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Immunolocalization of TAR DNA-Binding Protein of 43 kDa (TDP-43) in mouse seminiferous epithelium

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Abstract

TDP-43 (TAR DNA-binding protein of 43 kDa) is an evolutionarily conserved, ubiquitously expressed, multi-functional DNA/RNA-binding protein with roles in gene transcription, mRNA splicing, stability, transport, micro RNA biogenesis, and suppression of transposons. Aberrant expression of TDP-43 in testis and sperm was recently shown to be associated with male infertility, which highlights the need to understand better the expression of TDP-43 in the testis. We previously cloned TDP-43 from a mouse testis cDNA library, and showed that it functions as a transcriptional repressor, and regulates the precise spatiotemporal expression of the *Acrv1* gene, which encodes the acrosomal protein SP-10, during spermatogenesis. Here, we performed immunoblotting and immunohistochemistry of the mouse testis using four separate antibodies recognizing the amino and carboxyl termini of TDP-43. TDP-43 is present in the nuclei of germ cells as well as Sertoli cells. TDP-43 expression begins in type B / intermediate spermatogonia, peaks in preleptotene spermatocytes, and becomes undetectable in leptotene and zygotene spermatocytes. Pachytene spermatocytes and early round spermatids again express TDP-43, but its abundance diminishes later in spermatids (at steps 5 to 8). Interestingly, two of the four antibodies showed TDP-43 expression in spermatids at steps 9–10, which coincides with the initial phase of the histone-to-protamine transition. Immunoreactivity patterns observed in the study suggest that TDP-43 assumes different conformational states at different stages of spermatogenesis. TDP-43 pathology has been extensively studied in the context of neurodegenerative diseases; its role in spermatogenesis warrants further detailed investigation of the involvement of TDP-43 in male infertility.

Keywords

spermatogenesis; regulation of gene expression; testis; fertility

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1 Introduction

TDP-43 (TAR DNA-binding protein of 43 kDa) is a ubiquitously expressed and evolutionarily conserved multifunctional DNA/RNA-binding protein, with roles in gene transcription, mRNA splicing, stability, transposon silencing, and micro RNA biogenesis (Lagier-Tourenne and Cleveland, 2009). The human and mouse TDP-43 orthologues are 414 amino acids in length, and share 96% sequence identity. The primary structure of this protein includes two canonical RNA-recognition motifs (RRM1 and RRM2) in the amino terminal region, a nuclear localization signal and a nuclear export signal within the amino terminal region, and a Glycine-rich carboxy-terminal region.

TDP-43 was first cloned and named by a group interested in identifying transcription factors that bind to the human immunodeficiency virus (HIV) TAR DNA region, pulling the protein from a HeLa cell cDNA library probed with the HIV TAR double-stranded region (Ou et al, 1995). They further showed that TDP-43 represses transcription by binding to TAR and obstructing TAT protein binding. TDP-43 was cloned a second time by a group interested in identifying proteins binding to messenger RNAs corresponding to the intron region of *CFTR* (Cystic fibrosis transmembrane conductance regulator), consisting of a polymorphic (TG)m(T)n repeated sequence that is responsible for exon 9 skipping (Buratti et al, 2001). They also probed HeLa cell extract, identifying TDP-43 as well as its preference for UG/TG repeats in RNA/single stranded DNA and its participation in mRNA splicing (Buratti et al, 2004). We were the third group to clone TDP-43 from a screen to identify transcription factors that bind to the promoter of the spermatid-specific *Acrv1* gene, which codes for the sperm acrosomal protein SP-10. We screened a mouse testis cDNA library with radiolabeled *Acrv1* promoter (Acharya et al, 2006). Two canonical TGTGTG motifs were present within the promoter fragment probe, and electrophoretic mobility shift assays confirmed TDP-43 as the cognate binding protein. Mutation of TDP-43 binding sites in the *Acrv1* promoter led to premature expression of a reporter gene in spermatocytes, suggesting that TDP-43 may function as a repressor of *Acrv1* expression in these cells (Acharya et al, 2006). Indeed, Gal4-recruitment reporter assays demonstrated that TDP-43 acts as a transcriptional repressor, while chromatin immunoprecipitation studies confirmed TDP-43 promoter occupancy of *Acrv1* in spermatocytes along with components of RNA Polymerase II pause machinery (Lalmansingh et al, 2011). Thus, TDP-43 plays a key role in maintaining the precise spatiotemporal expression of *Acrv1* within the seminiferous epithelium. Furthermore, TDP-43 is partially responsible for silencing the testis-specific *Acrv1* gene in somatic tissues by tethering the proximal promoter to the nuclear matrix (Abhyankar et al, 2007).

