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Recapitulation of Pathological TDP-43 Features in Immortalized Lymphocytes from Sporadic ALS Patients

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal progressive neurodegenerative disorder of still unknown etiology that results in loss of motoneurons, paralysis, and death, usually between 2 and 4 years from onset. There are no currently available ALS biomarkers to support early diagnosis and to facilitate the assessment of the efficacy of new treatments. Since ALS is considered a multisystemic disease, here we have investigated the usefulness of immortalized lymphocytes from sporadic ALS patients to study TDP-43 homeostasis as well as to provide a convenient platform to evaluate TDP-43 phosphorylation as a novel therapeutic approach for ALS. We report here that lymphoblasts from ALS patients recapitulate the hallmarks of TDP-43 processing in affected motoneurons, such as increased phosphorylation, truncation, and mislocalization of TDP-43. Moreover, modulation of TDP-43 by an in-house designed protein casein kinase-1δ (CK-1δ) inhibitor, IGS3.27, reduced phosphorylation of TDP-43, and normalized the nucleo-cytosol translocation of TDP-43 in ALS lymphoblasts. Therefore, we conclude that lymphoblasts, easily accessible cells, from ALS patients could be a useful model to study pathological features of ALS disease and a suitable platform to test the effects of potential disease-modifying drugs even in a personalized manner.

Keywords ALS · Lymphoblasts · TDP-43 · CK-1δ

Introduction

ALS is a neurodegenerative disorder characterized by progressive degeneration of upper and lower motor neurons leading to weakness of affected muscles and eventually to muscular paralysis. Most cases of ALS are sporadic (sALS); however, about 5% of cases have a family history of ALS (fALS). About 20% of cases with autosomal dominant fALS and 2% of patients with sALS show mutations in superoxide dismutase (SOD1) gene [1]. Other genes causing fALS are *VCP*, *TARDBP*, *SQSTM1*, *C9ORF72*, *OPTN*, and *UBQLN2* [2–12]. The physiological functions and properties of these genes can be grouped according to their involvement in (1) protein quality control, (2) cytoskeletal dynamics, (3) RNA homeostasis, and (4) DNA damage response [13].

A hallmark of both sporadic and familial ALS is the presence of abnormal protein aggregates in the cytoplasm of neuronal and glial cells, which contain the trans-activating response region DNA binding protein of 43 kDa, known as TDP-43, as the main component [14]. The pathogenic role of TDP-43 in ALS was reinforced by the finding of mutations

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in the *TARDBP* gene in some ALS patients [15]. TDP-43 is a highly conserved 414-amino acid nuclear protein and ubiquitously expressed [16]. TDP-43 contains different RNA recognition motifs, such as a nuclear localization sequence (NLS), a nuclear export signal [17], and a glycine-rich C terminus that mediates protein–protein interactions [18]. The C terminus of TDP-43 is the site of the majority of ALS causing mutations identified in human disease, indicating this region of the protein may mediate pathological protein modifications and aggregation. The C terminus of TDP-43 is truncated, phosphorylated, and forms inclusions in tissues affected by ALS [2, 19, 20]. TDP-43 pre-dominantly resides in the nucleus but is capable of nucleo-cytoplasmic shuttling [17]. Indeed, abnormal localization of TDP-43 in the cytoplasmic compartment, either in its native form or post-translationally modified forms, had been extensively identified by histochemical studies on brain cortex and spinal cord of ALS patients [21]. The phosphorylation of TDP-43 in tandem serine 409 and 410 characterizes most TDP-43 proteinopathy cases [20]. Protein casein kinase-1 (CK-1) is considered the main kinase able to phosphorylate TDP-43 in vitro and in vivo [22, 23]. Moreover, the activity of CK-1 was found to be upregulated in spinal cord tissue in ALS [24] and therefore could play a role in disease pathogenesis.

There is increasing evidence that ALS has to be regarded as multisystem degeneration as it also presents non-motor symptoms [25, 26]. In particular, peripheral blood mononuclear cells (PBMCs) or skin fibroblasts display traits of the disease such as down-regulation of Bcl-2 [27, 28], increased nitrative stress [28], intracellular calcium dysregulation [29], glutamatergic dysfunction [30], and mitochondrial dysfunction [31, 32]. Regarding TDP-43 proteinopathy, mislocalization of TDP-43 in circulating lymphomonocytes was reported from ALS patients carrying *TARDBP* mutations as well as in some of sALS individuals [33], suggesting that cytoplasmic TDP-43 levels in peripheral cells could be a plausible biomarker for monitoring disease progression.

