The Role of Karyopherin-alpha in the Pathogenesis of TDP-43 Proteinopathy

by

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Abstract

Aberrant cellular processing and targeting of TDP-43 has been implicated in a wide variety of neurological diseases such as frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS). These diseases are characterized by the sequestration of TDP-43 into the cytoplasm of afflicted neurons, leading to the formation of ubiquitinated, cytoplasmic inclusions and an increased susceptibility to cellular insults. While the underlying causes of TDP-43 proteinopathy are unknown, we are investigating the role of a protein family known as the karyopherins in the nuclear targeting of TDP-43. Using co-immunoprecipitation in SH-SY5Y cells we determined that a major binding partner of TDP-43 is karyopherin-alpha 2 (KPNA2). Next, utilizing a high-density peptide array comprised of overlapping peptide sequences derived from TDP-43 we identified six regions where KPNA2 may directly interact with TDP-43. From these regions we developed six small, cell-penetrating peptides designed to specifically inhibit the interaction between KPNA2 and TDP-43. Through the use of these synthetic peptides, we were able to interfere with the binding of KPNA2 to TDP-43 in vitro. We found that the disruption of this specific protein-protein interaction was not sufficient to induce TDP-43 cytoplasmic sequestration, as determined by co-immunoprecipitation and subcellular fractionation assays.

As our research focused primarily on healthy SH-SY5Y cells, future studies will focus on investigating the effects of peptide-mediated TDP-43 nuclear import impairment paired alongside oxidative insult. We will also investigate whether compensatory mechanisms within SH-SY5Y cells are responsible for the nuclear localization of TDP-43 in the absence of KPNA2-mediated nuclear import.
Preface

This thesis is based on work conducted by Dr. Max Cynader, Dr. William Jia, and Bryson Armstrong in the Cynader laboratory of the UBC Brain Research Centre. Dr. Max Cynader, Dr. William Jia and I identified and designed the project. I was responsible for conducting all experiments and performing all data analysis. I was tasked with the writing of this thesis, and Dr. Max Cynader and Dr. William Jia both were involved in the editing process.
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Dedicated to my Mother and Grandmother
1: Introduction

1.1 Structure and Function of TDP-43

Transactive Response DNA-Binding Protein 43 (TDP-43) is a highly conserved, ubiquitously expressed nuclear protein encoded by the *TARDBP* gene located on human chromosome 1P36.2 (Banks, Kuta et al. 2008). TDP-43 was first described by Ou et al. (1995) as a transcriptional regulator which represses both basal and TAT-induced expression of the human immunodeficiency virus type 1 (HIV-1) gene. Subsequent studies revealed that TDP-43 plays a critical role in the alternative splicing of a variety of mammalian pre-mRNAs, such as those coding for CFTR (Buratti, Dörk et al. 2001; Buratti and Baralle 2001), SKAR (Fiesel, Weber et al. 2012), and ApoA-II (Mercado, Ayala et al. 2005) proteins.

More recent investigations have hinted at an increasingly complex role for TDP-43. In mice, Polymenidou et al. (2011) showed that TDP-43 is involved in regulating the largest set of cassette exons reported to date. They determined that the reduction of TDP-43 in the adult mouse nervous system was sufficient to alter the expression levels of 601 mRNAs, as well as the splicing patterns of 965 mRNAs (Polymenidou, Lagier-Tourenne et al. 2011). In humans, TDP-43 has been shown to influence the generation of a number of alternative mRNA isoforms, many of which encode for proteins that have been implicated in neuronal development and neurological diseases (Tollervey, Curk et al. 2011). Interestingly, TDP-43 has been
found to act in an autoregulatory manner, controlling its own expression by binding to \textit{TARDBP} mRNA (Tollervey, Curk et al. 2011).

TDP-43, which consists of 414 amino acids, structurally appears to belong to a group of proteins known as 2 RBD-Gly RNA Binding Proteins (Wang, Wang et al. 2004). This group of proteins, which also includes heterogeneous ribonucleoprotein particles (hnRNPs) and other factors involved in RNA splicing and transport, is characterized by the presence of two RNA recognition motifs (RRMs) along with a carboxy-terminal glycine-rich domain (Wang, Wang et al. 2004). TDP-43 has been shown to directly interact with transcripts via these RNA recognition motifs (Buratti and Baralle 2001). In addition to these motifs, TDP-43 contains two closely spaced, specific clusters of basic residues near the N-terminal (Winton, Igaz et al. 2008). This region is hypothesized to act as a bipartite nuclear localization signal.

Through the use of site directed mutagenesis, Winton et al. (2008) found that the bipartite signal sequence found on TDP-43 is necessary for the nuclear import of the protein. While TDP-43 is localized primarily within the nuclei of healthy neuronal and non-neuronal cells, it must be continuously shuttled between the nucleus and the cytoplasm in order for it to perform its cellular functions. Nuclear TDP-43 is believed to be targeted to the cytoplasm via a leucine-rich nuclear export signal located near the middle of the protein. Site directed mutagenesis studies have provided support for this hypothesis (Winton, Igaz et al. 2008).
1.2 TDP-43 and Disease

Aberrant cellular processing of TDP-43 has been implicated in a number of debilitating neurodegenerative diseases. In 2006, TDP-43 was discovered to be the primary protein component of the pathological inclusions observed in the affected neurons of patients suffering from either Amyotrophic Lateral Sclerosis (ALS) or Frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) (Neumann, Sampathu et al. 2006). In both diseases, TDP-43, which is primarily localized to the nuclei of healthy neurons, was found to redistribute to the cytoplasm where it aggregates into insoluble, ubiquitin-positive inclusions. The pathologic TDP-43 found in these inclusions has been shown to be ubiquitinated, hyperphosphorylated and cleaved into C-terminal fragments (Neumann, Sampathu et al. 2006).

While pathological TDP-43 is a primary feature of both ALS and FTLD-U, there are a number of other neurological diseases that exhibit signs of abnormal TDP-43 processing. Alzheimer’s disease, hippocampal sclerosis, corticobasal degeneration and the Parkinsonism-dementia complex of Guam have all been observed to present with secondary TDP-43 pathology (Geser, Martinez-lage et al. 2009). In these diseases, TDP-43 pathology is generally present to a lesser degree than is observed in primary TDP-43 proteinopathies such as ALS and FTLD-U (Geser, Martinez-lage et al. 2009). Additionally, while TDP-43 pathology exhibits a rather diffuse distribution in ALS and FTLD-U patients, the pathology observed in secondary TDP-
43 proteinopathies is generally localized within more discrete brain regions (Geser, Martinez-lage et al. 2009).