Over the past ten years, however, TDP-43 has emerged as clinically important in the context of neurodegenerative disorders. TDP-43 is aberrantly phosphorylated and/or ubiquitinated, and forms insoluble protein aggregates in the cytoplasm of motor neurons and glial cells of patients with a variety of neurodegenerative diseases, including amyotrophic laterals sclerosis and dementia (Neumann et al, 2006). Indeed, gain-of-function and loss-of-function phenotypes are believed to cause a variety of TDP-43-related proteinopathies (Lee et al, 2011). Regarding reproduction, TDP-43 is aberrantly expressed in germ cells and sperm

obtained from infertile men (Varghese et al, 2016). Thus, TDP-43 is an important protein to study from the point of view of male fertility.

The purpose of this study was to examine the expression and distribution of mouse TDP-43 within the seminiferous epithelium. Using antibodies to both amino and carboxyl termini of TDP-43, we showed that TDP-43 is expressed in both Sertoli cells and germ cells. Nuclear TDP-43 expression is prominent during critical points in male germ cell differentiation – i.e., during the spermatogonia-to-spermatocyte transition, meiosis, and histone-removal phase of spermatid nuclei.

2 RESULTS

2.1 Characterization of antibodies recognizing the amino-terminus of TDP-43

The majority of anti-TDP43 antibodies available commercially are made against the carboxyl terminus. Here we report characterization of two guinea pig polyclonal antibodies (GP#2 and GP#3) generated against the amino terminus (amino acid residues 3–271) of mouse TDP-43. The antigenic regions of GP#2 and GP#3 antibodies were identified by immunoblot analysis of recombinant TDP-43 representing different regions of TDP-43: Glutathione-S-transferase (GST)-tagged, full-length TDP-43; GST-tagged TDP-43 lacking the carboxyl-terminal Glycine-rich domain; two unrelated Histidine (His)-tagged recombinant proteins; and the immunogen, His-tagged mouse TDP43 protein.

GP#2 and GP#3 antisera reacted strongly with His-tagged immunogen, but not with unrelated His-tagged proteins (SP-10 or PUR α) (Figure 1), suggesting that these polyclonal antibodies are specific to TDP-43. Both antisera also reacted with both GST-tagged TDP-43 targets (full length as well as the version without the Glycine-rich domain [Gly]), but the intensity varied: GP#2 reactivity was stronger with the GST-fusion proteins compared to that of GP#3, whereas GP#3 reactivity was much stronger against the immunogen itself (Figure 1). One interpretation of this difference is that the epitopes recognized by GP#3 are hidden in the context of the GST fusions. Alternatively, GP#3 may recognize a conformational epitope that is predominant in the truncated version of TDP-43. Nevertheless, both GP#2 and GP#3 polyclonal antibodies specifically recognize the amino terminus of TDP-43.

2.2 Recognition of endogenous TDP-43 by the polyclonal guinea pig antibodies

TDP-43 is a ubiquitously expressed protein, so mouse brain and testis extracts were probed using GP#2 and GP#3 antibodies to identify endogenous TDP-43 protein by immunoblot. For comparison, we included two commercially available TDP-43 antibodies, from Proteintech and Abcam, which recognize the carboxyl terminus of TDP-43 (Table 1). Both commercial antibodies identify bands that migrate at 43 kDa and ~30 kDa, corresponding to the endogenous full-length and cleaved TDP-43 proteins, respectively (Figure 2). GP#2 and GP#3 antibodies reacted strongly with the canonical 43-kDa TDP-43 band in testis extract (Figure 2, arrowhead). All four antibodies identified a 43-kDa band in brain extracts, whereas preimmune sera or no-primary control blots were not reactive to either brain or testis extracts (data not shown).