This work was undertaken to further study TDP-43 pathological features in immortalized lymphocytes from sALS patients. To this end, phosphorylation status of full-length and truncated TDP-43, as well as the subcellular localization of TDP-43 were determined by Western blotting and confocal microscopy. Here, we report that lymphoblastoid cell lines (LCLs) from ALS patients recapitulate the pathogenic mechanisms thought to be involved in the neurodegenerative process in ALS, such as increased phosphorylation and truncation of TDP-43 as well as altered subcellular distribution of TDP-43. In addition, we investigated the effects of one in-house designed inhibitor of CK-1δ, IGS3.27 [23], modulating TDP-43 phosphorylation and processing of TDP-43. It is concluded that LCLs from sALS patients could serve as a human-cell-based screening platform for drug discovery.

Materials and Methods

Materials

All components for cell culture were obtained from Invitrogen (Barcelona, Spain). PVDF (polyvinylidene difluoride) membranes for Western blots were purchased from Bio-Rad (Richmond, CA, USA). The enhanced chemiluminescence (ECL) system was from Amersham (Uppsala, Sweden). The *N*-benzothiazolyl-2-phenyl-acetamide derivative, CK-1δ inhibitor, IGS3.27, was synthesized in our laboratory as previously described [23]. Antibodies against human TDP-43 (10782-2-AP) and phospho(409/410)-TDP-43 (22309-1AP) were obtained from Proteintech (Manchester, UK). Antibodies against β-actin (sc-81178), α-tubulin (sc-23948), and GAPDH (sc-25778) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and anti-Lamin B1 was purchased from Calbiochem (Billerica, MA, USA).

Cell Lines

Lymphoblastic Cell Lines

Peripheral blood samples of all the individuals enrolled in this study were collected after written informed consent of the patients or their relatives (demographic information is presented in Table 1) to establish the lymphoblastoid cell lines (LCLs) as previously described (Ibarreta et al. 1997), by infecting peripheral blood lymphocytes with the Epstein Barr virus (EBV). All study protocols were approved by the Hospital Doce de Octubre and the Spanish Council of Higher Research Institutional Review Board and are in accordance with National and European Union Guidelines. All patients were diagnosed by applying the revised El Escorial criteria [34]. All patients were negative for SOD1 mutations, and only one of them have a hexanucleotide expansion (1894 repeats) in the C9orf72 gene.

Table 1 Demographic and clinical characterization of subjects included in this study

	Control ($n = 6$)	ALS ($n = 8$)
Gender (M/F)	(2/4)	(5/3)
Family history	No	No
Age (years ± SD)		
At sampling	62 ± 7	62 ± 11
At onset	NA	60 ± 12
Site of onset (*n*)		
Bulbar	NA	4
Limb	NA	3
Respiratory	NA	1

M male, *F* female

Lymphoblastoid cells lines were grown in suspension in T flasks in an upright position, in approximately 8 ml of RPMI-1640 medium that contained 2 mM L-glutamine, 100 μg ml^{-1} streptomycin/penicillin and 10% (*v*/*v*) fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 incubator at 37 °C. Fluid was routinely changed every 3 days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

Immunoblotting Analysis

Cells were collected by centrifugation, washed with PBS, and total protein extracts were obtained by lysing them as previously described [35]. To separate the cytosolic and nuclear fractions, cells were harvested, washed in PBS, and then lysed in ice-cold hypotonic buffer as previously described [36]. After extraction on ice for 15 min, 0.5% Nonidet P-40 was added and the lysed cells were centrifuged at 4000 rpm for 10 min. Supernatants containing cytosolic proteins were separated and pellets were resuspended in hypertonic buffer to lysate the nucleus [36]. The protein content of the extracts was determined by the Pierce BCA Protein Assay kit (Thermo Scientific). Equal amounts of proteins were resolved by SDS–polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes and immunodetected, as previously described [35]. The following primary antibodies were used: TDP-43 (1:1000), phospho-(S409/410)–TDP-43 (1:500), β-actin (1:500), α-tubulin (1:1000), and Lamin B1 (1:1000). Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Bio-Rad) and detected with a chemiluminescent substrate detection system ECL. Relative band intensities were quantified using Image Studio Lite software (LI-COR Biotechnology, NE, USA).