In the primary TDP-43 proteinopathies ALS and FTLD-U, afflicted cells exhibit a substantial loss of nuclear TDP-43 along with an increase in pathologic TDP-43 and TDP-43 fragments within the cytoplasm (Neumann, Sampathu et al. 2006). While TDP-43 cytoplasmic sequestration and aggregation has been shown to occur in these neurodegenerative diseases, it is unclear what role pathological TDP-43 plays in cellular toxicity.

There is evidence to suggest that TDP-43 proteinopathies result in a toxic gain of function (GOF) in afflicted cells. A study utilizing cultured rodent neurons found that the degree of cytoplasmic TDP-43 expression correlated with neurotoxicity (Halliday, Bigio et al. 2012). In rodent models, overexpression of WT and mutated TDP-43 has been shown to be neurotoxic, and this toxicity was observed in a dose-dependent fashion (Halliday, Bigio et al. 2012). Additionally, disease progression has been found to correlate with levels of TDP-43 C-terminal fragments (CTFs) in some rodent models (Halliday, Bigio et al. 2012). Furthermore, a number of studies have shown that mutations in the TARDBP gene may lead to cellular toxicity in a gain of function manner, including elevated stress granule formation, enhanced toxic TDP-43 CTF generation and increased production of prion-like, protease-resistant fragments (Halliday, Bigio et al. 2012).

While there is evidence that TDP-43 proteinopathy results in a toxic GOF in affected cells, there is also reason to believe that pathogenesis may arise due to the
loss of functional TDP-43 (LOF). As TDP-43 is implicit in many critical cellular processes, it follows that a disruption in its normal functions may have drastic consequences. The sequestration of TDP-43 into cytoplasmic aggregates may prevent sufficient levels of TDP-43 from translocating into the nucleus, where it normally regulates a variety of mRNA processing (Halliday, Bigio et al. 2012). Although LOF and GOF mechanisms underlying the toxicity of TDP-43 are often presented as competing hypotheses, there is no evidence that these mechanisms are mutually exclusive, and both are likely to play a role in the etiology of TDP-43 proteinopathies.

1.3 TDP-43 and ALS

Amyotrophic Lateral Sclerosis (ALS) is a debilitating neurodegenerative disease that is characterized by progressive paralysis, muscular atrophy and death (Siddique and Ajroud-Driss 2011). Recent studies have estimated that the lifetime risk of acquiring spontaneous ALS before the age of 70 is between 1 in 1000 to 1 in 400, with males being slightly more at risk than females (Wijesekera and Leigh 2009). The average life expectancy of a patient suffering from ALS is 2-3 years after diagnosis, with respiratory failure being the leading cause of mortality (Pratt, Getzoff et al. 2012).

While the majority of ALS cases are sporadic in origin (sALS), it has been reported that approximately 10% are of the familial variety (fALS) (Siddique and Ajroud-Driss 2011). Familial ALS is inherited in either an autosomal dominant or autosomal recessive fashion (Siddique and Ajroud-Driss 2011). A number of genetic
mutations have been found to be associated with fALS. Mutations in SOD1, which encodes for the enzyme Superoxide dismutase (Rosen, Siddique et al. 1993), and FUS, a gene encoding for RNA-binding protein Fused in Sarcoma (FUS) (Vance, Rogelj et al. 2009), have both been observed in patients presenting with fALS.

A major pathological characteristic of ALS is the presence of ubiquitinated cytoplasmic inclusions in afflicted cells; most commonly, spinal motor neurons (Siddique and Ajroud-Driss 2011; Deng, Zhai et al. 2010). Recently, it was discovered that the major protein component of inclusions seen in non-SOD1 ALS is TDP-43, which is found to colocalize with FUS and the ubiquitin-binding protein p62 in the cytoplasm of diseased cells (Sreedharan, Blair et al. 2008; Deng, Zhai et al. 2010). Mutations in the TARDBP gene have been observed in cases of both sALS and fALS, and studies suggest that TARDBP mutations may be present in up to 4% of fALS cases (Sreedharan, Blair et al. 2008; Deng, Zhai et al. 2010).

While ALS and FTLD have historically been regarded as separate, distinct pathologies, it is becoming clear that these diseases may share similar etiologies. A 2003 review on motor neuron disease (MND) reported that as many as 20-40% of patients suffering from ALS exhibit cognitive impairments such as those seen in FTLD, and approximately 5% of MND patients are diagnosed as having motor neuron disease with dementia (MND-D), or FTLD-MND (Leigh, Abrahams et al. 2003). Interestingly, cognitive impairments associated with FTLD-MND are most commonly observed in patients with marked bulbar symptoms, although these
Impairments have been reported in patients exhibiting non-bulbar ALS as well (Leigh, Abrahams et al. 2003).

Currently, there are not many options available for the treatment of ALS. The majority of post-diagnosis support focuses on palliative care measures, such as providing non-invasive ventilation and supplying the patient with specially modified foodstuffs designed for easy ingestion (Pratt, Getzoff et al. 2012). While palliative care, in some cases, has been shown to improve the quality of life and the prognosis of the patient, there is a severe lack of pharmacological options targeting the progression of ALS (Pratt, Getzoff et al. 2012). A greater understanding of the pathophysiology of this disease is necessary for the development of more effective therapeutics.

1.4 TDP-43 and FTLD

Frontotemporal lobar degeneration (FTLD) is one of the leading causes of dementia, accounting for approximately 5%-10% of recorded cases (Rademakers, Baker et al. 2007). With an age of onset of as early as 30 years of age (Rabinovici and Miller 2010), it represents approximately 10%-20% of all dementia cases occurring before the age of 65 years (Rademakers, Baker et al. 2007). Interestingly, there appears to be a strong familial component associated with the risk of acquiring FTLD: it has been reported that nearly half of all FTLD patients have a family history of a resemblant dementia (Rademakers, Baker et al. 2007).
FTLD consists of three distinct clinical variants, which are defined by their initial and predominant symptoms (Rabinovici and Miller 2010). Behavioural-variant frontotemporal dementia (bvFTD) is characterized by significant changes of personality and behaviour in patients, as well as the progressive development of apathy and disinhibition (Rabinovici and Miller 2010). While the behavioural changes of these patients are substantial, this variant of FTLD has less of an impact on overall cognition (Rabinovici and Miller 2010). Notably, while ALS has been found to co-occur with any of the FTLD variants, it is most commonly coupled with bvFTD (Rabinovici and Miller 2010).

Semantic dementia (SD) is another variant of FTLD. Patients suffering from SD initially present with fluent, anomic aphasia, which, over time, may progress to multimodal agnosia (Rabinovici and Miller 2010). Semantic dementia, in some cases, is also characterized by marked behavioural changes that overlap with those seen in bvFTD patients (Rabinovici and Miller 2010). Patients exhibiting SD tend to perform poorly during tests on semantic memory, such as confrontation naming and category fluency tests, whereas their spatial abilities, executive functions and episodic memory remain intact (Rabinovici and Miller 2010).

