TDP-43 dysregulation and STMN-2 mis-splicing upon proteasomal inhibition in potential iPSC-derived neuronal ALS model

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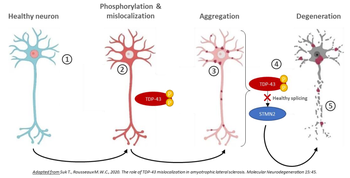
1 BACKGROUND & GOAL

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects both cortical and spinal motor neurons. The majority of ALS cases are characterized by TDP-43 pathology, being accumulation, nuclear-to-cytosolic mislocalization and phosphorylation of the transactivation response DNA binding protein (TDP)-43[1]. In rare genetic forms of ALS, this can be caused by mutations such as M337V in the TDP-43 (TARDBP) gene[1]. TDP-43 is involved in many mRNA processes, and its depletion results in mis-splicing of a number of mRNAs, including the neuronal growth associated factor, stathmin 2 (STMN-2)[2]. This mis-splicing contributes to axonal degeneration. To model the ALS phenotypes in vitro, proteasomal inhibition is often used to induce TDP-43-associated phenotypes[3].



Adapted from Suk T., Rousseaux M. W. C., 2020. The role of TDP-43 mislocalization in amyotrophic lateral sclerosis. Molecular Neurodegeneration 15:45.

Current consensus for ALS pathology:
1. Nuclear TDP-43
2. TDP-43 phosphorylation and mislocalization
3. (Phospho-) TDP-43 aggregation and inclusion
4. Mis-splicing of Stathmin-2 (STMN-2)
5. Lack of microtubule scaffolding & axodegeneration

Figure 1 | ALS pathology

Goal:
In our work we assessed if homozygous or heterozygous CRISPR-edited TDP-43 M337V mutations in induced pluripotent stem cell (iPSC)-derived glutamatergic neurons (a cortical neuron type), generated by bit.bio using the opti-ox technology[4-5], alone, or in combination with an MG-132 proteasomal inhibitor, would result in TDP-43 phosphorylation and aggregation and/or STMN-2 splicing, and could therefore be used as an ALS-relevant disease model.

2 METHODS

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Generated ioGlutamatergic Neurons: WT, TDP-43 M337V/WT, TDP-43 M337V/M337V → Cell culture according to bit.bio established protocols → Day 6 or 20; trigger 0.75 µM MG-132 → Day 7: Fixation, ICC and high content analysis → Day 21: ddPCR and analysis

Algorithm development: TDP-43 mislocalization and phosphorylation

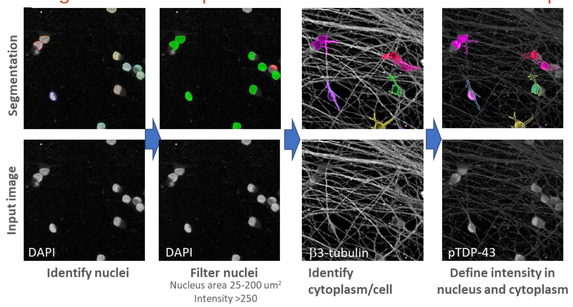

Figure 2 | High content analysis algorithm development for ICC analysis
Neuronal cell compartments were segmented using PE Columbus 2.9.1. Fluorescence intensity values of pTDP-43 and TDP-43 in those compartments were used to calculate mislocalization (nucleus/cytoplasmic TDP-43) and phosphorylation (cytoplasmic pTDP-43).

4 CONCLUSION

A relevant translational *in vitro* drug discovery model for ALS in hiPSC-derived neurons was developed:
- We generated ioGlutamatergic neurons with a homozygous or heterozygous TDP-43 M337V mutation [4-5], making it possible to directly compare the effect of the mutation on ALS-associated phenotypes with the isogenic control neurons
- An ICC and ddPCR assay was developed to assess TDP-43 mislocalization, phosphorylation, and STMN-2 expression
- A robust assay for drug development to assess TDP-43 mislocalization upon MG-132 treatment in bit.bio ioGlutamatergic Neurons was established
- An assay was established to assess MG-132-induced TDP-43 phosphorylation, which could be >90% prevented by using JNK inhibitor SP600125 as a tool compound
- An assay was established to assess MG-132-induced full-length STMN-2 mRNA reduction and STMN-2 trunc increase in mature neurons
- The homozygous M337V mutation resulted in a small but significant increase in TDP-43 mislocalization and phosphorylation without the need for MG-132 proteasomal inhibition
- The heterozygous M337V mutation resulted in a significant increase in STMN-2 missplicing compared with the isogenic wt control neurons after proteosomal inhibition

References:
1. *Scotter EL, et al, 2015. Neurotherapeutics 12*
2. *Melamed Z, et al, 2019. Nature neuroscience 22(2), 180–190*
3. *Klim JR, et al, 2019. Nature neuroscience 22(2),167-179*
4. *Pawlowski M, et al, 2017. Stem cell reports 8(4), 803-812*
5. *https://www.bit.bio/glutamatergic-neurons-tdp43-m337v*

