Phosphorylation of hnRNP K by cyclin-dependent kinase 2 controls cytosolic accumulation of TDP-43

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Abstract

Cytosolic accumulation of TAR DNA binding protein 43 (TDP-43) is a major neuropathological feature of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). However, the mechanisms involved in TDP-43 accumulation remain largely unknown. Previously, we reported that inhibitors of cyclin-dependent kinases (CDKs) prevented cytosolic stress granule accumulation of TDP-43, correlating with depletion of heterogeneous ribonucleoprotein (hnRNP) K from stress granules. In the present study, we further investigated the relationship between TDP-43 and hnRNP K and their control by CDKs. Inhibition of CDK2 abrogated the accumulation of TDP-43 into stress granules. Phosphorylated CDK2 co-localized with accumulated TDP-43 and phosphorylated hnRNP K in stress granules. Inhibition of CDK2 phosphorylation blocked phosphorylation of hnRNP K, preventing its incorporation into stress granules. Due to interaction between hnRNP K and TDP-43, the loss of hnRNP K from stress granules prevented accumulation of TDP-43. Mutation of Ser216 and Ser284 phosphorylation sites on hnRNP K inhibited hnRNP K- and TDP-43-positive stress granule formation in transfected cells. The interaction between hnRNP K and TDP-43 was further confirmed by the loss of TDP-43 accumulation following siRNA-mediated inhibition of hnRNP K expression. A substantial decrease of CDK2 and hnRNP K expression in spinal cord motor neurons in ALS patients demonstrates a potential key role for these proteins in ALS and TDP-43 accumulation, indicating that further investigation of the association between hnRNP K and TDP-43 is warranted. Understanding how kinase activity modulates TDP-43 accumulation may provide new pharmacological targets for disease intervention.
Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease associated with degeneration of motor neurons in the spinal cord and motor cortex. Frontotemporal lobar degeneration with ubiquitin inclusions (FTLD-U) is a form of dementia resulting from neuronal loss in the frontal and temporal lobes of the brain (1). Despite clear clinico-pathological differences, these disorders share a high degree of co-morbidity and may represent a spectrum of related disorders (2). Although the underlying pathological process in these diseases is not clear, the identification of the heterogeneous ribonucleoprotein (hnRNP), transactivation response DNA-binding protein-43 (TDP-43) as a major protein component of ubiquitinated inclusions in ALS and FTLD-U patients has focused research on the role of hnRNP accumulation in these diseases (3).

TDP-43 is a predominantly nuclear protein, although it can shuttle between the nucleus and cytosol. The protein has roles in RNA processing, including transcription, pre-mRNA splicing, transport and stabilization of mRNA (4,5). In ALS and FTLD-U post-mortem CNS tissues, TDP-43 accumulates predominantly as a C-terminal fragment of 25 or 35 kDa in cytosolic inclusions. The aggregated TDP-43 is also heavily ubiquitinated, and phosphorylated at the C-terminus (6). A key role for abnormal TDP-43 homeostasis in TDP-43 proteinopathies (ALS and FTLD-U) has been supported by the identification of more than 40 mutations to date in the TDP-43 gene (TARDBP) linked to disease (7).

Although cell studies have elegantly demonstrated that accumulation of C-terminal TDP-43 in the cytosol of neurons results in detrimental outcomes, potentially related to both loss of function and toxic gain of function, there is still a very poor understanding of the processes leading to TDP-43 accumulation and its role in disease progression of ALS and FTLD-U. A key role for TDP-43 localization to cytosolic stress granules (SGs) has been identified (7,8). Cytosolic RNA SGs are sites of stalled mRNA pre-initiation complexes and are formed by cells during certain stresses to allow cellular resources to be directed to up-regulation of stress-related proteins (7). Upon removal of stress, SGs rapidly disperse to allow normal mRNA processing to return. TDP-43, together with many additional hnRNPs localize to SGs in many cell types, including neurons during cell stress. Whether this is a preliminary step in TDP-43 cytosolic aggregation is unclear (7), however, TDP-43 has been shown to bind to various SG proteins both in cells and in post-mortem tissue from ALS and FTLD-U patients (9). Further support for the role of TDP-43 localization to SGs being an important aspect of disease progression comes from studies identifying additional hnRNPs including fused in sarcoma (FUS, also known as hnRNP P2), hnRNP A1 and hnRNP A3 in both SGs and cytosolic inclusions in ALS (10–13). TDP-43 accumulation in SGs can lead to subsequent abnormal protein aggregation (14).

The potential role for TDP-43 SG localization as a key step in ALS and FTLD-U neuropathology has led to major efforts to understand the mechanisms controlling TDP-43 accumulation in SGs. The movement of hnRNPs between the nucleus, cytosol and SGs is controlled by various signaling kinases including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. It has been shown that p38 controls stress-related accumulation of hnRNP A1 (15). JNK controls cytosolic movement of hnRNP K via phosphorylation of serine residues (16) and we have shown that JNK specifically controls TDP-43, but not HuR, localization to SGs in cells exposed to the mitochondrial inhibitor, paraquat (17). This was supported by Higashi et al. (18) who demonstrated that another mitochondrial toxin (sodium arsenite) also induced JNK-mediated TDP-43 phosphorylation and accumulation into SGs. Moreover, Suzuki et al. (19) reported that JNK was involved in TDP-43-mediated cell death. Other kinases also potentially control TDP-43 accumulation. Ayala et al. (20) have shown that ERK can co-localize with TDP-43 during cell stress. It was recently reported that AMP-activated kinase alpha 1 (AMPKα1) can control TDP-43 accumulation in vitro, while activated AMPKα1 was associated with mislocalized TDP-43 in ALS patients (21). CDC7, a kinase involved in control of the cell cycle also co-localizes with phosphorylated TDP-43 in FTLD patients (22). To expand our understanding of how kinases control TDP-43 accumulation in SGs, we recently screened a large number of kinase inhibitors for their ability to modulate TDP-43 SG localization in neuronal cultures. We demonstrated that in addition to JNK, inhibitors of cyclin-dependent kinases (CDKs) provided robust abrogation of TDP-43 accumulation in SGs. Moreover, these inhibitors were able to reverse accumulation of pre-formed TDP-43-positive SGs (23). We also observed that CDK inhibitors blocked aggregation of C-terminal TDP-43 in cells transfected with a C-terminal fragment of TDP-43, providing strong support for CDK control of disease-relevant TDP-43 accumulation. However, the mechanism by which CDKs control TDP-43 cytosolic accumulation during stress is not clear. Delin-eating this process is essential to understanding the early stages of TDP-43 accumulation in disease and offering potential targets to block accumulation at its earliest stage.

