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Olga M. Alexeeva April 11, 2012

Functional characterization of the interaction of TAR DNA binding protein 43 (TDP-43) and poly(A) binding protein nuclear 1 (PABPN1)

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Abstract

Functional characterization of the interaction of TAR DNA binding protein 43 (TDP-43) and poly(A) binding protein nuclear 1 (PABPN1)

By Olga M. Alexeeva

Abnormalities in RNA metabolism are emerging as a characteristic pathological feature of many neurodegenerative diseases, especially following the recent discovery of the RNA-binding protein TAR DNA binding protein 43 kDa (TDP-43) as the major component of disease-related protein aggregates in the brains of patients with amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). TDP-43 function in health and disease has not been fully characterized, which limits our understanding of the disease process. In this study, we investigate the physiological role of TDP-43 by characterizing its interaction with poly(A) binding protein nuclear 1 (PABPN1), identified previously as a putative interaction partner in a yeast two-hybrid screen. PABPN1 is an RNA-binding protein that regulates polyadenylation of nascent mRNA tails whose mutant version carrying a polyalanine expansion causes autosomal dominant oculopharyngeal muscular dystrophy (OPMD). Due to several similar features between TDP-43 and PABPN1, including ubiquitous expression, predominantly nuclear localization, involvement in mRNA transport, and association in respective protein-aggregation diseases, we hypothesized that TDP-43 and PABPN1 directly interact in a physiologically relevant way. TDP-43/PABPN1 interaction was confirmed by independent *in vitro* methods, as well as by colocalization analysis in intact primary neurons. PABPN1 was found for the first time to be present in axons and localized to cytoplasmic stress granules with TDP-43, suggesting the possibility of an extranuclear functional relationship in mRNA transport and/or protection. We also found that PABPN1 can alter the localization and aggregation properties of a pathological C-terminal fragment of TDP-43 when overexpressed in cells. These findings provide important first steps towards the characterization of a functional relationship between the two proteins, which will be critical in understanding their cellular roles in health and disease.

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INTRODUCTION

Neurodegenerative diseases carry an incalculable human cost to society. The loss of cognitive ability, normal mental functioning, and control over movement associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Huntington's disease, and others take away what many consider to be the essence of being human. Because age is the greatest risk factor for many neurodegenerative diseases, we can expect large increases in incidence rates as the geriatric population of the United States is projected to be the fastest-growing age cohort in the coming decades (Vincent and Velkoff, 2010). Financially, these diseases account for hundreds of billions of dollars in United States health care spending each year (Alzheimer's Association, 2012), and unfortunately, no successful cures or therapies to prevent their progression exist.

While we have a much greater understanding of the processes underlying some of these diseases today, and while we can pinpoint the genetic causes of some forms of these diseases, the causes for the sporadic forms and the exact pathomechanisms responsible for cell death and symptomatology remain unknown. In the past decade, however, much research has converged to reveal similar pathological features across many different neurodegenerative diseases. These similar features include presence of abnormally accumulated protein (often aberrantly processed, reviewed in Naeem et al., 2011), transmissibility of abnormal protein aggregates (Cushman et al., 2010), induction of programmed cell degeneration and death (Bredesen et al., 2006), and more recently, altered RNA metabolism (Hanson et al., 2012). It is tempting to speculate that central pathogenic mechanisms that link neurodegenerative diseases with different origins and clinical manifestations can be identified. Such mechanisms would provide targets for the treatment and cure of several heterogeneous diseases.

In recent years, a large body of evidence has arisen in support of the importance and centrality of altered RNA metabolism in various degenerative diseases, including diseases characterized by degeneration of nerve and muscle cells. Deregulation of RNA processing mechanisms has been suggested to be involved in Alzheimer's disease (Pascale and Govoni, 2012), frontotemporal dementia (FTD) (Barmada and Finkbeiner, 2010), Fragile X syndrome (Todd and Paulson, 2010), spinocerebellar ataxia type 2 (Liu-Yesucevitz et al., 2011), myotonic dystrophy types I and II (Sicot et al., 2011), and oculopharyngeal muscular dystrophy (OPMD) (Hanson et al., 2012). Some of the strongest evidence for the centrality of dysfunctional RNA processing in disease processes comes from research into motor neuron diseases, including spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS). In these diseases, mutations in genes coding for proteins involved in RNA metabolism (*SMN1* in SMA; *TARDBP* and *FUS* in ALS) directly cause degeneration of motor neurons (Hanson et al., 2012).

The recent focus on the implication of RNA binding proteins in degenerative disease arises partly from the identification in 2006 of the RNA-binding protein TAR DNA binding protein (TDP-43) as the major component of pathological protein aggregates in the brain tissue of nearly all ALS and FTD patients (Neumann et al., 2006; Arai et al., 2006). ALS and FTD are closely related conditions with overlapping clinical and neuropathological features that many now consider to represent a clinical continuum of TDP-43 proteinopathies (Mackenzie, 2007). ALS, also known as Lou Gehrig's disease, is the most common form of motor neuron disease, affecting up to 30,000 people in the United States at any given time (ALS Association, 2012). Only about 5 - 10 % of all ALS cases are caused by inherited genetic defects (familial ALS) whereas for the large majority of patients without a family history of the disease (sporadic ALS) there is no known cause for ALS. The disease is characterized by progressive degeneration of motor neurons typically beginning late in life (with nearly all patients experiencing symptom onset between the ages of 40 and 70), resulting in paralysis and death usually within 3 to 5 years. It is the third leading neurodegenerative cause of death after Alzheimer's and Parkinson's diseases, and, as with other

neurodegenerative diseases, there is no cure for it. In ALS, TDP-43 is abnormally processed, and a truncated 25 kDa C-terminal fragment is abnormally phosphorylated and ubiquitinated, forming cytoplasmic aggregates (Arai et al., 2006; Neumann et al., 2006). These aggregates have been found to be detergent-insoluble and biochemically distinct from the wild-type protein. Further support for the significance of TDP-43 in ALS pathology was presented in 2008, when multiple groups reported that mutations in the *TARDBP* gene, which encodes TDP-43, can cause ALS disease in both sporadic and familial forms (Gitcho et al., 2008; Kabashi et al. 2008; Sreedharan et al. 2008; Van Deerlin et al. 2008; Yokoseki et al. 2008). Since then, much work has been done to examine the function and behavior of TDP-43 in both health and disease to elucidate its role in the ALS disease process. Interestingly, since the 2006 discovery of pathologic TDP-43-containing inclusions in ALS and FTD patients, abnormal TDP-43 aggregates have also been found in patients with other neurodegenerative diseases such as Alzheimer's disease , Parkinson's disease, and Huntington's disease, suggesting that ALS pathology may be relevant to the development of other neurodegenerative diseases (Da Cruz and Cleveland, 2011).

TDP-43 is a 43-kiloDalton (kDa) RNA binding protein that belongs to the family of heterogenous nuclear ribonucleoproteins (hnRNPs) and is essential for cell survival, ubiquitously expressed, and highly conserved in evolution, with human, fly, and worm protein orthologs showing striking similarities in their nucleic acid binding specificity (Ayala et al., 2005). Because TDP-43 is predominantly localized to the nucleus, most studies have focused on its nuclear role in regulating transcription, splicing, and miRNA processing (Lagier-Tourenne et al., 2010), but it has also been shown to be present in dendrites and to localize to spines in response to neuronal stimulation (Wang et al., 2008). Proposed cytoplasmic functions include control of mRNA transport, stability, translation and mRNA silencing in cellular stress response (Lee et al., 2012). Recently, Dr. Rossoll and coworkers have for the first time identified the presence of mobile TDP-43-containing granules in the axons of primary motor and cortical neurons, expanding the site of action of TDP-43 beyond the soma and dendrites (Fallini et al., under revision).

In an attempt to further investigate possible cellular roles of TDP-43, Rossoll and coworkers identified Poly(A) binding protein nuclear 1 (PABPN1/PABP2) as a candidate interaction partner of TDP-43 in a yeast-two-hybrid screen. PABPN1 is an RNA binding protein known to be involved in polyadenylation of mRNA transcripts (Wahle, 1991), and shown to be present during various stages of mRNA metabolism, including nucleocytoplasmic shuttling, the pioneer round of translation, and nonsense-mediated decay (NMD) (Lemay et al., 2010). PABPN1 appears to be associated predominantly with untranslated mRNAs and it is replaced by the cytoplasmic poly(A) binding protein PABPC1/PABP1 upon translation. PABPN1 shares several similarities with TDP-43, including presence of an RNA recognition motif (RRM) and nuclear localization signal (NLS), predominantly nuclear localization, ubiquitous expression across all cells and tissues, and roles in RNA metabolism and transport. Mutated PABPN1, like mutated TDP-43, causes a dominant degenerative disease characterized by the presence of insoluble protein aggregates: genetic expansions of the N-terminal poly-alanine repeat region of PABPN1 cause oculopharyngeal muscular dystrophy (OPMD), a late-onset degenerative disease affecting facial and neck muscle cells (Brais and Rouleau, 1993). Expanded-PABPN1 forms nuclear aggregates in OPMD, which have been shown to be detergent- and salt-insoluble, in contrast to the wild-type form of PABPN1 (Fan et al., 2007; Wang et al., 2005). While OPMD is not primarily a neurodegenerative disease, OPMD cases with neurogenic involvement have been reported (Boukriche et al., 2002). Interestingly, cytoplasmic TDP-43 aggregates have also been found in OPMD muscle tissue (Kusters et al., 2009).

