Transactive response DNA-binding Protein-43 (TDP-43) involvement in the Phosphatidylinositol-3-Kinase pathway.

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Certifico:

- Que el Trabajo de Fin de Máster (TFM) titulado Transactive response DNA-binding Protein-43 (TDP-43) involvement in the Phosphatidylinositol-3-kinase pathway ha sido realizado bajo mi supervisión por Dª Laura Santana Cordón, matriculada en el Mastér en Biomedicina, durante el curso académico 2019/2020.

- Que una vez revisada la memoria final del TFM, doy mi consentimiento para ser presentado a la evaluación (lectura y defensa) por el Tribunal designado por la Comisión Académica de la Titulación.

Para que conste, firmo el presente certificado en La Laguna a 6 de Julio del 2020

Dr. Ángel Acebes Vindel

Dr. Abraham Acevedo Arozena

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Contents

Abstract

Amyotrophic lateral sclerosis (ALS) is a mortal neurodegenerative disease and together with frontotemporal dementia (FTD) they are considered a part of the same spectrum of diseases collectively called TDP-43 proteinopathies, since they have several pathological characteristics in common. One of the main characteristics that define ALS is the loss of TDP-43 from the nucleus and its accumulation in the cytoplasm, forming inclusions. Since the PI3K/AKT pathway is neuroprotective and controls a prosynaptogenic program, this pathway is important in the study of ALS pathogenesis. The usage of a PI3K pathway activating transduction peptide (PTD4-PI3KAc) as a pharmacological tool offers us the possibility of studying the possible functional interactions between TDP-43 and the PI3K pathway. Thanks to the use of a an allelic series of Knock-In mice with point mutations in the *Tardbp*/TDP-43 gene and the use of the activating peptide, we can study how the activation of the PI3K pathway can influence the different mutations in TDP-43.

Resumen

La esclerosis lateral amiotrófica (ELA) es una enfermedad neurodegenerativa mortal y junto con la demencia frontotemporal (DFT) se consideran parte del mismo espectro de enfermedades llamadas colectivamente proteinopatías TDP-43, ya que tienen en común varias características patológicas. Una de las características principales que definen a la ELA es la pérdida de TDP-43 del núcleo y su acumulación en el citoplasma formando inclusiones. Dado que la vía PI3K/AKT es neuroprotectora y controla un programa prosinaptogénico, esta vía tiene importancia en el estudio de la patogénesis de la ELA. El uso de un péptido de transducción activador de la vía PI3K (PTD4-PI3KAc) como herramienta farmacológica nos ofrece la posibilidad de estudiar las posibles implicaciones entre TDP-43 y la vía PI3K. Gracias al uso de una serie alélica de ratones Knock-In con mutaciones puntuales en el gen *Tardbp*/TDP-43 y al uso del péptido activador, podemos estudiar cómo la activación de la vía PI3K puede influir en las distintas mutaciones en TDP-43.

Keywords: ALS, TDP-43, PI3K/AKT pathway, *Tardbp* mutations.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive fatal neurodegenerative disorder [1, 2, 3]. This disorder is characterized by the progressive degenerations of motor neurons in the brain and spinal cord, which causes progressive muscle weakness that, finally leads to death, usually from respiratory failure [1, 3]. This pathology was first described by the neurologist Jean-Martin Charcot in 1869 [2, 3] and its name make reference to both the degeneration of corticospinal motor neurons, whose descending axons in the spinal cord seems like scarred (lateral sclerosis), and the loss of spinal motor neurons, with secondary denervation and muscle wasting (amyotrophy). Commonly, the clinical symptoms of ALS start in mid-life, usually in the 6th decade, however, it can start as soon as in the first or second decade of life [3]. The patients manifesting this disorder generally start with localized symptoms, such as a unilateral limb weakness or bulbar impairment, that are spreading later, a feature found in the most common neurodegenerative diseases [2, 3]. The clinical symptoms are a consequence of the dysfunction and death of motor neurons in the primary motor cortex, brainstem and spinal cord, causing spasticity, weakness and muscle wasting. All these conditions gradually lead to paralysis and death of the patient, typically 3-5 years after diagnosis [2, 3], although some forms of the disease show prolonged survival [3].

ALS is an orphan disease - diagnosed in 1-2 individuals per 100.000 cases each year in most countries with a prevalence of around 5 cases per 100.000 total cases each year [2, 3]. Approximately a 5-10% of ALS cases are inherited (familiar ALS, fALS) and generally show monogenic autosomal dominant inheritance [1, 2]. 90% of patients diagnosed with ALS present a sporadic disease (sALS) without apparent family history. Both familiar ALS (fALS) and sporadic (sALS) can appear together with frontotemporal dementia (FTD). Frontotemporal dementia (FTD) is a related neurodegenerative disease affecting frontal and temporal lobes, leading to progressive behavioural and language changes that result in dementia. The clinical manifestations of ALS and FTD can co-exist and both diseases have in common several pathologic and genetics characteristics, therefore they are considered part of the same disease spectrum: TDP-43 proteinopathies [1, 4, 5].

At present, there is no effective treatment for ALS with substantial clinical benefit to patients. At this time, there are only two neuroprotective drugs approved by US Food and Drug Administration (FDA): Riluzol and Edaravone. Both drugs are able to

increase the lifespan of some patients just a few months. Riluzol blocks excessive glutamatergic neurotransmission, whereas Edaravone prevents oxidative stress damage [2].

1.1. Transactive response DNA-binding protein 43

In 2006, Neumann et al. reported that the nuclear RNA binding protein (RBP) Transactive response DNA-binding protein of 43 kDa (TDP-43) is the main component of the characteristic aggregates in the affected motor neurons of patients with ALS [6] and the major component of pathological cytoplasmic inclusions characteristic of frontotemporal lobar degeneration (FTLD) [7]. Along the years, the involvement of TDP-43 in ALS has been described in several investigations and reviews pointing out the functional importance of TDP-43 mutations in ALS and FTD [8].

TDP-43 is a 414-amino acid protein encoded by the *TARDBP* gene on chromosome 1 (reference sequence NM_007375.3) [8], containing two highly conserved RNA recognition motifs (RRM1 and RRM2), a nuclear localization signal (NLS) at the N-terminal domain and a glycine-rich region mediating protein-protein interactions at the C-terminal domain [9]. The TDP-43 protein is highly conserved and a ubiquitously expressed heterogeneous ribonucleoprotein (hnRNP) that is primarily nuclear but shuttles between the cytoplasm and nucleus [7]. The C-terminal and N-terminal domain are the main TDP-43 regions that interact whit others proteins, and the RRMs are necessary to bind RNA mainly in a sequence-specific manner, preferentially binding (UG)n-enriched sequences [8, 10].

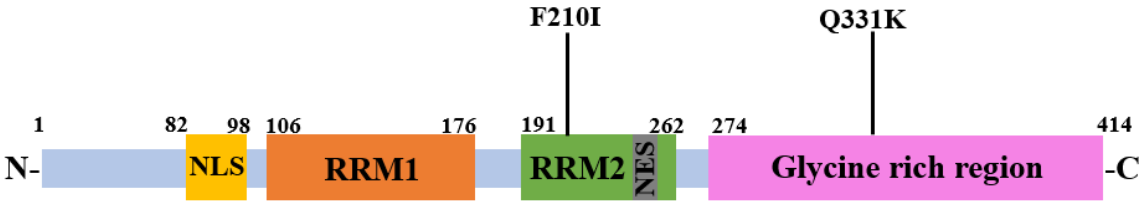

Figure 1. Linear structure of TDP-43 protein. It has is indicated the location of the different regions. Also shown the location of the two mutations used in this study (Q331K and F210I). NLS (Nuclear Localization Signal); RRM1 and RRM2 (RNA Recognition Motifs 1 and 2); NES (Nuclear Export Sequence). This figure has been adapted from the following bibliographical references: 4, 10, 11.

As a RNA/DNA-binding protein, TDP-43 is a multifunctional factor that plays an important role in the regulation of RNA splicing, in the stability, in the transport and in the microRNA processing [8, 9]. It has a self-regulatory mechanism that determines its level of synthesis.

The direct link of TDP-43 to the disease was established by the discovery of mutations in the *TARDBP* gene [6]. A large number of genetic factors have been identified that drive motor neurons degeneration in ALS, increase susceptibility to disease or influence the speed of its progression [3]. The genomic analysis has allowed identification of causal mutations of disease that have also subsequently been found in some sporadic patients. In general, known pathogenic mutations can be identified in ~15% of ALS cases. Several of these mutations are in genes that encode RNA-binding proteins (RBP), being the two most frequent TDP-43 and FUS (Fused in Sarcoma). Furthermore, TDP-43 is found in the cytoplasmic neuronal inclusions in more than 97% of ALS cases and in more than 40% FTD cases [1]. Finally, *TARDBP* mutations beside other well know "ALS genes" are found in ~5% of fALS and ~2% of sALS [2].