In this study, we found that selective inhibitors of CDK2 abrogated the cytosolic accumulation of TDP-43. Phosphorylated (active) CDK2 co-localized with accumulated TDP-43 and hnRNP K in SGs. Inhibition of CDK2 phosphorylation blocked phosphorylation of hnRNP K, preventing its incorporation into SGs. Due to interaction between hnRNP K and TDP-43, the loss of hnRNP K from SGs prevented accumulation of TDP-43 and also affected formation of SGs positive for HuR. Confirmation of the importance of hnRNP K in TDP-43-positive SG formation was obtained through mutation of key phosphorylation sites (Ser216 and Ser284) on hnRNP K, and siRNA-mediated inhibition of hnRNP K expression. We also demonstrated a substantial decrease in CDK2 and hnRNP K expression in spinal cord motor neurons from ALS patients. Due to the recently identified role for hnRNPs in ALS and frontotemporal dementia, further investigation of the association between hnRNP K and TDP-43 is warranted. Understanding how kinase activity modulates TDP-43 accumulation may provide new pharmacological targets for disease intervention.

Results

TDP-43 consistently co-localizes with hnRNP K in SGs

We have reported recently that inhibitors of CDKs can prevent and reverse formation of TDP-43-positive SGs including endogenous TDP-43 and cells transfected with C-terminal constructs (23). The mechanism controlling this is unclear as TDP-43 does not contain classical CDK phosphorylation consensus sites in its primary sequence. However, we found in our previous study that CDK (and additional kinase) inhibitors induced almost identical changes to both TDP-43 and hnRNP K when added to paraquat-treated cells (23). hnRNP K does have consensus sites for CDK2; S/T-P-x-K/R (i.e. residues Ser216, Pro217 and Ser284, Pro285) both of which are known to be phosphorylated in cells (16,24–26) supporting the possibility that interaction between TDP-43 and hnRNP K may be an important factor in controlling TDP-43 accumulation. To examine this further, we
initially determined whether TDP-43 and hnRNP K consistently co-accumulate in the cytosol during cell stress.

SH-SY5Y cells treated with 1 mM paraquat overnight revealed a high level of co-occurrence of TDP-43 and hnRNP K in SGs as assessed using immunofluorescence analysis (Figs 1 and 2A). We observed that 89 ± 12% of hnRNP K-positive SGs were also positive for TDP-43. In contrast, 63 ± 8% of HuR-positive SGs were positive for TDP-43 demonstrating a significantly lower level of concordance between HuR and TDP-43 compared with hnRNP K and TDP-43 (P < 0.01) as previously reported (23). To determine if the co-localization of hnRNP K and TDP-43 was consistent in other cell models, we examined NSC34 motor neuron hybrid cells stably transfected with mCherry-tagged full length human TDP-43. As paraquat did not induce robust SGs in this cell-type (data not shown), we treated the cells with 300 μM sodium arsenite for 3 h. mCherry vector alone showed diffuse localization throughout the cell and this was largely unchanged upon treatment with sodium arsenite. In mCherry-TDP-43 expressing cells, without treatment, some accumulation of TDP-43 was occasionally observed in the perinuclear region of transfected cells (Supplementary Material, Fig. S1), although no accumulation of hnRNP K was observed. After treatment with sodium arsenite, robust cytosolic accumulation of TDP-43 was observed and this strongly co-localized with accumulated hnRNP K (Supplementary Material, Fig. S1, white arrows). Interestingly, TDP-43 accumulation was greater than hnRNP K, perhaps associated with a higher propensity to aggregate as we have demonstrated previously (14). No accumulation of hnRNP K was observed with this treatment in vector control cells, demonstrating that the presence of the human TDP-43 expression enhanced the cytosolic hnRNP K accumulation during stress. Primary murine cortical neurons were also treated with sodium arsenite for 3 h and again, co-localization of TDP-43 and hnRNP K was observed in the cytosol of treated cells (Supplementary Material, Fig. S2).

These findings clearly demonstrate that TDP-43 and hnRNP K co-localize in the cytosol in different cell types and during different stresses and this is consistent with an important association between these proteins.

Figure 1. Inhibitors of CDKs block formation of TDP-43-positive SGs in SH-SY5Y cells treated with paraquat. Cells were exposed to 1 mM paraquat overnight with or without CDK inhibitors, Olomoucine, aminopurvalanol A or roscovitine, or the src inhibitor, PP1. Cells were examined for the presence of TDP-43 and/or hnRNPK-positive SGs. Green = TDP-43, red = hnRNPK, blue = DAPI. Right hand panels are merged images of TDP-43 and hnRNPK. Arrows indicate SGs, bar = 10 μm.
Figure 2. Effect of CDK inhibitors on TDP-43, HuR and hnRNP K-positive SG formation in SH-SY5Y cells after treatment with paraquat. (A) Cells were exposed to 1 mM paraquat overnight with or without CDK inhibitors, Olomoucine, aminopurvalanol A, CDK2 Inhibitor I, or roscovitine, or the src inhibitor, PP1 or CDK1/5 Inhibitor. Cells were examined for the presence of TDP-43, HuR, and/or hnRNP K-positive SGs. *P < 0.05 compared with untreated control, ANOVA and Dunnett post-hoc analysis. (B) Cells were exposed to 1 mM paraquat overnight with or without the CDK inhibitor, Olomoucine. Cells were examined for the presence of SGs positive for TDP-43, phospho-CDK2 (p-CDK2), total CDK2 (t-CDK2) or total CDK5 (t-CDK5). **P < 0.01 compared with paraquat only, Student’s t-test. (C) Cells were exposed to 1 mM paraquat overnight with or without the CDK inhibitor, Olomoucine. Cells were examined for the presence of SGs positive for hnRNP K and phospho-CDK2 (p-CDK2). **P < 0.01 compared with paraquat only, Student’s t-test. (D) Cells were exposed to 1 mM paraquat overnight with or without the CDK inhibitor, Olomoucine. Cells were examined for the presence of SGs positive for TDP-43 and phospho-hnRNP K Ser284 (p-hnRNP K). **P < 0.01 compared with paraquat only, Student’s t-test. (E) Cells were exposed to 1 mM paraquat overnight with or without the CDK inhibitor, Olomoucine. Cells were examined for the presence of SGs positive for TDP-43 and phospho-hnRNP K Ser216 (p-hnRNP K). **P < 0.01 compared with paraquat only, Student’s t-test. (F) Cells were exposed to 1 mM paraquat overnight with or without the CDK inhibitor, Olomoucine. Cells were examined for the presence of SGs positive for CDK2, CDK5 and phospho-hnRNP K Ser284 (p-hnRNP K). **P < 0.01 compared with paraquat only, Student’s t-test.
Cytosolic TDP-43 binds to hnRNPK