Like altered RNA metabolism, aberrant protein aggregation is believed to be a key pathological feature in many different neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, frontotemporal dementia (FTD), and many others (reviewed in Naeem et al., 2011). Both ALS and OPMD, like many other degenerative diseases, are surrounded by controversy over the roles of their respective aggregates. Do the aggregates contribute a toxic gain-of-function which directly causes disease, or are they "innocent" correlates of the disease process and remain

separate from toxic pathomechanisms? Or, do they sequester potentially toxic aberrant protein species, thereby serving as a defense against disease mechanisms? In ALS, localization of TDP-43 shifts from the nucleus to the cytoplasm, where it eventually forms insoluble aggregates. This mislocalization of the protein to the cytoplasm suggests that perhaps a depletion of TDP-43 from the nucleus prevents the protein from fulfilling its normal function (Dormann and Haass, 2011). Alternatively, in the case of PABPN1, perhaps the change in solubility (from the soluble wild-type species to the insoluble mutated species) is what leads to disease (Raz et al., 2011). In both cases, however, it is often suggested that the key to disease is the inability of the protein to function normally, which compels further research into the respective wild type functions of both proteins.

As more researchers begin to think of neurodegenerative diseases also as diseases of RNA processing, insight into the functions and dysfunctions of RNA-binding proteins is becoming more critical. In this study, I investigated the potential relationship between TDP-43 and PABPN1 in an attempt to gain insight into their respective functions. Based on their putative interaction found in a yeast two-hybrid screen and their similar features, I hypothesized that the two proteins have a physiologically relevant functional relationship, and that they will interact and co-localize, and perhaps even co-aggregate in mutant forms. My first aim was to confirm the interaction using several different biochemical and immunocytochemical techniques, including co-immunoprecipitation and co-localization analysis of overexpressed constructs and endogenous proteins. After successful confirmation of the interaction, I analyzed the aggregation of the mutant forms of the two proteins upon overexpression *in vitro*, and started to characterize the aggregates in terms of solubility and to quantify them in terms of localization. Lastly, I began to investigate cellular processes in which TDP-43 and PABPN1 could both participate, proposing stress granule sequestration, mRNA transport, and local translation as having potential for being arenas for the interaction of TDP-43 and PABPN1.

METHODS

Constructs

Several human TDP-43 and PABPN1 cDNA constructs were used (Figure 1). TDP-43 tagged at the N-terminus with the triple FLAG epitope (FLAG-TDP-43); TDP-43 fused at the N-terminus to the monomeric fluorescent red protein mCherry (mCh-TDP-43); the C-terminal 25kDa fragment of TDP-43 found in pathological aggregates of ALS and FTD patients fused at the N-terminus to mCherry (mCh-TDP-208); wild-type PABPN1 tagged with the double HA epitope at the N-terminus; wild-type PABPN1 and a patient-derived expanded version of PABPN1 (17 instead of 10 alanine residues at the N-terminus) fused at the N-terminus to monomeric enhanced green fluorescent protein GFP (GFP-PABPN1 and GFP-EX-PABPN1, respectively).

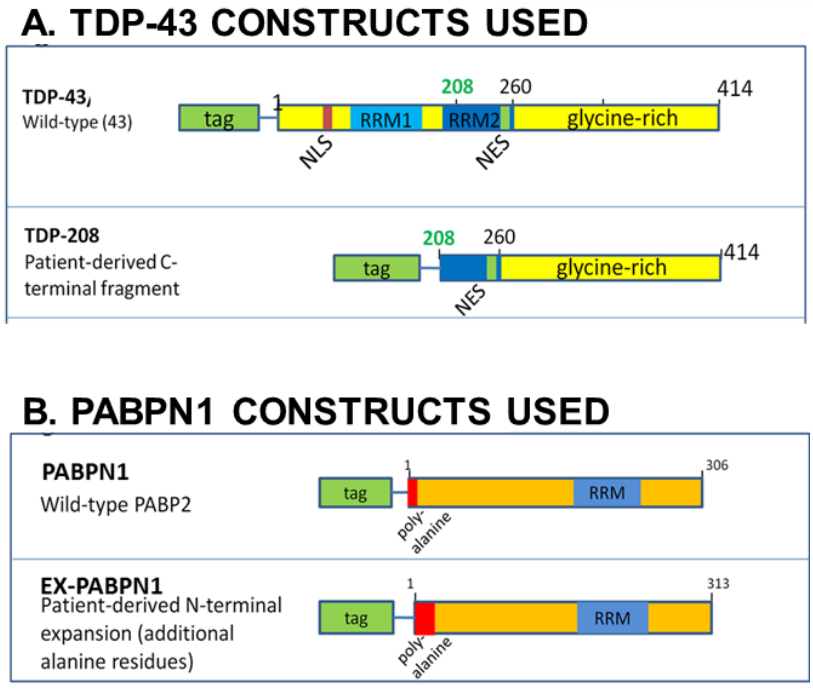


Figure 1, A: TDP-43 constructs used. *Top:* Full-length, wild type human TDP-43 tagged at the N-terminus with either mCherry or triple FLAG tag. *Bottom*: truncated C-terminal fragment found in patients tagged at N-terminus with mCherry.
Figure 1, B: PABPN1 constructs used. *Top:* Full-length, wild type human PABPN1 tagged at the N-terminus with double HA tag or GFP. *Bottom:* mutant patient-derived PABPN1 containing 17-alanine expansion at its N-terminus. Numbers represent amino acid count from N-terminus.

Yeast two-hybrid assay

Human TDP-43 and PABPN1 cDNAs cloned into yeast-two-hybrid vectors pGBKT7 (binding domain "bait" vector, Clontech) and pGADT7 (activating domain "prey" vector, Clontech) were transformed into the *S. cerevisiae* two-hybrid reporter strain AH109 (Clontech). Cells were plated onto selective (-leu, -trp) plates to isolate transformants, then transformants were streaked out onto selective (SD/-leu/-trp/-ade/-his/+ X-α-Gal) plates to screen for expression of the *ADE2*, *HIS3*, and *MEL1* reporter genes and to visualize colonies positive for yeast-two-hybrid interaction, appearing in blue.

Co-immunoprecipitation of HA- and FLAG-tagged constructs from cell lines

HEK293 cells, cultured in DMEM medium containing 10% FBS, were co- transfected with FLAG-TDP-43 and HA-PABPN1 using Turbofect (Fermentas) according to manufacturer's recommendations. Two days after transfection, cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 with protease inhibitor cocktail, Sigma), centrifuged for 15 minutes at 20,000 g, and the supernatant was incubated with either anti-HA (HA PrecipHen, Aves Labs Inc) or anti-FLAG (Sigma) agarose beads overnight at 4°C with rotation. Cells transfected with either FLAG- or HA-constructs were used as controls. Beads were washed 5 times with lysis buffer, boiled with Laemmli buffer and bound proteins were separated on a 10% SDS-PAGE gel for Western blot analysis. Samples were blotted onto nitrocellulose membranes and blocked with Odyssey blocking buffer (LiCor). Monoclonal antibodies (mouse anti-TARDBP clone 3H8, Abcam; and rabbit anti-PABPN1 EP3000Y, Epitomics) were used for detection.

Co-immunoprecipitation of endogenous protein from brain tissue.

Brains from E-13.5 and E-17 mouse embryos were flash frozen in liquid nitrogen immediately following dissection and kept at -80° until needed. Brains were homogenized with a dounce

homogenizer and lysed on ice in 1 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) with protease inhibior cocktail (Sigma), then sonicated and spun down at 20,000 g for 15 minutes. A small amount of input from the supernatant was set aside, and the rest was used for co-immunoprecipitation. Immunoprecipitation of PABNP1 was done with rabbit monoclonal anti-PABPN1 (EP3000y, Epitomics) and Protein A-agarose beads (Calbiochem) according to manufacturer's instructions. Normal rabbit serum (Millipore) was used as a control. Beads were washed 5 times with lysis buffer and boiled in Laemmli sample buffer. Samples were separated on a 10% SDS-PAGE gel , blotted onto nitrocellulose membranes, blocked with Odyssey blocking buffer (LiCor) and detected with the same anti-PABPN1 and anti-TARDBP antibodies listed above (1:2000).