GP#2 and GP#3 antibodies reacted with more bands compared to the Proteintech and Abcam antibodies (Figure 2). GP#2 and GP#3 identified bands in the 30–15 kDa range, which may represent amino-terminal breakdown products of TDP-43. The pattern of recognition for both guinea pig antisera was overlapping, but the intensity or specific bands varied. For example, the 25-kDa band is stronger using GP#3 whereas the 15-kDa band is stronger using GP#2, suggesting differences in epitope recognition (Figure 2). TDP-43 is known to form multimeric aggregates of high molecular weight (Shiina et al, 2010), so detection of a 250-kDa band in the brain and testis and a 100-kDa band unique to the brain was not surprising. On the other hand, GP#3 reacted with a prominent ~130-kDa band in the testis extract. These differences in reactivity likely reflect variation in inter-domain interaction and aggregate formation by TDP-43. One reason why Proteintech and Abcam antibodies failed to pick up the higher-molecular-weight bands could be that the carboxyl terminus is not exposed or because these aggregates predominantly consist of amino-terminal fragments of TDP-43.

In sum, the pattern detected with GP#2 and GP#3 antibodies is consistent with the reported heterogeneity of TDP-43 (Sampathu et al, 2006; Shiina et al, 2010). The differences in reactivity between the antisera are likely due to alternative epitope recognition and/or availability in these blotted species.

2.3 TDP-43 expression in mouse seminiferous epithelium

Immunohistochemistry was performed on Bouin's-fixed, paraffin-embedded adult mouse testis sections using antibodies that recognize the amino (GP#2 and GP#3) or carboxyl termini (Proteintech and Abcam) of TDP-43. No signal was detected when only secondary antibodies were used (data not shown). The staining pattern obtained with GP#2 (recognizes amino acids 3–271) and Proteintech (recognizes amino acids 264–414) antibodies was identical, and represents prototypical TDP-43 immunolocalization in the mouse testis, so we presented only the data obtained with GP#2 (Figure 3). Overall, TDP-43 was detected in both the germ cells as well as Sertoli cells of the seminiferous epithelium, indicating that TDP-43 function might be critical for both cell types. The majority of TDP-43 immunostaining was nuclear, which is consistent with the presence of nuclear localization and nuclear export signals within TDP-43; some signal was also visible in the cytoplasm.

We next examined TDP-43 immunolocalization at all 12 stages of the mouse seminiferous epithelium cycle to determine the spatiotemporal expression of TDP-43 (Figure 3). The undifferentiated spermatogonial stem cells or type A progenitor spermatogonial cells did not contain TDP-43 staining (A₃ in Figure 3l and A₄ in Figure 3b). TDP-43 staining first appeared in the differentiating intermediate spermatogonia at Stage IV, became stronger in type B spermatogonia at Stages V and VI, and peaked in preleptotene spermatocytes at Stages VII and VIII (Figure 3e–h). Interestingly, the levels of TDP-43 diminished in leptotene and zygotene spermatocytes at Stages IX–XI, but became prominent again in pachytene spermatocytes. Pachytene spermatocytes of Stages II–XII expressed TDP-43 with intensity matching that of preleptotene spermatocytes (Figure 3). Among haploid male germ cells, TDP-43 expression was high in early round spermatids of Stages I through IV, but gradually tapered off in later stages. The fact that two separate antibodies, raised against the

amino (GP#2) and carboxyl (Proteintech) termini of TDP-43 displayed identical staining pattern validated the above immunolocalization data within the seminiferous epithelium.

2.4 Conformational changes of TDP-43 revealed by immunohistochemistry

We also used GP#3 and Abcam antibodies (Table 1) for immunohistochemical localization of TDP-43 in mouse testis. Unlike their counterparts (see Section 2.3), these two antibodies exhibited highly restricted reactivity in the seminiferous epithelium. GP#3 predominantly stained the Sertoli cell nuclei, but was absent from germ cells (Figure 4a–d) until they reached preleptotene spermatocytes at Stages VII–VIII (Figure 4b) and elongating spermatids of Stage X (Figure 4c). This contrasting staining pattern between GP#2 and GP#3 is interesting because both polyclonal antibodies were generated using the same antigen (His-tagged TDP-43, amino acids 3–271), and is consistent with the immunoblot data (Figures 1 and 2). Our interpretation is that the GP#3 antibody recognizes a conformational epitope of TDP-43 that is enriched in Sertoli cells (Figure 4), preleptotene spermatocytes (Figure 4b), and Step-10 spermatids (Figure 4c).