Immunofluorescence

Cells ($1 \times 10^6 \times ml^{-1}$) were fixed for 30 min in 4% paraformaldehyde in PBS and blocked and permeabilized with 0.5% Triton X-100 in PBS–0.5% BSA for 60 min at room temperature. Cells were attached to poly-L-lysine-coated coverslips using the Cytospin centrifuge at 700 rpm for 7 min before they were incubated overnight with anti-TDP43 polyclonal antibody. After removing the primary antibody, cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit antibody. For nuclear staining, the preparations were mounted on ProLong® Gold Antifade Reagent with DAPI (Thermo Fisher) allowing nuclear visualization. High-resolution images were acquired for ~ 30 cells per group in $n = 3$ independent experiments using a confocal microscope Zeiss 510 equipped with a META detection system and a ×63 oil immersion objective. Cytosolic TDP-43 levels per cell were quantified using the *Volocity* software (PerkinElmer, Waltham, MA).

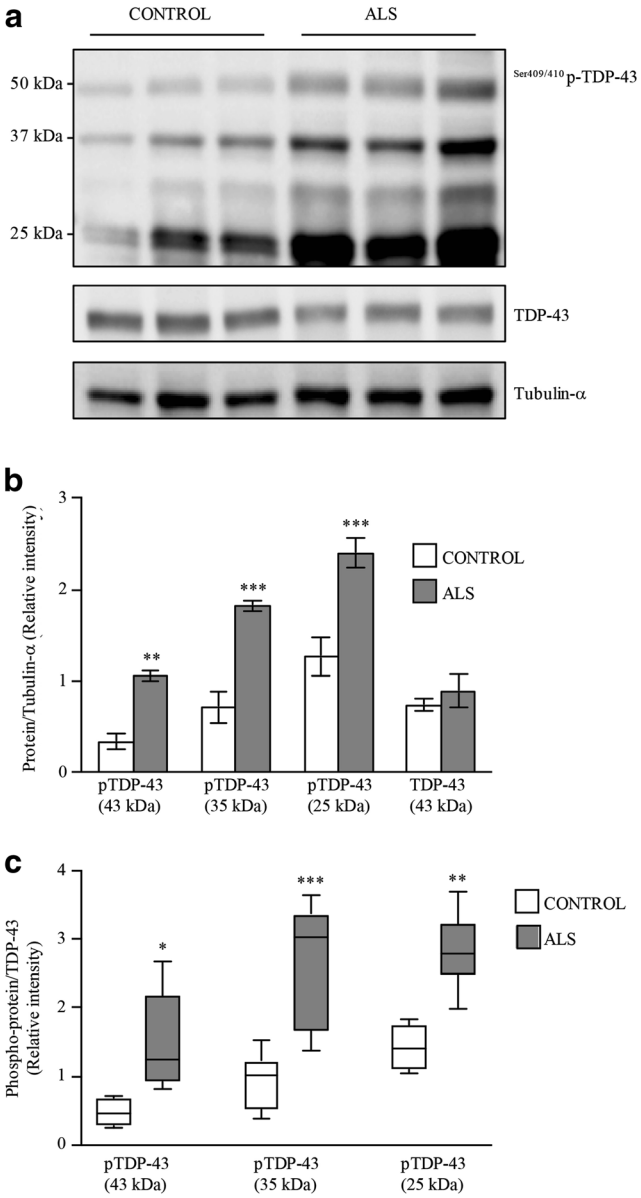

Fig. 1 Cellular content and phosphorylation status of TDP-43 in lymphoblasts from control and ALS patients. **a** Immortalized lymphocytes from control and sporadic ALS individuals were seeded at an initial density of $1 \times 10^6 \times ml^{-1}$ in RPMI containing 10% FBS; 24 h later, cells were collected and processed to detect phospho- and total-TDP-43 protein levels by Western blotting. Representative immunoblots are shown. Tubulin-α was used as loading control. **b** Levels of phospho-TDP-43 (full length and truncated) and total TDP-43. Data represent mean ± SEM of different experiments carried out with lymphoblastoid cell lines from six control individuals and eight ALS patients. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$ significantly different from control cells). **c** Box plots showing the ratios of phospho-TPP-43/total TDP-43

