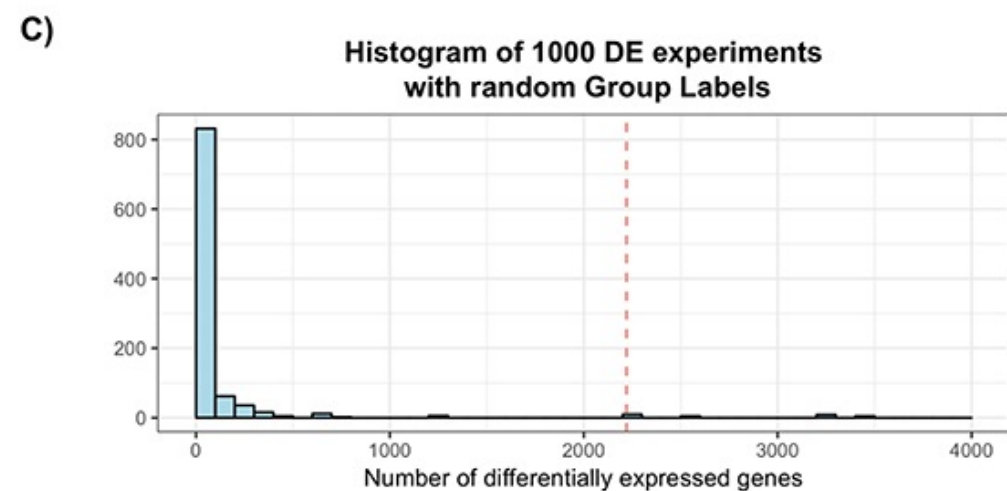
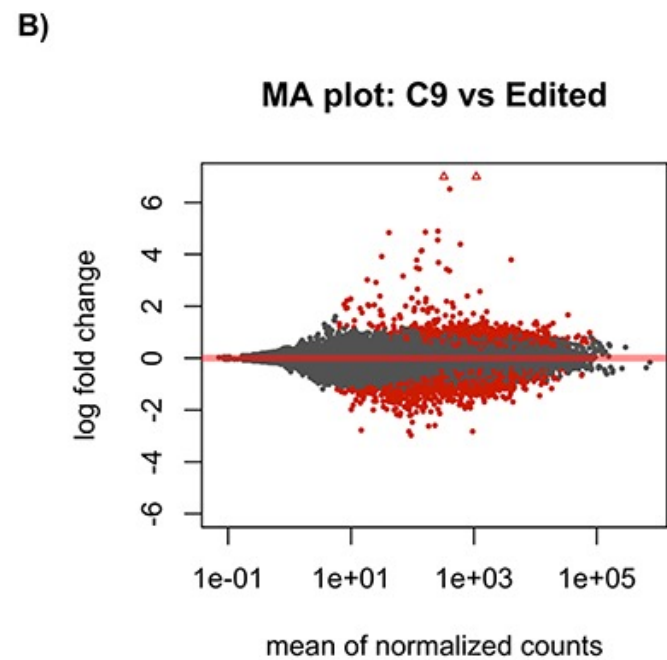
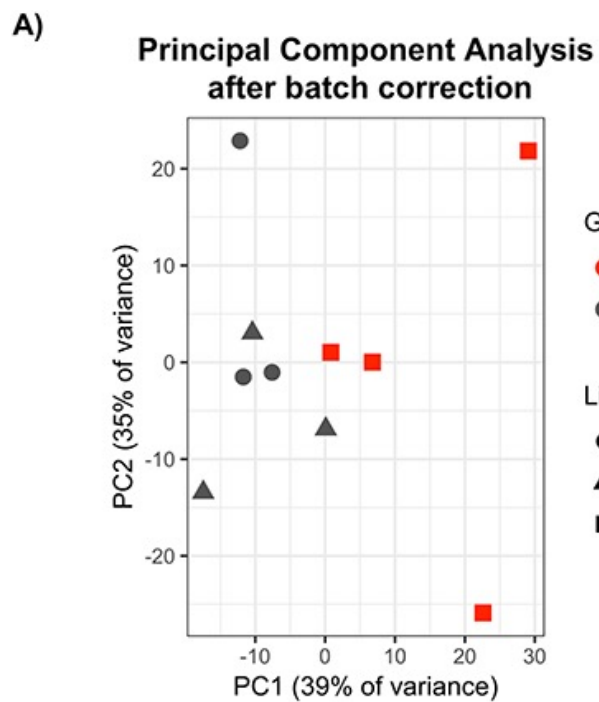