The Abcam antibody, raised against the carboxyl terminus of TDP-43 (amino acids 250–350), also showed highly restricted immunoreactivity (Figure 4e–h). Nuclei of intermediate (Figure 4b–d) and type B spermatogonia (Figure 4g–h), preleptotene and leptotene spermatocytes (Figure 4e), and Step-9 to -10 spermatids (Figure 4f–g), suggesting that the recognized epitope of TDP-43 is exposed only at specific stages during germ cell differentiation. Indeed, the Abcam antibody picked up TDP-43 in intermediate spermatogonia from an earlier stage (Stage II; Figure 4e) than GP#2 (Stage IV; Figure 3). While reactivity with spermatogonial cells was generally consistent with that of GP#2, we noted the absence of staining of pachytene spermatocytes and Sertoli cell nuclei by the Abcam antibody. Interestingly, Step-10 spermatid nuclei stained intensely by both the Abcam and GP#3 antibodies.

The differential immunoreactivity of GP#2, GP#3, and Abcam polyclonal antisera in the seminiferous epithelium suggests that TDP-43 assumes multiple conformational states in testicular cells. We consider the immunolocalization obtained by GP#2 as prototypical TDP-43 pattern for the mouse testis (Figure 5).

3 DISCUSSION

The present study established the spatiotemporal pattern of expression of TDP-43 within the mouse seminiferous epithelium. Immunoblot experiments performed with antibodies recognizing the amino and carboxyl termini of TDP-43 revealed its multiple molecular weight forms in testis extracts, while immunohistochemistry suggested that TDP-43 exists in multiple conformational states within testicular cells. Furthermore, TDP-43 is expressed in both somatic and germ cells of the testis, residing predominantly in the nucleus, although occasional cytoplasmic localization was observed, and its presence is stage- and cell-specific within the seminiferous epithelium.

The use of four different antibodies that recognize different regions of TDP-43 ensured that TDP-43 expression was detected, and provided verification of immunoreactivity. The

Proteintech antibody (raised against the carboxyl terminus of TDP-43) and GP#2 (raised against the amino terminus of TDP-43) showed identical immunohistochemistry patterns in mouse testis sections (Figure 3 and data not shown). None of the four antibodies stained undifferentiated or progenitor type A spermatogonia, suggesting that TDP-43 might not participate in the maintenance of spermatogonial stem cells or the differentiating A₁–A₄ spermatogonia. TDP-43 first appeared in intermediate and type B spermatogonia, reaching maximum levels in preleptotene spermatocytes and pachytene spermatocytes (Figures 3 and 4). This peak abundance in preleptotene spermatocyte suggests a role for TDP-43 as these cells enter meiotic prophase. Interestingly, TDP-43 staining abruptly diminished in the leptotene and zygotene spermatocytes. This decreased abundance was true for all four antibodies tested, suggesting that temporary down-regulation of TDP-43 may be critical for the differentiation of leptotene and zygotene spermatocytes. Indeed, subsequent pachytene spermatocytes from Stage II to stage XII showed strong TDP-43 reactivity (Figure 3). This latter increase is consistent with a role for TDP-43 during the wave of transcription and splicing that occurs in pachytene spermatocytes. Our previous work showed that TDP-43 plays a role in repression of *Acrv1* gene transcription in spermatocytes (Lalmansingh et al, 2011). TDP-43 expression within round spermatid nuclei varied: Steps 1–4 spermatid nuclei showed appreciable TDP-43 staining that diminished in Steps 5–8 spermatid nuclei (Figure 3). This profile suggests a role for TDP-43 in early events of spermiogenesis, such as acrosome biogenesis. GP#3 and Abcam antibodies, however, revealed the presence of TDP-43 in the nuclei of Steps 9–10 spermatids, whereas GP#2 and Proteintech antibodies did not. This differential recognition suggests that TDP-43 undergoes a conformational change in Steps 9–10 spermatids, which corresponds to the stage when spermatid nuclei begin the transition from a histone-bound to protamine-bound state. Whether or not TDP-43 plays a role in initiating chromatin remodeling in male germ cells remains undetermined.

Sertoli cells provide nutrition and support to the differentiating germ cells. Three out of the four antibodies (GP#2, GP#3, and Proteintech) detected nuclear TDP-43 in Sertoli cells within the seminiferous epithelium. The fact that GP#3 antibody stained TDP-43 predominantly in the Sertoli cells, but not in germ cells, supports the model that TDP-43 assumes different conformation states. Future work using Sertoli cell-specific deletion of TDP-43 should clarify how this nuclear protein contributes to Sertoli cell function.