The third variant of FTLD is known as progressive nonfluent aphasia (PNFA), which is characterized by the gradual deterioration of motor speech and expression of language (Rabinovici and Miller 2010). PNFA is frequently associated with corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP); patients
with PNFA tend to exhibit Parkinsonism and limb apraxia, as well as supranuclear gaze palsies (Rabinovici and Miller 2010).

A major component of FTLD pathology is the presence of inclusion bodies within cells located in the frontal and temporal lobes of afflicted patients. There are two distinct types of inclusion bodies observed, which are defined by their immunoreactivities: tau-positive inclusions and tau-negative, ubiquitin-positive inclusions (Rabinovici and Miller 2010). The majority of FTLD cases can be subclassified as either tau-positive FTLD (FTLD-Tau) or ubiquitin-positive FTLD (FTLD-U) based on these inclusions, with FTLD-U presenting as the most common clinical form of FTLD (Dickson, Kouri et al. 2011).

Tau-positive FTLD is characterized by the presence of tau-positive inclusions in the cytoplasm of neurons and glia. Examples of FTLD-related tauopathies include Pick’s disease, CBD, PSP and argyrophilic grain disease (Rabinovici and Miller 2010). The majority of familial FTLD-Tau patients, as well as some sporadic FTLD-Tau patients, have been found to harbor mutations in the microtubule-associated protein tau (MAPT) gene, which encodes for the tau protein (Dickson, Kouri et al. 2011).

While FTLD-Tau represents a large proportion of cases of FTLD, there are many patients suffering from FTLD that do not exhibit tau immunoreactivity. Once immunohistochemical techniques became more refined, it was discovered that a majority of tau-negative FTLD cases exhibit staining for ubiquitin-positive
inclusions; this subcategory of FTLD was termed FTLD-U (Rabinovici and Miller 2010).

In 2006, Neumann et al. discovered that, for most cases of FTLD-U, the major protein component of the observed ubiquitinated inclusions is hyperphosphorylated, truncated TDP-43. Unlike in ALS, where ubiquitin-positive inclusions are found primarily in spinal motor neurons, the neuronal inclusions observed in FTLD-U are characteristically observed in both frontal and temporal cortices, as well as in the hippocampus (Baker, Mackenzie et al. 2006).

As frontotemporal dementia exhibits a strong familial inheritance pattern, the pathogenesis of this disease is believed to contain a genetic component. In 2006, a study by Baker et al. revealed an association between FTLD-U and mutations in GRN, a gene encoding for the protein progranulin (PGRN). Further epidemiologic studies have revealed that mutations in GRN may account for 5%-11% of all cases of FTD, and up to 11%-26% of cases of familial FTD (Mackenzie 2007).

Progranulin, also known as proepithelin, is a heavily glycosylated, 593-amino acid protein involved in a wide variety of physiological processes, such as embryonic development, inflammation and wound healing (Ahmed, Mackenzie et al. 2007). PGRN has an estimated molecular weight of 68.5 kDa; due to glycosylation, however, its weight is observed to be closer to 90 kDa (Ahmed, Mackenzie et al. 2007). Progranulin consists of seven granulin-like domains and is cleaved by extracellular proteases, resulting in the generation of small peptides known as granulins (GRNs). These GRNs have been shown to mediate numerous biological
functions such as cell cycle progression, tumorigenesis and inflammation (He and Bateman 2003).

While the function of PGRN in the periphery has been the subject of numerous studies, there is little known about the role of PGRN in the central nervous system. In a recent study, Tang et al. (2011) discovered that progranulin binds directly to TNF-receptors (TNFR) in both mouse and human cells. They found that PGRN acts to reduce inflammatory signaling by blocking the interaction between TNF-α and TNFRs, leading to increased cell viability during periods of inflammation (Tang, Lu et al. 2011).

PGRN mutations linked to FTLD-U have been shown to lead to the nonsense-mediated decay (NMD) of mutant transcripts, resulting in PGRN haploinsufficiency (Baker, Mackenzie et al. 2006). It follows that PGRN haploinsufficiency, and the resulting disinhibition of neuronal TNF-α signaling, may lead to decreased neuronal survivability over time. By utilizing RNAi technology, Guo et al. (2010) discovered that PGRN haploinsufficiency, induced in mouse primary cortical neurons, was sufficient to induce cytoplasmic mislocalization of TDP-43, increase cellular vulnerability to H₂O₂ and NDMA insults and enhance caspase-3 activation.

While it is clear that cytoplasmic mislocalization of TDP-43 and the pathogenesis of clinical FTLD-U are strongly linked, the mechanisms behind the cytoplasmic sequestration of TDP-43 are still unknown. Recent studies suggest that an impairment of the nuclear transport system, responsible for returning cytoplasmic TDP-43 to the nucleus, may be involved in the development of TDP-43
proteinopathy (Nishimura, Zupunski et al. 2010). The proteins implicated in the nuclear transport of TDP-43 are known as karyopherins (KPNs) (Nishimura, Zupunski et al. 2010).

1.5 Karyopherins

The transportation of a protein from the cytoplasm to the nucleus is a highly selective, regulated process. There are a number of different pathways involved in the nucleocytoplasmic transport of proteins within a cell, the most well understood being classical nuclear localization. Classical nuclear localization utilizes monopartite and bipartite classical nuclear localization signals (cNLS) and is mediated by a superfamily of proteins known as karyopherins (Conti, Muller et al. 2006).

The label karyopherin encompasses a family of conserved proteins comprised of both importins and exportins, proteins involved in shuttling target proteins to and from the nucleus, respectively (Goldfarb, Corbett et al. 2004). There are a number of proteins involved in classical nucleocytoplasmic shuttling, primarily the karyopherin-αs (KPNA) and karyopherin-βs (KPNB) (Goldfarb, Corbett et al. 2004).

The karyopherin-α family consists of three distinct subfamilies: Importin αP (ImpαP), Importin αQ (ImpαQ), and Importin αS (ImpαS) (Yasuhara, Oka et al. 2009). Each KPNA is encoded by a distinct gene in mammals (Tejomurtula, Lee et al. 2009). To date, there have been seven KPNA-encoding genes discovered in humans, and six found in mice (Tejomurtula, Lee et al. 2009). The ImpαP subfamily consists
of karyopherin-α2 (KPNA2) and the recently discovered karyopherin-α7 (KPNA7), the ImpαQ subfamily consists of karyopherin-α3 (KPNA3), karyopherin-α4 (KPNA4) and karyopherin-α1 (KPNA1), and the subfamily ImpαS consists of karyopherin-α5 (KPNA5) and karyopherin-α6 (KPNA6) (Yasuhara, Oka et al. 2009; Tejomurtula, Lee et al. 2009). While the majority of human karyopherins exist as mouse orthologues, the presence of a human KPNA5 orthologue has not been found to be expressed in mice (Tejomurtula, Lee et al. 2009).