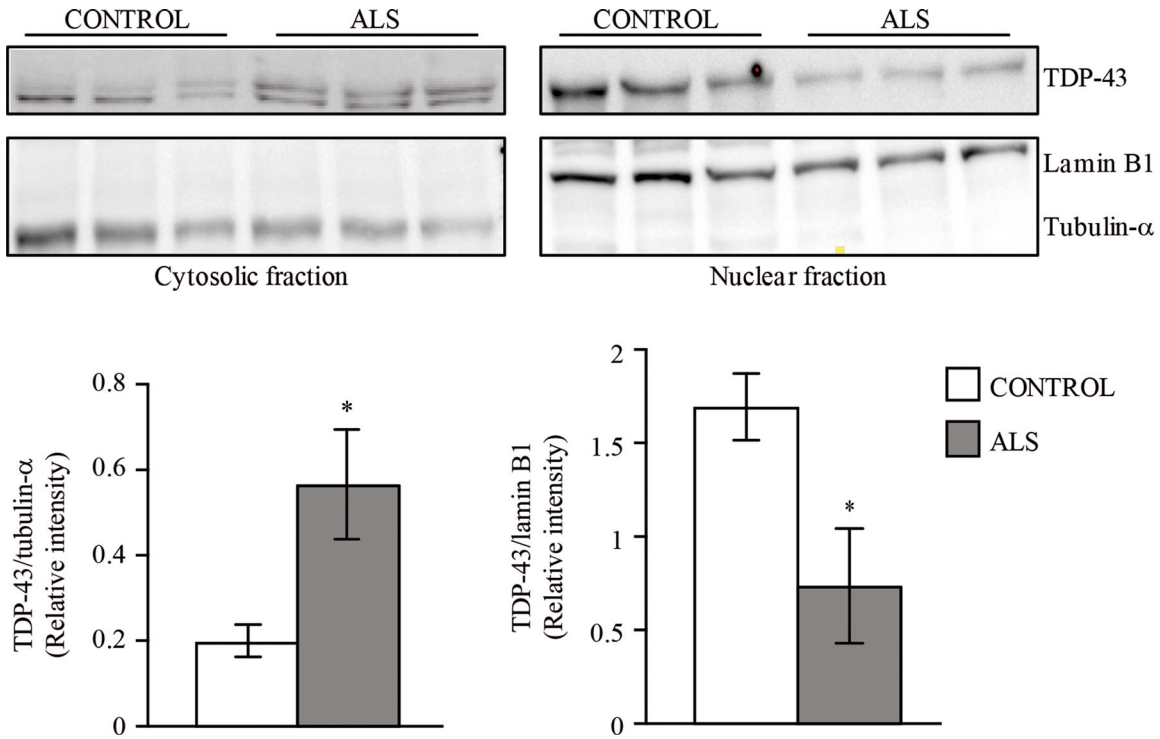

Fig. 2 Subcellular localization of TDP-43 in control and ALS lymphoblasts. Lymphoblasts from control and ALS patients were seeded and incubated as above. After harvesting, lymphoblasts were lysed to obtain cytosolic and nuclear fragments that were analyzed by Western blotting. Tubulin-α and Lamin B1 antibodies were used as loading and purity control of the cytosolic and nuclear fractions, respectively. A representative experiment is shown. Densitometric analyses, shown below, represent the mean ± SEM of different observations carried out in four cell lines from each group (*$p < 0.05$, significantly different from control cells;)

Statistical Analysis

Statistical analyses were performed with Graph Pad Prism 6 (La Jolla, CA, USA). All the statistical data are presented as mean ± standard error of the mean (SEM). Normality was checked with the Shapiro–Wilk test. Parametric tests were therefore used in the statistical analysis. Statistical significance was estimated by Student's *t* test or by analysis of variance (ANOVA) followed by the Fisher's LSD test for multiple comparisons. A value of $p < 0.05$ was considered significant.

Results

TDP-43 Phosphorylation in Control and ALS Lymphoblasts

TDP-43 phosphorylation was assessed by Western blotting using a phospho-specific (Ser 409/410) anti-TDP-43 antibody in lymphoblasts from control and ALS patients. As shown in Fig. 1, this antibody recognizes three major bands corresponding to the sizes 43, 35, and 25 kDa. It can be observed that there is a clear increase in the phosphorylation of the full length as well as in the truncated TDP-43 fragments in lymphoblasts from ALS patients (Fig. 1a, b). In contrast, we did not find differences in total TDP-43 levels between control or ALS cells (Fig. 1b). Figure 1c summarizes the ratio pTDP-43/TDP-43 (full length and truncated) obtained in all the LCLs used in this work.

Subcellular Distribution of TDP-43 in Control and ALS Lymphoblasts

Since the balance between nuclear and cytosolic content of TDP-43 is thought to be disrupted in the nervous system (SN) of ALS patients, we seek to elucidate whether TDP-43 also accumulates in lymphoblasts from ALS patients. For this purpose, we performed nuclear and cytoplasmic fractionation and analyzed the protein extracts by Western blot analysis. As shown in Fig. 2, there was an increase in the cytosolic content of TDP-43 in ALS cells compared with control lymphoblasts, whereas opposite changes occur in nuclear TPD-43. Similar results were obtained by immunofluorescence analyzing cytosolic TDP-43 levels in ALS cells compared to lymphoblasts from controls (Fig. 3).

