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Review Article

RNA-binding Motif Protein 45 (Rbm45)/Developmentally Regulated RNA-binding Protein-1 (Drbp1): Association with Neurodegenerative Disorders

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Neurodegenerative disorders are caused by the progressive loss of the structure and/or function of neurons, often through cell death, contributing significantly to morbidity and mortality. Cytoplasmic aggregation of proteins into inclusion bodies is a pathological characteristic of amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and Alzheimer's disorder (AD). These inclusion bodies have been shown to contain RNA-binding proteins participating in RNA-dependent and RNA–independent protein:protein interactions. RNA-binding motif protein 45 (RBM45), also known as developmentally regulated RNA-binding protein-1 (Drbp1), was first identified as a novel RNA binding protein in rat that functions in neural development. Advancing research has indicated a connection between the presence of human RBM45 protein cytosolic aggregates and degenerative neurological diseases. This review considers the structure, function, and distribution of RBM45 along with a look into potential future research on this multifunctional RNA-binding protein.

Keywords: Alzheimer's disorder (AD), amyotrophic lateral sclerosis (ALS), Drbp1, frontotemporal lobar degeneration (FTLD), Rbm45

Davis and Temple (1994) demonstrated that neurons and glial cells originate from neural stem cells which underwent differentiation during fetal neural development. Changes in stem cell properties are controlled by both intrinsic and extrinsic cues (Bertrand *et al*., 2002). An example of an intrinsic cue would be post-transcriptional gene regulation by neural RNA-binding proteins. Musashi-1, an RNA-binding protein, interacts with mRNA of mammalian *NUMB* gene to regulate gene expression at the level of translation (Imai *et al.*, 2001). Ultimately, Musashi-1 determines cell fate by maintenance of stem cell state, cell differentiation, and tumorigenesis via repressing translation of specific mRNAs (Okano *et al.*, 2002). Additionally, Musashi-1 has been found to mediate the posttranscriptional regulation of the infamous tau proteins which are defective in Alzheimer's disease (AD) neuropathology (Cuadrado *et al*., 2002). Musashi-1 is a quintessential example of the capabilities of an RNA-binding protein to regulate cellular physiology and, therefore, affirms that further research on RNA-binding proteins would be a beneficial endeavor.

RNA-binding motif protein 45 (Rbm45), also known as developmentally regulated RNA-binding protein-1 (Drbp1), is an RNA-binding protein first characterized by Tamada and coworkers (2002) in rats (*Rattus norvegicus*). Rbm45 contains RNA-binding motifs similar to those found in Musashi-1 and other RNA-binding proteins such as Elav (Nakamura *et al*., 1994) in *Drosphila* (fruit fly) and its mammalian homolog, Hu (Kasashima *et al*., 1999, 2002). Rbm45 belongs to the RNA recognition motif-type RNA (RRM)-binding domain (RBD) containing protein (RBDP) family (Tamada *et al*., 2002). Many eukaryotic proteins that are known or predicted to bind single-strand RNA contain one or more RBDs of about 90 amino acids (Burd and Dreyfuss, 1994). RBDs are also known as RNA-binding region RNP-1 signature domains (Letunic *et al*., 2011) and are found in a variety of proteins including heterogeneous nuclear ribonucleoproteins (hnRNPs), the protein component of small nuclear ribonucleoproteins (snRNPs)– both implicated in regulation of alternative splicing– and musashi-1 involved in posttranscriptional regulation (Schultz *et al*., 1998). The RBD structure consists of four β-strands and two α- helices arranged in an α/β sandwich, with a third helix present during RNA-binding (Schultz *et al*., 1998). Characterization of Rbm45 protein orthologues from human, rat, and mouse reveal that it contains 3 (Li *et al.*, 2015; Mashiko *et al*., 2016) or 4 RBDs (Tamada *et al*., 2002; HomoloGene:15560 [https://www.ncbi.nlm.nih.gov/homologene/?term=Rbm45; Accessed 11.13.2017]).