Figure 6

A)

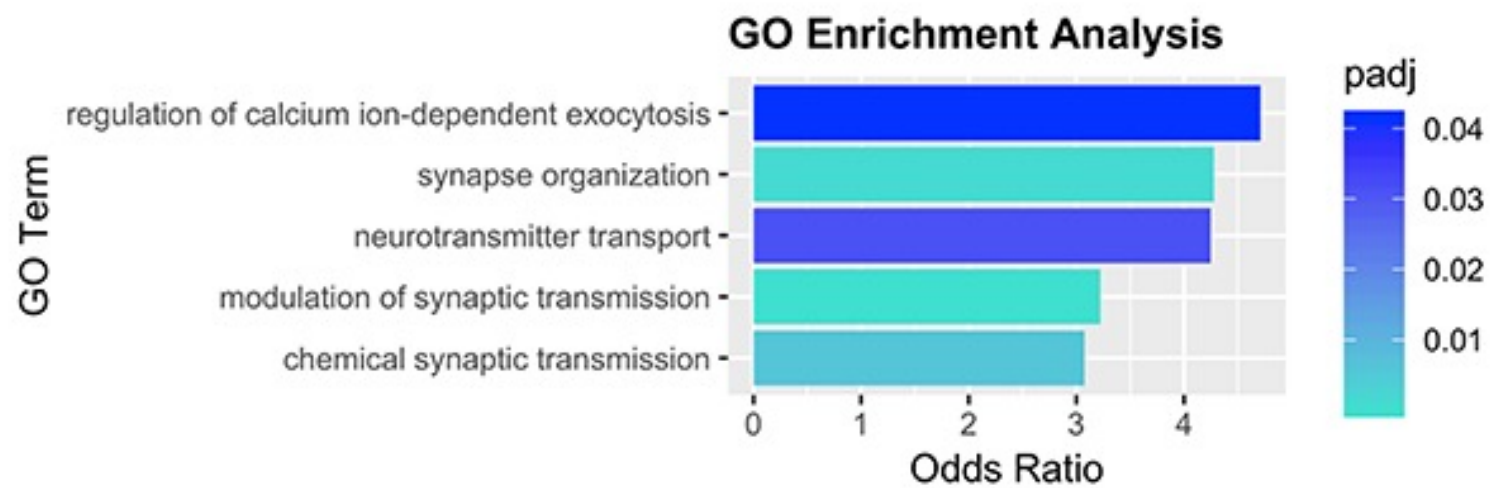
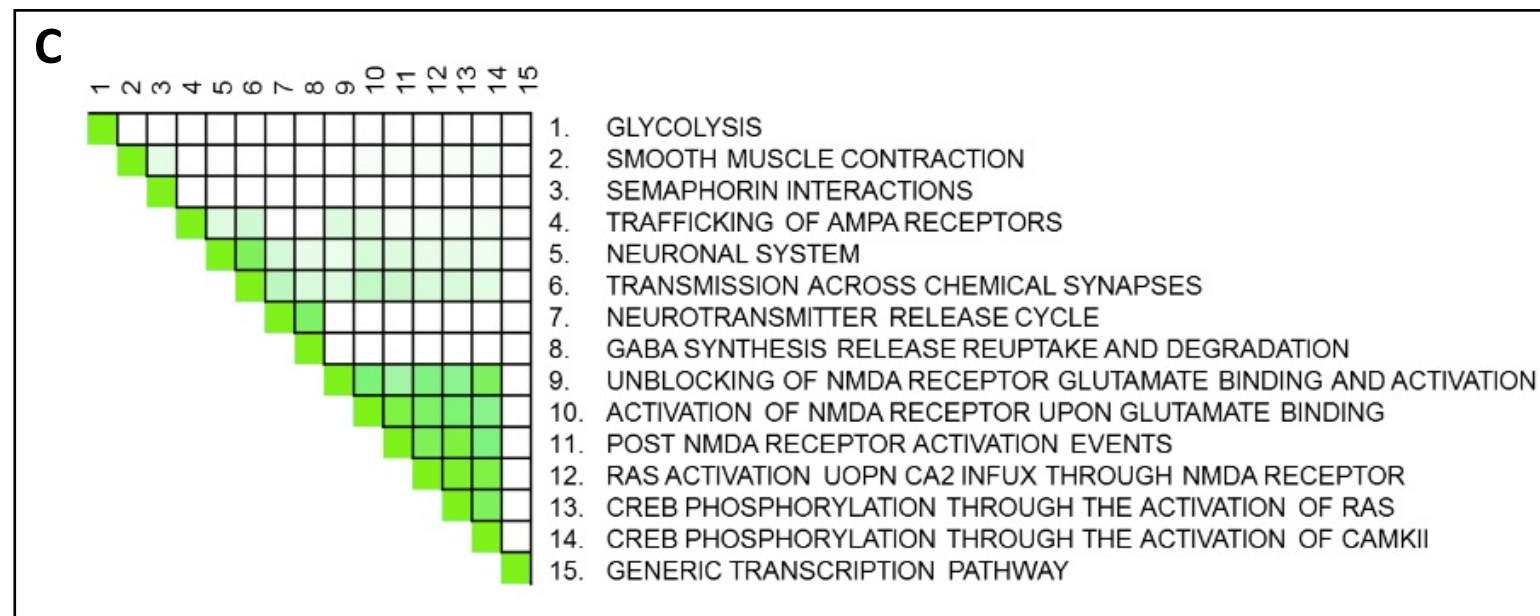