The functional importance of these mutations remains largely unknown. Most of these mutations are localized in C-terminal end of the protein, so it is probable that they are associated with characteristics that may be directly related to the pathology [8]. In ALS, TDP-43 loses its normal nuclear localization and it accumulates in the cytoplasm forming inclusions, the most common neuropathological marker for ALS [2, 12]. There are two hypotheses about the mechanism of the disease based on the formation of TDP-43 inclusions: a "loss of function" in the nucleus by the delocalization of TDP-43, and a cytoplasmic level "gain of function" produced by the eventual toxicity of the aggregates themselves or by toxic gain of function of cytoplasmic TDP-43. These two mechanisms are not mutually exclusive and may occur at the same time [8, 13]. In human disease, pathological TDP-43 is abnormally phosphorylated, ubiquitinated and cleaved to produce C-terminal fragments [2, 13, 14], these C-terminal TDP-43 fragments accumulate in inclusions [5]. Although under normal stress conditions TDP-43 leaves the nucleus, the aggregation of TDP-43 together with its nuclear elimination can lead to a deregulation of many splicing events that will causes a constant level of stress in the affected cells [6].

1.2. PI3K/AKT pathway

The phosphatidylinositol-3-kinase (PI3K) signaling pathway is highly conserved in evolution [15]. The activation of the PI3K/AKT signaling pathway plays a crucial role in fundamental cellular functions [16] such as cell proliferation and survival by phosphorylating many downstream effectors [17, 18] and regulates a wide spectrum of

cellular mechanisms including metabolism, growth, motility, genomic instability, angiogenesis, metastasis and synaptogenesis [17-20].

The PI3Ks constitute a unique family of three classes of intracellular lipid kinases that form phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) from phosphatidylinositol-4, 5-biphosphate (PIP_2). In this study we focus on class IA [15, 20, 21]. PIP_3 acts a secondary messenger [20, 21] and provides docking sites for 3-phosphoinositide dependent kinases (PDK1) and mTOR complex 2 (mTORC2). Both phosphorylate AKT at Thr-308 and Ser-473 residues, respectively, thereby leading to its full activation [17]. In turn, AKT activation leads to phosphorylation of downstream targets, inhibiting glycogen synthase kinase 3 (GSK3) [22] and activating mTOR complex 1 (mTORC1) [23]. GSK3 is a serine/threonine kinase and its activity has been implicated in axon formation, neuronal polarization, neuritogenesis, migration and guidance among other cellular processes [21, 22]. Specifically, in mammalian brains, GSK3 is required for proliferation, neurotransmission in adult neurons and differentiation [22].

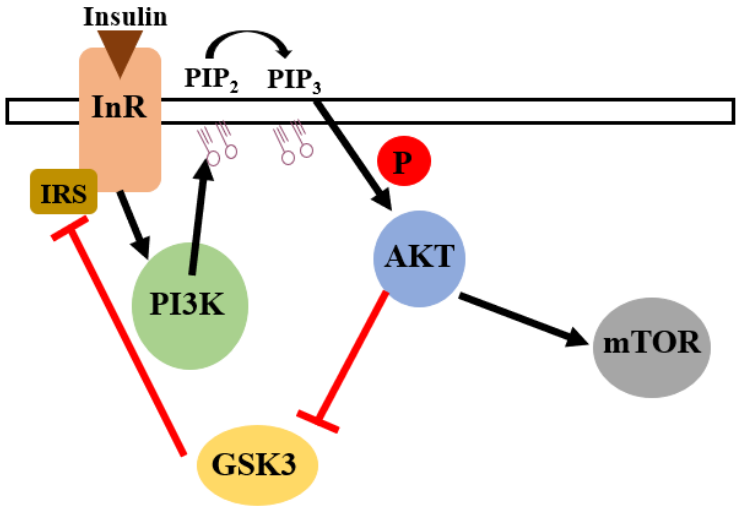

Figure 2. The PI3K/AKT signaling pathway. InR (Insulin Receptor); IRS (Insulin Receptor Substrate) This figure has been adapted from the following bibliographical references: 20, 22, 23, 24.

The importance of the PI3K/AKT pathway in neuronal survival has been highlighted in the neurodegeneration induced by neurodegenerative diseases such as ALS [16]. There is evidence to suggest that the PI3K/AKT pathway is involved in the pathogenesis of ALS. In a study made with neurons derived from iPS cells from patients with mutations in *TARDBP*/TDP-43, they show alterations in this pathway that possibly contribute to their neurotoxicity [25].

In the last years, the PI3K pathway has been involved in a signaling cascade that leads to synapse formation and spinogenesis in *Drosophila melanogaster* [24] and in vertebrates, both *in vitro* and *in vivo* [21, 22]. The effect of PI3K on synaptic density is independent of the stage of development of the culture or the type of synapse [21]. Conversely, inhibitors of the PI3K pathway reduce the number of synapses and spines in flies and mammals [22]. Therefore, it is known that the activity of PI3K and the downstream AKT tightly control the formation and maintenance of synapses in a cell autonomous manner [24]. The synaptogenic effect of this signaling pathway is primarily mediated by AKT activation and its overexpression has been shown to produce synaptic augmentation while GSK3 overexpression reduces the number of synapses [21, 24]. The study of the PI3K/AKT pathway together with the study of GSK3 could establish the possible causes that produce the different neurodegenerative diseases since it has been seen that, in many of these diseases, the number of synapses is an important determinant of behaviour [26] and the loss of synapses is related to cognitive deterioration in aging and most neurological pathologies [23].

The most recent data on this prosynaptogenic pathway show that changes in the number of synapses can lead to notable changes in behaviour. This could be key to neurodegenerative diseases such as Alzheimer's disease [26] and ALS, among others. Recent data from *Drosophila melanogaster* indicate that the PI3K synaptogenic pathway involves the type II BMP receptor Wit (*wishfull thinking*) and several MAPKs, but not mTOR or S6K, which are characteristic of other PI3K classical signaling pathways [27]. In a more recent study on this prosynaptogenic pathway, it is shown that the restoration of PI3K activity is a potential new pharmacological strategy against Alzheimer's disease [27], so studying the mechanisms underlying the effects that PI3K has on synaptogenesis and neuroprotection would be of potential therapeutic interest for diseases like ALS.

Thanks to generation of a series of mutant KI strains of mouse which have different mutations in the *Tardbp*/TDP-43 gene that differentially affect TDP-43 functions, we can study the effect of these point mutations in TDP-43 on PI3K/AKT signaling by using a pharmacological tool such as a PI3K pathway activating transduction peptide called PTD4-PI3KAc.

2. Hypothesis and Objectives

Our study hypothesis is that there is a functional interaction between TDP-43 and the PI3K/AKT pathway. To confirm this hypothesis, we will study if mutations in TDP-43 affect the PI3K pathway both at baseline and when we inoculate a transduction peptide that activates the PI3K pathway called PTD4-PI3KAc. On the other hand, we will assess if activation of the PI3K pathway affects TDP-43 functions, such as in splicing.

There is preliminary laboratory data suggesting that TDP-43 mutations affect the PI3K pathway. For this study we are going to make use of two specific mutations, one is an ALS causative mutation that leads to a splicing gain of function (*Tardbp*^{Q331K}) and the other is a TDP-43 loss of function (*Tardbp*^{F210I}). The different mutations are explained in **section 3.1.**

The objectives of this study are:

1. Study the possible relationship between TDP-43 and the PI3K/AKT pathway.
2. Describe the behaviour of different mutations in the *Tardbp*/TDP-43 gene against treatment with a PI3K pathway activating peptide.
3. Study whether the PI3K pathway activating peptide affects the functionality of TDP-43 in splicing in endogenous genes.

3. Materials and Methods

In this study we performed experiments with immortalized Mouse Embryonic Fibroblast (MEFs) which carry points mutations in the *Tardbp* (TDP-43) gene. We used a pharmacological tool which is a PI3K/AKT pathway activating transduction peptide called PTD4-PI3KAc in MEFs. Other experiments were planned, which are discussed in **section 3.10** and that could not be carried out due to the situation generated by the Covid-19.