To examine whether there was a direct interaction between TDP-43 and hnRNPK in the cell cytosol, we immunoprecipitated TDP-43 from cytosolic extracts of SH-SY5Y cells. Probing the extracts revealed the presence of hnRNPK (as well as TIAR, which we have also reported to be controlled by kinases in a manner analogous to TDP-43) (23) (Fig. 3A and B). The interaction between TDP-43 and hnRNPK was further confirmed by reverse immunoprecipitation of hnRNPK (Fig. 3C, showing co-immunoprecipitation of TDP-43) from untreated SH-SY5Y cell lysates. These findings are consistent with an interaction between TDP-43 and hnRNPK. No binding of TDP-43 to HuR or hnRNPA1 in the immunoprecipitation was observed suggesting that the co-localization of TDP-43 with these proteins may not involve direct strong binding between the proteins. In this case, the co-localization observed between TDP-43 and HuR in SGs may represent different mechanisms or pathways of accumulation into the same SGs. We observed no consistent change in the level of TDP-43 or hnRNPK in the immunoblots of the immunoprecipitated cytosolic lysates after paraquat treatment. This suggested that under normal conditions TDP-43 and hnRNPK may already be bound together in the cytosol. Upon paraquat treatment, these proteins then move or aggregate into SGs. Unfortunately, due to the fact that SGs cannot be purified, we were unable to investigate differences in TDP-43/hnRNPK interactions in SGs compared with non-SG cytosolic protein by using immunoprecipitation. However, to provide further evidence for a direct binding of TDP-43 to hnRNPK, we transfected HEK293 cells with WT-hnRNPK-GFP. An immunoprecipitation was performed with a GFP-trap and immunoblotted for TDP-43 (Fig. 3D). The observation of co-immunoprecipitated TDP-43 with hnRNPK-GFP supports interaction between these proteins in different cell-types.

Figure 3. Co-localization of TDP-43 and hnRNPK by immunoprecipitation of cytosolic extracts of SH-SY5Y and HEK293 cells. Cytosolic extracts of SH-SY5Y cells untreated or treated with 1 mM paraquat overnight were immunoprecipitated (IP) with antisera to TDP-43 and immunoblotted (IB) with antisera to TDP-43, hnRNPK, TIAR, HuR or hnRNPA1 (A). To confirm cytosolic enrichment, original lysate was probed for the nuclear marker Ki67 and the cytosolic marker tubulin. (B) Immunoprecipitation of TDP-43 from untreated SH-SY5Y cell lysates and immunoblotted for TDP-43 and hnRNPK, compared with no primary antibody in the immunoprecipitation, and input lane. (C) Immunoprecipitation of hnRNPK from untreated SH-SY5Y cell lysates and immunoblotted for hnRNPK and TDP-43, compared with no primary antibody in the immunoprecipitation, and input lane. (D) HEK293 cells were transfected with GFP-constructs for WT-hnRNPK, Ser216Ala/Ser284Ala hnRNPK and Ser216Ala/Ser284Ala hnRNPK. Cytosolic extracts were immunoprecipitated (IP) with GFP-trap and immunoblotted (IB) with antisera to GFP and TDP-43. n = 2-3 biological replicates for each immunoprecipitation.
CDK2 inhibitors block accumulation of TDP-43 and hnRNP K

Our previous study demonstrated that CDK inhibitors decreased cytosolic SG localization of TDP-43 and hnRNP K (23). As hnRNP K has CDK2 phosphorylation sites, we further examined if inhibitors of this kinase modulated accumulation of TDP-43 and hnRNP K during cell stress. Co-treatment of SH-SY5Y cells with paraquat and inhibitors with known activity or selectivity towards CDK2, Olomoucine, aminopurvalanol A, CDK2 Inhibitor I and roscovitine, resulted in a strong inhibitory effect on SG accumulation of both TDP-43 and hnRNP K (Figs. 1 and 2A). As reported recently (23), alternative kinase inhibitors such as CDK1/5 inhibitor and PP1 did not have this effect.

Phosphorylated CDK2 co-localizes with TDP-43 and hnRNP K in SGs

Although some of the inhibitors used, particularly roscovitine and CDK2 Inhibitor I, have a selective inhibitory action on CDK2 over CDK1 and CDK5, it remained a possibility that the inhibitory activity was via modulation of alternative CDKs. Therefore, we investigated whether CDK2 co-localizes with TDP-43 and hnRNP K in SGs as co-localization would be expected if CDK2 modulates SG localization of these hnRNPs. In fact, we observed strong co-localization of total and, importantly, phosphorylated (active) CDK2 with TDP-43 and hnRNP K in SGs (Figs. 2B and 4). As expected, inhibition of CDK2 activity abrogated detectable levels of phosphorylated CDK2 and this resulted in loss of TDP-43 localization.

Figure 4. CDK inhibition blocks co-localization of TDP-43 and CDK2 in cytosolic SGs in SH-SY5Y cells treated with paraquat. Cells were exposed to 1 mM paraquat overnight with or without the CDK inhibitor, Olomoucine and examined for the presence of SGs positive for TDP-43, total CDK2 (t-CDK2), phospho-CDK2 (p-CDK2) or total CDK5 (t-CDK5). Green = CDK2, red = TDP-43, blue = DAPI. Right hand panels are merged images of CDK2 and TDP-43. Arrows indicate SGs, bar = 10 μm.
and hnRNP K-positive SGs (Fig. 4). No co-localization of CDK5 was observed with TDP-43 and hnRNP K in SGs during paraquat treatment (Figs. 2B and 4). These findings support a role for direct interaction between CDK2 and TDP-43 and/or hnRNP K resulting in SG localization of the latter.