The predicted molecular weight of TDP-43 monomer is 43 kDa, but we observed immunoreactive bands both higher and lower than this mass (Figure 2). TDP-43 is known to interact with itself, so several of the high-molecular-weight bands identified by GP#2 and GP#3 antibodies may reflect these multimers (Sampathu et al, 2006; Shiina et al, 2010; Cragnez et al, 2014). Detection of these high-molecular-weight aggregates by GP#2 and GP#3 antibodies also suggest that they contain the amino-terminus of TDP-43. Not all the GP#2-reactive bands were detected by antibodies recognizing the carboxyl terminus, so these aggregates likely represent partial fragments of TDP-43.

TDP-43 contains an amino-terminal region, two RNA recognition motifs (RRM), and a carboxyl-terminal, prion-like domain. Nuclear magnetic resonance data showed that TDP-43 has dynamic inter-domain interactions coordinated by the intrinsically disordered prion-like domain (Wei et al, 2016); these interactions are proposed to alter the aggregation

mechanism. Such a model is consistent with the heterogeneity of high-molecular-weight bands seen by Western blot (Figure 2). GP#2 and GP#3, but not Proteintech and Abcam antibodies, identified these bands, which could be due to exposure of the amino-terminal but not carboxy-terminal regions of TDP-43. Inter-domain interactions are of relatively low affinity which could foster the conformational exchange between open and closed states (Wei et al, 2016). We predict that physiological conditions influence the conformational changes of TDP-43. This could account for the observed Sertoli cell- and germ cell-specific reactivity by GP#3 and Abcam antibodies, respectively. Such dynamic conformational states are expected to have implications for TDP-43 function.

TDP-43 is known to undergo post-translational modification, including ubiquitination, phosphorylation, and sumoylation (Hasegawa et al, 2008; Neumann, 2009; Buratti and Baralle, 2009; Xiao et al, 2016), all of which are known to cause proteins to migrate slower in polyacrylamide gels. Conversely, several caspase cleavage sites have been identified, and blockage with specific caspase inhibitors confirmed role for caspases in cleaving TDP-43 to produce 35-, 25-, and 15-kDa fragments (Dormann et al, 2009; Zhang et al, 2009). TDP-43 is also a target of calpain (Yamashita et al, 2012). Based on the immunoreactivity patterns seen in the immunoblots (Figure 2), we suggest that the cleaved products of TDP-43 retain both amino and carboxyl termini, while the persistence of these modified forms of TDP-43 under physiological conditions suggest that they might be functionally important.

In summary, TDP-43 is expressed by both germ cells and Sertoli cells of the mouse testis and appears to possess functions that align with specific stages of spermatogenesis. Studies using the conditional knockout approach should help address the exact role(s) for this protein during spermatogenesis and for maintaining male fertility.

4 MATERIALS & METHODS

4.1 Recombinant protein production

Generation of recombinant proteins corresponding to TDP-43, PUR α , and SP-10 were described previously (Acharya et al, 2006; Osuru et al, 2014). GST-tagged TDP-43 proteins were generated by cloning the mouse *Tdp43* cDNA, corresponding to amino acids 1-414 (GST-tagged full-length TDP-43) or to amino acids 3-271 (GST-tagged Gly TDP-43), into pGEX (GE Healthcare Life Sciences, PA, USA). GST fusion proteins were purified using the Pierce Glutathione agarose resin Kit (Thermo Scientific, MA, USA), as per manufacturer's instructions.

4.2 Polyclonal antibody production in guinea pigs

Generation of polyclonal antibodies to the amino terminus of mouse TDP-43 protein was previously described (Acharya et al, 2006). Briefly, His-tagged recombinant mouse TDP-43 (amino acids 3-271, fused to His-tag at the carboxyl terminus), cloned in pET22b+, was produced and purified as previously described (Reddi et al, 1994). The purified recombinant protein was mixed with Incomplete Freund's Adjuvant (Sigma-Aldrich, MO, USA), and three separate guinea pigs (#2, #3, and #4) were immunized as described previously

(Acharya et al, 2006). After two booster injections at one-month intervals, final bleeds were collected and aliquots were stored at -80°C .