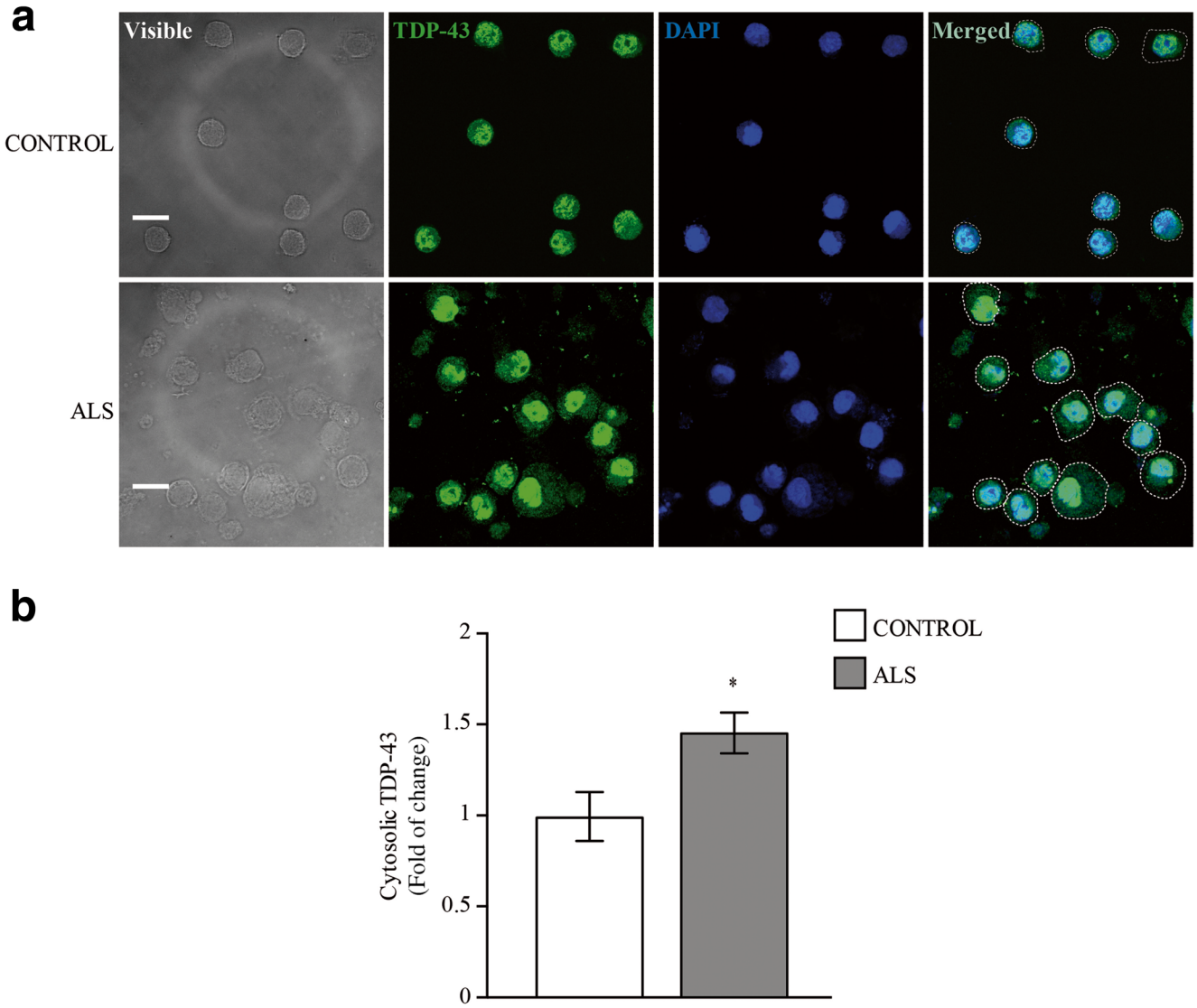

Fig. 3 Immunofluorescence analysis of the subcellular localization of TDP-43 in control and ALS subjects. Lymphoblasts were seeded at 1×10^6 cells × ml^{-1} and incubated for 24 h. TDP-43 protein localization was assessed by confocal laser scanning microscopy. Cells were stained with anti-TDP-43 antibody followed by secondary antibody labeled with Alexa Fluor 488. DAPI was included in the mounting media to stain the nucleus. Merged images show that ALS cells present higher cytosolic localization of TPD-43 protein. Dashed lines marked out the cellular area using visible light. Scale bars = 15 μM. **b** Quantification of TDP-43 cytosolic localization in lymphoblasts from ALS patients compared to controls. Data are expressed as mean ± SEM for experiments carried out with four different cell lines for each group. *$p < 0.01$ significantly different from control cells