3.1. Mouse lines

The Acevedo-Arozena's laboratory generated and characterized new Knock-In (KI) mouse models carrying different points mutations in the endogenous mouse *Tardbp*/TDP-43 gene leading to physiological levels of expression. These mutations are characterized in that *Tardbp*^{F210I} mutation partially inhibit binding capacity of TDP-43 to RNA without affecting protein expression levels, therefore this mutation would represent a partial loss of TDP-43 function. On the other hand, *Tardbp*^{Q331K} mutation is a pathogenic mutation that it is present in ALS and/or FTD patients and previous work by the Acevedo-Arozena laboratory has shown that it leads to a gain of function of TDP-43 in RNA splicing function.

Genetically modified mice have a C57BL/6J gene background. For the generation of these mice, with respect to the F210I mutation (phenylalanine to isoleucine substitution at amino acid position 210), the Acevedo-Arozena's laboratory produced founder mice from the N-ethyl-N-nitrosourea (ENU) programmes' sperm archive and generated litters by *in vitro* fertilisation. They also generated a new KI model through genome editing using CRISPR/Cas9 with the Q331K mutation (glutamine to lysine substitution at amino acid position 331) where it has been confirmed that there is a gain of function in the splicing in these mice. The F210I mutation is found in RRM2 and the Q331K mutation is found in the glycine-rich region of TDP-43 (**Fig. 1**). With respect to the WT genotype MEFs these are different from each other, that is, the WT of the *Tardbp*^{F210I} mutation are different from the WT of the *Tardbp*^{Q331K} mutation. This is so because the WT of each mutation comes from embryos that are littermates of the mice with the different mutations.

All the information about how these mice generated is available in the bibliographic reference [10].

3.2. Mouse Embryonic Fibroblast

The used MEFs are immortalized via transfection with the T antigen form the SV40 virus. These immortalized MEFs have point mutations in the *Tardbp* gene. For the experiments we used two genotypes for each mutation: Wild-Type (WT: *Tardbp*$^{+/+}$) and Homozygotes (HOM: *Tardbp*$^{Q331K/Q331K}$ or *Tardbp*$^{F210I/F210I}$) in correspondence with the mutant genotype.

3.3. Cell Culture

For each experiment, the maintenance and culture of the cells was done the same way. Sterility conditions were always kept during work, provided by a biosafety cabinet type II.

Cells were seeded in 60mm Petri plates (Thermo Fisher Scientific) and the growth medium for maintenance of MEFs is the Dulbecco's modified Eagle's Medium (DMEM) (Thermo Fisher Scientific), supplemented with 10% Fetal Bovine Serum (FBS) and penicillin and streptomycin. All cells were maintained in an incubator at 37ºC with a percentage of relative humidity of 95% and 5% level of CO_2. Each plate contains a concentration of 250.000 cells/ml in a final volume of 4ml. Once the cells are seeded, they were allowed to growth for two days to reach 70-80% confluence prior to add the PTD4-PI3K activating peptide (see below). In each experiment there will be cells in wells incubated with the activating peptide and control wells incubated with Dimethyl sulfoxide (DMSO). We prepare two Master Mix with DMEM and peptide or DMSO. PTD4-PI3K stock is at a concentration of 10mM and for the inoculation we use a final concentration of 20µM. We added to each plate 2.5ml of the correspondent Master Mix and we incubate during 6 hours.

3.3.1. PTD4-PI3KAc peptide

PTD4-PI3KAc is a synthetic transduction peptide capable of transiently activate the PI3K pathway in mammals. The peptide sequence is the following: YARAAARQARAGSDGGpYMDMS; consisting of a transduction domain, PTD4 [YARAAARQARA], fused to a phosphopeptide containing the intracellular phosphorylated domain of the Platelet Derived Growth (PDGF) receptor C-terminus [GSDGGpYMDMS] [21, 22]. This transduction domain is that allows the peptide to enter in cell while the activator domain mimics the intracellular phosphorylated domain of the PDGF receptor C-terminus. Therefore, when the peptide enters in the cell, it produces conformational changes and finally initiates the phosphorylation cascade without the

tyrosine kinase receptor [21, 28]. In conclusion, PTD4-PI3KAc is able to activate PI3K in a ligand-independent manner.

PTD4-PI3KAc peptide was purchased from BIOSIGMA, S. L. We prepare a stock of the peptide at a concentration of 10mM in aliquots with Phosphate Buffered Saline (PBS) and DMSO. This is kept at -20ºC. As control, we also prepare aliquots of DMSO in the same way, containing in this case only PBS and DMSO.

3.4. Protein extraction

After 6 hours of incubation with the PI3K activating peptide we proceed to protein extraction. To do this, we wash the plates with PBS 1x and place the plates on ice or on a cold surface. For the protein extraction we use Urea 7M extraction buffer. We prepare a lysis buffer with Urea 7M together with protease and phosphatase inhibitors and we keep it on ice. To each plate we add 80µl of lysis buffer and we lift the cells with a cell scraper. Once this is done, we transfer the contents of the plates to Eppendorf tubes and we keep them in ice. The next step is to sonicate during 15 seconds with the conditions of cycle 1 and amplitude 80, this step is repeat 3 times for each sample and we keep them in ice. Finally, we centrifuge the samples at maximum speed for 3 minutes at 4ºC and transfer the supernatants to new Eppendorf tubes.

Protein concentration was determined by Bicinchoninic Acid assay (BCA, Sigma-Aldrich) where we use Bovine Serum Albumin (BSA) as standard. In this method, the total protein concentration is exhibited by a change in colour of the sample solution from green to purple in proportion to the protein concentration. For this, we prepare serial dilutions in a 96 well plate with the next volumes:

	1	**2**	**3**	**4**	**5**	**6**
BSA (1mg/ml)	0µl	1µl	2µl	4µl	8µl	16µl
MiliQ water	19µl	18µl	17µl	15µl	11µl	3µl
Lysis buffer (Urea 7M)	1µl	1µl	1µl	1µl	1µl	1µl
Quantification reagent	140µl	140µl	140µl	140µl	140µl	140µl

In addition to the samples for the calibration curve, we prepare the study samples where in each well will be added: 19µl MiliQ water, 1µl of the sample and 140µl

quantification reagent. The quantification reagent is prepared by making a 50/1 BSA/Copper Sulfate mixture.

Once the calibration curve is prepared, the plate is incubated at 37ºC for 30 minutes. Next, we read the absorbances at 562 nm in the plate reader Multiskan™ FC (Thermo Scientific) where we chose the BCA program. The absorbance readings are taken to an Excel file and the concentration of the different samples is calculated. We mix the appropriate amount of protein and loading buffer (Laemmli (x4)) according to the volumes indicated in the Excel and it is prepared in such a way that in each well of the Western blot gel will be loaded 35µl volume where there will be 70µg of proteins. Finally, the proteins are boiled for 5 minutes at 92ºC and next step is the Western blot.

3.5. Western blot

We employ Laemmli loading buffer (x4) (1.2ml Tris-HCl pH 6.8, glycerol, 10% Sodium Dodecyl Sulfate (SDS), 0.0006% bromophenol blue, 2% β-mercaptoethanol and MiliQ water). To load the proteins, we make two or three gels depending on whether we are going to use two antibodies (AKT and pAKT Ser-473) or three antibodies (AKT, pAKT Ser-473 and pAKT Thr-308). The gels we make are 10% with a depth of 1.5mm, first we do the separating gel (normally for two gels) which has 7.4ml autoclaved water, 3.2ml buffer low, 5.4ml acrylamide, 20µl TEMED and 40µl APS. We let this gel polymerize for about half an hour and then prepare the stacking gel which has 6.6ml autoclaved water, 2ml buffer up, 1.34ml acrylamide, 20µl TEMED and 60µl APS. This is allowed to polymerize and the gels we use are 10-well. Next, proteins are loaded onto a gel and electrophoresis is set to 70V until they are aligned on the stacking gel (~20 minutes) and then allowed to run at 120V (~1 hour). In the first stage of the electrophoresis the proteins are aligned and when they reach the separating gel, we raised the voltage and the phase begins in which the proteins are going to separate according to their size. Once the electrophoresis is completed, we made the transfer.

The transfer is made in such a way that we assembled a system, colloquially called “sandwich”, in the following order: negative pole, sponge, filter paper, gel, nitrocellulose membrane, filter paper, sponge and positive pole. When assembling this “sandwich” we must be very careful not to leave bubbles. Once assembled, the electrotransfer is carried out, for this, the different “sandwiches” are placed in a cuvette,

filled with transfer buffer 1x (Trizma Base, glycine, 96% methanol and MiliQ water) and the transfer is made at 300mA for one hour and kept on ice.