4.3 Western Blot

Purified recombinant proteins (1–2 μg) were separated by denaturing gel electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, MA, USA). Testis and brain tissues were quickly dissected, snap-frozen and stored at -80°C until use. Testis and brain tissues were sonicated in RIPA lysis buffer (Thermo Scientific, MA, USA) containing protease inhibitors (Thermo Scientific, MA, USA) (each mini tablet was dissolved freshly in 10mL of RIPA buffer). Protein concentrations in the lysate were determined using a BCA Protein Assay kit (Thermo Scientific, MA, USA). Total protein (10–20 μg) was heated at 90°C for 5 minutes, separated by denaturing gel electrophoresis on 4–20% Tris-glycine polyacrylamide mini gels (Bio-Rad Laboratories, CA, USA), and then transferred to PVDF membranes (EMD Millipore, MA, USA). Membranes were then blocked at room temperature for 1 h in 3% bovine serum albumin, and incubated at 4°C overnight with primary antibodies (Table 1) using the following dilutions: Proteintech anti-TDP-43 (1:1200 dilution of 12892-1-AP) (Proteintech, IL, USA), Abcam anti-TDP-43 (1:3000 dilution of ab41972), GP#2 and #3 anti-TDP-43 (1:8000 dilution of each) (see Section 4.2), or mouse monoclonal anti-tubulin (1: 5000 dilution of T0198) (Sigma-Aldrich, MO, USA). Membranes were then incubated for 1 h at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies (1:15000) (Santa Cruz Biotechnology, CA, USA). Immunoreactivity was detected using Super Signal West Femto enhanced chemiluminescence substrate (Thermo Scientific). Images were captured using a Syngene Chemi XR5 G:BOX (Integrated Scientific Solutions, San Diego, CA, USA), with 1-min exposure time, or on X-ray film, with 5-min exposure time.

4.4 Immunohistochemistry

Testes harvested from C57BL/6 males (10–12 weeks of age) were fixed in Bouin's fixative (Sigma-Aldrich) for 16–20 hours. Five-micron-thick sections were cut from paraffin-embedded blocks, deparaffinized in xylene, and rehydrated through a graded series of ethanol baths. An Autostainer robotic platform (Dako, Glostrup, Denmark) was used to perform the immunohistochemistry. Endogenous peroxidases were blocked using Peroxidase and Alkaline Phosphatase Blocking Reagent (Dako); antigen-retrieval steps were not performed.

Primary antibodies GP#2 and #3 (see Section 4.2) were used at 1:1600 dilution; Proteintech and Abcam anti-TDP-43 polyclonal antibodies were used at 1:100 and 1:200 dilutions, respectively (see Table 1). Peroxidase-AffiniPure goat anti-guinea pig IgG (H+L) (106-035-003) or goat anti-rabbit IgG antibodies (___) (Jackson ImmunoResearch Laboratories, PA, USA) were each used at 1:200 dilutions. Antigen-antibody binding was detected by incubation with 3,3'-diaminobenzidinetetrahydrochloride (DAB+) chromogen (Dako), as per the manufacturer's instructions. All the slides were then counterstained with hematoxylin, dehydrated, cleared, and mounted for assessment and imaging. Bright-field images were captured using a BX51 microscope with a 40 $\times$ objective and QColor3 image processing software (Olympus Life Sciences, Tokyo, Japan).

Cross sections obtained from four independent age-matched C57B6 mice were used for immunohistochemistry. Each slide contained two to four sections, and at least four slides obtained from different depths of tissue from each mouse testis were assessed per antibody. We observed remarkable consistency in the staining pattern.

4.5 Peptide blocking with immunizing peptide

Two aliquots each of GP#2 and GP#3 (1:1600 dilution each) were prepared: One aliquot was incubated at 4°C for 1 hour with 5 µg of His-tagged TDP-43 (amino acids 3–271) to block the immunogen reactive antibodies; the second aliquot served as the “antibody alone” control. These dilutions were then used for immunohistochemistry, as described above (Section 4.4). While the “antibody alone” controls showed typical staining patterns, the blocked antibodies yielded no signal, indicating that the GP#2 and GP#3 antibodies were specific to TDP-43 (Figure S1). The commercial antibodies (Proteintech and Abcam) were supplied as immunogen affinity-purified immunoglobulins, so peptide-blocking assays were not performed to demonstrate specificity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

<i>Acrv1</i>	Acrosomal vesicle protein 1
GST	Glutathione-S-transferase
His	histidine (6×)
TDP-43	TAR DNA-binding protein of 43 kDa