Cell culture, staining and imaging

Neuroblastoma N2A cells or HEK293 cells, cultured in DMEM containing 10% FBS, were co-transfected with expression plasmids for mCh-TDP-43, mCh-TDP-208 or mCherry, together with either EX-PABPN1-GFP or PABPN1-GFP or GFP, using Turbofect (Fermentas) per manufacturer's instructions. Cells were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature 48 hours after transfection and washed with PBS. Cells were permeabilized in 0.25% Triton X-100 in PBS (5 minutes) and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) on glass slides. For KCl treatment, cells were washed once in PBS and treated with the KCl solution buffer described in Messaed et al., 2007 (1.5 M KCl in 30 mM HEPES, 65 mM PIPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) for 20 minutes prior to fixation. For Triton X-100 treatment, cells were washed twice in chilled PBS and incubated on ice with 0.5% Triton X-100 in sucrose buffer (10 mM PIPES, 100 mM NaCl, 2.5 mM magnesium acetate, 0.3 M sucrose, pH 6.8) for 5 minutes and washed again in PBS before fixation.

Motor neurons from E-13.5 mouse embryos, as well as hippocampal neurons from E-17 mouse embryos, were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature and washed

with PBS. Cells were permeabilized in 0.25% Triton X-100 in PBS (5 minutes) and stained with monoclonal antibodies to TDP-43 (mouse anti-TARDBP clone 3H8, Abcam, 1:1000) and PABPN1 (rabbit anti-PABPN1 EP3000Y, Epitomics; 1:500). Axons of hippocampal neurons were visualized by microtubule-associated protein tau staining (polyclonal chicken anti-tau, ab75714, Abcam; 1:20,000). For arsenite treatment, cells were incubated at 37°C for 1 hour with 0.5 mM sodium arsenite prior to fixation. Visualization of cytoplasmic stress granules was done using goat anti-eIF3η (N-20, Santa Cruz Biotechnology; 1:200).

All images were acquired on an epifluorescence microscope (Ti, Nikon) equipped with a cooled CCD camera (HQ2, Photometrics). Image stacks (5-10 slices, 0.2 µm step size) were deconvolved using AutoQuant 3D Deconvolution software (Media Cybernetics) and processed with Imaris 3D image processing software (Bitplane).

RESULTS

TDP-43 and PABPN1 interact in yeast two-hybrid assay

A yeast two-hybrid assay was done to confirm the original yeast two-hybrid screen result suggesting interaction between TDP-43 and PAPBN1. Full length human TDP-43 and PABPN1 were cloned into pGBKT7 and pGADT7 vectors and transformed into competent cells of a yeast two-hybrid reporter strain, and colonies were allowed to grow for one week before scoring. Blue colored colonies indicated interaction and were scored on a scale from + (weak interaction) to +++ (strong interaction) based on colony size and color development. TDP-43 in pGADT7 showed weak but clear interaction with PABPN1 in pGBKT7 (+), and the reverse combination also exhibited a weak interaction (+) (Table 1). TDP-43 was observed to dimerize strongly (+++), and PABPN1 was observed to dimerize with moderate strength (++). Negative controls in the form of yeast cells transfected with at least one empty vector (pGBKT7 or pGADT7) did not grow colonies, confirming that overexpression of exogenous constructs alone would not activate the reporter genes and produce a false positive result.

	pGADT7	PABPN1	TDP-43
pGBKT7	-	-	-
PABPN1	-	++	+
TDP-43	-	+	+++

Table 1. A yeast two-hybrid assay confirms the interaction of TDP-43 and PABPN1. The table shows plasmids used for co-transfection. Columns represent empty pGADT7 prey vector and PABPN1 and TDP-43 cloned into pGADT7, respectively; rows represent empty pGBKT7 bait vector and PABPN1 and TDP-43 cloned into pGBKT7, respectively. Strength of interaction is reported on a scale from strong (+++) to weak (+).

Overexpressed FLAG-TDP-43 and HA-PABPN1 interact in cell lysates

While the yeast two-hybrid assay with the full length cDNAs confirmed the putative interaction observed in the yeast two-hybrid screen, this method may yield false positive results and independent methods are necessary to substantiate the finding. We further established the interaction through co-immunoprecipitation of overexpressed epitope-tagged constructs. Immunoprecipitation (IP) of HA-PABPN1 was done in lysates from human embryonic kidney 293 (HEK293) cells transfected with HA-PABPN1 and FLAG-TDP-43 with agarose beads coated with anti-HA antibodies. Both FLAG-TDP-43 and HA-PABPN1 were detected in the precipitate, showing an association of the two proteins in the cell lysate. Cells transfected with only FLAG-TDP-43 as a negative control did not show presence of FLAG-TDP-43 in the anti-HA IP pellet, suggesting that the co-immunoprecipitation was specific. Endogenous proteins as well as tagged constructs were detected in IP inputs in Western blots with anti-TDP-43 and anti-PABPN1 antibodies.

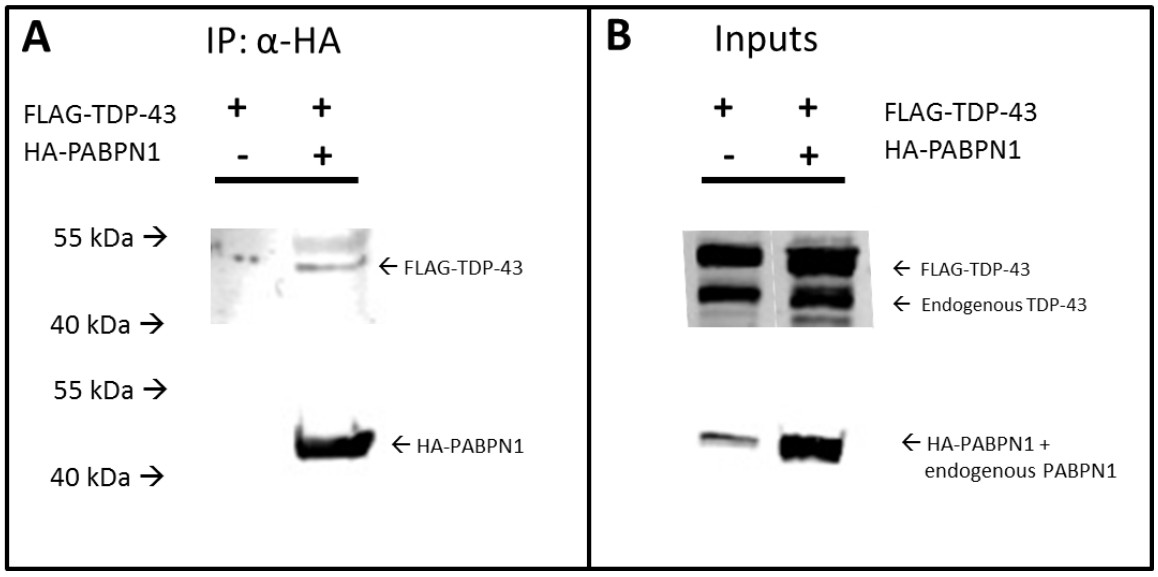


Figure 2. Co-immunoprecipitation of FLAG-TDP-43 with HA-PABPN1 in transfected HEK293 cells. HEK293 cells were co-transfected with HA-PABPN1/FLAG-TDP-43 and only with FLAG-TDP-43 as a control. Lysates were used for Western blot analysis and co-immunoprecipitation with anti-HA beads. *A:* Western blot analysis of immune precipitates from lysates shown in *B* after pull-down with anti-HA beads. FLAG-TDP-43 is co-immunoprecipitated with HA-PABPN1 but not from control lysates. *B:* Lysates of cells transfected with expression vectors for HA-PABPN1/FLAG-TDP-43 or only FLAG-TDP-43. Proteins are detected with anti-TDP-43 and anti-PABPN1 antibodies.

Endogenous TDP-43 and PABPN1 interact in brain tissue lysates

To rule out overexpression artifacts, I then wanted to demonstrate interaction of endogenous proteins in tissue, which is a more physiologically relevant demonstration of interaction. Homogenized brain tissue of embryonic mice was used for immunoprecipitation of endogenous PABPN1. Both PABPN1 and TDP-43 were detected in the pull-down of PABPN1, whereas neither protein was detected after a control immunoprecipitation with normal rabbit serum, showing that the endogenous proteins specifically associate in brain lysates (Figure 3).