Finally, the membranes are blocked with 5% BSA in Tris-buffered saline and 0.1% Tween-20 (TBS-T) for 1 hour. After blocking, we added 0.5% BSA (5% BSA and TBS-T) with the primary antibodies: anti-AKT (1:1.000) (Cell Signaling Technology, Inc.), anti-pAKT Ser-473 1:1.000 (Cell Signaling Technology, Inc), anti-pAKT Thr-308 (1:1.000) (Cell Signaling Technology, Inc.) and anti-β-actin (1:15.000) (Proteintech Group, Inc.). Membranes with primary antibodies are incubated overnight at 4ºC. The membranes were washed with TBS-T (10 minutes x3) and then incubated with the secondary antibodies (1:5.000): Goat anti-Mouse IgG (H+L) and Goat anti-Rabbit IgG (H+L) (Invitrogen, Thermo Scientific) for 1 hour. The membranes are again washed with TBS-T (10 minutes x3) and then we develop the membranes.

3.6. Western blot quantification

The membranes are developed through chemiluminescence. We incubated each membrane with a mix of luminol and peroxidase during 4 minutes. Afterwards, we developed the membranes using of the ImageQuant™ LAS 4000 (GE Healthcare) and we quantified the proteins using the ImageQuantTL program. Once the bands of each membrane have been quantified, the data is taken to an Excel file where the statistical study of the possible results obtained will be carried out.

3.7. RNA extraction and complementary DNA synthesis

For the RNA extraction from cells we use Trizol. After treatment, the plates are washed with PBS and we proceed to extract with the Trizol reagent (Thermo Fisher Scientific). This process is carried out with care not to contaminate the samples. In the first place, we add 500µl of Trizol to samples and we incubate for 5 minutes at room temperature. Next, we add chloroform, mix them by inversion and let them settle for 3 minutes. After that, we centrifuge the samples at 12.000rmp for 5 minutes at 4ºC and collect the upper aqueous phase that contains the RNA carefully to avoid carry over of the interphase and put them in new tubes. To precipitate the RNA, we add to each sample 250µl of isopropanol, mix them and we let them for 10 minutes at room temperature. The samples are then centrifuged at maximum speed for 10 minutes and the supernatant is discarded. To the pellet we add 75% ethanol and mix it. We centrifuge the samples at 7.500rpm for 5 minutes at 4ºC and discard the supernatant.

Finally, we resuspend the pellet of the different samples in 25µl of molecular biology-grade water.

For complementary DNA (cDNA) synthesis, first, we quantify the RNAs of the different samples with a NanoDrop™ 2000/c Spectrophotometers (Thermo Scientific). Once quantified, all the RNA samples were brought to a concentration of 200ng/µl. We prepare the synthesis reaction mix using the Quanta Bio qScript cDNA synthesis kit. This reaction mix carried molecular biology water, buffer (qScript reaction), retrotranscriptase and, finally, we add the different samples of RNAs with random primers. In this step we add a synthesis control tube (without RNA). Once this is done, we start the synthesis reaction in the T100™ Thermal Cycler (Bio-Rad) with the following conditions:

- 22ºC, 5 minutes.
- 42ºC, 30 seconds.
- 58ºC, 5 minutes.

3.8. Splicing analysis by RT-PCR and quantification

To perform the RT-PCR, we are going to use two endogenous genes that are *Sortilin* and Eif4h for which we will amplify exon 18 of *Sortilin* and exon 5 of *Eif4h*:

- Mouse *Sortilin1*-Reverse: TGGCCAGGATAATAGGGACA.
- Mouse *Sortilin1*-Forward: CAGGAGACAAATGCCAAGGT.
- *Eif4h* Exon 5- Reverse: GGTCTCTGTGCTCGTTCCTC.
- *Eif4h* Exon 5-Forward: TGGATTCAGGAAAGGTGGAC.

We prepare the PCR mix using Green DreamTaq (K1081, Thermo Scientific), this mix contain molecular biology-grade water, buffer (GreenTaq x2), primers mix and cDNA of the different samples. The next step is start synthesis reaction in the T100™ Thermal Cycler (Bio-Rad) with the following conditions for 35 cycles:

- 95ºC, 3 minutes.
- 95ºC, 30 seconds.
- 60ºC, 1 minute (annealing temperature for both assays).
- 72ºC, 1 minute.
- 72ºC, 10 minutes.

We add a PCR control tube. Finally, we load PCRs in a 2% agarose gel, and visualized by ethidium bromide staining, and quantitated using the Molecular Imager System (Bio-Rad). Once the bands of each agarose gel have been quantified, the data is taken to an Excel file and we use GraphPad for the statistical study of the possible results obtained will be carried out.

3.9. Statistics

From the obtained data, we calculate in Excel the values of the mean and the standard deviation. We carry out the different statistical studies with the GraphPad Prism program to see if the results obtained from the different experiments are significant or not.

3.10. Experiments that could not be performed

Given the situation that began in the month of march in which was declared the state of alarm by the Covid-19, we were not able to do more experiments that were planned for the study. With respect to the experiments discussed in this work, it was not possible to carry out more times the experiment with the peptide with the *Tardbp*^{F210I} strain to obtain valid results to present in this work. Regarding the experiment to test the functionality of TDP-43 in splicing only two experiments could be carried out.

We wanted to conduct experiments on primary motor neurons and in cortical primary neurons from the TDP-43 KI mice used in the study (*Tardbp*^{Q331K} and *Tardbp*^{F210I} mutations). These experiments could not be performed, and we discuss them in **section 5.3**.

4. Results

4.1. Experiment with the PI3K activating peptide (PTDA-PI3KAc) and its study in Western blot

For these experiments, MEFs were grown to approximately 70-80% confluence and we inoculate PTD4-PI3KAc at concentration of 20µM into each sample and incubated for 6 hours. In each experiment, from the lysate with the extracted proteins, we made two or three gels depending on whether we used the 3 study antibodies (anti-AKT, anti-pAKT Ser-473 and anti-pAKT Thr-308), or two gels if we only used anti-AKT and anti-pAKT Ser-473 antibodies since with the anti-pAKT Thr-308 we were unable to obtain clear results due to different problems. To measure activation of the pathway, PI3K uses the ratio pAKT (Ser-473 or Thr-308)/AKT/β-actin.

Regarding the experiment carried out with both MEFs with the *Tardbp*^{Q331K} and *Tardbp*^{F210I} mutation, this experiment was carried out as a test in which only the different strains without treatment with the peptide (DMSO) were studied and it was not done in duplicate. Next, the result obtained from the Western blot is shown in **Fig 3**:

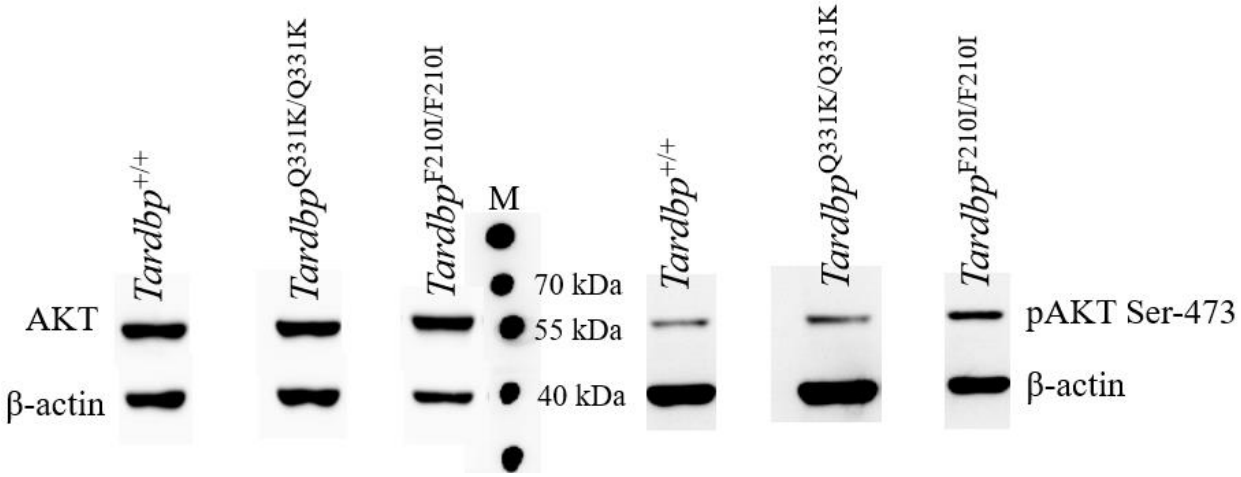

Figure 3. Western blot of a culture of MEFs with the *Tardbp*^{Q331K} and *Tardbp*^{F210I} mutation against total AKT and pAKT Ser-473. The AKT band and pAKT Ser-473 band goes out at 60kDa and the β-actin goes out at 43kDa. *Tardbp*$^{+/+}$ belongs to the WT genotype of the *Tardbp*^{F210I} mutation. All samples belong to the same gel, some bands were erased to simplify the result. M (Molecular Weight Marker).