References

- Abhyankar MM, Urekar C, Reddi PP. A novel CpG-free vertebrate insulator silences the testis-specific SP-10 gene in somatic tissues: role for TDP-43 in insulator function. *J Biol Chem*. 2007; 282:36143–36154. [PubMed: 17932037]
- Acharya KK, Govind CK, Shore AN, Stoler MH, Reddi PP. Cis-Requirement for the Maintenance of Round Spermatid-Specific Transcription. *Dev Biol*. 2006; 295:781–790. [PubMed: 16730344]
- Buratti E, Baralle FE. The molecular links between TDP-43 dysfunction and neurodegeneration. *Adv Genet*. 2009; 66:1–34. [PubMed: 19737636]
- Buratti E, Brindisi A, Pagani F, Baralle FE. Nuclear factor TDP-43 binds to the polymorphic TG repeats in CFTR intron 8 and causes skipping of exon 9: a functional link with disease penetrance. *Am J Hum Genet*. 2004; 74:1322–1325. [PubMed: 15195661]
- Buratti E, Dork T, Zuccato E, Pagani F, Romano M, Baralle FE. Nuclear factor TDP-43 and SR proteins promote in vitro and in vivo CFTR exon 9 skipping. *EMBO J*. 2001; 20:1774–1784. [PubMed: 11285240]

- Cragnez L, Klima R, Skoko N, Budini M, Feiguin F, Baralle FE. Aggregate formation prevents dTDP-43 neurotoxicity in the *Drosophila melanogaster* eye. *Neurobiol Dis.* 2014; 71:74–80. [PubMed: 25088712]
- Dormann D, Capell A, Carlson AM, Shankaran SS, Rodde R, Neumann M, Kremmer E, Matsuwaki T, Yamanouchi K, Nishihara M, Haass C. Proteolytic processing of TAR DNA binding protein-43 by caspases produces C-terminal fragments with disease defining properties independent of progranulin. *J Neurochem.* 2009; 110:1082–1094. [PubMed: 19522733]
- Hasegawa M, Arai T, Nonaka T, Kametani F, Yoshida M, Hashizume Y, Beach TG, Buratti E, Baralle F, Morita M, Nakano I, Oda T, Tsuchiya K, Akiyama H. Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Ann Neurol.* 2008; 64:60–70. [PubMed: 18546284]
- Lagier-Tourenne C, Cleveland DW. Rethinking ALS: the FUS about TDP-43. *Cell.* 2009; 136:1001–1004. [PubMed: 19303844]
- Lalmansingh AS, Urekar CJ, Reddi PP. TDP-43 is a transcriptional repressor; the testis-specific mouse ACRV1 gene is a TDP-43 target in vivo. *J Biol Chem.* 2011; 286:10970–10982. [PubMed: 21252238]
- Lee EB, Lee VM, Trojanowski JQ. Gains or losses: molecular mechanisms of TDP43-mediated neurodegeneration. *Nat Rev Neurosci.* 2011; 13:38–50. [PubMed: 22127299]
- Neumann M. Molecular Neuropathology of TDP-43 Proteinopathies. *Int J Mol Sci.* 2009; 10:232–246. [PubMed: 19333444]
- Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, Schuck T, Grossman M, Clark CM, McCluskey LF, Miller BL, Masliah E, Mackenzie IR, Feldman H, Feiden W, Kretzschmar HA, Trojanowski JQ, Lee VM. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science.* 2006; 314:130–133. [PubMed: 17023659]
- Osuru HP, Monroe JE, Chebolu AP, Akamune J, Pramoongjago P, Ranpura SA, Reddi PP. The acrosomal protein SP-10 (Acrv1) is an ideal marker for staging of the cycle of seminiferous epithelium in the mouse. *Mol Reprod Dev.* 2014; 81:896–907. [PubMed: 25158006]
- Ou SH, Wu F, Harrich D, Garcia-Martinez LF, Gaynor RB. Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs. *J Virol.* 1995; 69:3584–3596. [PubMed: 7745706]
- Reddi PP, Castillo JR, Klotz K, Flickinger CJ, Herr JC. Production in *Escherichia coli*, purification and immunogenicity of acrosomal protein SP-10, a candidate contraceptive vaccine. *Gene.* 1994; 147:189–195. [PubMed: 7926798]
- Sampathu DM, Neumann M, Kwong LK, Chou TT, Micsenyi M, Truax A, Bruce J, Grossman M, Trojanowski JQ, Lee VM. Pathological heterogeneity of frontotemporal lobar degeneration with ubiquitin-positive inclusions delineated by ubiquitin immunohistochemistry and novel monoclonal antibodies. *Am J Pathol.* 2006; 169:1343–1352. [PubMed: 17003490]
- Shiina Y, Arima K, Tabunoki H, Satoh J. TDP-43 dimerizes in human cells in culture. *Cell Mol Neurobiol.* 2010; 30:641–652. [PubMed: 20043239]
- Varghese DS, Chandran U, Soumya A, Pillai SM, Jayakrishnan K, Reddi PP, Kumar PG. Aberrant expression of TAR DNA binding protein-43 is associated with spermatogenic disorders in men. *Reprod Fertil Dev.* 2016; 28:713–722. [PubMed: 25359468]
- Wei Y, Lim L, Wang L, Song J. Inter-domain interactions of TDP-43 as decoded by NMR. *Biochem Biophys Res Commun.* 2016; 473:614–619. [PubMed: 27040765]
- Xiao Y, Pollack D, Andrusier M, Levy A, Callaway M, Nieves E, Reddi P, Vigodner M. Identification of cell-specific targets of sumoylation during mouse spermatogenesis. *Reproduction.* 2016; 151:149–166. [PubMed: 26701181]
- Yamashita T, Hideyama T, Hachiga K, Teramoto S, Takano J, Iwata N, Saido TC, Kwak S. A role for calpain-dependent cleavage of TDP-43 in amyotrophic lateral sclerosis pathology. *Nat Commun.* 2012; 3:1307. [PubMed: 23250437]
- Zhang YJ, Xu YF, Cook C, Gendron TF, Roettges P, Link CD, Lin WL, Tong J, Castaneda-Casey M, Ash P, Gass J, Rangachari V, Buratti E, Baralle F, Golde TE, Dickson DW, Petrucelli L. Aberrant cleavage of TDP-43 enhances aggregation and cellular toxicity. *Proc Natl Acad Sci U S A.* 2009; 106:7607–7612. [PubMed: 19383787]