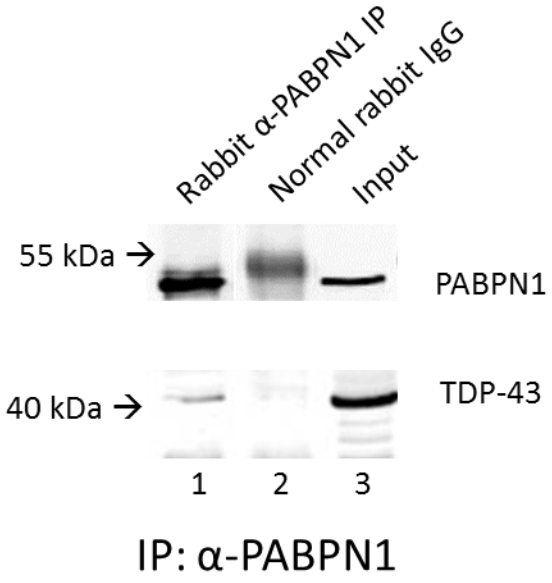


Figure 3. Endogenous TDP-43 and PABPN1 interact in embryonic mouse brain tissue lysate. Mouse brain lysates were used for immunoprecipitation with anti-PABPN1 antibodies. Western blot analysis with anti-PABPN1 and anti-TDP-43 detects both proteins in the immune precipitate (lane 1), whereas control immunoprecipitation with normal rabbit serum (lane 2) yielded neither PABPN1 (top) nor TDP-43 (bottom). Both PABPN1 and TDP-43 were present in the lysate (lane 3).

Co-localization of endogenous TDP-43 and PABPN1 in hippocampal neuronal axons

Showing that proteins are capable of interaction in biochemical assays, however, does not necessarily indicate that they interact functionally under physiological conditions, nor does it reveal in what subcellular compartment the interaction takes place. Thus, I moved on to studying the localization

of the proteins in intact cells by immunocytochemical analysis. Endogenous proteins in hippocampal neurons were detected by immunocytochemistry with anti-PABPN1 and anti-TDP-43 antibodies and visualized using fluorescence microscopy. Localization of both proteins was mainly nuclear, as expected (not shown). Interestingly, however, endogenous TDP-43 and PABPN1 were also detected in neuronal axons (Figure 4). This is the first time PABPN1 is reported to be present in axons, while the axonal localization of TDP-43 has recently been established in the lab (Fallini et al., under revision). Moreover, a fraction of the axonal TDP-43 and PABPN1 appeared to be colocalized within the same granular structures (Figure 4, white arrows), although a quantitative analysis of the colocalization is necessary to rule out the possibility of random overlap. This novel finding of PABPN1 in axons, potentially colocalized with TDP-43, supports a functional interaction between PABPN1 and TDP-43 outside of the nucleus.

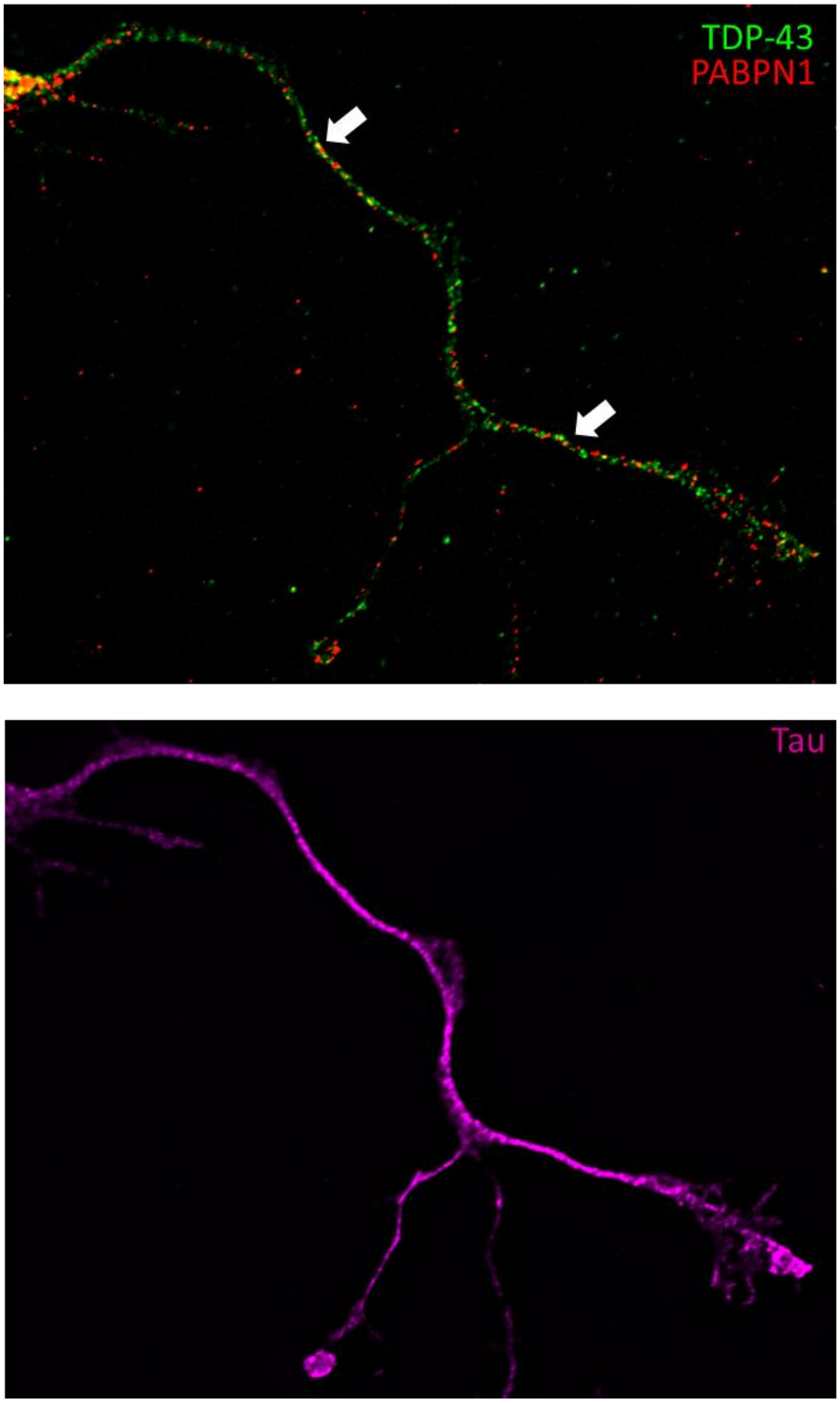


Figure 4. Co-localization of endogenous TDP-43 and PABPN1 in hippocampal neuron axons. Cultured primary hippocampal neurons were fixed, permeabilized, and stained for TDP-43 (green), PABPN1 (red), and axonal marker MAP tau (magenta). A single optical section from a deconvolved image stack is shown. TDP-43 and PABPN1 were both detected in axonal granules. White arrows indicate areas of co-localization to the same granular structures. Scale bar: 15 µm.

Localization of endogenous TDP-43 and PABPN1 in cytoplasmic stress granules

To further investigate the potential of an extranuclear physiological interaction of TDP-43 and PABPN1, I looked for colocalization of TDP-43 and PABPN1 in cytoplasmic stress granules, which are known to contain TDP-43 and other RNA-associated proteins (Colombrita et al., 2009). Because PABPN1 and TDP-43 are known to be involved in nucleocytoplasmic shuttling, and because both TDP-43 and PABPN1 have been shown to be present in the cytoplasm and axons, it is plausible that they may both be present in stress granules. Primary motor neurons exposed to arsenite stress for one hour showed formation of cytoplasmic stress granules, and the localization of both TDP-43 and PABPN1 was indeed shifted to cytoplasmic granules that were positive for a specific stress marker (Figure 5). This novel finding indicates a potential for stress granule sequestration to be an arena for TDP-43/PABPN1 interaction.