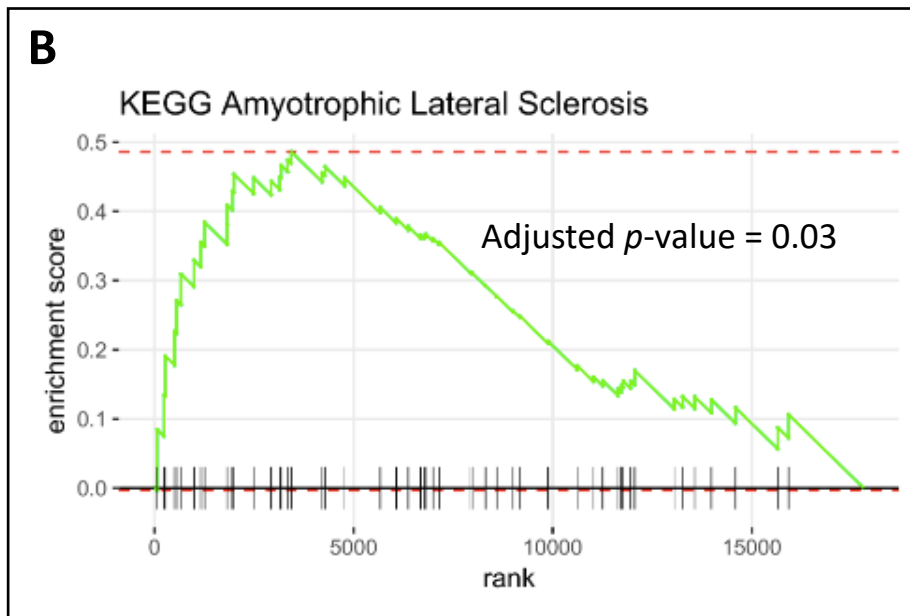
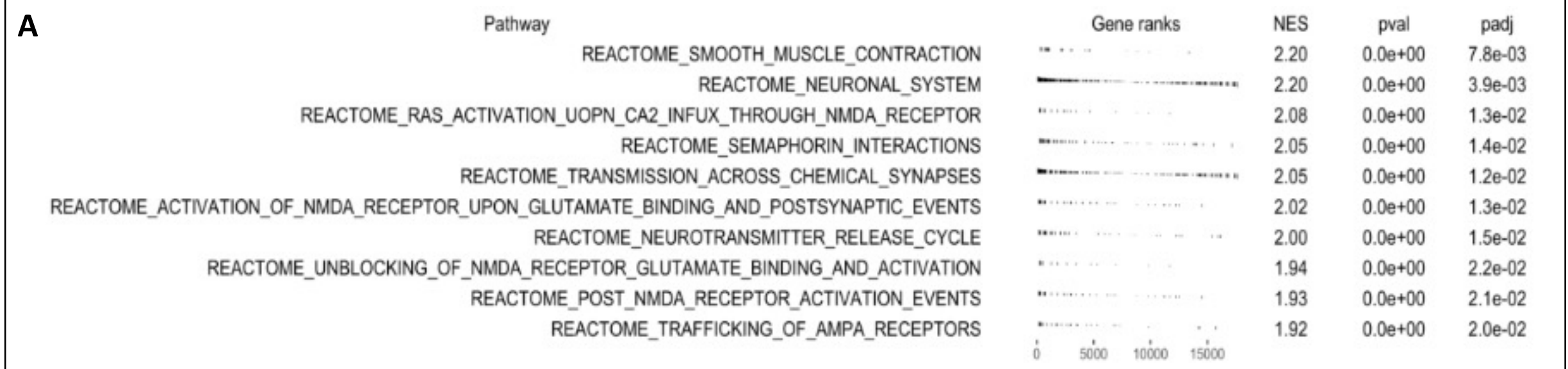