We perform the quantification and obtain the total values and phosphorylation of the AKT effector for both treatments in these cells. We obtain a table with the quantification values of the total AKT and pAKT Ser-473 bands and it is shown below **(Table 1)**:

Table 1. Quantification of the total and phosphorylated AKT bands of *Tardbp*$^{+/+}$, *Tardbp*^{Q331K} and *Tardbp*^{F210I}. In this table we measure densitometry (arbitrary units). F210I WT (*Tardbp*$^{+/+}$); Q331K HOM (*Tardbp*$^{Q331K/Q331K}$); F210I HOM (*Tardbp*$^{F210I/F210I}$)

	AKT	Actin AKT	pAKT Ser-473	Actin pAKT Ser-473
F210I WT DMSO	39146688	35681333	5156499	7755888
Q331K HOM DMSO	41022668	42856653	6583636	10120876
F210I HOM DMSO	34604803	21588200	7926250	5714422

Since we do not have replicates (n= 1), in this case we cannot calculate the different statistics since the results would not give us significant because they have a small sample size. We calculate the pAKT/AKT/β-actin value of each sample from the values obtained in **Table 1**, and obtain the following values (arbitrary units):

- F210I WT (*Tardbp*$^{+/+}$): 0.606
- Q331K HOM (*Tardbp*$^{Q331K/Q331K}$): 0.680
- F210I HOM (*Tardbp*$^{F210I/F210I}$): 0.865

We represent these data obtained in a bar diagram shown below **(Fig. 4)**:

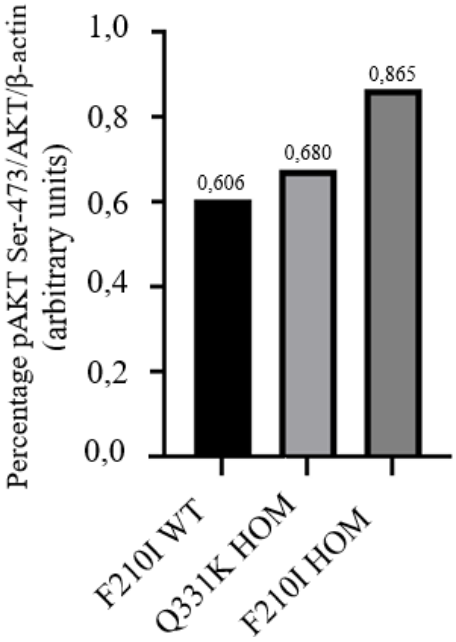

Figure 4. Histogram representing the differences between the genotypes. We can observe a potential baseline overactivation with respect to the WT genotype. F210I WT (*Tardbp*$^{+/+}$); Q331K HOM (*Tardbp*$^{Q331K/Q331K}$); F210I HOM (*Tardbp*^{F210I7F210I}).

In this **Fig. 4** we can see how there is a slight basal activation on the *Tardbp*$^{Q331K/Q331K}$ and a larger activation on the *Tardbp*$^{F210I/F210I}$ when compared to the WT genotype. Based on previous data from the laboratory, the basal *Tardbp*$^{Q331K/Q331K}$ overactivation

should be greater. Failure to not observe a larger overactivation may be due to problems during the procedure, such as not loading enough protein.

The experiments with *Tardbp*^{Q331K} strain was carried a number of times, but due to different problems, I only obtained results from one experiment to present here. The experiment was done in duplicate and we show the results with AKT **(Fig. 5)** and pAKT Ser-473 **(Fig. 6)**:

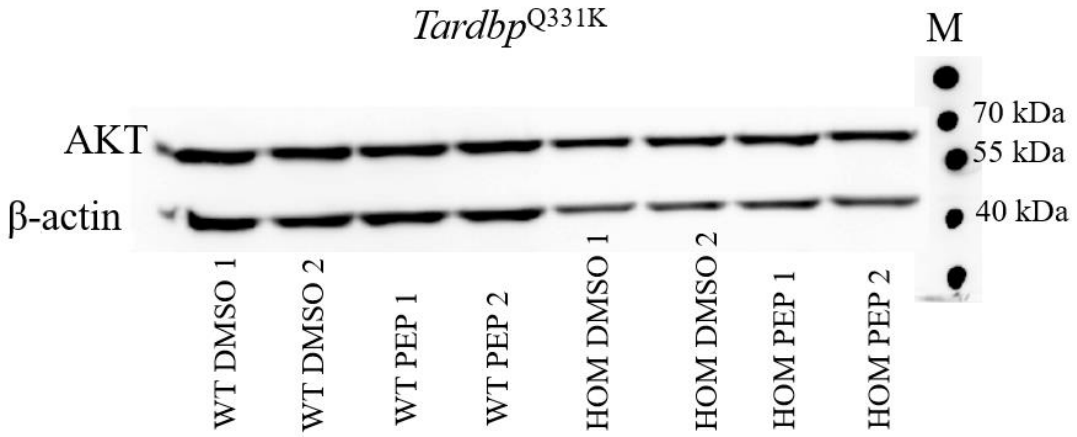

Figure 5. Western blot of a culture of MEFs with the *Tardbp*^{Q331K} mutation against total AKT. The experiment was performed in duplicate. The AKT band goes out at 60kDa and the β-actin goes out at 43kDa. WT (Wild-Type; *Tardbp*$^{+/+}$); HOM (Homozygous; *Tardbp*$^{Q331K/Q331K}$); PEP (PTD4-PI3KAc peptide); M (Molecular Weight Marker); 1 (replica 1); 2 (replica 2).

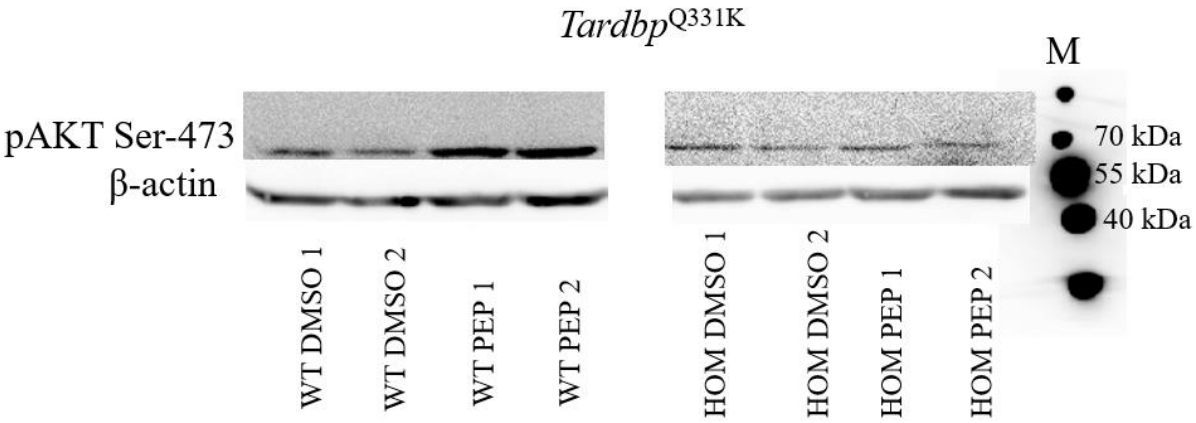

Figure 6. Western blot of a culture of MEFs with the *Tardbp*^{Q331K} mutation against pAKT Ser-473. The experiment was performed in duplicate. The pAKT Ser-473 band goes out at 60kDa and the β-actin goes out at 43kDa. All the samples belong to the same gel, they were separated in two since the samples belonging to the HOM genotype were given more contrast to better observe the bands. WT (Wild-Type; *Tardbp*$^{+/+}$); HOM (Homozygous; *Tardbp*$^{Q331K/Q331K}$); PEP (PTD4-PI3KAc peptide); M (Molecular Weight Marker); 1 (replica 1); 2 (replica 2).

In both **Fig. 5** and **Fig. 6** we can see that there is a lower concentration of proteins for the HOM (*Tardbp*$^{Q331K/Q331K}$) genotype, this may be due to errors during processing. There is less quantity since in **Fig. 5** we see that the β-actin bands corresponding to this

genotype have less intensity than those of the WT genotype, and in **Fig. 6** the bands of the pAKT Ser-473 are very poorly defined.