Effects of IGS 3.27 on Phosphorylation Status of TDP-43 in Control and ALS Lymphoblasts

The isoform δ of CK-1 (CK1-δ) is known to phosphorylate different serine and threonine sites on TDP-43 protein, and thus qualifies as a potential target for ALS treatment [22]. For this reason, we sought to evaluate the effects of a selective in-house designed inhibitor of CK-1δ [23], IGS3.27. For these experiments, cells were incubated in the absence or presence of 5 μM inhibitor for 24 h. This concentration was chosen based in previous work from this laboratory [37]. Figure 4 shows that IGS3.27 was able to partially block the enhanced TDP-43 phosphorylation of TDP-43 in ALS lymphoblasts, without affecting total levels of non-phosphorylated TDP-43. Moreover, treatment with IGS3.27 decreased the cytosolic TDP-43 levels, preventing the cytosolic accumulation of TDP-43 in ALS lymphoblasts, without affecting significantly TDP-43 levels in control cells (Fig. 5). Finally, we sought to evaluate the effects of IGS3.27 on subcellular localization of TDP-43. As shown in Fig. 5, IGS 3.27 treatment had no appreciable effects on control cells, but it was able to decrease the accumulation of cytosolic TDP-43 in ALS lymphoblasts, restoring in addition normal nuclear levels of this protein. Together, these results suggest that IGS3.27 prevents the