Figure 7



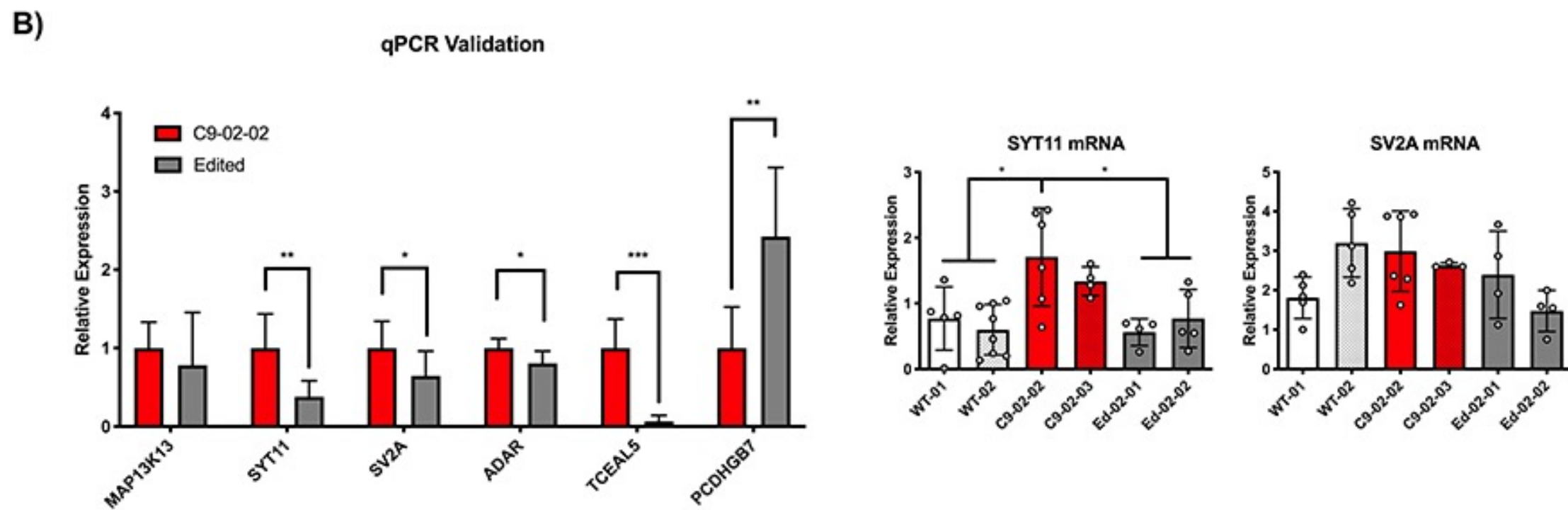
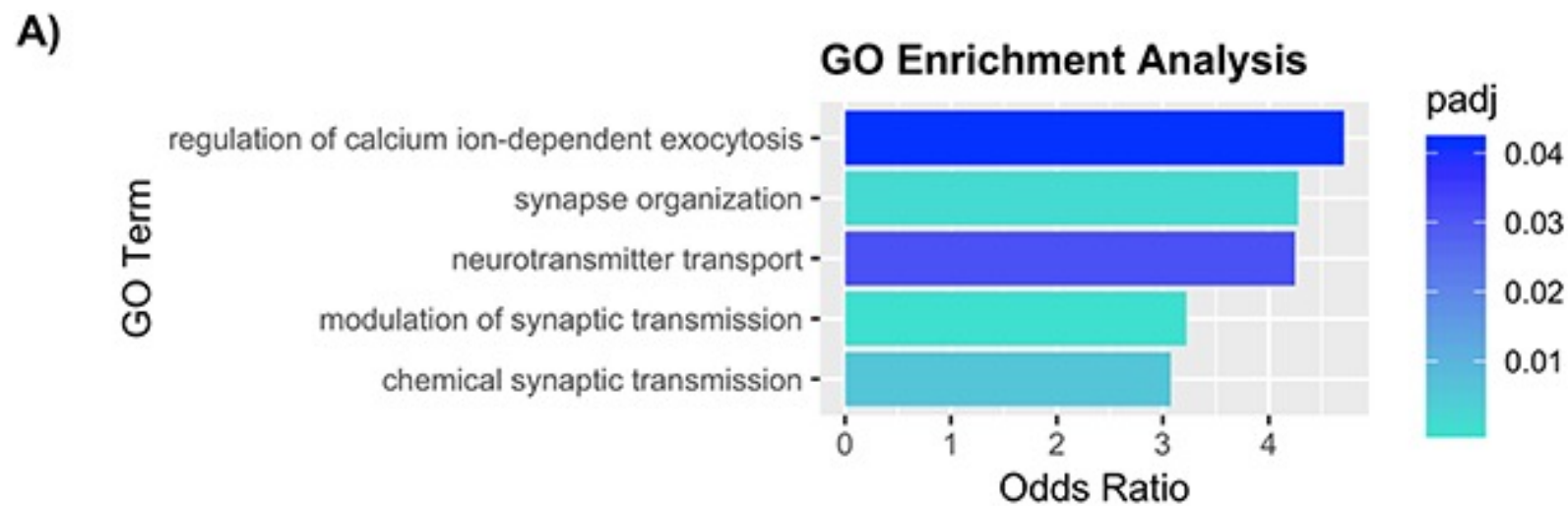