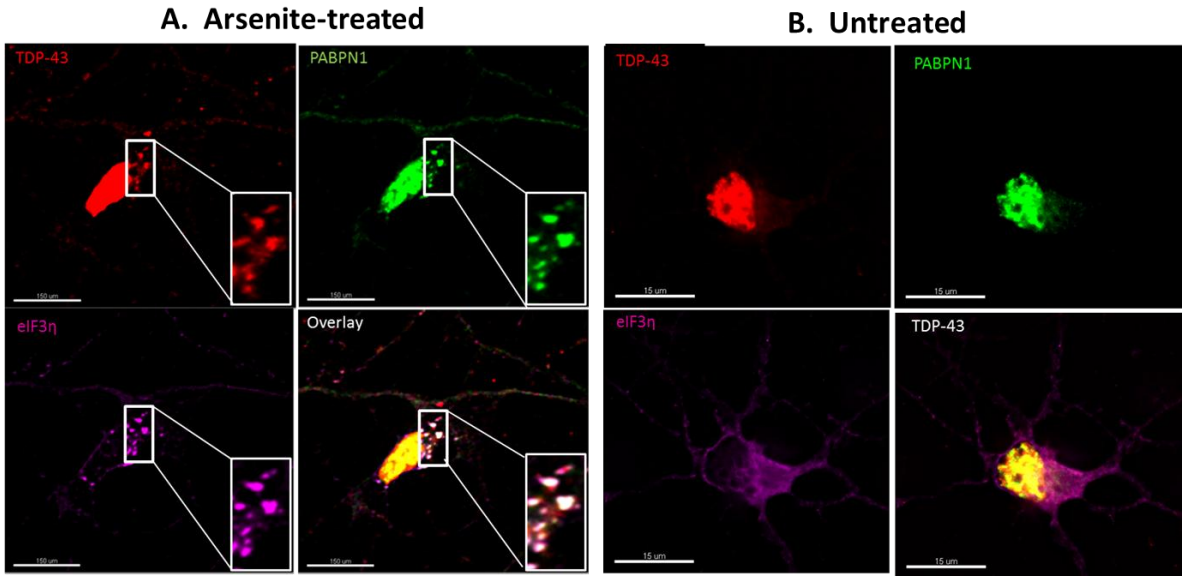


Figure 5. Endogenous TDP-43 and PABPN1 localize to cytoplasmic stress granules in response to arsenite treatment in motor neurons. A: Primary motor neurons were treated with 0.5 mM sodium arsenite for 1h to induce oxidative stress and the formation of stress granules. Anti-eIF3 was used as a marker for cytoplasmic stress granules (magenta). Close overlap between TDP-43 staining (red) and PABPN1 staining (green) is evident within stress granules. B: Untreated cells shown as control; no stress granules seen. Scale bar: 15 µm.

Nuclear recruitment of the cytoplasmic aggregation-prone TDP fragment by PABPN1

Aggregation-prone proteins have been shown to sometimes recruit associated proteins into insoluble inclusions. Since pathogenic forms of both TDP-43 and PABPN1 are known to form cytoplasmic or nuclear aggregates in patients, we decided to investigate a potential co-aggregation as evidence for their association. I used TDP-208, the patient-derived C-terminal fragment from amino acid 208 to 414 found in ALS patients, fused to the red fluorescent protein mCherry (mCh-TDP-208), and EX-PABPN1, the 17-alanine expansion mutant found in some patients with OPMD fused to green fluorescent protein (GFP-EX-PABPN1). Both of these constructs have been shown to recapitulate pathological aggregation found in ALS and OPMD patients when overexpressed *in vitro* (Igaz et al., 2009; Abu-Baker and Rouleau, 2007). Control constructs were full length TDP-43 fused to mCherry (mCh-TDP-43) and wild-type PABPN1 fused to GFP (GFP-PABPN1). Negative controls for the fused proteins were expression plasmids for mCherry and GFP.

Neuro 2A (N2A) murine neuroblastoma and HEK293 cells were co- transfected with one GFP construct and one mCherry construct in a total of nine combinations (see Table 2) and fixed after 48 hours, after which localization of the fluorescent proteins was analyzed by fluorescence microscopy. The wild type and pathological forms of the proteins respectively behaved as reported in the literature when co-expressed with a GFP or mCherry control (not shown). GFP-PABPN1, GFP-EX-PABPN1 and mCh-TDP-43 remained localized to the nucleus, while mCh-TDP-208 was found either diffusely localized throughout the cytoplasm or concentrated in cytoplasmic aggregates. When the two mutant proteins were co-expressed together, however, the localization of mCh-TDP-208 was dramatically shifted to the nucleus. In the majority of cells, mCh-TDP-208 was found either equally distributed between nucleus and cytoplasm, or predominantly in the nucleus, and cytoplasmic aggregates were absent. Moreover, the nuclear mCh-TDP-208 often seemed to co-localize with the nuclear mutant PABPN1, with both

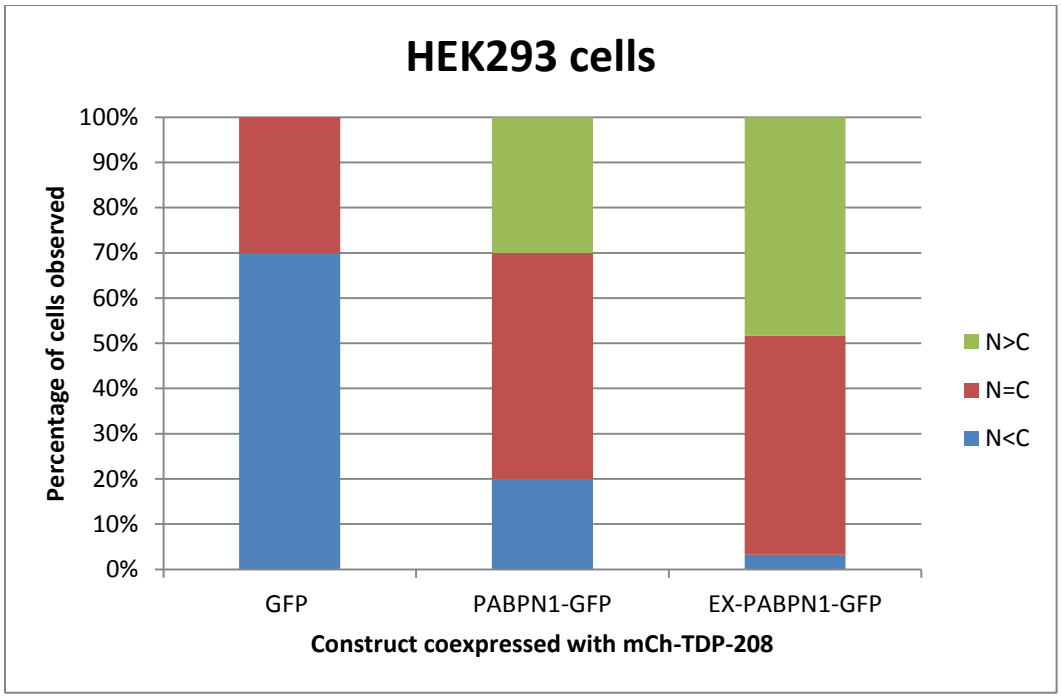
proteins showing similar patterns of fluorescence within the nucleus. A congruent, but less dramatic, shift in localization was also seen when mCh-TDP-208 was co-expressed with the wild-type GFP-PABPN1. It appeared that both GFP-PABPN1 and GFP-EX-PABPN1 were capable of recruiting cytoplasmic mCh-TDP-208 into the nucleus, to lesser and greater degrees, respectively.

PABPN1 construct	TDP-construct
GFP-PABPN1	mCh-TDP-43
GFP-EX-PABPN1	mCh-TDP-43
GFP	mCh-TDP-43
GFP-PABPN1	mCh-TDP-208
GFP-EX-PABPN1	mCh-TDP-208
GFP	mCh-TDP-208
GFP-PABPN1	mCh
GFP-EX-PABPN1	mCh
GFP	mCh

Table 2. Combinations of fluorescently tagged expression plasmids used in co-transfections.

To quantify this shift in localization of mCh-TDP-208 from cytoplasm to nucleus, I scored mCh-TDP-208 localization in cells co-transfected with GFP-PAPBN1, GFP-EX-PABPN1 or GFP by judging whether the majority of a cell's observed mCh-TDP-208 fluorescence was localized in the nucleus (N>C), the cytoplasm (N<C), or both equally (N=C). In 70% of scored cells expressing GFP, mCh-TDP-208 was predominantly cytoplasmic (Figure 6A). This percentage dropped to only about 20% when cells

expressed GFP-PABPN1, and to 3% for cells expressing the mutant GFP-EX-PABPN1. Accordingly, the percentage of cells showing predominantly nuclear presence of mCh-TDP-208 increased from 0% in the GFP-expressing cells to 30% in the GFP-PABPN1-expressing cells to almost 50% in the GFP-EX-PABPN1-expressing cells. Similar results were obtained with both HEK293 and N2A cells. These data are indicative of nuclear recruitment of mCh-TDP-208 by GFP-PABPN1 and GFP-EX-PABPN1. This is remarkable, since the pathological TDP fragment lacks an NLS and its expression mimics the loss of nuclear TDP-43 followed by aggregation of this C-terminal fragment in cytoplasmic aggregates that is a hallmark of ALS and FTD.