Although each karyopherin-α is distinct, there exists a great deal of homology between members of this protein family. Karyopherin-α isoforms within the same subfamily exhibit roughly 85% sequence homology, while KPNA proteins within different subfamilies exhibit approximately 50% sequence homology (Tejomurtula, Lee et al. 2009). All known KPNA proteins contain the same structural motifs. The two major motifs seen are the importin-β binding (IBB) domain, located near the N-terminal, and multiple repeated armadillo (ARM) motifs (Tejomurtula, Lee et al. 2009). The ARM motifs have been shown to form an anionic NLS-binding pocket (Tejomurtula, Lee et al. 2009; Yasuhara, Shibazaki et al. 2007). Notably, when not bound to KPNB, the IBB domain of KPNA is bound to its own ARM domain in an autoinhibitory fashion (Chook and Blobel 2001).

Karyopherin expression is highly regulated, and the expression levels of individual KPNA isoforms have been shown to vary between both the tissue type and the stage of development of the organism (Kohler, Ansieau et al. 1997; Yasuhara, Shibazaki et al. 2007). Karyopherin-α proteins are ubiquitously expressed
and, while each KPNA recognizes both classical bipartite and monopartite NLSs, each exhibits distinct affinities and transport efficiencies for specific proteins (Kohler, Speck et al. 1999). The variety of KPNA proteins involved in nuclear transport allows for redundancy within the system. A number of studies suggest that some proteins may be targeted to the nucleus by more than one KPNA (Nishimura, Zupunski et al. 2010; Ma and Cao 2006).

Karyopherin-β1 (KPNB1) is a member of the karyopherin-β family, a family of proteins involved in both classical and non-classical nuclear localization (Chook and Blobel 2001). KPNB1 has been shown to directly interact with nucleoporins, a family of proteins that make up nuclear pore complexes (NPC) (Kobe 1999). NPCs are large, multimeric protein channels that allow the passive movement of ions and small molecules across the nuclear envelope while restricting the exchange of macromolecules, such as proteins over 20 kDa (Kobe 1999). Macromolecules must contain the appropriate signals, such as a cNLS, in order to cross through the NPC into the nucleus (Kobe 1999).

While some KPNBs have been shown to bind directly to non-classical NLSs, in the case of classical nuclear localization KPNB1 binds to the IBB domain of the target KPNA within the cytoplasm. This binding results in the disinhibition of the KPNA ARM domain, allowing the KPNA to bind the cNLS of the target protein (Kobe 1999).
1.6 Classical Nuclear Import Cycle

The nuclear import of a protein begins in the cytoplasm of the cell and utilizes the RanGTP-RanGDP nucleocytoplasmic gradient to drive the process (Kutay, Bischoff et al. 1997). First, KPNB1 binds to the IBB region of KPNA within the cytoplasm, disinhibiting the KPNA ARM domain and increasing its binding affinity for cNLSs (Kobe 1999). After KPNA binds the cNLS of the protein destined for nuclear import, the resulting trimeric complex is shuttled to the nuclear envelope where KPNB1 mediates the translocation of the protein complex through the NPC (Lange, Mills et al. 2007). Once inside the nucleus, KPNB1 binds to RanGTP. This binding induces a conformational change, causing KPNB1 to release the IBB domain of KPNA (Lee, Matsuura et al. 2005). The IBB domain, now free to bind the ARM domain, initiates the release of the cargo protein from KPNA (Lee, Matsuura et al. 2005).

In order for a cell to continue to shuttle proteins into the nucleus, the karyopherins involved must first be recycled back to the cytoplasm. Kutay et al. (1997) found that, after the dissociation of the trimeric nuclear import complex, nuclear KPNA rapidly associates with a protein dimer consisting of cellular apoptosis susceptibility protein (CAS) and RanGTP. The binding of KPNA and RanGTP to CAS was observed to be highly cooperative, and CAS was found to have a higher affinity for NLS-free KPNA as opposed to NLS-occupied KPNA (Kutay, Bischoff et al. 1997)
Cellular apoptosis susceptibility protein is a nuclear-export factor that interacts with the nuclear pore complexes, allowing it to cross the nuclear membrane without the need for other soluble factors (Kutay, Bischoff et al. 1997). Once the KPNA-CAS-RanGTP trimer leaves the nucleus via the NPC, cytoplasmic proteins Ran-specific binding protein 1 (RanBP1) and Ran GTPase-activating protein (RanGAP1) catalyze the hydrolysis of Ran-GTP to Ran-GDP (Kutay, Bischoff et al. 1997). As CAS has very low affinity for Ran-GDP, the cytosolic hydrolysis of Ran-GTP causes the dissociation of the trimeric complex into the individual protein components (Kutay, Bischoff et al. 1997). The newly released, cytoplasmic KPNA is free to bind KPNB1 and the NLS of cargo proteins, reinitiating the classical nuclear import cycle.

### 1.7 KPNA and TDP-43

In 2010, Nishimura et al. utilized a SMARTpool human small interfering RNA library, targeted against 82 known nuclear transport proteins, in order to determine which proteins were involved in shuttling TDP-43 to the nucleus of SH-SY5Y cells. They found that knocking down either CAS or KPNB1 in SH-SY5Y cells led to the cytoplasmic mislocalization and aggregation of TDP-43. Similar findings were reported utilizing mouse neuroblastoma N2a cells and mouse primary cortical neurons (Nishimura, Zupunski et al. 2010).

Nishimura et al. (2010) went on to investigate the effects of CAS and KPNB1 knockdowns on the subcellular distribution of Karyopherin-αs. They discovered that the knockdown of CAS results in the accumulation of KPNAs within the nucleus
while, conversely, the knockdown of KPNB1 leads to the cytoplasmic sequestration of KPNAs (Nishimura, Zupunski et al. 2010).

In order to determine which KPNA isoforms are involved in the subcellular transport of TDP-43, Nishimura et al. (2010) performed a glutathione S-transferase-pull-down on SH-SY5Y cell lysate, utilizing GST-karyopherin-α fusion proteins. They found that endogenous TDP-43 was able to bind to the five KPNA isoforms examined: KPNA1, KPNA2, KPNA3, KPNA4, and KPNA6. This suggested that multiple KPNA isoforms may be involved in the nuclear import of TDP-43. Nishimura et al. went on to discover that GST-KPNA fusion proteins do not bind to TDP-43ΔNLS, a modified form of TDP-43 lacking an NLS; this demonstrated that the association between KPNA and TDP-43 requires a cNLS (Nishimura, Zupunski et al. 2010).