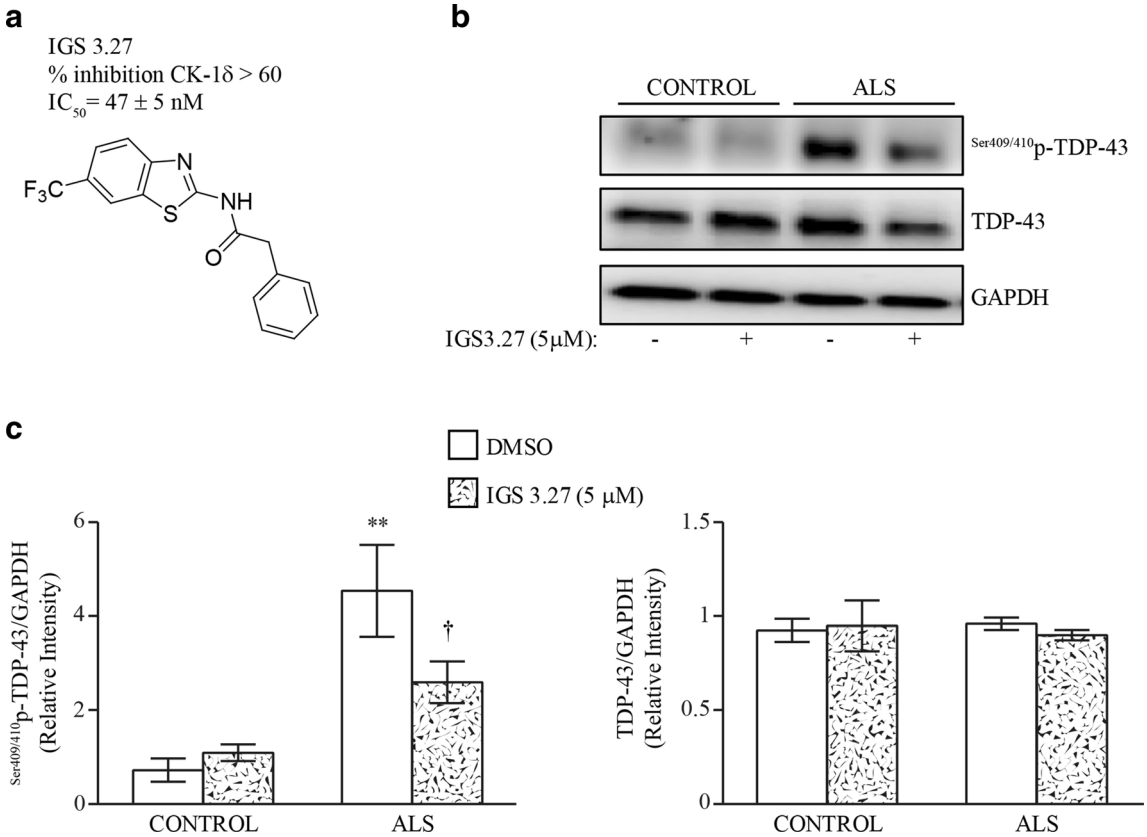

Fig. 4 Effects of the CK-1δ inhibitor, IGS 3.27, on TDP-43 phosphorylation. **a** The molecular structure and % of inhibition of CK-1δ and IC_{50} are shown. **b** Immortalized lymphocytes from control and sporadic ALS individuals were seeded at an initial density of $1 \times 10^6 \times ml^{-1}$ in absence or presence of IGS3.27 (5 μM). 24 h after drug addition, cells were harvested and processed for Western blotting analysis. The image represents an immunoblot showing the effect of the CK-1δ inhibitors decreasing the phosphorylation status of TDP-43 in control and ALS lymphoblasts. **c** The plots below represent quantified 43 kDa bands of phosphorylated TDP-43 (left panel) or TDP-43 (right panel) normalized by GAPDH. Densitometric analyses represent the mean ± SEM of different observations carried out in three cell lines from each group (**$p < 0.01$ significantly different from control cells, †$p < 0.05$ significantly different from untreated cells)

pathological exit of TDP-43 from the nucleus in lymphoblasts from ALS patients.

Discussion

ALS is a multisystemic disease in which pathological processes extend beyond the CNS [38]. In particular, PBMCs from ALS patients were reported to display traits of the disease including oxidative stress, Ca^{2+} fluxes, or mitochondrial dysfunction (29–32). Recent studies have used this type of cells to look for transcriptional alterations to assess pathogenesis in ALS, and for example, an expression analysis of protein homeostasis pathways in the PBMCs of sALS patients had revealed alterations in the transcription of genes involved in the proteasome and autophagy processes [39]. Here, we show that immortalized lymphocytes from sporadic ALS patients recapitulate the TDP-43 abnormalities observed in neuronal cells of ALS cases. TDP-43 pathological processing includes translocation from nucleus to cytoplasm, truncation, and hyperphosphorylation. These observations support the usefulness of lymphoblasts from ALS patients to mechanistic studies, particularly in sporadic cases, for which no experimental models are available.

By using a phosphorylation-dependent antibody, we detected quantitative changes in phosphorylated full-length and truncated TDP-43 protein in lymphoblasts from sporadic ALS patients. In contrast, no significant changes were found in non-phosphorylated TDP-43 levels on ALS cells when compared with control lymphoblasts. Both full-length pTDP-43/TDP-43 and fragmented pTDP-43/TDP-43 ratios are higher in ALS than in control cells. The changes in TDP-43 phosphorylation were accompanied by alteration in the balance between cytosolic and nuclear localization of the TDP-43 protein. Our results indicate that there is a significant reduction in the levels of nuclear TDP-43 in ALS lymphoblasts, together with TDP-43 accumulation in the cytosolic compartment. As far as we know, this is the first report showing increased levels of full and truncated pTDP-43 in lymphoblasts derived from sALS patients. Our results confirm a previous report showing the accumulation of TDP-43 in the cytoplasm of circulating lymphomonocytes [33]. Together, these observations