The gene encoding Rbm45 is highly conserved evolutionarily and has orthologs in many species including sponges (*Amphimedon queenslandica*), fruit fly (*Drosophila melanogaster*), sea urchin (*Strongylocentrotus purpuratus*), bony fish (*Danio rerio*), frog (*Xenopus laevis*), chicken (*Gallus gallus*), and human (*Homo sapiens*) (Li *et al.*, 2015; Mashiko *etal.*,2016; www.ncbi.nlm.nih.gov/homologene/?term=Rbm45 [accessed 12.13.2017]). In the rat, Rbm45 shows highest expression in the early embryonic brain with expression peaking between embryonic day 12 and 16 and gradually declining into adulthood (Tamada *et al*., 2002). Human RBM45 is normally expressed predominantly in the nucleus of neurons and glia in the hippocampus and spinal cord (Collins *et al*., 2012; Li *et al.*, 2016). There is also an abundance of RBM45 in cerebrospinal fluid indicating that this protein may have extracellular functions (Collins *et al*., 2012). Recent research (Bakkar *et al.*, 2015; Collins *et al*. 2012; Li *et al.*, 2015, 2016; Mashiko *et al*., 2016) has indicated a connection between RBM45 inclusions and the degenerative neural diseases amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and AD. Unfortunately, at this time, there is little known about the function(s) of RBM45 or how it contributes to the progression of these neurodegenerative disorders. RBDPs are typically multifunctional and act in both the nucleus and cytoplasm, influencing transcription, RNA splicing, RNA export, translation, and transport of mRNAs. Current research indicates that RBM45 shares many of these same functions and also interacts with other RNA binding proteins to modulate responses to oxidative stress and modulate extracellular signaling (Li *et al.*, 2015).

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Human RBM45 Structure

RBM45 contains at least three RBDs, a C-terminal nuclear localization sequence (NLS), a nuclear export signal (NES), and a homo-oligomerization association (HOA) domain (Li *et al.*, 2015; Mashiko *et al.*, 2016; Tamada *et al*., 2002). RBM45 is distinct from other neural inclusion-forming RNA-binding proteins, such as TDP-43, FUS, TAF15, and hnRNP-A1 because it does not contain a prion-like domain (King *et al*., 2012; Li *et al*., 2016). The N-terminal two RBDs are sufficient to bind Kelch-like ECH-associated protein1 (KEAP1), a protein critical in oxidative stress response, in a RNA-independent manner; however, the role of the RBDs in granule accumulation is unclear (Bakkar *et al*., 2015). The NLS localizes this protein to the nucleus, but RBM45 is able to be shuttled between the cytoplasm and the nucleus by a double leucine motif that serves as the NES (Mashiko *et al*., 2016). Li and coworkers (2015, 2016) determined that the HOA domain is located at residues 258–318 within RBM45 and is highly conserved across species, suggesting an important functional role. The HOA domain confers both structural and functional attributes to RBM45 including self-association and cytoplasmic stress granule localization, but its role in mediating RBM45 oligomerization and association with other RBDPs suggests this domain also contributes to the pathological aggregation of RBM45 with other RBDPs in ALS, FTLD, and AD.

RBM45 Role in Neurodegenerative diseases

The aggregation of RNA-binding proteins into inclusion bodies is one of the most prevalent and well-documented pathologies associated with ALS, FTLD, and AD (reviewed in Li *et al*., 2016). In ALS and FTLD patients, RBM45 is contained in cytoplasmic inclusions in motor neurons that were immunoreactive for TDP-43 and ubiquitin. The RBM45 inclusions were similar to TDP-43 inclusion patterns, but could be distinguished by their speckled nuclear immunostaining and lack of incorporation into hippocampal dystrophic neurons (Collins *et al*., 2012). The most abundant RBM45 pathology occurred in patients harboring the *C9ORF72* repeat expansion, a hexanucleotide repeat (GGGGCC) demonstrated to be the cause of chromosome 9q21-linked ALS-FTLD (DeJesus-Hernandez *et al*., 2001; Renton *et al*., 2011).

Genetic (yeast two-hybrid screen; Hans *et al*., 2014), cytological and biochemical studies (Immunoprecipitation [IP] and high throughput immunoprecipitation-coupled-mass spectrometry [IP-MS]; Hans *et al*., 2014; Li *et al.,* 2015, 2016) detected and validated RBM45 interaction with a number of ALS- and FTLD-linked proteins, including TDP-43, FUS, Matrin-3, hnRNP-A1, and hnRNP-A2/B1. In a study by Collins and colleagues (2012), 91% of patients with ALS, 100% of FTLD patients and 25% of AD patients had RBM45 cytoplasmic inclusion bodies. RBM45 inclusions were observed in the cytoplasm of spinal cord motor neurons, glia, and neurons of the dentate gyrus. No RBM45 inclusions were detected in any region or cell type examined in control subjects (Collins *et al.*, 2012). The actual mechanism(s) for how the protein incorporates itself into the inclusion bodies is not well understood; however, the HOA domain allows RBM45 to self-associate into homo-pentamers and/or decamers and bind to other inclusion forming proteins (e.g. TDP-43; Li *et al*., 2016).