Nishimura et al. (2010) went on to analyze the expression levels of nuclear transport factors in both the affected cortical regions of FTLD-U patients and the spinal cords of ALS patients. Interestingly, they found that there was a dramatic reduction of CAS and KPNA2 expression in the medial temporal lobes of FTLD-U patients. In contrast, they found no significant difference in the expression of KPNB, KPNA1 or KPNA6 when compared to healthy controls. Upon analysis of spinal cord lysates derived from ALS patients, the team found that there was an increase in KPNA2 expression, a decrease in KPNA6 expression, and no observable change in KPNB and KPNA1 expression. CAS expression was also unchanged. Through the use of immunohistochemistry, they found that cytoplasmic KPNA2 staining was
significantly higher in the spinal cords of ALS patients (Nishimura, Zupunski et al. 2010).

1.8 Specific Aims

While the findings by Nishimura et al. (2010) suggest that disruption of the nucleocytoplasmic transport system may be involved in the pathogenesis of TDP-43 proteinopathies, any cause and effect relationship remains unclear. As pathological TDP-43 is observed in a number of devastating neurological diseases, an understanding of the mechanisms underlying its nuclear import is of great importance.

Aim 1: Identify the endogenous KPNA isoforms that interact with TDP-43 in SH-SY5Y cells

Aim 2: Utilizing a high-density peptide array, determine the regions of TDP-43 that interact with KPNA isoforms expressed in SH-SY5Y cells and, from these sequences, develop small interfering peptides that are able to specifically block the interaction between relevant KPNA isoforms and TDP-43

Aim 3: Utilizing our putative interference peptides, determine whether blocking the interaction between TDP-43 and KPNA is sufficient to cause cytoplasmic mislocalization and aggregation of TDP-43 in SH-SY5Y cells
2: Peptide-Mediated Inhibition of TDP-43 Nuclear Targeting

2.1 Introduction

The karyopherin-α isotypes involved in classical nuclear import within a cell depends upon both the cell type and the developmental stage of the cell (Kohler, Ansieau et al. 1997; Yasuhara, Shibazaki et al. 2007). While Nishimura et al. (2010) determined that endogenous TDP-43, derived from SH-SY5Y cell lysate, is able to bind to recombinant GST-tagged KPNA1, KPNA2, KPNA3, KPNA4 and KPNA6, in order to fully investigate the mechanisms underlying the nuclear import of TDP-43 the identities of the endogenous KPNA isotypes involved must be elucidated.

Furthermore, in order to explore a cause and effect relationship between the nuclear import of TDP-43 and the pathogenesis of TDP-43 proteinopathy, it is essential that one is able to disrupt the nuclear import of TDP-43 in such a way that reduces the risk of compensatory cellular mechanisms taking over. Utilizing RNA interference, Nishimura et al. (2010) showed that the knockdown of expression of individual KPNA isoforms was not sufficient to induce TDP-43 cytoplasmic mislocalization. These results suggest that redundancies within the karyopherin family allow for nuclear import to continue in the absence of a single KPNA isoform, but these redundancies may be a result of compensatory changes in KPNA expression rather than a normal cellular mechanism; RNA interference has been
shown to induce compensatory changes in cellular physiology (Villarroya, Lara et al. 2011).

To investigate the effect of inhibiting the nuclear import of TDP-43, while minimizing the potential for cellular compensation, we set out to develop small interference peptides, comprised of short stretches of amino acids derived from TDP-43, that could be utilized to rapidly and specifically block the interaction between TDP-43 and relevant karyopherin-α isoforms.

First, we performed Western blotting on cell lysate to determine the identities of the KPNA isoforms expressed in SH-SY5Y cells. We then performed a co-immunoprecipitation on SH-SY5Y cell lysate, utilizing endogenous KPNA isoforms as bait, in order to elucidate which isoforms were able to bind TDP-43 in vitro.

Once we determined the KPNA isoform involved in TDP-43 nuclear import, we utilized a high-density peptide array to identify putative regions of TDP-43 that were able to directly bind to the recombinant KPNA isoform. Our peptide array consisted of a series of overlapping, sequential 12-14mer peptides with a frameshift of two amino acids, which, all together, encompassed the entire primary sequence of human TDP-43. From these KPNA-binding regions of TDP-43 we designed six putative interference peptides, ranging from 8-11mer, and tested their blocking efficacy in vitro. After establishing our peptides as a useful tool for blocking the binding of TDP-43 and its nuclear import protein, we went on to investigate
whether the inhibition of this interaction is sufficient to induce TDP-43 proteinopathy \textit{in vitro}.

\section*{2.2 Materials and Methods}

\subsection*{2.2.1 Cell Culture}

A human neuroblastoma cell line, SH-SY5Y, was procured from American Type Culture Collection (Manassas, VA). The cell line was maintained as per the guidelines of American Type Culture Collection, and cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM: Sigma Chemical Co., St. Louis, MO) supplemented with 10\% fetal bovine serum (Gibco-BRL, Grand Island, NY) and 1\% streptomycin/penicillin (Gibco-BRL, Grand Island, NY). Cell cultures were cultivated in a humidified incubator (NuAir, Plymouth, MN) with a 5\% CO\textsubscript{2} environment at 37\degree C.

\subsection*{2.2.2 High-Density Peptide Array Synthesis}

Blank cellulose membranes were acquired from Intavis AG (Köln, Germany). High-density peptide arrays were synthesized by the UBC Peptide Synthesis facility, utilizing cellulose membranes as per Hilpert, Winkler et al. (2007). Peptides were synthesized from C-terminal to N-terminal, with the C-terminal end anchored to the membrane via an ester bond. First, pentafluorophenyl ester (PFP) activated amino acids were loaded on their appropriate membrane coordinate, at 0.11uL of a single amino acid per spot. The unused portions of the membranes were blocked via acetylation. Fluorenlymethyloxycarbonyl (Fmoc) protection groups were removed
by four short, successive washes with dimethylformamide (DMF), followed by two five-minute washes with a solution of 20% piperidine in DMF, four more quick DMF washes and two methanol washes. Free amino groups were stained using 0.02% bromophenol blue (BPB) in methanol. Membranes were then subjected to a final methanol wash and subsequently air-dried, after which they were ready for the next cycle of amino acid coupling. Once the final amino acids were coupled to the membrane-bound peptides, side-chain protecting groups were removed via treatment with trifluoroacetic acid (TFA) in a solution of 3% triisopropylsilane, 2% water and 1% phenol. A finished membrane represented the full-length sequence of human TDP-43 and was composed of multiple spots. Each spot consisted of sequential, overlapping peptides of either 12-mer or 14-mer with a frame shift of two amino acids between neighboring spots.