Interestingly, analysis of total p-CDK2 levels in paraquat-treated cells did not reveal a substantial change in detectable levels (Supplementary Material, Fig. S3). Induction of cell stress was confirmed by elevated levels of phosphorylated p38, a known stress-responsive kinase (Supplementary Material, Fig. S3). The lack of a substantial change in phosphorylated CDK2 levels during stress could either indicate that the changes are highly localized and not easily determined in cell extracts, or that constitutively phosphorylated CDK2 localizes with hnRNP K and TDP-43 in SGs during stress.

Commitment to CDK2 Inhibition

CDK2 inhibitors block hnRNP K phosphorylation and hnRNP K and TDP-43 accumulation in SGs

From these studies, we theorized that CDK2 may control localization of hnRNP K and associated TDP-43 to SGs through phosphorylation of the hnRNP K consensus sites for CDK2 (e.g. residues Ser216 and/or Ser284). In support of this, we observed SG co-localization of phosphorylated hnRNP K using antisera to p-hnRNP K Ser216 or p-hnRNP K Ser284 with TDP-43 (Figs 2D and E and 5A) and CDK2 (Fig. 2F). Two out of three CDK2 inhibitors tested reduced total cellular levels of phosphorylated hnRNP K (Fig. 5B) and blocked accumulation of phosphorylated (and total) hnRNP K and TDP-43 in SGs (Figs 2A, D and E and 5A). Olopatadine had limited effect on total CDK2 phosphorylation in cell lysates (Fig. 5B) but still inhibited hnRNP K localization to SGs (Fig. 5A). This may reflect action on cytosolic CDK2 that cannot be detected in total cell lysates or could involve inhibition of alternative CDKs which we have not yet been able to determine. CDK2 inhibition also abrogated co-localization of total CDK2 and p-hnRNP K (Fig. 2F). Similar effects were not observed for CDK5. This provided strong support for a mechanism involving CDK2 phosphorylation of hnRNP K in controlling co-localization of both hnRNP K and TDP-43 to SGs.

Mutation of hnRNP K phosphorylation sites (Ser216 and Ser284) inhibits hnRNP K and TDP-43 co-localization to SGs

To provide further support that phosphorylation of key residues on hnRNP K is essential for controlling TDP-43 localization to SGs, we generated phosphorylation site mutant hnRNP Ks containing Ser284Ala and Ser216Ala/Ser284Ala and transfected HEK293 cells with the GFP-tagged constructs (compared with cells transfected with wild-type (WT) hnRNP K-GFP). As shown in Figure 3D, compared with WT hnRNP K-GFP, both mutant forms of hnRNP K revealed reduced capacity to bind TDP-43 in co-immunoprecipitation experiments. Transfected cells were then either untreated or treated with sodium arsenite for 3 h to induce formation of SGs (sodium arsenite was used as paraquat did not readily induce strong SG formation in HEK293 cells). Cells transfected with WT-hnRNP K-GFP revealed formation of hnRNP K-GFP- and TDP-43 positive SGs upon treatment with sodium arsenite (Fig. 6 and Supplementary Material, Fig. S4A), as seen in other cell types containing endogenous hnRNP K expression. Additional SGs containing only TDP-43 were also observed and were likely formed by association with endogenous hnRNP K. In cells transfected with Ser284Ala-hnRNP K-GFP, there was a reduction in the numbers of hnRNP K-GFP- and TDP-43-positive SGs upon sodium arsenite treatment (Fig. 6 and Supplementary Material, Fig. S4B). This effect was substantially stronger in the Ser216Ala/Ser284Ala-hnRNP K-GFP transfected cells (Fig. 6 and Supplementary Material, Fig. S4C). As expected, additional TDP-43-positive SGs that likely formed with endogenous hnRNP K were still evident in mutant hnRNP K-transfected cells. However, these results do provide further evidence that phosphorylation of specific serine residues on hnRNP K has a key role in modulating hnRNP K and also TDP-43 localization to SGs.

Depletion of hnRNP K inhibits formation of TDP-43 SGs

If hnRNP K is essential for controlling TDP-43 localization to SGs, then its depletion should inhibit TDP-43 SG accumulation. We investigated this by using siRNA against hnRNP K to deplete levels of the protein in SH-SYSY cells. siRNA against hnRNP K significantly depleted levels of hnRNP K in treated cells (Fig. 7A), while control siRNA had no effect. Knockdown of hnRNP K did not alter the level of cell viability under basal and paraquat-treated conditions demonstrating that the loss of hnRNP K was not toxic to the cells (Supplementary Material, Fig. S5). siRNA treatment prevented formation of hnRNP K-positive SGs as expected. Likewise, rarely were TDP-43-positive SG observed in siRNA treated cells after paraquat exposure (Fig. 7B and C). In fact, there was also an analogous loss of HuR SGs (Fig. 7B). This contrasted with our finding above that inhibition of hnRNP K phosphorylation reduced formation of TDP-43 positive SGs but not SGs per se (i.e. HuR positive). This suggests that while phosphorylated hnRNP K may be associated with TDP-43 SG accumulation, the hnRNP K protein may have additional roles in controlling SG dynamics. Our findings, however, demonstrated that hnRNP K expression was required for robust SG formation in response to paraquat and is consistent with the need for hnRNP K to control TDP-43 accumulation in SGs.

Loss of CDK2 and hnRNP K expression in motor neurons is a feature of ALS

To examine whether there was a similar relationship between hnRNP K and TDP-43 ex vivo, we examined cervical spinal cord motor neurons in control and ALS cases for the expression of hnRNP K. We observed a robust decrease in total cellular hnRNP K levels in motor neurons of both SALS (sporadic) and FALS (familial, with unknown mutations) cases of ALS. This decrease was indicative of a parallel decrease in both cytosolic and nuclear hnRNP K in these cells (Fig. 8A–F). No hnRNP K-positive cytosolic inclusions were observed. To determine if these changes were associated with altered CDK2 activity, we also examined the same tissues for total CDK2 expression (antiserum to phospho-CDK2 did not produce a robust signal on the tissue sections). We observed that nuclear, cytosolic and total CDK2 levels were decreased in the SALS and FALS tissues in a pattern very similar to the decrease in hnRNP K (Fig. 8G–I). To ensure that these changes were not associated with general protein loss, we compared the staining to neurofilament H (NF H) in the same tissues and found that the NF H was not significantly altered (Fig. 8). These findings demonstrate that CDK2 and hnRNP K expression are substantially lower in spinal cord motor neurons of ALS cases.