Figure 7

c)

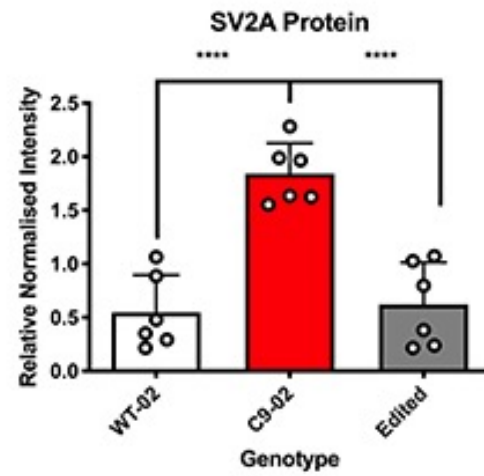
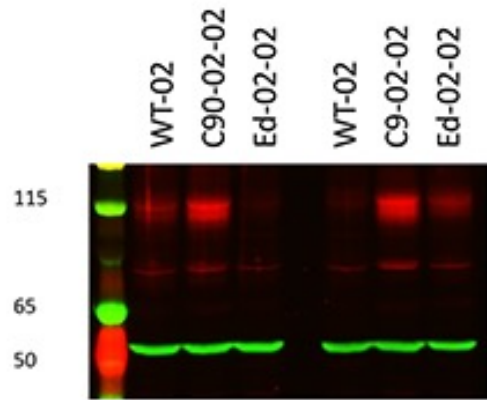
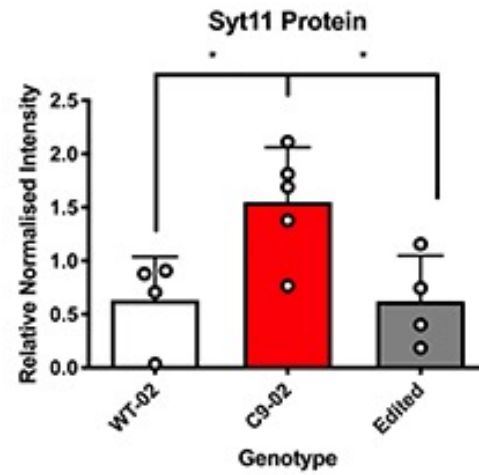