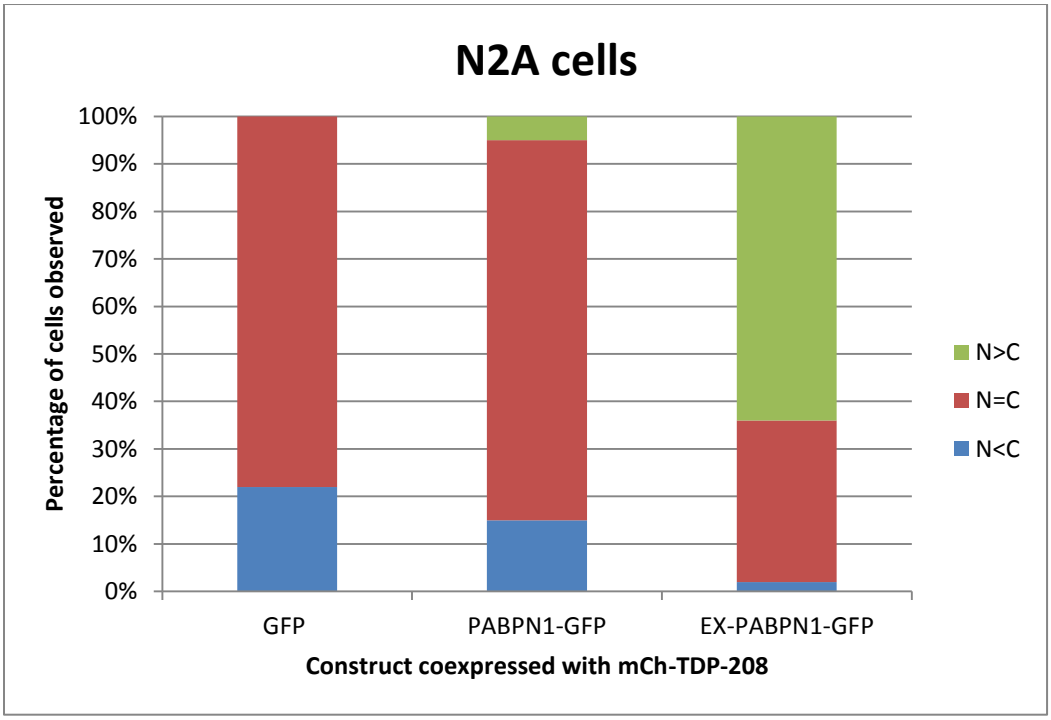


Figure 6A. mCh-TDP-208 is recruited into nucleus by GFP-EX-PABPN1. *Top:* HEK293 cells transfected with mCh-TDP-208 and GFP, GFP-PABPN1, or GFP-EX-PABPN1 were scored based on whether the mCh-TDP-208 fluorescent signal was predominantly nuclear (N>C), cytoplasmic (N<C), or approximately equal in both (N=C). While mCh-TDP-208 is predominantly cytoplasmic in the majority of cells when co-expressed with GFP, its localization becomes more nuclear upon co-expression with GFP-PABPN1. When co-expressed with GFP-EX-PABPN1, this change in localization becomes even more apparent as mCh-TDP-208 becomes predominantly nuclear in almost 50% of cells. *Bottom:* Similar result was observed in N2A cells.

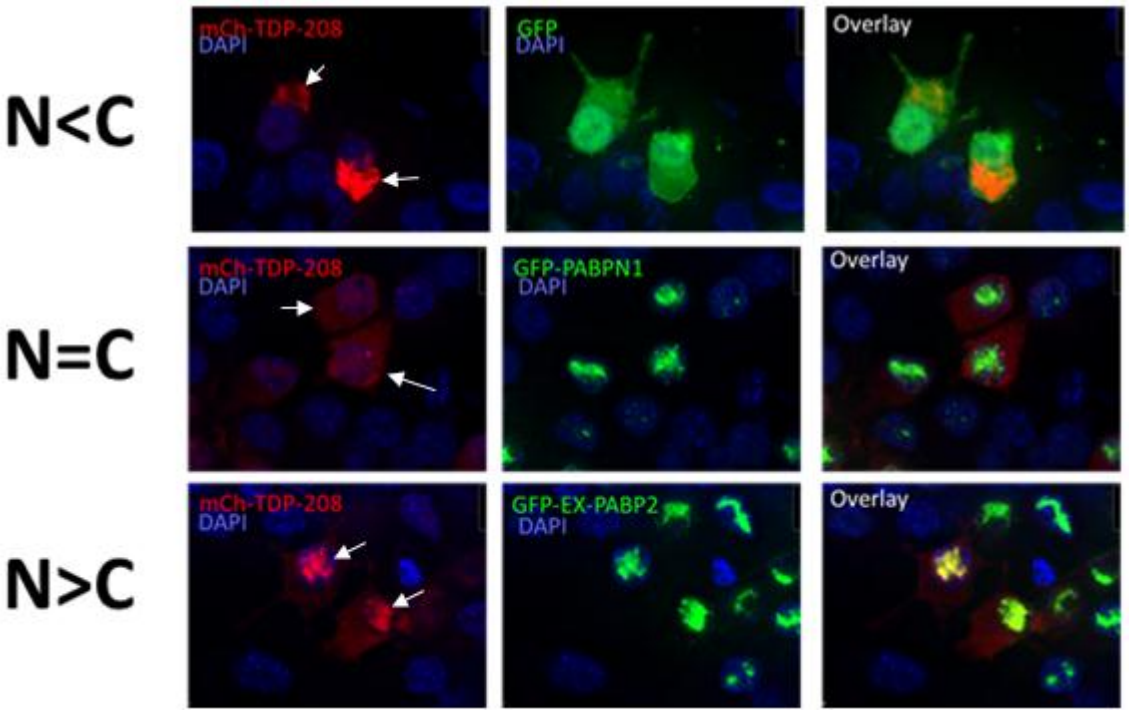


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Figure 6B. Representative visual examples of Neuro 2A (N2A) cells for each of three scoring categories in Figure 6A. *Top:* N2A cells coexpressing GFP showing predominantly cytoplasmic (N<C) fluorescence of mCh-TDP-208. *Middle:* Cells coexpressing GFP-PABPN1 showing approximately equal cytoplasmic and nuclear fluorescence of mCh-TDP-208 (N=C). Bottom: Cells coexpressing GFP-EX-PABPN1 showing predominantly nuclear fluorescence of mCh-TDP-208 (N>C).

Salt and detergent solubility of GFP-EX-PABPN1 and mCh-TDP-208 aggregates

TDP-208 recruited into the nucleus by EX-PABPN1 often appeared to strongly co-localize within the same nuclear domains, suggesting a possible co-aggregation of the proteins. Therefore, we decided to characterize the mutant aggregates in terms of their solubility. It was reported by Fan et al. that live-cell KCl treatment washes out overexpressed PABPN1 but leaves overexpressed EX-PABPN1 aggregates unaffected, suggesting observable biochemical differences in solubility under high salt conditions (2001). We were unable to reproduce this phenomenon, as we saw no consistent difference between GFP-PABPN1 and GFP-EX-PABPN1 presence in treated cells, nor any difference in mCh-TDP-208 (Figure 7).

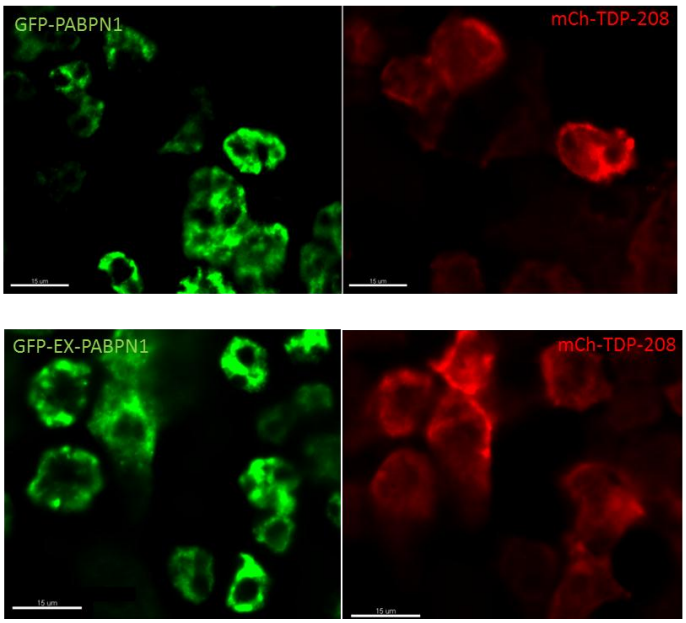

Figure 7. KCl treatment of live HEK293 cells. Both GFP-PABPN1 (top left) and GFP-EX-PABPN1 (bottom left) were resistant to KCl treatment when coexpressed with TDP-208. Resistance was also seen in mCh-TDP-208 (top and bottom, right). Scale bar: 15 µm.