2.2.3 Protein-Protein Interaction Assay

The TDP-43 high-density peptide membrane was activated by a 10-minute ethanol wash with light rocking. Membrane was washed three times in TBS-T (50mM Tris-base, 27mM KCl, 136mM NaCl, 0.2% Tween-20, pH 8.0) for five minutes, and blocked at room temperature for 3 hours in blocking buffer (5% sucrose, 4% non-fat milk, TBS-T). Membrane was then washed once in TBS-T for 5 minutes and probed with primary protein solution consisting of either blank blocking buffer (for negative control) or recombinant human KPNA2 protein (5ug/mL, Abcam, Toronto, ON) in blocking buffer, overnight at 4°C. The following day, membrane was washed three times for 20 minutes in TBS-T. Membrane was
then incubated overnight at 4°C in Goat polyclonal antibody against KPNA2 (Abnova, Taipei, Taiwan) diluted in blocking buffer (0.25ug/mL). The peptide membrane was washed at room temperature three times for 20 minutes in TBS-T, and immediately incubated in donkey anti-goat HRP-labeled secondary antibody (1:2000, Santa Cruz Biotechnology, Dallas, TX) in blocking solution for two hours at room temperature. After three more 20-minute washes in TBS-T, the membrane was gently blotted dry with tissue and developed using Western Lightning-ECL Enhanced Chemiluminescence Substrate (PerkinElmer, Waltham, MA). After visualization, the membrane was washed for five minutes with TBS-T and twice briefly with deionized water. If membrane was probed with negative control, regeneration procedure was performed (as per 2.2.4) and above procedure was repeated, without initial ethanol activation, using recombinant KPNA2. Membranes were unable to be regenerated for further use after performing KPNA2 protein probe, so three separate membranes were utilized during experimentation.

2.2.4 Peptide Array Regeneration

After visualization, peptide membrane was washed three times for 5 minutes with TBS-T followed by one brief wash with deionized water. Membrane was incubated in fresh Regeneration Buffer A (8M Urea, 1% SDS, 0.1% β-ME, deionized water) overnight at 37°C. The following day, membrane was briefly washed twice with deionized water and treated with Regeneration Buffer B (10% Acetic Acid, 40% deionized water, 50% ethanol) for 90 minutes at room temperature, with
gentle shaking. Membrane was then rinsed twice with deionized water and washed three times for five minutes with TBS-T.

2.2.5 Peptide Synthesis

Seven short cell-penetrating peptides were synthesized by GL Biochem Ltd. (Shanghai, China) and refined to greater than 90% purity by HPLC. Purity was verified by mass spectrometry. Six putative blocking peptides (p1-p6), derived from segments of TDP-43, and one scrambled control peptide (pS) were developed with a cell penetrating, truncated N-terminal TAT domain. The scrambled control peptide contains the same amino acid composition and charge density as p1. The peptides were dissolved in distilled water and stored at -80°C.

2.2.6 Peptide Treatments

Peptides were combined and diluted to appropriate composition and concentration in warm DMEM, with 10% FBS and 1% antibiotics. The media of near-confluent, 100mm plates of SH-SY5Y cells was replaced with peptide-rich media, and plates were placed back in incubator. Cells were allowed to grow for either four hours (Co-immunoprecipitation) or 24 hours (Fractionation and Immunocytochemistry) before harvesting for experimentation.

2.2.7 Protein Extraction for Western Blotting

Confluent 6-well plates of SH-SY5Y cells were washed two times, on ice, with cold PBS and lysed with 100uL of 2x sample buffer (62.5mM Tris-HCl at pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate, 0.01% Bromophenol blue, 5% β-
mercaptoethanol). Lysate was collected, boiled at 100°C for 5 minutes, and stored at -80°C.

### 2.2.8 Co-Immunoprecipitation

Confluent 100mm plates of SH-SY5Y cells were washed two times, on ice, with cold PBS. Cells were collected in 1mL of cold PBS and centrifuged at 3000g for 5 minutes at 4°C. The supernatant was discarded, and cell pellets were resuspended in 1mL of iced lysis buffer (0.5% deoxycholate, 0.5% Triton X-100, 10uM PMSF and protease inhibitor cocktail tablet in PBS) and incubated on ice for 15 minutes. Samples were then centrifuged at 3000g for 5 minutes. From each, 400uL of supernatant was collected and diluted to 1mL with PBS (supplemented with 10uM PMSF and protease inhibitor cocktail tablet). We added 30uL from each sample to 10uL of 4X sample buffer, boiled for 5 minutes and stored at -20°C to serve as lysate controls. Samples were then precleared for one hour at 4°C with 10uL of a 1:1 solution of Protein A/G Sepharose beads (GE Healthcare, Piscataway Township, NJ) and lysis buffer. Beads were pelleted at 300g for 30 seconds and supernatant was collected. Samples were then incubated overnight at 4°C with either 4ug of normal goat IgG (Santa Cruz Biotech, Dallas, Texas), 4ug of normal rabbit IgG (Santa Cruz Biotech, Dallas, Texas), or 4ug of primary antibody. Primary antibodies utilized: anti-KPNA1, anti-KPNA4, anti-KPNA6 (Abcam, Toronto, ON) and anti-KPNA2 (Abnova, Taipei, Taiwan). The following day, 50uL of a 1:1 solution of Protein A/G Sepharose beads and lysis buffer were added to each sample. Samples were rotated gently for 4 hours at 4°C. Beads were pelleted via centrifugation at 300g for 30
seconds, supernatant was discarded, and beads were washed with 1mL of cold PBS. Centrifugation and washes were repeated three more times. Beads were dried using a fine needle and syringe, and proteins were eluted by 60uL of 2x sample buffer per sample. Samples were boiled at 100°C for 5 minutes, and stored at -20°C.

2.2.9 Western Blot

Samples were boiled at 100°C for 5 minutes and briefly centrifuged at 13000g. 30uL of each sample was electrophoretically resolved on a 10% SDS-polyacrylamide gel using a Bio-Rad Gel electrophoresis system (Bio-Rad, Hercules, CA) at 100V. Samples were run alongside PageRuler™ Plus Prestained Protein Ladder (Fermentas, CA) for determination of molecular weight. Resolved proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) using the Bio-Rad Wet Transfer system (Bio-Rad, Hercules, CA) at 100V for 90 minutes at 4°C. Nitrocellulose membranes were washed with TBST three times before blocking for one hour at room temperature in 5% non-fat milk, dissolved in TBST. After blocking, membranes were briefly washed three times in TBST and incubated overnight at 4°C in primary antibody, diluted 1:1000 in a solution of 3% bovine serum albumin (BSA) and 0.3% sodium azide in TBST. Primary antibodies used: anti-KPNA1, anti-KPNA4, anti-KPNA6 (1:1000, Abcam, Toronto, Ontario), anti-KPNA2 (1:1000, Abnova, Taipei, Taiwan) and anti-TDP-43 (1:1000, Proteintech, Chicago, IL). Membranes were subsequently washed three times in TBST and incubated in an appropriate HRP-conjugated secondary antibody at a 1:1000 dilution in 5% BSA/TBST, for one hour at room temperature. Secondary antibodies used: anti-goat
(Santa Cruz, Dallas, TX) and anti-rabbit (PerkinElmer, Waltham, MA). After washing membranes 3 more times with TBST, membranes were developed using Western Lightning-ECL Enhanced Chemiluminescence Substrate (PerkinElmer, Waltham, MA). Protein band densities were analyzed with ImageJ software (NIH) and normalized against internal actin controls.