Discussion

The loss of nuclear TDP-43 and accumulation and ultimately aggregation of C-terminal TDP-43 fragments in the cytosol is...
believed to be an important phenomenon contributing to the pathophysiology of ALS and FTLD-U. However, the precise molecular pathways controlling this process are not well understood. TDP-43 is an hnRNP and co-localizes with additional hnRNPs in cytosolic SGs, where it may subsequently progress to larger insoluble aggregates. The mechanisms controlling early TDP-43 accumulation in SGs are being investigated. Our recent studies have identified a key role for specific kinases controlling cytosolic accumulation of TDP-43 (23). We have demonstrated that JNK, CDKs and GSK3 are likely to have important roles in the formation and/or maintenance of TDP-43 positive SGs (14,17,23). This has been supported by subsequent reports describing a role for JNK controlling SG accumulation of C-terminal TDP-43 and TDP-43 toxicity (18,19) and the presence of kinases associated with TDP-43 aggregates in ALS or FTLD-U patients (20,22).

While these studies clearly demonstrate a key role for kinases in modulating TDP-43 accumulation, it is not clear how this is mediated at the molecular level. TDP-43 does not contain known phosphorylation consensus sites for CDKs (or JNK) (27). While it remains a possibility that TDP-43 could be phosphorylated via non-classical sites, it is perhaps more likely that TDP-43 movement is controlled through interaction with additional SG proteins. We report here evidence that TDP-43 consistently

Figure 5. CDK inhibition blocks co-localization of TDP-43 and p-hnRNP K in SH-SY5Y cells treated with paraquat. (A) Cells were exposed to 1 mM paraquat overnight with or without the CDK inhibitors, Olomoucine or roscovitine and examined for the presence of SGs positive for TDP-43, or phospho-hnRNP K Ser284 (p-hnRNP K Ser284). Green = TDP-43, red = p-hnRNP K Ser284, blue = DAPI. Right hand panels are merged images of TDP-43 and p-hnRNP K Ser284. Arrows indicate SGs, bar = 10 μm. (B) Cells were exposed to 1 mM paraquat overnight with or without the CDK inhibitors, Olomoucine, aminopurvalanol A or arcyriaflavin A and examined for the expression of total hnRNP K and p-hnRNP K Ser284.
neuron-like morphology as we have done here. We also observed in the cytosol including in some patients with C9orf72 mutations. This strongly suggests that co-localization of active CDK2 in hnRNP K and TDP-43 positive SGs is important for the maintenance of the SGs and was further supported by the observation that mutation of key CDK2 phosphorylation sites or knockdown of hnRNP K abrogated TDP-43-positive SG formation (Fig. 9).

Other kinases including JNK appear to be involved at an earlier stage, perhaps directing hnRNP K (and TDP-43) to SGs or SG ‘seeds’, but are not required for the maintenance of the mature SG. While we cannot rule out the involvement of additional CKDs in the hnRNP K/TDP-43 accumulation pathway, we did not observe any co-localization of CDK5/35 with hnRNP K or TDP-43 in our model. These or additional kinases could have key roles in other models of SG formation.

Whether hnRNP K has a significant role in TDP-43 proteinopathies is not known. The protein has key roles in chromatin remodeling, transcription, splicing and translation processes (31) and abnormal metabolism has been associated with several disorders including cancers and the neurodegenerative disorder, spinocerebellar ataxia type 10 (32,33) suggesting that changes to its metabolism could have significant outcomes in neurological disorders. Recently, additional hnRNP molecules, including hnRNP A1 and hnRNP A3 have been linked to ALS and FTLD (11,13). In the study by Mori et al. (11), hnRNP A3 was found to interact with the hexanucleotide expanded repeat associated with C9orf72 and aggregated in the cytosol including in some patients with C9orf72 mutations. These hnRNPs are known to be controlled by kinases including p38, ERK and JNK and it will be important to determine if these or alternative kinases control accumulation of the hnRNPs during ALS or FTLD. Interestingly, the study by Mori et al. also found interaction between hnRNP K and the C9orf72 hexanucleotide repeat (GGGGCC) but this was not examined in detail. These studies do, however, provide strong support for a common pathological process in ALS and FTLD involving cytosolic accumulation of hnRNPs. It remains to be determined if hnRNP K has a central role in any subset of these patients and if modulation of hnRNP K (and subsequently, TDP-43) can be achieved through pharmacological inhibition of key kinases.

Materials and Methods

Materials

4,6-Diamino-2-phenylindole dihydrochloride (DAPI) was obtained from Invitrogen (Mount Waverley, Victoria, Australia). N,N′-dimethyl-4,4′-bipyridinium dichloride (paraquat) and sodium arsenite, were from Sigma-Aldrich (Sydney, NSW, Australia). Tocriscreen kinase inhibitor toolbox was from Tocris Bioscience (Ellisville, Melbourne, Victoria, Australia).

Figure 6. Mutation of hnRNP K phosphorylation sites inhibit hnRNP K- and TDP-43-positive SG formation. HEK293 cells were transfected with GFP-constructs for WT hnRNP K, Ser284Ala hnRNP K and Ser216Ala/Ser284Ala hnRNP K. Cells were exposed to 500 μM sodium arsenite for 3 h and examined for numbers of SGs positive for TDP-43 alone, hnRNP K-GFP alone or TDP-43 and hnRNP K-GFP. *p = 0.05 compared with WT-hnRNP-GFP, Student’s t-test.

co-localizes with hnRNP K in cytosolic SGs in a range of cell types and with different stress inducers. We also confirm that TDP-43 and hnRNP K appear to interact directly or indirectly in the cytosol. This is consistent with previous reports indicating a direct association between these proteins as determined by proteomic analysis (28). Moreover, we have shown that expression of hnRNP K is substantially diminished in spinal cord motor neurons in ALS cases, along with CDK2. This is associated with a loss throughout the cell and is not restricted to nucleus or cytosol (Fig. 8). Whether there is a relationship between changes to hnRNP K and TDP-43 remains to be determined and will require assessment of levels in the same cells of serial sections using co-localization immunohistochemistry. Our findings are however, the first to describe a prominent change to hnRNP K in ALS spinal cord tissue. The reason this has not been reported previously is again likely to represent past examination of cells specifically expressing cytoplasmic inclusions of TDP-43 (29), rather than examination of cytosolic and nuclear hnRNP K in all cells with a motor neuron-like morphology as we have done here. We also observed that ~12% of cells contained cytosolic TDP-43-positive inclusions in sections from the same tissue samples, although we did not identify any hnRNP K-positive inclusions in the ALS tissues (hnRNP K may be lost from these inclusions at an early stage as we propose in Fig. 9 and have reported for HuR in vitro previously).