RBM45 serves two other very important functions that can assist in the formation of inclusion bodies when there is a mutation. RBM45 appears to play a role in the regulation of RNA splicing events and translation by eukaryotic initiation factors 2 and 4 pathways (Li *et al*., 2016). The dysregulation of RNA splicing is a very common characteristic of both ALS and FTLD and can lead to the formation of inclusion bodies. Cellular transportation is a very important function for RBM45 in both normal and disease functioning; RBM45 appears to indirectly assist in translation by transporting mRNA (Li *et al*., 2016). Another contributing factor to the formation of inclusion bodies appears to be the NLS and NES of RBM45. A recent study (Mashiko *et al*., 2016) indicates that only when the NLS and NES signals are both mutated do cytoplasmic aggregates form. These experiments demonstrate that each of these mutations is necessary but not sufficient for formation of inclusion bodies. Mashiko and coworkers (2016) propose two complementary hypothesis as to why this occurs. First, they propose that the double leucine motif of RBM45 is sensitive, and when disrupted, there is protein misfolding. Second, the HOA domain may create aggregation by facilitating the misfolding of the double leucine motif.

RBM45 in Antioxidant Response

Neurons are particularly susceptible to degeneration by reduction-oxidation dysregulation, especially neurons in the brain where there is high oxygen consumption, creating high levels of reactive oxygen species (ROS; Halliwell, 2006). Production of ROS predicts a role for oxidative stress in neurodegenerative disease progression. In fact, ROS have been shown to damage proteins and lipids in the CNS of ALS patients as well as in various organ systems of a transgenic mouse model of ALS (reviewed in Bakkar *et al*., 2015). The NRF-E2-related factor 2 (NRF2)/ KEAP1 pathway is a critical regulator of the responses to oxidative stress (Zhang, 2006). Bakkar and coworkers (2015) have shown that mis-localized (cytosolic) RBM45 physically interacts with KEAP1 during oxidative stress both *ex vivo*, in ALS patient spinal cords, and *in vitro*, using neuroblastoma cell lines. KEAP1 normally negatively regulates NRF2 by targeting it for proteasomal degradation via a KEAP1-Cul3 ubiquitin ligase complex. Under oxidative stress, KEAP1 is oxidized, disrupting protein structure, and dissociates from NRF2; KEAP1 then undergoes proteasomal degradation. NRF2 is free to translocate to the nucleus where it positively regulates expression of antioxidant response genes (reviewed in Bakkar *et al*., 2015; Tong *et al.*, 2006).

Bakkar and colleagues (2015) offer a model for the role of mis-localized RBM45 in the perturbation of the NRF2-KEAP1 oxidative stress response pathway. These authors propose that the cytoplasmic RNA-independent RBM45/KEAP1 interaction prevents the oxidation of KEAP1 under oxidative stress conditions reducing proteasomal mediated KEAP1 degradation. Excess levels of KEAP1 ultimately lead to the inappropriate degradation of NRF2 and a reduction in the amount of NRF2 available to translocate into the nucleus to activate antioxidant defense proteins. Reduced nuclear NRF2, compared to wild-type oxidative stress conditions, blunts the NRF2-KEAP1 oxidative response pathway, increasing ROS, and contributing to ALS pathogenesis.

Prospectus

RBM45 containing cytoplasmic aggregates have been strongly correlated with pathogenesis in the neurodegenerative

disorders ALS, FTLD, and AD (Bakkar *et al*., 2015; Collins *et al*., 2012; Li *et al*., 2015; Mashiko *et al*., 2016). Robert Bowser's group (Bakkar *et al*., 2015; Collins *et al*., 2012; Li *et al*., 2015, 2016) has proposed several avenues of research to further understand RBM45's role in neurodegenerative disorders. They propose using both differentiated and undifferentiated neuronal cell culture to understand the temporal pattern of RBM45 and TDP-43 (a well-established marker of ALS and FTLD pathogenesis) co-localization in inclusion bodies, RBM45 oxidative stress response, whether RBM45 cytoplasmic inclusions and/or loss of RBM45 from the nucleus cause cytotoxicity, and to tease out the fundamental question of whether RBM45 protein aggregation is a driver or a marker of neurodegeneration. Additionally, they (Li *et al*., 2016) have identified, using IP-MS, 131 high confidence protein:protein interactions (PPIs) with RBM45. These authors identified two major biological pathways for future investigation: RNA processing/splicing and cytoplasmic translation. They note that dysregulation of RNA splicing has been well-documented in ALS and FTLD and can result from RBDP mis-localization, aggregation, or both; these are characteristics shared by RBM45 (reviewed in Li *et al*., 2016).