We started from two control groups for the WT genotype (WT (*Tardbp*$^{+/+}$) DMSO 1, WT (*Tardbp*$^{+/+}$) DMSO 2) followed by two groups that were administered the PTD4-PI3KAc peptide (WT (*Tardbp*$^{+/+}$) PEP 1, WT (*Tardbp*$^{+/+}$) PEP 2). We did the same for the mutant genotype where we first have two control groups (HOM (*Tardbp*$^{Q331K/Q331K}$) DMSO 1, HOM (*Tardbp*$^{Q331K/Q331K}$) DMSO 2) and then the groups that were administered the PTD4-PI3KAc peptide (HOM (*Tardbp*$^{Q331K/Q331K}$) PEP 1, HOM (*Tardbp*$^{Q331K/Q331K}$) PEP 2). From the Western blots we perform the quantification and obtain the total values and phosphorylation of the AKT effector for both types of genotypes and treatments in this *Tardbp*^{Q331K} mutation. We obtain a table with the quantification values of the total AKT and pAKT Ser-473 bands and it is shown below **(Table 2)**:

Table2. Quantification of the total and phosphorylated AKT bands of *Tardbp*^{Q331K}. In this table we measure densitometry (arbitrary units).

	AKT	Actin AKT	pAKT Ser-473	Actin pAKT Ser-473
Q331K WT DMSO 1	37875279	32539499	1977031	17723400
Q331K WT DMSO 2	30029288	25086390	1928652	17290355
Q331K WT PEP 1	30235864	27929855	2793546	19554063
Q331K WT PEP 2	30452278	29020908	3506216	23062019
Q331K HOM DMSO 1	20970239	13699181	1758019	12674723
Q331K HOM DMSO 2	21966182	13518789	1585702	12563198
Q331K HOM PEP 1	22063840	15963941	1735466	13765226
Q331K HOM PEP 2	19986286	15576949	1481440	17104586

Next, with the Excel program, we calculate the average and standard deviation. In this case, we average between the replicates and calculate their standard deviation. We obtained the following table **(Table 3)**:

Table 3. Statistical analysis of the results where the average and standard deviation are calculated. The mean of the data was calculated from the pAKT/AKT/β-actin values of each sample. WT (Wild-Type; *Tardbp*$^{+/+}$); HOM (Homozygous; *Tardbp*$^{Q331K/Q331K}$)

	Average	Standard deviation
Q331K WT DMSO	0.095	0.002
Q331K WT PEP	0.138	0.009
Q331K HOM DMSO	0.084	0.009
Q331K HOM PEP	0.079	0.017

Once these data are obtained, we proceed to carry out the statistical study with the GraphPad Prism program. For this study, we performed a Student's t, analysed the data with an unpaired two-tailed t test and obtained the following ***p values:***

- Q331K WT PEP vs. Q331K WT DMSO: ***p*= 0.0299 (*)**
- Q331K HOM PEP vs. Q331K HOM DMSO: ***p*= 0.6985 (n.s.)**
- Q331K HOM DMSO vs. Q331K WT DMSO: ***p*= 0.2929 (n.s.)**

Finally, we represent these data obtained in a bar diagram shown below **(Fig. 7)**:

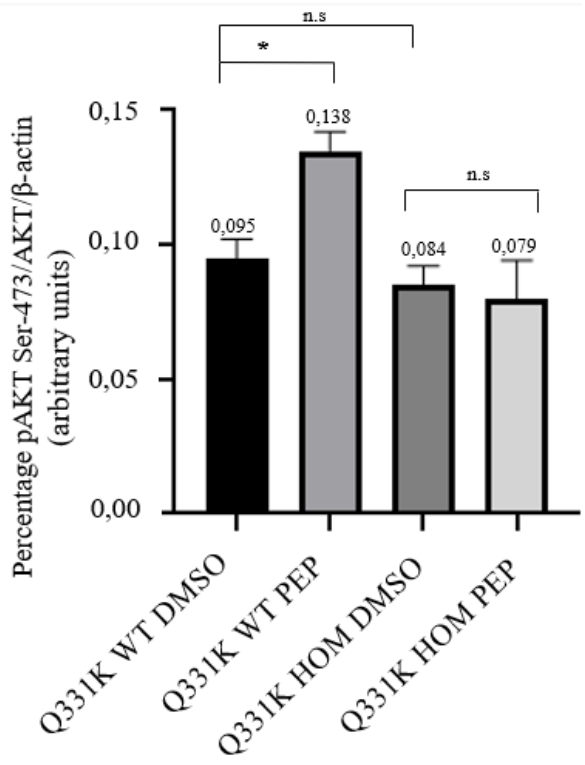

Figure 7. Histogram representing the differences between the genotypes of the *Tardbp*^{Q331K} mutation for the control groups and the groups treated with the PTD4-PI3KAc peptide. We can observe an activation by the PTD4-PI3KAc peptide in Q331K WT cells. In Q331K HOM cells, there is no activation by the peptide. * (significative); n.s. (no significative). WT (Wild-Type; *Tardbp*$^{+/+}$); HOM (Homozygous; *Tardbp*$^{Q331K/Q331K}$).

Regarding this experiment (**Fig. 7**), we can say that we see an activation of the PI3K pathway by the peptide in Q331K WT treated with PTD4-PI3KAc, and that we did not observe this in the mutant genotype.

4.2. Experiment to test the functionality of TDP-43 in splicing

For these experiments, MEFs were grown to approximately 70-80% confluence and we inoculate PTD4-PI3KAc at concentration of 20μM into each sample and incubated

for 6 hours. We wanted to test whether PTD4-PI3KAc affects the functionality of TDP-43 in splicing of some endogenous genes and the study was to made in two endogenous genes, *Sortilin* and *Eif4h*, from MEFs that present point mutations in *Tardbp*/TDP-43 and as the normal function of TDP-43 in splicing is impaired.

This experiment could only be performed twice. The first time was a test that was performed only with WT and HOM (*Tardbp*^{F210I}) MEFs and was not done in duplicate. It was performed a second time with MEFs with the *Tardbp*^{F210I} mutation and we were unable to obtain any results. Here we show the results of the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of the MEFs with the *Tardbp*^{Q331K} mutation. The experiment was done in quadruplicate for *Sortilin* **(Fig. 10)** and in triplicate for *Eif4h* **(Fig. 11)**:

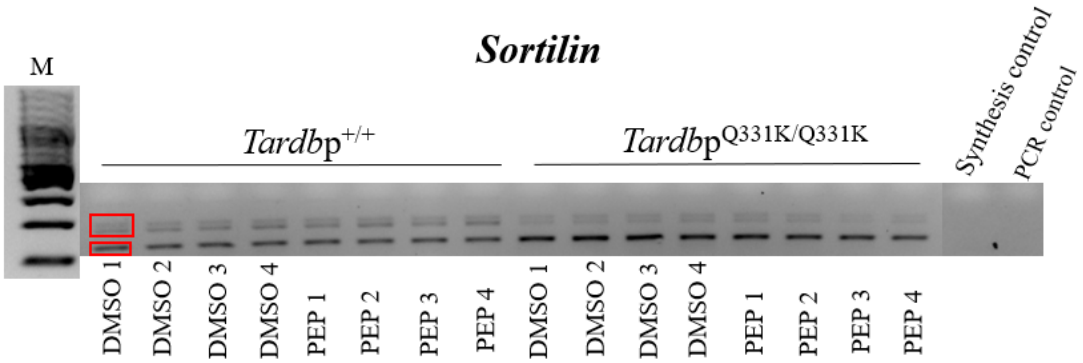

Figure 8. Quantification of RT-PCR of a culture of MEFs with the *Tardbp*^{Q331K} mutation in endogenous gene *Sortilin*. The experiment was performed in quadruplicate. The bands are quantified so that the intensity of the upper band is compared with the intensity of the lower band and the difference is calculated. We quantified the bands located in the red squares. PEP (PTD4-PI3KAc peptide); M (Molecular Weight Marker); 1-4 (replica 1-4). WT (Wild-Type; *Tardbp*$^{+/+}$); HOM (Homozygous; *Tardbp*$^{Q331K/Q331K}$).