Similar problems with KCl treatment were also reported by Wang et al., who showed that instead, Triton X-100 treatment could be used to distinguish wild-type PABPN1 and EX-PABPN1 based on their detergent solubility (2005). To test this alternative method, we first co-transfected HEK293 cells as shown in Table 2. After incubation of live HEK293 cells on ice with 0.5% Triton X-100 in sucrose buffer, we saw striking differences between GFP-PABPN1 and GFP-EX-PABPN1 in cells (Figure 8). GFP and GFP-PABPN1 were completely washed out of cells following treatment (Panels A and B), while nuclear GFP-EX-PABPN1 remained intact (Panel C). As expected, cytoplasmic mCh-TDP-208 aggregates were resistant to the Triton X-100 treatment (Panel A and B). Interestingly and unexpectedly, nuclear mCh-TDP-208 present in cells expressing GFP- PABPN1 and even more in cells expressing GFP-EX-PABPN1 was removed by Triton X-100 treatment (Panels B and C). The disappearance of nuclear mCh-TDP-208

staining suggests that the mCh-TDP-208 that is recruited into the nucleus by GFP-EX-PABPN1 (and to a lesser degree by GFP-PABPN1) is detergent-soluble.

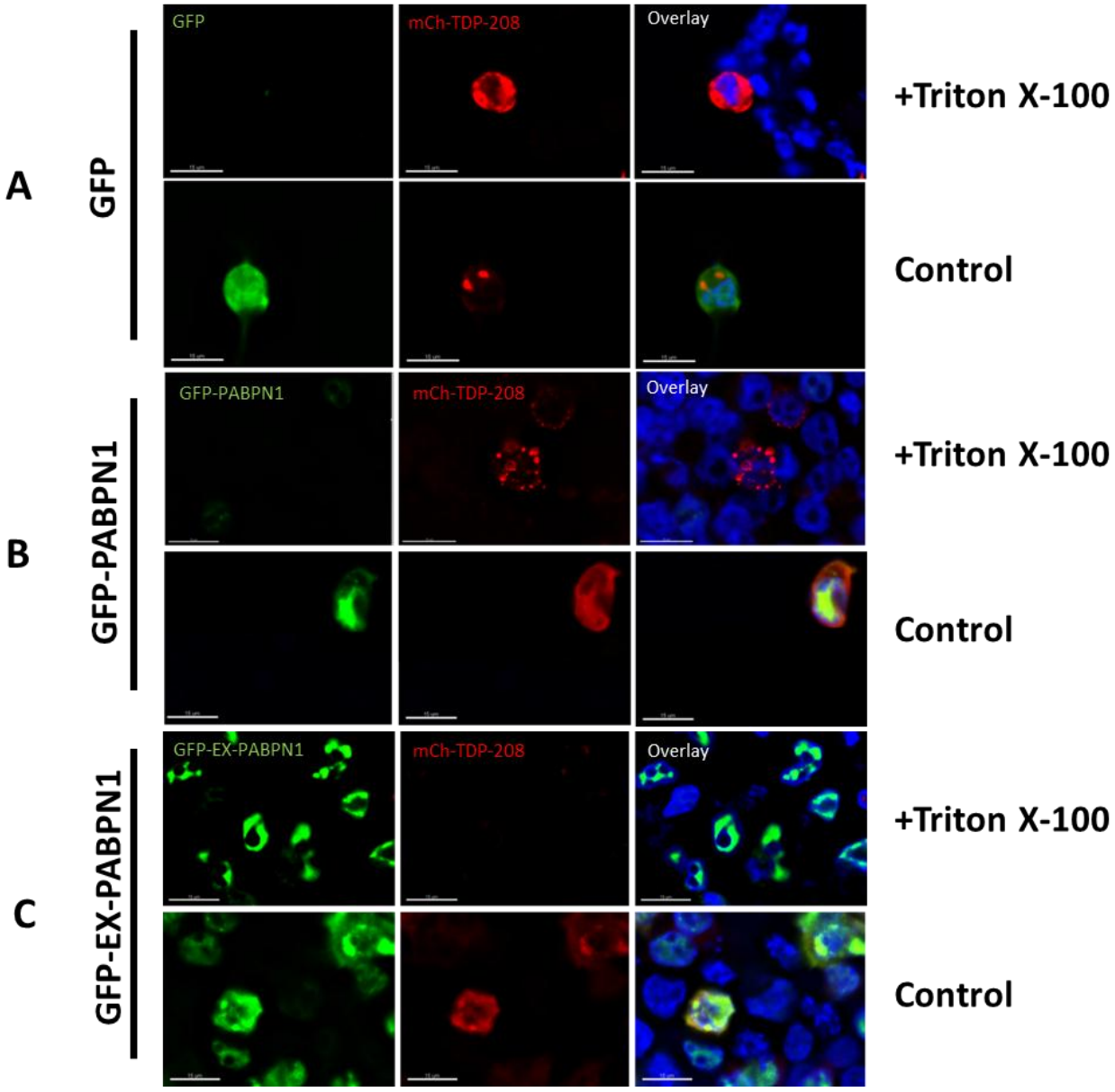

Figure 8. Triton X-100 treatment of live HEK293 cells. Triton X-100 treated (+Triton X-100) and untreated (control) cells are shown for three cotransfections (Panels A, B, and C). *Panel A.* GFP is washed out by treatment (top left), but persistence of cytoplasmic mCh-TDP-208 aggregates is seen (top middle). *Panel B*: GFP-PABPN1 and nuclear and diffuse cytoplasmic mCh-TDP-208 is washed out by treatment (top left), but rare cytoplasmic mCh-TDP-208 aggregates remain (top middle). *Panel C*: GFP-EX-PABPN1 is resistant to detergent treatment (left), but coexpressed mCh-TDP-208 is mostly nuclear and disappears in response to treatment (middle). DAPI (blue) shows nuclear staining and presence of cells. Scale bar: 15 µm

DISCUSSION

Abnormal RNA metabolism is becoming increasingly recognized as an important aspect of the pathology of different degenerative diseases, making investigation of RNA binding proteins critical to understanding disease pathomechanisms. In this study, we provide evidence for the interaction of two disease-associated RNA binding proteins, TDP-43 and PABPN1, in both their wild type and pathological forms. Using yeast two-hybrid and co-immunoprecipitation studies as independent complementary methods, we confirmed that wild-type TDP-43 and PABPN1 are capable of interacting. Both yeast two-hybrid and co-immunoprecipitation, however, are not necessarily indicators of direct protein-protein interaction, since RNA-mediated interaction (both proteins bound to the same RNA molecule, but not each other) may also be detected by these analyses. Thus, a confirmation of this interaction in the presence of RNA-digesting enzymes, or through the usage of purified recombinant proteins, is still necessary to show a direct interaction.

While showing the interaction in *in vitro* assays does suggest physiological protein interaction, it is also necessary to show that potential interaction partners are co-localized in living cells to provide strong evidence for *in vivo* interaction. We observed that endogenous PABPN1 and TDP-43 co-localize to the same cellular compartments in primary neurons. This provides further support for our hypothesis of a physiologically relevant association between PABPN1 and TDP-43, as it shows that PABPN1 and TDP-43 are not only capable of interacting, but also are found in close proximity in intact cells. Importantly, we show for the first time that PABPN1 is localized to neuronal axons and confirmed the unpublished axonal localization of TDP-43, expanding the possibility of a functional interaction beyond the nucleus and soma. A fraction of the observed axonal TDP-43 and PABPN1 were found colocalized within the same granule-like structures. Future studies will be required to quantify this axonal colocalization to evaluate whether it is specific or simply due to random overlap. If confirmed to be specific, it would be

interesting to compare axonal colocalization to nuclear colocalization to determine if there is potentially a preference for interaction in either cellular compartment. In addition, it would be interesting to determine whether stimulation of neurons by BDNF or KCl can influence levels of TDP-43 or PABPN1 found in axons, or whether it can change the extent to which they colocalize. If neuronal stimulation increases axonal TDP-43 or PABPN1 levels, this may support a role for both proteins in mRNA trafficking and/or the regulation of local translation.