However, our analysis of hnRNP K expression was done on all cells, not just those potentially containing TDP-43 inclusions. A global reduction to hnRNP K is clearly a prominent feature across the ALS tissue. The consequences in vivo of a robust loss of hnRNP K remain to be determined but could have important consequences for RNA processing.

Direct interactions between TDP-43 and hnRNP K could facilitate the control of TDP-43 movement by hnRNP K (and vice versa) (Fig. 9). hnRNP K is phosphorylated at several sites including Ser216 and Ser284. Habelhah et al. (16) reported that hnRNP K is phosphorylated by JNK at Ser284 and this controls movement and accumulation of the protein. Additional kinases have been shown to control the cellular trafficking of hnRNPs including ERK and p38-mediated control of hnRNP K and hnRNP A1, respectively (15,16,30). It is therefore not surprising that kinases modulate the movement of another hnRNP, TDP-43, during cell stress and this is supported by the report that AMPKα1 also controls TDP-43 (21). Our data strongly suggest that a key process in control of TDP-43 trafficking involves phosphorylation of hnRNP K by CDK2 (and potentially additional CDKs) at Ser216 and/or Ser284 during cell stress. CDK2 inhibitors reduced hnRNP K phosphorylation and abrogated accumulation of phosphorylated hnRNP K and TDP-43 during stress. Further support for this mechanistic pathway was shown by the accumulation of phosphorylated CDK2 in the same SGs as phosphorylated hnRNP K and TDP-43. This was consistent with our previous report demonstrating that only CDK2 inhibitors could abrogate pre-formed TDP-43-positive SGs (as distinct from preventing initial formation). Whether hnRNP K has a significant role in TDP-43 proteinopathies is not known. The protein has key roles in chromatin remodeling, transcription, splicing and translation processes (31) and abnormal metabolism has been associated with several disorders including cancers and the neurodegenerative disorder, spinocerebellar ataxia type 10 (32–35). In the latter, changes to hnRNP K metabolism appear to modulate PKC delta interaction with mitochondria, resulting in neuronal cell death (34). hnRNP K also has important roles in neuronal development and axonogenesis (36,37) suggesting that changes to its metabolism could have significant outcomes in neurological disorders. Recently, additional hnRNP molecules, including hnRNP A1 and hnRNP A3 have been linked to ALS and FTLD (11,13). In the study by Mori et al. (11), hnRNP A3 was found to interact with the hexanucleotide expanded repeat associated with C9orf72 and aggregated in the cytosol including in some patients with C9orf72 mutations. These hnRNPs are known to be controlled by kinases including p38, ERK and JNK and it will be important to determine if these or alternative kinases control accumulation of the hnRNPs during ALS or FTLD. Interestingly, the study by Mori et al. also found interaction between hnRNP K and the C9orf72 hexanucleotide repeat (GGGGCC) but this was not examined in detail. These studies do, however, provide strong support for a common pathological process in ALS and FTLD involving cytosolic accumulation of hnRNPs. It remains to be determined if hnRNP K has a central role in any subset of these patients and if modulation of hnRNP K (and subsequently, TDP-43) can be achieved through pharmacological inhibition of key kinases.
Polyclonal TDP-43 antisera were purchased from Proteintech Group (Chicago, IL, USA) to TDP-43 (10782-2-AP). Polyclonal antisera to total and phosphorylated hnRNP K, total hnRNP A1 and GFP were purchased from Abcam (Waterloo, Australia) and Enogene (Cherrybrook, NSW, Australia). Polyclonal antiserum to TIAR was from Cell Signaling Technology (Genesearch, Arundel, Queensland, Australia). Polyclonal antisera to CDK5 and p35, total and phospho-protein kinase C, total and phospho-p38 and total and phospho-CDK2 were from Santa Cruz Biotechnology (Scoresby, Victoria, Australia). Monoclonal antisera to HuR were obtained from Invitrogen (Mount Waverley, Victoria, Australia).

**SH-SY5Y cell line**

Neuroblastoma SH-SY5Y cell line was passaged and maintained in DMEM/F12 plus 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were maintained in 5% CO₂ at 37°C.

Figure 7. Knockdown of hnRNP K inhibits formation of TDP-43-positive SGs in SH-SY5Y cells treated with paraquat. (A) Cells were treated with siRNA to hnRNP K or with control siRNA. hnRNP K siRNA significantly reduced the expression of hnRNP K but not TDP-43. Treatment with 1 mM paraquat overnight did not alter the expression of hnRNP K with or without siRNA treatment. (B and C) Treatment of SH-SY5Y cells with hnRNP K siRNA blocked expression of hnRNP K and formation of hnRNP K SGs after treatment with 1 mM paraquat overnight. hnRNP K but not control siRNA also inhibited formation of TDP-43 and HuR-positive SGs (C). Red = hnRNP K, green = HuR, magenta = TDP-43, blue = DAPI. Right hand panels are merged images of hnRNP K, TDP-43, HuR and DAPI. Arrows indicate localization of SGs, bar = 10 μm.
**HEK 293 cell line**

Human Embryonic Kidney 293 cells were maintained in DMEM media (25 mM glucose) supplemented with 10% (v/v) FBS and penicillin/streptomycin and were maintained in 5% CO2 at 37°C.