Figure 1.

Characterization of polyclonal antibodies GP#2 and GP#3, generated against the amino terminus of TDP-43. Two (lane 1) or one microgram (lane 3) of GST-tagged full-length TDP-43, or 2 µg each of GST- gly TDP-43 (amino acids 3–271) (lane 2); His-tagged PURα (lane 4); His-tagged SP-10 (lane 5); and His-tagged gly (amino acids 3–271) (lane 6) were separated in duplicate gels, blotted, and probed with GP#2 (a) or GP#3 (b).

Figure 2.

Reactivity of amino- and carboxyl-terminus antibodies to endogenous TDP-43. Ten micrograms of protein in mouse brain and testis extract were separated by polyacrylamide gel electrophoresis in quadruplicate, and probed with antibodies recognizing the amino (GP#2 and GP#3) and carboxyl (Proteintech and Abcam) termini of TDP-43. All the antibodies recognized the 43 kDa band of TDP-43 (arrowhead

Figure 3.

Immunohistochemistry of mouse testis with anti-TDP-43 polyclonal antibody GP#2. Each panel corresponds to seminiferous tubule Stages I–XII (a–l, respectively). Scale bar, 30 μ m. A₃ and A₄, differentiating spermatogonia; ES, elongated spermatozoa; In, intermediate spermatogonia; L, leptotene; and MII, spermatocytes undergoing second meiotic division; P, pachytene spermatocytes; PL, preleptotene spermatocytes; R, round spermatids; RB, residual body; Z, zygotene.

Figure 4.

Immunohistochemistry of mouse testis with anti TDP-43 polyclonal GP#3 or Abcam antibodies. Sections stained with (a–d) GP#3 (recognizes amino acids 3–271 of TDP-43) or (e–h) Abcam antibody (recognizes amino acids 250–350 of TDP-43). Stages are indicated with Roman numerals. Scale bar, 30 μ m. A₃ and A₄, differentiating spermatogonia; ES, elongated spermatogonia; In, intermediate spermatogonia; L, leptotene; and MII, spermatocytes undergoing second meiotic division; P, pachytene spermatocytes; PL, preleptotene spermatocytes; R, round spermatids, RB, residual body; st9, step 9 spermatid; st10, step 10 spermatid; Z, zygotene.

Figure 5. Schematic of TDP-43 expression through the seminiferous epithelium cycle stages in the mouse. Darker and lighter shades represent higher and lower abundance of TDP-43, respectively.

Table 1

Polyclonal anti-TDP-43 antibodies used in this study

Name	Source	Animal host	TDP-43 peptide immunogen	Homology to mouse TDP-43
GP#2	In-house	Guinea pig	Mouse 3–271	100
GP#3	In-house	Guinea pig	Mouse 3–271	100
Proteintech	Proteintech	Rabbit	Human 264–414	97
Abcam	Abcam	Rabbit	Human 250–350	100