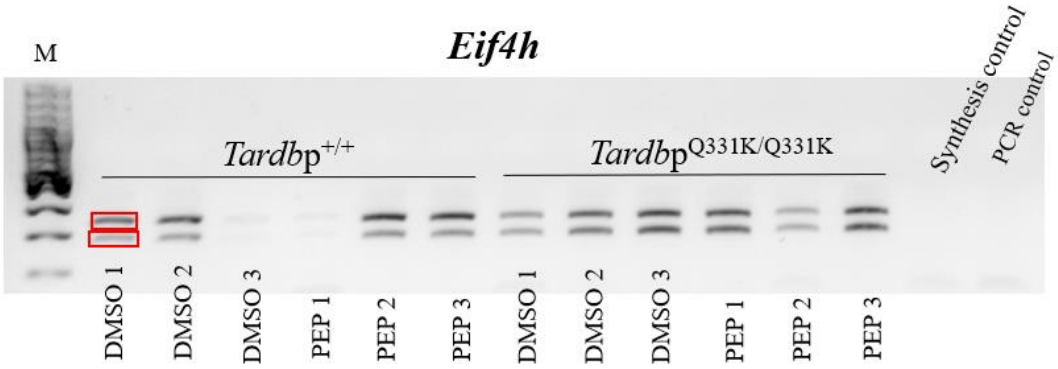

Figure 9. Quantification of RT-PCR of a culture of MEFs with the *Tardbp*^{Q331K} mutation in endogenous gene *Eif4h*. The experiment was performed in triplicate. The bands are quantified so that the intensity of the upper band is compared with the intensity of the lower band and the difference is calculated. We quantified the bands located in the red squares. PEP (PTD4-PI3KAc peptide); M (Molecular Weight Marker); 1-3 (replica 1-3). WT (Wild-Type; *Tardbp*$^{+/+}$); HOM (Homozygous; *Tardbp*$^{Q331K/Q331K}$).

In the study with *Sortilin* we started from four control groups for the wild genotype (DMSO 1, DMSO 2, DMSO 3, DMSO 4) followed by four groups that were administered the PTD4-PI3KAc peptide (PEP 1, PEP 2, PEP 3, PEP 4). We did the same for the mutant genotype where we started with four control groups (DMSO 1, DMSO 2, DMSO 3, DMSO 4) and then the groups that were administered the PTD4-PI3KAc peptide (PEP 1, PEP 2, PEP 3, PEP 4). In the study with *Eif4h* we started from three control groups for the wild genotype followed by three groups that were administered the PTD4-PI3KAc. We do the same for the mutant genotype. In this experiment we see in **Fig. 9** that the DMSO 3 and PEP 1 bands did not come out, these data do not appear when the ANOVA is performed. From the agarose gels of RT-PCR we perform the quantification for both types of genotypes and treatments in this *Tardbp*^{Q331K} mutation. To see if the data is significant or not, we performed an ANOVA with a Bonferroni correction and we found that the differences are not significant. In these results, as we can see in **Fig. 8** and **Fig. 9,** we see that the *Tardbp*^{Q331K} mutation produces a gain of function in splicing of TDP-43. This is a result that was already known, and it is published in paper [10]. What we do not get is whether the peptide affects this splicing function of TDP-43 which give us non-significant results.

Finally, we represent these data obtained in a point cloud shown below **(Fig. 10** and **Fig. 11)**:

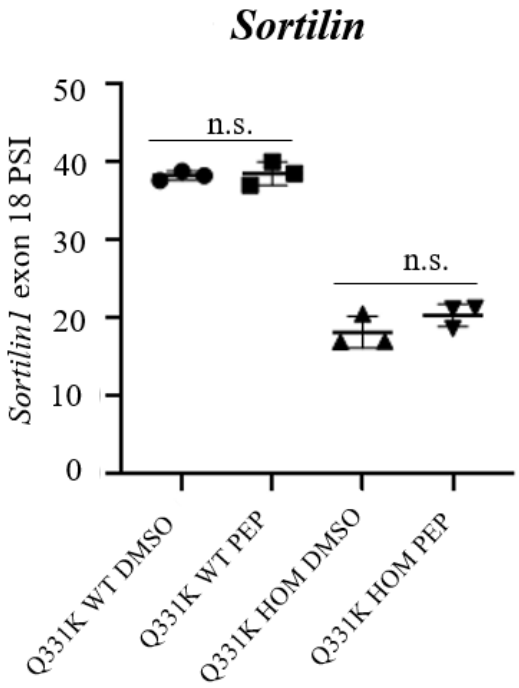

Figure 10. Point cloud of the results obtained in the endogenous gene *Sortilin* in MEFs with *Tardbp*^{Q331K}. This was done in quadruplicate, but data was removed from each group for deviating the mean. ANOVA with a Bonferroni correction was performed, and the data are not significant. n.s. (not significative), PSI (Percentage of Inclusion). WT (Wild-Type; *Tardbp*$^{+/+}$); HOM (Homozygous; *Tardbp*$^{Q331K/Q331K}$).

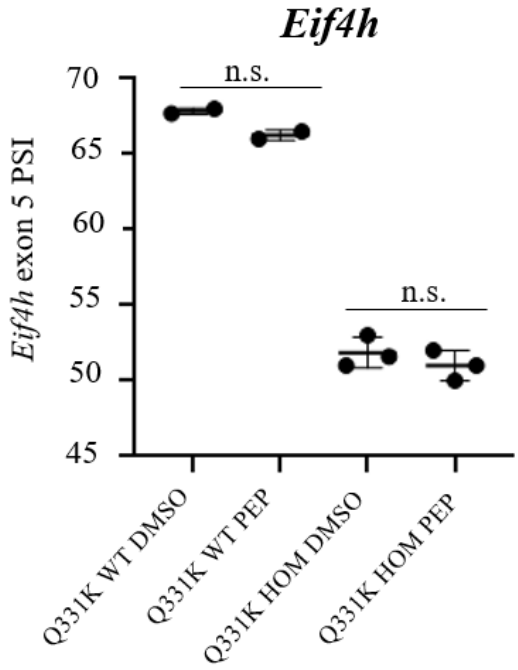

Figure 11. Point cloud of the results obtained in the endogenous gene *Eif4h* in MEFs with *Tardbp*^{Q331K}. This was done in triplicate, but of the WT genotype two data did not come out. ANOVA with a Bonferroni correction was performed, and the data are not significant. n.s. (not significative), PSI (Percentage of Inclusion). WT (Wild-Type; *Tardbp*$^{+/+}$); HOM (Homozygous; *Tardbp*$^{Q331K/Q331K}$).

5. Discussion

Different studies have shown that there is a functional interaction between TDP-43 and the PI3K pathway, it is currently being studied and there are studies that confirm that there may be an interaction between the two. The hypothesis raised in this work is that there might be a functional interaction between TDP-43 and the PI3K pathway. That is, that the PI3K pathway may somehow influence the function of TDP-43, or that mutations in TDP-43 may influence the PI3K pathway. Preliminary laboratory data suggests that TDP-43 mutations may affect the PI3K/AKT pathway at multiple levels.

5.1. Experiment with the PI3K activating peptide (PTDA-PI3KAc) and its study in Western blot

This experiment was performed to study the activation of the PI3K/AKT pathway using the PTD4-PI3KAc peptide. They were performed in MEFs with two endogenous point mutations in *Tardbp*/TDP-43. These experiments were intended to be performed in primary cultures of cortical neurons from the KI mice used in this study to validate the results in this cell type as they are key to the study of ALS. Given the situation that occurred with Covid-19, these experiments on primary neurons were not possible to be performed.

In these experiments we checked the total protein (AKT) levels as well as the levels of activated AKT via phosphorylation at Ser-473. In the first result presented here (**Fig. 3**) we observed a potential baseline overactivation of both the mutant genotypes (*Tardbp*$^{Q331K/Q331K}$ and *Tardbp*$^{F210I/F210I}$) when compared to the WT genotype (*Tardbp*$^{+/+}$). In previous data from the laboratory on the *Tardbp*$^{Q331K/Q331K}$ mutation, it was shown that this overactivation should be much greater than that obtained in this work, and this may be due to problems during the development of the experiment. As we can see in **Fig. 3** both in the mutant genotype *Tardbp*$^{Q331K/Q331K}$ and in *Tardbp*$^{F210I/F210I}$ we see the bands of the pAKT Ser-473 more saturated compared to the band of the genotype WT (*Tardbp*$^{+/+}$). Given that we have a small sample size (n= 1), we cannot say that this result is significant, that is, we cannot conclude that the potential baseline overactivation that we see both in **Fig. 3** as in **Fig. 4** is always so since we cannot statistically study whether this result is significant or not. With respect to **Fig. 5** and **Fig. 6**, we did not observe this baseline overactivation in the mutant genotype (*Tardbp*$^{Q331K/Q331K}$), this may be due to the fact that in this experiment less protein of the

mutant genotype was loaded. It may be for this reason that the baseline overactivation that has been seen in preliminary laboratory data cannot be observed.