**NSC34-TDP-43 cell line**

These are a hybrid cell line generated by the fusion of motor neuron enriched embryonic spinal cord cultures with a neuroblastoma cell line (38). Cells were transfected with the pm-Cherry-N1

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**Figure 8.** hnRNPK and CDK2 expression in motor neurons of SALS (sporadic) and FALS (familial) cases. Expression of hnRNPK was examined in spinal cord motor neurons of control (A), sporadic (B) and familial (C) cases. Arrows indicate motor neurons. Bar = 20 μm. Expression of hnRNPK was quantitated using Image J 1.46r software in nucleus (D), cytosol (E) and total (F). Expression of CDK2 was examined in spinal cord motor neurons of control (G), sporadic (H) and familial (I) cases. Arrows indicate motor neurons. Bar = 20 μm. Expression of CDK2 was quantitated using Image J 1.46r software in nucleus. As a control, tissues from the same controls and cases were examined for expression of the general neuronal marker, neurofilament H (NF H). *P < 0.05 compared with control, using one-way ANOVA.
expression vector alone or the vector containing human wild-type TDP-43 (WT-TDP43). The multiple cloning site of the pm-Cherry-N1 vector (Clontech Laboratories, Mountain View, USA) allows expression of fusion proteins on the N-terminus of the fluorescent mCherry protein (39). Use of this vector therefore adds a fluorescent 29 kDa mCherry tag to the C-terminal end of the TDP43. Additional sequences in the vector include a Kozak consensus site to enhance translational efficiency (40), an SV40 origin for replication in mammalian cells, a pUC origin for propagation in E.coli and SV40 polyadenylation signals. The vector also contains a neomycin resistance cassette, which was utilized to select stably transfected cells by growing cells in media supplemented with G418 (700 μg ml⁻¹) for 2 weeks. Limiting dilution was used to generate monoclonal cell lines. Stably transfected lines were maintained in DMEM media (25 mM glucose) supplemented with 10% (v/v) FBS, penicillin/streptomycin and G418.

Primary cortical neurons

Cortices were isolated from mouse embryos at Day 14 gestation in Krebs solution, comprising 124.06 mM NaCl, 5.37 mM KCl, 1.17 mM NaH₂PO₄, 14.43 mM glucose, 0.05 mM phenol red, 24.93 mM HEPES, 0.05 mM bovine serum albumin and 2.05 mM MgSO₄. Cortices were then incubated for 20 min at 37°C (in a shaking water bath) in Krebs solution containing an added 0.11 mM trypsin (Sigma). Subsequently, Krebs solution containing 25.8 nM DNase (Sigma) and 129.38 nM soy bean trypsin inhibitor (Sigma) was added to the suspension, and cells were mixed for a further 2 min. Cortices were then centrifuged for 3 min at 259 RCF. Supernatant was removed; 1 ml of Krebs solution containing 25.8 nM DNase (Sigma) and 129.38 nM soy bean trypsin inhibitor (Sigma) was added to the pellet. Pellet was re-suspended to create a cell suspension. Cells were then further centrifuged at 259 RCF for 3 min, supernatant removed and cells re-suspended in cortical plating medium comprising 10% 10xMEM, 1 mM l-glutamine, 6.56 mM NaHCO₃, 10 mg/ml gentamicin, 10% FBS and 5% horse serum (Life Technologies). Cells were plated at a density of 6 × 10⁴ cells per cm² on coverslips coated with 20 μg/ml poly-d-lysine. Cells were incubated at 37°C for 24 h. After this time, plating medium was removed and replaced with Neurobasal medium supplemented with 2% B-27 supplement, and 500 μM l-glutamine. After 5 days in culture at 37°C, 5% CO₂, half of the medium was changed to new supplemented Neurobasal medium. Cells were used for experiments after 7 days in culture.

Exposure of cells to stress

Cells were grown in 24- or 6-well plates or on 12 mm coverslips (for immunofluorescence). Paraquat or sodium arsenite was prepared in dH₂O and added at indicated concentrations and the medium was briefly mixed by aspiration. Incubations were performed for periods stated in individual experiments. Where indicated, cells were co-treated with kinase inhibitors at 10 μM from stock solutions prepared at 10 mM in DMSO (23). Control cultures were treated with vehicle alone. For immunoblotting, cells were harvested into Phosphosafe Extraction Buffer (Merck Biosciences, San Diego, CA, USA) containing protease inhibitor cocktail (Roche Diagnostics, Hawthorn, Victoria, Australia) and stored at −80°C until use. For immunofluorescence studies, cells were grown on glass coverslips and fixed by treating with 4% paraformaldehyde for 30 min.

Western blot analysis of protein expression and phosphorylation

Cell lysates prepared in Phosphosafe Extraction Buffer at equal protein concentration were mixed with electrophoresis SDS sample buffer and separated on 12% SDS–PAGE Tris–Glycine gels. Proteins were transferred to PVDF membranes and blocked with 4% skim milk solution in PBST before immunoblotting. Membranes were probed with polyclonal antisera against TDP-43 (1:5000), total or phospho-hnRNP K, (1:1000), total or phospho-CDK2 (1:1000), CDK5 and p25/35 (1:1000) or HuR (1:1000). Secondary antiserum was rabbit-HRP at 1:5000 dilution. Blots were developed using GE Healthcare ECL Advance Chemiluminescence (Rydalmere, NSW, Australia) and imaged on a Fujiﬁlm LAS3000 imager (Burntholm, Bundoora, Victoria, Australia). Expression of GAPDH was determined using antisera at 1:5000 for protein loading controls where necessary.

Expression vectors and site-directed mutagenesis

WT full-length hnRNP K GFP fusion construct cloned into the pEGFP-C1 vector was used for site-directed mutagenesis of...
hnRNP K phosphorylation sites. To mutate serine CDK2 phosphorylation sites (Ser216 and Ser284) to alanine residues in hnRNP K, the GeneArt® Site-Directed Mutagenesis System (Life technologies) was employed according to the manufacturer’s instructions. The mutagenic primers used for Ser216Ala were 5’ forward: AATCTTGAGCATATAGCTAGCATCCAT and 3’ reverse: TGAAGGAGAGTCTAATAGGTCAGGAT. The mutagenic primers used for Ser284Ala were 5’ forward: GATTATGATATGCCCTGGTCAGGACC and 3’ reverse: GCCTGACGAAGGGCCATATCATATACT. All mutants were verified by sequencing.

**Calcium phosphate transfection of HEK293 cells**

HEK293T cells were transiently transfected with WT hnRNP K-GFP, Ser284Ala hnRNP K-GFP and Ser216Ala/Ser284Ala hnRNP K-GFP using the calcium phosphate transfection method. Briefly, cells were cultured in 60 mm dishes in serum and antibiotic-containing media for 24 h prior to transfection. The media was changed 3–4 h prior to transfection. A transfection mixture containing 300 μl 1× HBS, 18 μl 2 M CaCl2, and 10 μg hnRNP K-GFP DNA was added drop-wise to each 60 mm dish. Cultures were incubated overnight at 37°C and glycerol shock performed with 15% glycerol in 1X HBS. Cultures were incubated for a further 24 h prior to treatment and fixation.