2.2.10 Subcellular Fractionation

Confluent 100mm plates of SH-SY5Y cells were placed on ice and washed twice with 5mL of iced PBS. Each plate was incubated in 1mL of Buffer A (10mM HEPES-KOH, 10mM KCl, 10mM EDTA, 0.4% NP-40, 1.5mM MgCl₂, 1mM DTT, Protease inhibitor cocktail tablet, at pH 7.9) for 20 minutes, with gentle rocking at 4°C. Cells were gently collected into microcentrifuge tubes and centrifuged at 300g for 5 minutes, at 4°C. Nuclear pellets were set on ice and supernatant was collected, centrifuged at 17000g to clear nuclear debris, and transferred to clean tubes as cytoplasmic fractions. Nuclear pellets were resuspended in 1mL of Buffer A, vortexed briefly, and centrifuged at 300g for 5 minutes at 4°C. Supernatant was discarded. Nuclear pellets were resuspended in iced PBS, spun down at 300g for 5 minutes at 4°C, and supernatant was again discarded. Pellets were then subjected to 150uL of Buffer B (20mM HEPES-KOH, 400mM NaCl, 1mM EDTA, 10% glycerol, 1mM DTT and protease inhibitor cocktail tablet, at pH 7.9) on ice for two hours, with brief vortexing every 30 minutes. Nuclear samples were then centrifuged at 17000g for 10 minutes at 4°C, and supernatant was transferred to clean tubes as nuclear fractions. Both cytoplasmic and nuclear fractions were combined in a 3:1
ratio with 4X sample buffer, boiled at 100°C for 5 minutes, and stored at -20°C. Purity of fractions was confirmed by Western blotting for the presence of Lamin-b1, a nuclear envelope protein (Abcam, Toronto, ON) and heat shock protein 90 (HSP-90), a cytoplasmic-specific protein (BD Biosciences, Mississauga, ON)

2.2.11 Immunocytochemistry

SH-SY5Y cells were grown to approximately 60% confluency on untreated glass coverslips in 12-well plates. Coverslips were placed directly into a warmed fixation solution of 4% PFA and 4% sucrose in PBS. Coverslips were incubated in fixation solution for 45 minutes at 37°C, then immediately washed with PBS three times for two minutes. Cells were permeabilized in .25% TritonX-100 in PBS for five minutes at room temperature, and briefly washed once for five minutes in PBS. Coverslips were blocked in a solution of 10% BSA in PBS for 30 minutes at 37°C. Blocking solution was carefully replaced with a primary antibody solution composed of 3% BSA in PBS and mouse polyclonal HSP-90 (1:150, BD Biosciences, Mississauga, ON), and coverslips were left to incubate in a sealed container overnight at 4°C. The following day, coverslips were briefly washed five times in PBS. Secondary antibody solution, consisting of 3% BSA in PBS and Alexa Fluor 488 goat anti-mouse fluorescent antibody (1:1000, Invitrogen, Burlington, ON) was placed on coverslips for 45 minutes at 37°C. After five brief washes in PBS, coverslips were probed with rabbit polyclonal antibody against TDP-43 (1:150, Proteintech, Chicago, IL) in 3% BSA/PBS overnight at 4°C. Coverslips were washed five times in PBS, then incubated in Alexa Fluor 633 goat anti-rabbit antibody
(1:1000, Invitrogen, Burlington, ON) at 37°C for 30 minutes. After washing five times in PBS, coverslips were gently patted dry and mounted on glass slides with Prolong Gold Anti-fade Reagent with DAPI (Invitrogen, Burlington, ON). Samples were imaged with an Olympus Fluoview FV1000 Confocal scanning microscope. Mean cytoplasmic and nuclear TDP-43 staining was quantified using ImageJ software.

2.3 Results

2.3.1 Human Neuroblastoma SH-SY5Y Cells Express KPNA2 and KPNA4

Our Western immunoblot analysis of SH-SY5Y whole cell lysate revealed significant expression of two karyopherin-α isoforms, KPNA2 and KPNA4, while KPNA1 and KPNA6 expression was minimal (data not shown).

2.3.2 Karyopherin-α2 Interacts with TDP-43 in SH-SY5Y Cells

The interaction between TDP-43 and endogenous KPNA isoforms in SH-SY5Y cells was determined by utilizing one of either KPNA1, KPNA2, KPNA4 or KPNA6 isoforms as bait proteins in a co-immunoprecipitation assay against endogenous TDP-43. After performing an immunoblot against TDP-43 we found that, out of the four isoforms examined, only KPNA2 was able to pull TDP-43 out of the cell lysate in an appreciable amount (Fig. 2A). The isoforms KPNA1, KPNA4 and KPNA6 did not exhibit binding with TDP-43 suggesting that, under normal conditions, the principal karyopherin-α isoform responsible for transporting TDP-43 within SH-SY5Y cells is KPNA2. The immunoprecipitation samples were then probed for the presence of the
individual KPNA isoforms. The isoform KPNA2 was present in high quantities in the cell lysate both before and after immunoprecipitation (IP), while KPNA1, KPNA4 and KPNA6 were barely detected (Fig. 2B).

2.3.3 Recombinant Karyopherin-α2 Binds with Six Distinct Regions of TDP-43 on High-Density Peptide Array

High-density peptide array technology enables the identification of putative interaction domains between two proteins. Our membrane consisted of a series of spots, each comprised of a 12-14mer peptide derived from TDP-43. After incubation with KPNA2 protein, peptides that exhibited binding developed as dark spots, while non-binding peptides remained undeveloped. The developed spots on the KPNA2-treated membrane were compared with those of the KPNA2-absent control membrane in order to rule out any non-specific antibody interactions. As each sequential peptide spot has a frame shift of two amino acids, putative binding regions were determined by the presence of multiple, consecutive darkened spots. We found that KPNA2 strongly interacts with six distinct regions of the TDP-43 peptide array (Fig. 3), and identified a unique sequence of 8-10 amino acids within each region. All six sequences, as well as a scrambled derivative of the first sequence, were analyzed via basic local alignment search tool (BLAST) and were found in no other proteins within the database. Putative interference peptides were developed from these sequences, each with a conjugated TAT-domain to allow for cellular penetration.
2.3.4 The Interaction Between Endogenous Karyopherin-α2 and TDP-43 Can Be Disrupted by Interference Peptides In Vitro