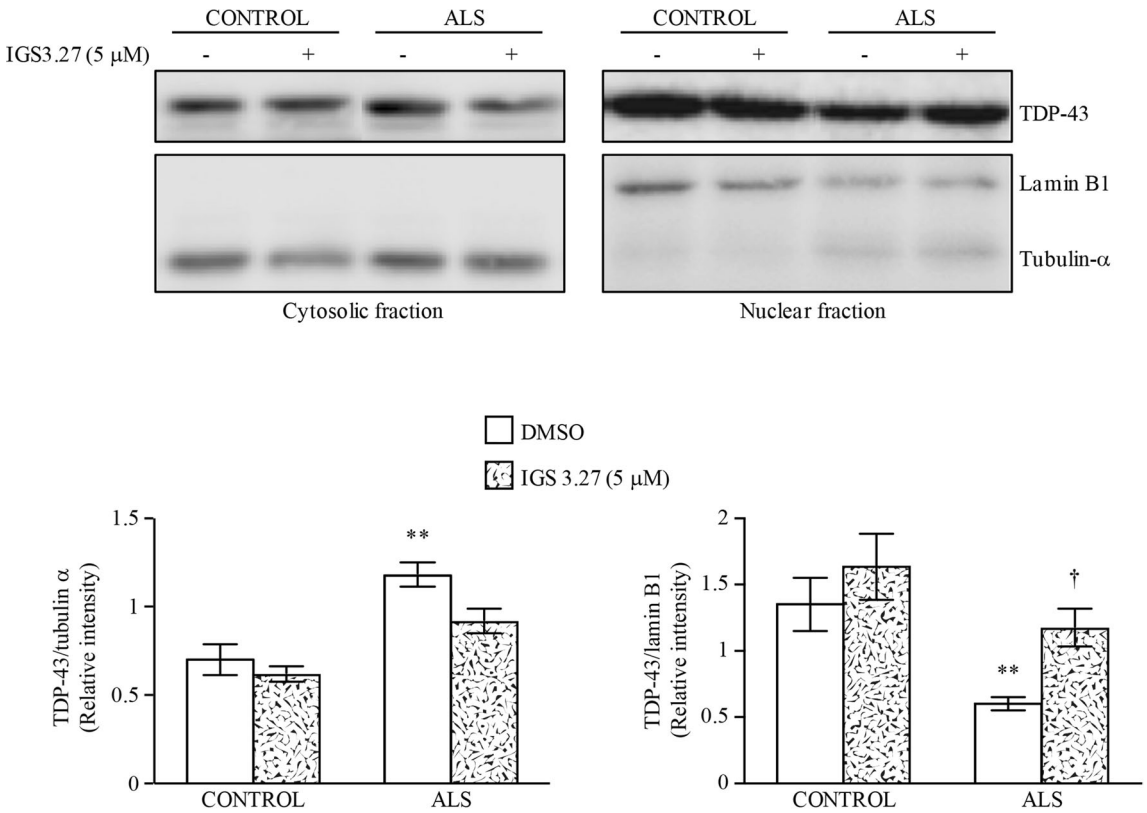

Fig. 5 Effects of CK-1δ inhibitor, IGS 3.27, on the subcellular localization of TDP-43 in control and ALS lymphoblasts. The experimental conditions are identical to those described in the legend to Fig. 4: 24 h after treatment with IGS 3.27, lymphoblasts were lysed to obtain both cytosolic and nuclear fragments that were analyzed by Western blotting. Tubulin-α and Lamin B1 antibodies were used as loading and purity control of the cytosolic and nuclear fractions, respectively. A representative experiment is shown. Densitometric analyses, shown below, represent the mean ± SEM of different observations carried out in three to five different cell lines from each group (*$p < 0.05$, significantly different from control cells, †$p < 0.05$ significantly different from untreated cells)

demonstrate that peripheral cells from ALS patients recapitulate the increased phosphorylation and subcellular mislocalization of TDP-43 that characterizes the altered motor neurons of ALS patients [19–21]. On the other hand, the results presented herein are in accordance with previous reports from this laboratory showing alterations in TDP-43 phosphorylation and in TDP-43 translocation from the nucleus to cytosol in lymphoblasts of FTLD-TDP patients [37, 40, 41]. These observations give further support to the idea of an ALS-FTLD continuum [42, 43].

It is believed that abnormal phosphorylation of TDP-43 at the Ser 409/410 is a critical step in ALS and other neurodegenerative diseases. Protein casein kinase 1δ is considered the main kinase involved in TDP-43 phosphorylation [22]. For these reasons, the search for specific inhibitors of these enzymes has become a challenge for the treatment of these proteinopathies [44]. On these grounds, we developed a number of potent CK-1δ inhibitors, able to prevent TDP-43 phosphorylation in vitro and neurotoxicity in vivo [23]. Here, we have evaluated the efficacy of one of these kinase inhibitors in abrogating the pathological TDP-43 changes of ALS lymphoblasts. The benzothiazole IGS3.27 was shown to significantly inhibit the endogenous TDP-43 phosphorylation, and that it is important to reduce TDP-43 accumulation in ALS cells, restoring the normal ratio of nuclear/cytosolic levels of TDP-43. These findings are in accordance with a previous report showing that overexpression of active CK-1δ promotes mislocalization and accumulation of cytosolic phosphorylated TDP-43 in cultured neuroblastoma SH-SY5Y cells [45]. Therefore, it seems that modulating TDP-43 phosphorylation may contribute to regulate the nucleo-cytoplasm shuttle of TDP-43. Similar results were obtained in peripheral cells from FTLD-TDP patients [37]. Thus, immortalized lymphocytes from ALS or FTLD-TDP patients recapitulate the pathological features of these diseases, hyperphosphorylation, truncation, and cytosolic accumulation of TDP-43. Moreover, our results indicate that inhibiting the enzymatic activity of CK-1δ rescue the normal levels of phosphorylated TDP-43 and prevents its cytosolic accumulation, recovering its usual homeostasis. Taken together, these observations reinforce the use of lymphoblasts from patients to test disease-modifying drugs, adding further support to the idea that CK-1δ inhibitors could be considered as a useful pharmacological approach for treatment of ALS/FTLD-TDP.

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Compliance with Ethical Standards

Conflict of Interest None of the authors has any conflict of interest to report.

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