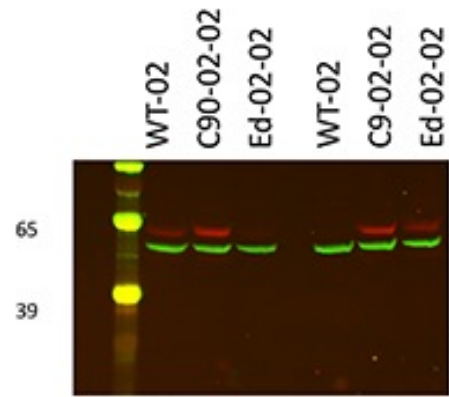
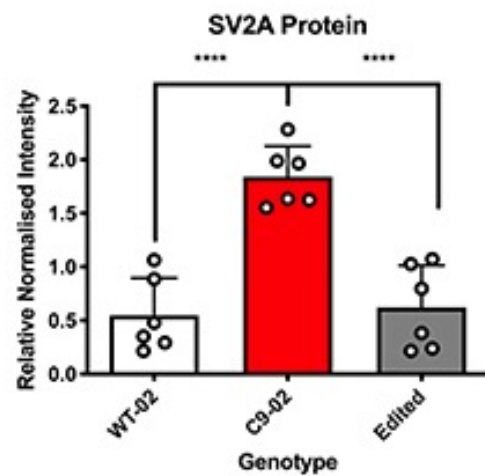
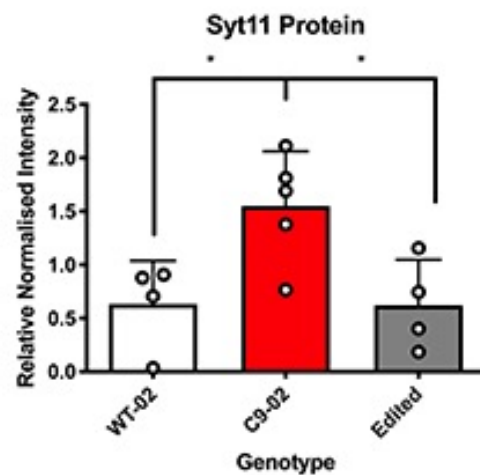
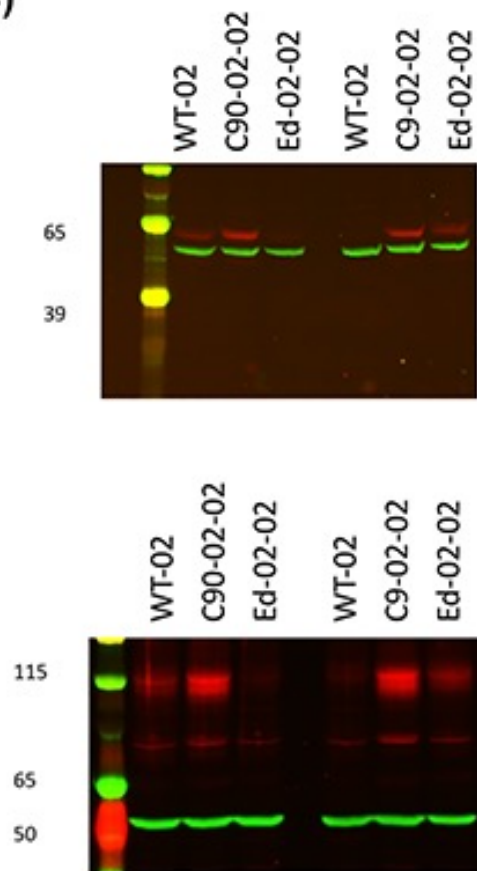