The last result presented was the only one that indicated that there are significant differences when comparing the means in the cells with the WT genotype *Tardbp*$^{+/+}$ of the control group (DMSO) and with the treatment with the PTD4-PI3KAc peptide (PEP) (**Fig. 7**) of MEFs cells with the *Tardbp*^{Q331K} mutation. This indicates that there has been an increase in phosphorylated AKT in the WT strain treated with the peptide compared to the WT strain used with control (DMSO), which means that activation of the PI3K/AKT pathway has occurred following treatment with the peptide. This only occurs in the WT genotype since with the homozygotes no activation is seen by the peptide, with both the homozygous group and the group treated with PTD4-PI3KAc maintaining similar values. These data indicate that the peptide PTD4-PI3KAc in the WT genotype (*Tardbp*$^{+/+}$) produces an activation of the PI3K pathway while in the mutant genotype (*Tardbp*$^{Q331K/Q331K}$) it does not produce this activation. Therefore, this mutation (*Tardbp*^{Q331K}) causes the PI3K pathway to not respond to activation when we inoculate PTD4-PI3KAc, so that its activation in response to external stimuli could not be modulated and would lose its neuroprotective potential. Finally, with respect to the *Tardbp*^{F210I} mutation that represents a loss of function of TDP-43, what we might have expected is that by inoculating the peptide, TDP-43 it would recover part of its function. This could be not true since as seen in **Fig. 3** and **Fig. 4** we see that in the *Tardbp*^{F210I} mutation the PI3K pathway is activated without the treatment of the peptide (DMSO), with the data we have we cannot conclude anything about what would happen in this mutation.

5.2. Experiment to test the functionality of TDP-43 in splicing

Changes in splicing function have been proposed as a possible crucial mechanism in the pathogenesis of ALS [10]. This experiment was performed to test whether the PTD4-PI3KAc peptide affects the functionality of TDP-43 in the splicing of some endogenous genes. Initially, it was performed in MEFs, but the possible results we obtained were going to be validated in primary cultures of cortical neurons from the KI mice used in this study. Given the situation that occurred with Covid-19, these experiments on primary neurons were not possible to perform.

We performed a RT-PCR and what we were going to observe is if the addition of the PTD4-PI3KAc peptide modulates or modifies in any way the way in which TDP-43 includes or excludes exons, that is, the splicing function of TDP-43. In the case of these two endogenous genes used for this study (*Sortilin* and *Eif4h*), when TDP-43 presents a loss of function (*Tardbp*^{F210I}), inclusion of exon 18 (in *Sortilin*) and exon 5 (in *Eif4h*) is promoted. However, when TDP-43 present a gain of function (*Tardbp*^{Q331K}) exclusion of exon 18 (in *Sortilin*) and exon 5 (in *Eif4h*) is promoted [10]. These exons that are included or excluded are called "cassettes exons" and this is dependent on the function of TDP-43. In the results of the experiment carried out presented in this work, we did not obtain any significant differences using *Tardbp*$^{+/+}$ and *Tardbp*$^{Q331K/Q331K}$ MEFs (**Fig. 10** and **Fig. 11**), and therefore, under these conditions, we cannot conclude that PI3K activation using the activating peptide PTD4-PI3KAC affects TDP-43 splicing function.

5.3. Experiments that could not be performed

Given the situation that occurred with Covid-19, these experiments on primary neurons were not possible to perform. These experiments were intended to be performed on primary cultures of cortical neurons from the KI mice used in this study.

5.3.1. Experiment with the PI3K activating peptide (PTDA-PI3KAc) and its study in Western blot

Regarding the experiment performed with the peptide the experiment made with the *Tardbp*^{F210I} strain (WT and HOM) was carried out a number of times, but in no one could we obtain a clear result since different problems occurred during the experiments. At the beginning of the experiments made in the laboratory, problems arose, for example, that we did not know if the antibodies were working correctly (data of these experiments not shown in this memory). Another failure that occurred during the experiments was my lack of laboratory experience and various problems during the procedure. I consider that the problem that I did not manage to solve with respect to the experiments carried out with the TardbpF210 strain was that when inoculating the peptide, it did so when the cells were not at the ideal confluence for this study, which is 70-80%.

I planned to do all the results in primary cortical neurons to validate if the results from MEFs from both TDP-43 mutant genotypes.

5.3.2. Study of TDP-43 inclusions formation

TDP-43 is the main component of the characteristic aggregates in the affected motor neurons of ALS patients and is the main component of the characteristic cytoplasmic pathological inclusions of FTLD (**section 1.1**). Since this formation of cytoplasmic inclusions and the normal delocalization of TDP-43 from the nucleus represents the most common neuropathological marker for ALS, our study hypothesis for this experiment is that the PI3K pathway regulates the nuclear-cytoplasmic transport of TDP-43 and PTD4-PI3KAc could modulate this transport in some way and affect the formation of these inclusions.

We wanted to study how the use of the PI3K pathway activating peptide (PTD4-PI3KAc) could affect the formation of the cytoplasmic inclusions of TDP-43 and their delocalization in primary cortical neurons with point mutations in *Tardbp*/TDP-43. For this we were going to carry out experiments on this cell type where we were going to inoculate the peptide. We were going to perform immunofluorescences against TDP-43 and obtain the possible results seeing the possible differences that may exist between TDP-43 located in the nucleus and TDP-43 forming cytoplasmic inclusions in the WT and HOM genotypes (*Tardbp*$^{Q331K/Q331K}$ and *Tardbp*$^{F210I/F210I}$).

Neurons with the *Tardbp*^{Q331K} mutation have a phenotype which we can potentially modulate since part of TDP-43 leaves the nucleus for having this mutation. The plan was to use the PTD4-PI3KAc peptide to ask if PI3K activation affects TDP43 nuclear-cytoplasmic transport since there are studies that show that the PI3K signaling pathway regulates this transport [30].

5.3.3. Prosynaptogenic program and spinogenesis study

Previous studies obtained in *Drosophila melanogaster* and rodents [21, 22] show the critical role that PI3K has in the regulation of synapse number and spinogenesis producing functional and behavioural effects *in vivo* [21, 22, 24].

PTD4-PI3KAc increases the number of synapses both *in vivo* and *in vitro* and the role for PI3K signaling in controlling the number of synapses in mammals has been demonstrated [21]. In the PI3K pathway when AKT is activated it inhibits GSK3. GSK3 has activity in axon formation and in neuritogenesis among other functions [22]. In addition to studying the PI3K/AKT pathway, if we also study GSK3 we could establish the possible causes that it produces in certain neurodegenerative diseases since it is

known that overexpression of GSK3 reduces the number of synapses (see **section 1.2**, Introduction).

Our hypothesis proposed for this study is that, since the loss of synapses is related to neurodegeneration, the use of PTD4-PI3KAc could prevent this neurodegeneration in some way since we can induce synaptogenesis through activation of the PI3K/AKT pathway.

We wanted to study how these primary cortical neurons reacted *in vitro* to the prosynaptogenic effect of the PI3K/AKT pathway. For this we were going to carry out primary cultures of cortical neurons which would be allowed to grow and when they are in the growth period, the peptide will be inoculated. These cultures would have been grown for approximately 21 days and then immunofluorescence would have been made against Bassoon and Synapsin (both are presynaptic proteins [22]. As a control, cultures would be made to which instead of inoculating PTD4-PI3K we would inoculate the same concentration of DMSO. To visualize the spines, a transfection will be performed with a plasmid encoding the GFP protein fused to chick β-actin under the control of the platelet-derived growth factor promoter region [22]. Finally, images will be visualized under a confocal microscope and spines will be counted.

Based on our results from MEFs, from these experiments we might expected to see a prosynaptogenic effect on the WT genotype when we inoculated the peptide, but no response from the mutant genotype (HOM *Tardbp*^{Q331K}). The fundamental thing is that we wanted to measure the prosynaptic effect of PI3K/AKT pathway activation on cortical neurons in these KI mice and see if it actually worked or not because this is a functional measure to see if mutations in TDP-43 functionally affect the PI3K/AKT pathway.

6. Conclusions

The conclusion that we can draw from the limited work presented here is the following:

1. The Q331K TDP-43 mutation is affecting the activation of the PI3K pathway since the use of a PTD4-PI3KAc peptide does not yield any response in mutant MEFs.

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