**Immunofluorescence analysis**

Cells were grown on 12 mm diameter coverslips and treated with stress inducers and kinase inhibitors as indicated. Cells were fixed with 4% w/v paraformaldehyde in PBS for 30 min and permeabilized with 90% chilled methanol for 5 min. After blocking for 1 h with 10% normal goat serum, cells were incubated with primary antibody for total TDP-43 (1:1500), HuR (1:50), total or phospho-CDK2 (1:200), CDK5 or p25/35 (1:200) or total or phospho-hnRNP K (1:200) for 2 h at room temperature or overnight at 4°C. This was followed by labeling with secondary AlexaFluor or FITC goat anti-mouse or anti-rabbit antisera at 1:500 for 2 h at room temperature or overnight at 4°C. After washing, the coverslips were incubated with DAPI at 0.5 μg/ml for 5 min and analyzed using a Leica inverted microscope with Zeiss Axiocam digital camera or Zeiss META upright confocal LSM710 microscope (Carl Zeiss, Jena, Germany) using Zeiss ZEN Black software. Coverslips were examined blinded to the inhibitor treatment and the numbers of TDP-43, HuR, hnRNP K or CDK-positive SGs were manually counted in pre-determined multiple fields of view of two to three coverslips for each treatment as recently reported (23). SGs were determined as positive if they were distinctly brighter than the surrounding cytoplasmic or nuclear immunofluorescence and/or were clearly separated from surrounding immunofluorescence to allow positive identification of a discrete structure. Images shown are representative of multiple fields and replicate or triplicate coverslips per experiment (23).

**Immunoprecipitation**

SH-SY5Y cell cytosolic fractions were prepared as previously described (41) and protein concentrations were determined by BCA protein assay (Pierce). Immunoprecipitations were carried out as previously described (41) by using Dynabeads® Protein G beads (Life Technologies) according to the manufacturer’s instructions. Briefly, equal amounts of lysate (300 μg) were cleared by protein G Dynabeads for 1 h at 4°C. One microgram of N-terminus TDP-43 antibody (ProteinTech) was bound to 50 μl of Dynabeads protein G beads for 20 min at RT on a rotating wheel. Dynabeads/TDP-43 complexes were incubated for a further 20 min with cleared lysates at RT. To remove unbound and non-specifically bound proteins, the Dynabeads were pulled down magnetically and washed three times with washing buffer. The bound proteins were eluted by boiling in elution buffer containing 4× SDS sample buffer for 5 min at 95°C. Portions of immunoprecipitates were separated by SDS–PAGE and immunblotted to probe for TDP-43, Ki67, hnRNP K, hnRNP A1, TIAR and HuR.

**Immunoprecipitation of GFP fusion proteins**

HEK 293 cells were transiently transfected with WT hnRNP K-GFP, Ser284Ala hnRNP K-GFP and Ser216Ala/Ser284Ala hnRNP K-GFP using lipofectamine 2000 (Life technologies) according to manufacturer’s instructions. To immunoprecipitate GFP fusion proteins GFP-Trap® A kit (Chromotek, Germany) was used. Briefly, HEK 293 cells were lysed in Phosphosafe extraction buffer (Millipore) with protease inhibitors. Lysates were incubated on ice for 30 min and subsequently centrifuged at 20 000g for 10 min at 4°C. The cleared lysates were then diluted to a final volume of 1 ml in dilution buffer containing 10 mm Tris/HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA and supplemented with protease inhibitors. Lysates were then incubated under rotation for 2 h at RT with GFP-Trap® A beads. Beads were washed three times in dilution buffer, resuspended in 50 μl of 2× SDS sample buffer, boiled and analyzed by western blot analysis.

**siRNA**

SH-SY5Y cells were cultured in antibiotic-free media 24 h prior to transfection. The next day, cells were washed twice with OptiMEM media and incubated in Opti-MEM media for 30 min to condition cells. Cells were transfected with negative siRNA or siRNA targeting hnRNP K (OriGene) using DharmaFECT 4 transfection reagent (Thermo Scientific) in antibiotic free media to a final concentration of 10 nm. Cells were harvested 72 h post-transfection for western blot analysis, FACS or immunofluorescence analysis.

**FACS**

Cell death was quantified by staining with propidium iodide (2.5 μg/ml), followed by flow cytometric analysis in a FACScan (Becton Dickinson). In each sample 10 000 events were measured, and the survival (% PI-negative cells) quantified.

**Immunohistochemistry**

Immunohistochemical staining was performed on 5 μm thick paraffin embedded cervical spinal cord sections from two sporadic ALS patients, one familial ALS patient without any known mutations, and matched control patients. Ethics approval for use of tissue was obtained through the University of Sydney Human Research Ethics Committee. The sections were deparaffinized with xylene and rehydrated in a series of descending concentrations of ethanol and water. The sections were heated in 10 mm citrate buffer (pH 6.0), followed by 3% hydrogen peroxide incubation at room temperature. Non-specific binding of antibody was blocked using 3% goat serum (Vector Laboratories, Inc.). The sections were incubated with primary rabbit anti-total hnRNP K (1:500), or anti-CDK2 (1:200) at 4°C overnight, followed by incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc.). For detection of neurofilament H, sections were incubated with mouse monoclonal antibodies to neurofilament H (SMI-32) (1:2500) at 4°C overnight, followed by incubation with
biotinylated goat anti-mouse IgG (Vector Laboratories, Inc.). The immunoreactivity was stained by avidin-biotin peroxidase complex detection system (Vector Laboratories, Inc.) with 3,3′-diaminobenzidine as the chromogen. After counter staining with hematoxylin, sections were mounted with DPX (Sigma-Aldrich) and coverslipped. For CDK2, hnRNPA1 and neurofilament H analysis, 10 images were examined from controls, nine from sporadic ALS and five from familial ALS cases. The average number of cells analyzed per case was 23 using Image J 1.46r software to quantify the cytosolic, nuclear and total stain of CDK2, hnRNPA1 and neurofilament by a blinded observer.

Statistical analysis

All data described in graphical representations are mean ± standard error of the mean of three or more separate experiments unless stated otherwise. Results were analyzed using a Student’s t-test or a two-way ANOVA and Dunnett post-hoc test as appropriate.

Supplementary Material

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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