Figure 7

C)



D)

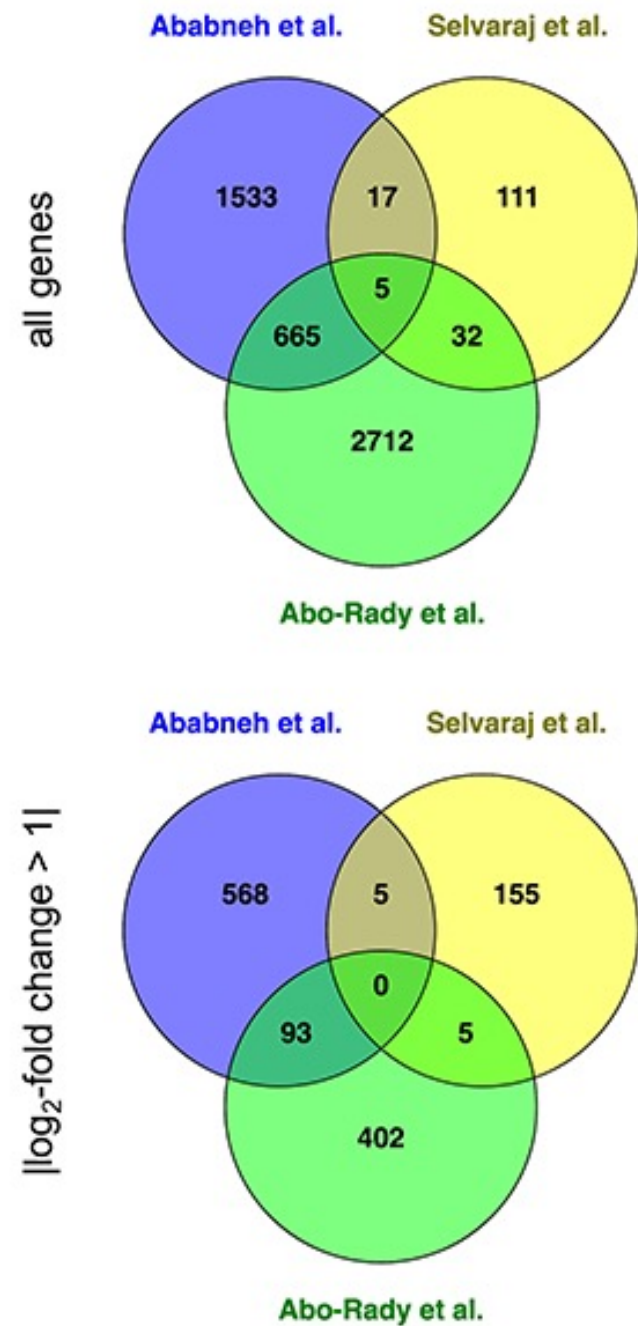


Figure 7

Strengths of this paper

- Novel strategy for targeting the repeat for correction – not just removal.
- Thorough validation of the edited iPSCs
- Thorough characterization of the differentiated iPSCs

Overall: excellent proof-of-concept

Areas for improvement

- Only a single patient cell line used
- Percentage of clones that were successfully edited?
 - Hard to know due to early puromycin selection – has implication for therapeutic use
- RAN-translated DPRs: none of the proline-rich DPRs examined
- Transcript analysis: not sure about conclusions for individual variants
- DE analysis comparison - reveals that small differences can create widely divergent results
 - highlights that gene expression profiling is very much specific to a study
- Off-target effects – less than one sentence addressing this in discussion

Future Directions

- Methylate the (GGGGCC)₂ repeat tract and then assess upstream methylation in the promoter region
 - Could help to deconvolute cause of promoter methylation: methylation of the repeat tract or expansion of the repeat tract?
- Suggest CRISPR/Cas9 HR as a future therapeutic strategy
 - Need to overcome low HR in mature neurons
- Simple model organism – phenotype reversal?
- Excellent tool - many possibilities for *in cellulo* work to create isogenic lines