3 RESULTS

TDP-43 mislocalization

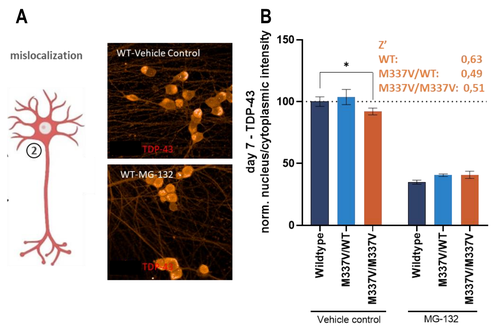

Figure 3 | A robust assay for TDP-43 mislocalization upon MG-132 treatment in bit.bio ioGlutamatergic Neurons day 7
The TDP-43 nuclear to cytoplasmic ratio (mislocalization) was assessed by ICC and high content analysis (**A**) and plotted (**B**). bit.bio ioGlutamatergic Neurons WT, TDP-43 M337V/WT and TDP-43 M337V/M337V were treated with DMSO or 0.75 µM MG-132 for 24 hours on day 6, and fixed on day 7. Statistics were analysed by two-way ANOVA (table), and Z' was calculated for each cell type by comparing vehicle control (DMSO) and MG-132. Error bars indicate SD, n=5 technical repeats. Table: Significance of two way ANOVA is indicated for (**B**); VC (vehicle control), TR (MG-132).

Two way ANOVA with Sidak's posthoc analysis

Comp 1	Comp 2	Significance
WT VC	DS VC	0.0158
WT VC	WT TR	<0.0001
C20 VC	C20 TR	<0.0001
DS VC	DS TR	<0.0001

VC (Vehicle control), TR (Treated)

TDP-43 phosphorylation

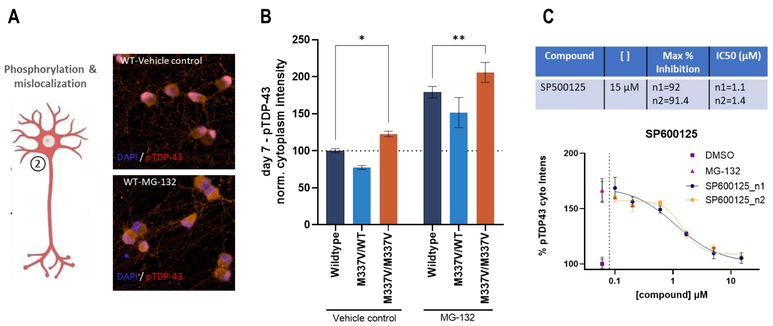

Figure 4 | MG-132-induced TDP-43 phosphorylation can be >90% prevented by JNK inhibitor SP600125
pTDP-43 cytoplasmic intensity normalized to WT vehicle control (TDP-43 phosphorylation and mislocalization) was assessed by ICC (**A**) and plotted (**B**). bit.bio ioGlutamatergic Neurons WT, TDP-43 M337V/WT and TDP-43 M337V/M337V were treated with DMSO or 0.75 µM MG-132 (a proteasome inhibitor) for 24 hours on day 6, and fixed on day 7. (**C**) ioGlutamatergic Neurons WT were 2 hours pre-treated with 15 µM SP600125 (a JNK inhibitor), after which 0.6 µM MG-132 was added for 24 hours. DMSO only was used as a control. n1 and n2 indicate two independent biological experiments. Error bars indicate SD, n=5 technical repeats. Table: Significance of two way ANOVA is indicated for (**B**); VC (vehicle control), TR (MG-132).

Two way ANOVA with Sidak's posthoc analysis

Comp 1	Comp 2	Significance
WT VC	DS VC	0.0109
WT VC	WT TR	<0.0001
WT TR	C20 TR	0.0011
WT TR	DS TR	0.0009
C20 VC	C20 TR	<0.0001
DS VC	DS TR	<0.0001

VC (Vehicle control), TR (Treated)

STMN-2 misregulation

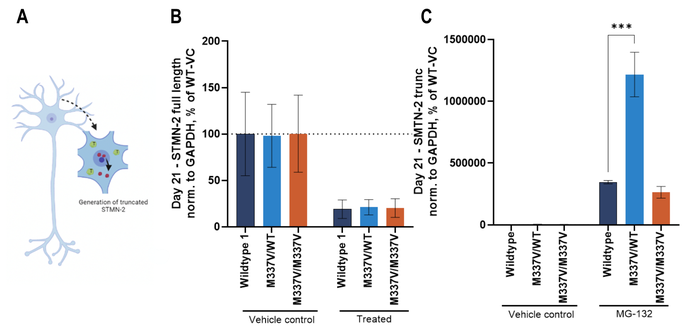

Figure 5 | MG-132-induced FL STMN-2 reduction and mis-splicing in bit.bio ioGlutamatergic Neurons day 21
STMN-2 full length and truncated mRNA (mis-spliced) (**A**) normalized to GAPDH and displayed as a % of WT vehicle control as measured by ddPCR and plotted (**B-C**). bit.bio ioGlutamatergic Neurons WT, TDP-43 M337V/WT and TDP-43 M337V/M337V were treated with DMSO or 0,75 µM MG-132 for 24 hours on day 20, and harvested on day 21. Error bars indicate SD, n=4 technical repeats. Table: Significance of two way ANOVA is indicated for (**B/C**); VC (vehicle control), TR (MG-132).

Two-way ANOVA

	full	trunc
VC WT - VC C20	ns	ns
VC WT - VC DS	ns	ns
VC WT - TR WT	*	*
VC C20 - TR C20	*	****
VC DS - TR DS	*	ns
TR WT - TR C20	ns	***
TR WT - TR DS	ns	ns

VC = vehicle control, TR = MG-132
* P < 0.05, ** P < 0.01, *** P < 0.001