We speculated that the granule-like structures in which TDP-43 and PABPN1 appear to colocalize in axons might be neuronal RNA transport granules. In neurons, RNAs are thought to be packaged together with RNA-binding proteins into translationally silent RNA granules and transported along dendrites and axons to growth cones and synapses where their protein products are required – only then are they activated and translated. TDP-43 has been observed to be transported into dendritic spines in response to neuronal stimulation and to co-localize with RNA binding proteins such as FMRP and Staufen 1 that are known to be involved in regulating mRNA transport and local translation (Wang et al., 2008). This has led to the hypothesis that TDP-43 may be a neuronal activity-responsive factor functioning in the regulation of neuronal plasticity, perhaps by acting as a translational repressor. Currently, nothing is known about a localization or functional role of PABPN1 in dendrites and axons. Noteworthy, PABPN1 has been identified in a proteomic study as a component of RNA granules containing the mRNA binding protein insulin-like growth factor II mRNA-binding protein (IMP1) / zip code-binding protein (ZBP1) (Jonson and Nielsen et al., 2007). IMP1/ZBP1 plays an important role in axonal mRNA transport and local translation of mRNA cargos required for axonal regeneration *in vitro* and *in vivo* (Donnelly et al., 2011). Since PABPN1 is associated with untranslated mRNAs, and since mRNAs in transport granules are believed to be translationally silent, it is attractive to speculate that PABPN1 may participate in mRNA trafficking in axons through a presence in these transport granules.

Our discovery of axonal PABPN1 presented here, in addition to the discovery of axonal TDP-43 by Dr. Rossoll and co-workers (Fallini et al., under revision), suggests that the arena for a TDP-43/PABPN1 functional relationship need not be limited to the nucleus, where the bulk of endogenous PABPN1 and TDP-43 are found. This notion is further supported by our finding of PABPN1 and TDP-43 in cytoplasmic stress granules. Similar to neuronal RNA transport granules in composition (Anderson and Kedersha, 2006), stress granules are dynamic, transient structures, formed in response to environmental stress such as oxidative stress and heat shock, in which inactivated mRNAs are packaged. These structures contain a wide variety of RNAs and RNA-associated proteins and are believed to be a protective cellular response to stress during which translation is repressed and mRNAs are tightly regulated (reviewed in Anderson and Kedersha, 2006). While TDP-43 is already known to be present in stress granules (Colombrita et al., 2009), this has not been reported for PABPN1 to our knowledge. This novel finding indicates a potential physiological TDP-43/PABPN1 interaction in stress granules: TDP-43 and PABPN1 may associate and, together with other mRNA binding proteins, determine the fate of mRNAs recruited into stress granules.

More studies are needed to investigate the possibility of other functional relationships of TDP-43 and PABPN1 in addition to further characterizing the mRNA transport role suggested by axonal colocalization and the translational repression role suggested by involvement in cytoplasmic stress granules. Other roles for a TDP-43 and PABPN1 interaction may be found by looking at other RNA-related cytoplasmic processes, such as the first round of translation of freshly synthesized mRNA (named the “pioneer round of translation”), which is known to involve PABPN1 (Lemay et al., 2010). During the pioneer round of translation, faulty mRNAs with premature stop codons are identified as a part of a quality control mechanism prior to the recruitment of bulk-translation proteins (reviewed in Maquat et al., 2010). Before entering the pioneer round of translation, newly synthesized mRNAs are bound by cap binding proteins (CBP) CBP20 and CPB80 and by both poly(A) binding protein PABPN1 and

its cytoplasmic homologue poly(A) binding protein cytoplasmic 1 (PABPC1) and exported to the cytoplasm, where the pioneer round takes place. The fate of the new mRNA appears to be decided by the proteins associated with it during and after the pioneer round. If an mRNA is destined for steady-state levels of translation, PABPN1 dissociates from it, and the cap binding proteins CBP20 and CBP80 are replaced by eukaryotic initiation factor 4E (eIF4E), which recruits transcription factors and promotes bulk translation. Because TDP-43 is known to 1) act as a translational repressor *in vitro* (Wang et al., 2008) and 2) to be associated with silenced mRNAs in transport granules, looking for colocalization of TDP-43 with pioneer-round markers like CBP20/80 vs. steady-state translation markers like eIF4E may provide evidence for a possible role of TDP-43 in the pioneer round.

Another putative role for both TDP-43 and PABPN1 is the regulation of long non-coding RNAs (ncRNAs) such as the nuclear enriched abundant transcript 1 (NEAT1), which plays an important role in the assembly of nuclear structures termed paraspeckles (Clemson et al., 2009). TDP-43 has been shown to associate with NEAT1 ncRNA, and NEAT1 levels are increased in brain tissue from FTD cases (Tollervey et al., 2011). Interestingly, a proteomic study validated by immunocytochemistry and Western blot in Dr. Seyfried's lab in collaboration with the Bassell lab has shown that TDP-43 co-aggregates with the paraspeckle markers RBM14, PSF and NonO (Dammer et al., under revision). NEAT1 also appears to be regulated by PABPN1 (Dr. Bachand, personal communication). Quantitative fluorescent *in situ* experiments with NEAT1-specific probes and immunocytochemistry with paraspeckle markers will show whether overexpression or knock-down of TDP-43 and PABPN1 affects NEAT1 levels and the formation of paraspeckles, which would suggest another potential functional relationship between these proteins.

When characterizing the behavior of PABPN1 and TDP-43 mutants, we observed a change in localization of mutant mCh-TDP-208 from the cytoplasm to the nucleus upon co-expression with either GFP-PABPN1 or GFP-EX-PABPN1, to different degrees. This suggests that the TDP-43/PABPN1 interaction

we observed is maintained even between the pathogenic forms of the proteins, and more specifically, that PABPN1 is capable of recruiting aberrant TDP-43 from the cytoplasm to the nucleus. It appears likely that this recruitment follows as a result of the interaction of TDP-208-mCh and GFP-EX-PABPN1 in the cytoplasm, since mCh-TDP-208 lacks a nuclear localization signal (NLS). This provides more evidence in support of a functional relationship between TDP-43 and PABPN1 in the cytoplasm, which was also suggested by RNA granule and stress granule localization (discussed above). Quantitative analysis of protein content of nuclear vs. cytoplasmic lysate fractions of cells coexpressing different combinations of mutant protein constructs and controls is still needed to better characterize this change in localization.

It is well known that both TDP-208, the pathological C-terminal fragment of TDP-43, and EX-PABPN1, the mutant version of PABPN1 responsible for OPMD, form detergent- and salt-insoluble aggregates in their respective patients (Neumann et al., 2006; Fan et al., 2001). However, we showed that while mCh-TDP-208 does form insoluble aggregates in the cytoplasm when coexpressed with a GFP control or with GFP-PABPN1, the frequency of these aggregates is reduced with GFP-PABPN1 coexpression and completely eliminated with GFP-EX-PABPN1 coexpression. Instead, TDP-43 seems to be found more in the nucleus in cells coexpressing wild-type or mutant PABPN1, where it appears to exist in a more soluble form, as demonstrated by Triton X-100 treatment of cells (Figure 8). The reduction of observable mCh-TDP-208 fluorescence in cells coexpressing GFP-EX-PABPN1 or GFP-PABPN1, but not in cells coexpressing GFP, following Triton X-100 treatment suggests that overexpression of PABPN1 is capable not only of recruiting the aggregate-prone pathological C-terminal TDP-43 into the nucleus, but also of reducing its insolubility and ability to aggregate. This opens the possibility of reversing the loss of nuclear TDP-43 and clearing pathological TDP-208 aggregates from cells. Understanding this process may present an avenue for therapy for patients with disease characterized by aberrant TDP-43 aggregation. The differential solubility of TDP-43 can still be confirmed

with quantitative biochemical assays for detergent solubility of cell extracts from mutant-expressing cells and controls. Interestingly, the yeast gene *PBP1* (poly(A)-binding protein (Pab1p)-Binding Protein) and its human ortholog Ataxin-2, the disease protein of the neurodegenerative disease spinocerebellar ataxia type 2, have been identified as a potent modifier of TDP-43 toxicity in animal and cellular models (Elden et al., 2010). Therefore, it would be interesting to see whether PABPN1 can modulate TDP-43 toxicity in yeast and mammalian cells.

This study provided a series of important first steps in the confirmation and further characterization of a putative PABPN1/TDP-43 interaction identified during a previous honors project (Bi Mo). We present evidence supporting TDP-43 and PABPN1 association in cytoplasmic stress granules and axonal mRNA transport, and discuss the possibility of other roles for a TDP-43/PABPN1 interaction. More detailed studies may venture to further characterize the functional and physiological relevance of the interaction between TDP-43 and PABPN1 in these and other cytoplasmic processes. A better understanding of the influence of PABPN1 on the localization of pathological C-terminal TDP-43 may pave the way for therapeutic interventions in diseases characterized by TDP-43 aggregation. Full characterization of this novel protein-protein interaction will provide invaluable insights into the functions of both disease proteins TDP-43 and PABPN1, which is critical for understanding their respective diseases.

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