As mentioned above, RBM45 has been implicated in oxidative stress response (Bakkar *et al*., 2015). Mashiko and coworkers (2016) produced a mutant RBM45 protein (mtLL/R470G) that induced cytoplasmic aggregation and reduced mitochondrial membrane potential. As these authors point out, mitochondria are a main source of ROS, which can induce the intrinsic apoptotic cascade. They propose that since dysregulation of mitochondrial function is evident in ALS (Cozzolino and Carrì, 2012) and a decrease in mitochondrial membrane potential precedes mitochondrial failure, RBM45 cytoplasmic aggregation causes mitochondrial dysfunction leading to bioenergetic malfunction and perhaps apoptosis. Buoying the proposal that RBM45 mutation may lead to mitochondrial failure and apoptosis is the observation that several PPIs occur between RBM45 and proteins involved in apoptotic nuclear changes (Li *et al*., 2016). Furthermore, mitochondria play a pivotal role in Ca^{2+} homeostasis, and Ca^{2+} overload is associated with neuronal cell death during hypoxia. Interestingly, RBM45 has been demonstrated to protect cardiomyocytes from hypoxia-induced cell death (Pyo *et al*., 2008) and may play a similar role in neurons. Taken together, these data suggest mitochondrial dysfunction via a RBM45 dependent pathway may lead to neuronal toxicity.

Surprisingly, though much progress has been made identifying protein-binding partners of RBM45 (Li *et al*., 2016), nothing is known about RNA binding partners of RBM45. Tamada and coworkers (2002), who first cloned and identified Rbm45 from rat brain, showed that RBM45 has high affinity for poly (C) RNA (binding reduced only ~50% in the presence of 1 M KCl vs. 0.1 M KCl) with poly (G) having the next highest affinity. Intriguingly, the most abundant pathology in ALS patients was observed in *C9ORF72* hexanucleotide (GGGGCC) repeat-expansion positive tissue. The *C9ORF72* hexanucleotide would encode a GC-rich RNA, suggesting it may be a binding partner of RBM45, though it has not been identified as such to date (Cooper-Knock *et al*., 2014; Li *et al.*, 2016; Mori *et al*., 2013). Similarly, RBM45 has been implicated in the binding of a GACGAC motif in a comprehensive RBDP gene regulation study (Ray *et al.*, 2013). This motif is associated with enhancement of splicing (Tian and Cole, 2001) during exon inclusion complementing the work of Li and coauthors (2016), who identified the splicing factor hnRNP-L as an RBM45 PPI through IP-MS and verified with IP and immunocytochemistry. The binding of RBM45 to the GACGAC motif needs to be determined empirically alongside poly (C) as a positive control and poly (A) as a negative control using a KCl gradient (Tamada *et al*., 2002). Pull-down assays or affinity chromatography using recombinant RBM45 and purified mRNA or total RNA from mouse brain can be used to identify putative RNA partners for RBM45. Concurrently, FOLDALIGN (Gorodkin *et al*., 1997) analysis of mouse and human RNAs from the UniGene database (https://www.ncbi.nlm.nih.gov/) could be used to identify predicted targets (De Silanes *et al.*, 2004).

RBDPs are multifunctional proteins participating in numerous biological processes such as RNA metabolism (e.g. RNA splicing, translation, mRNA stability), oxidative stress, bioenergetics, DNA damage response, and disease pathogenesis (Li *et al*., 2016; Lukong *et al*., 2008; Mashiko *et al*., 2016). RBM45 is no exception. As enumerated above, RBM45 has been implicated in neuropathologies, mitochondrial dysfunction, apoptosis, and RNA metabolism. Despite the work described herein, the *in vivo* function of RBM45 is not known. Now, recent work (Gong *et al*., 2017) demonstrates a function for RBM45 in genome stability by regulating DNA damage response (DDR) through interaction with histone deacetylase 1 (HDAC1). Gong and coworkers' (2017) data indicate that RBM45 negatively regulates its protein binding partner, FUS, from over-recruiting HDAC1 to damaged DNA. These data suggest the first clear *in vivo* role for RBM45 as a regulator of genotoxic stress. Further elucidation of *in vivo* RBM45 activity may be modeled with an *Rbm45* knock-out mouse. Predicted defects in brain development may result in embryonic lethality; therefore, a neuronal-specific conditional knock-out mouse could be created to bypass potential problems with embryonic lethality from a total knock-out of RBM45 function and provide insight into RBM45 activity in the adult animal.

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