To determine the efficacy of our interference peptides, we performed a co-immunoprecipitation on peptide treated SH-SY5Y cells, using KPNA2 as bait and immunoblotting for TDP-43. Briefly, cells were treated with all six blocking peptides (pALL), an equal solution of peptides 2, 5 and 6 (p256), or the scrambled control peptide (pS) for four hours, at a final concentration of either 15μM or 40μM in FBS-supplemented DMEM. A CoIP was then performed on cell lysates as previously described (2.3.2). The TDP-43 pull-down bands were quantified and normalized to TDP-43 levels detected within lysate controls. The normalized TDP-43 levels for each sample were then compared to the IgG control, the TDP-43 levels of which were assigned a baseline value of one. At 15μM, only peptide cocktail pALL was able to significantly inhibit the interaction between KPNA2 and TDP-43 relative to the untreated control (PD) and the scrambled peptide control (pS) (p<0.05, student’s t-test). The 15μM scrambled peptide control treatment did not significantly inhibit the protein-protein interaction when compared with PD (Fig. 4). The 40μM peptide cocktails were observed to have no significant effect on the ability of KPNA2 to pull TDP-43 out of solution.
2.3.5 Inhibition of the Interaction Between KPNA2 and TDP-43 is Not Sufficient to Induce Cytoplasmic Mislocalization of TDP-43

To determine whether the inhibition of the KPNA2-TDP-43 nuclear import complex is sufficient to induce TDP-43 mislocalization, we treated SH-SY5Y cells with our peptide cocktails for 24 hours and examined the subcellular distribution of TDP-43 via nuclear fractionation (Fig. 5) and immunocytochemistry (ICC) (Fig. 6). Here, peptide cocktails pS, p256, and pALL and were tested at concentrations of either 15uM or 40uM on healthy SH-SY5Y cells. For immunocytochemical analysis, peptide cocktails p26 and p6 were also utilized. For each interference peptide cocktail examined, we found that the subcellular distribution of TDP-43 remained unchanged when compared to controls treated with scrambled peptide (fractionation data for 40uM peptide treatment not shown).

2.4 Discussion

Under normal cellular conditions, TDP-43 is transported to the nucleus via the classical nuclear import system. During classical import, cytosolic TDP-43 binds directly to one of seven different karyopherin-α isoforms that mediate its passage through the nuclear pore complex. Although each recombinant karyopherin-α isoform has been shown to bind to TDP-43 in vitro, the endogenous isoform(s) responsible for the nuclear import of TDP-43 in a given cell likely depends upon the expression level of each KPNA isoform, as well as their individual binding affinities for TDP-43 (Nishimura, Zupunski et al. 2010). Here, we determined the expression patterns of KPNA isoforms in SH-SY5Y cells by performing immunoblotting on
whole-cell lysates utilizing antibodies directed against four KPNA isoforms: KPNA1, KPNA2, KPNA4 and KPNA6. Our results showed that, out of the four isoforms examined, only KPNA2 and KPNA4 were expressed in detectable amounts. While our data confirmed the presence of these two isoforms, it does not rule out the presence of other KPNA proteins. However, rather than complete inhibition of TDP-43 nuclear translocation, our goal is to examine the functional consequences of deficient KPNA-mediated TDP-43 nuclear targeting. Hence we set out to identify the major KPNA isoforms responsible for TDP-43 binding and nuclear targeting.

Using the four aforementioned KPNA isoforms as bait, we sought to determine which, if any, strongly interacted with TDP-43 in our cell line. While it is likely that multiple KPNA isoforms are involved in the nuclear transport of proteins, only KPNA2 was found to bind TDP-43 with such an affinity as to be detectable via co-immunoprecipitation (CoIP).

We went on to examine the specific interaction domains involved in the binding of KPNA2 and TDP-43 using our TDP-43-derived high-density peptide arrays. High-density peptide array technology has been shown to be an effective method of investigating discrete domains involved in protein-protein interactions (Lizcano, Deak et al. 2002). We found that recombinant human KPNA2 interacted with TDP-43 at six specific regions, with the strongest binding occurring near the glycine-rich region at the C-terminal end of TDP-43. Interestingly, one of the regions of TDP-43 that exhibited the strongest interaction with KPNA2 was a region that contains five documented point mutations that have been observed to be associated
with familial ALS. If this region is crucial for the binding of KPNA2 to TDP-43, it follows that genotypes exhibiting point mutations in this region may display deficiencies in the nuclear import of TDP-43, resulting in the subsequent development of TDP-43 cytoplasmic accumulation and proteinopathy.

Although it has been shown that a functional NLS is necessary for the nuclear import of TDP-43 (Nishimura, Zupunski et al. 2010), we did not observe any binding of KPNA2 to the NLS region of TDP-43. We suspect this may be due to the fact that we excluded KPNB1 from our protein-protein interaction assay and, in the absence of KPNB1, the IBB-domain of KPNA2 autoinhibits its NLS-binding domain. While it is likely that KPNA2 would exhibit strong binding at the NLS of TDP-43 in the presence of KPNB1, the NLS would not provide a suitable target for blocking the specific nuclear import of TDP-43 due to the ubiquity of this signal within nuclear-destined proteins. The strength of binding observed between KPNA2 and non-NLS regions of TDP-43 indicate that other binding regions are important for this interaction, and these regions may prove to be more suitable targets for the inhibition of this specific protein interaction.

We analyzed the putative KPNA2-interaction regions of TDP-43 and, from these sequences, designed six short, 8-10mer TAT-conjugated blocking peptides: p1-p6. A TAT-conjugated, scrambled-sequence peptide, containing the same amino acid composition as p1, was designed to serve as a control. To determine the efficacy of our peptides, we examined their ability to interfere with the binding of KPNA2 to TDP-43 in vitro using our established CoIP assay. Our data indicates that, at a final
peptide concentration of 15uM, the combination of all six peptides (pALL) was able to significantly inhibit the binding of TDP-43 to KPNA2 within four hours of application when compared to both the mock treatment (PD) and the scrambled control peptide (Fig. 4). The peptide cocktail p256 appeared to disrupt the interaction at a 15uM concentration, but it did not do so to a significant amount. At the same concentration, the scrambled control did not appear to significantly inhibit this protein-protein interaction relative to the mock treatment. At a final concentration of 40uM, our peptide cocktails did not appear to block the interaction between TDP-43 and KPNA2. It is unclear as to why the peptides did not exhibit interference at higher concentrations. One reason for this finding could be that, at 40uM, the peptide cocktails may have become toxic to the cells. If the cells that were heavily affected by the 40uM cocktails merely succumbed to apoptosis, the co-immunoprecipitation performed would be sampling from the surviving cellular population. This incomplete sampling may have masked any minor peptide-mediated interference.

To determine the physiological consequences of blocking the interaction between KPNA2 and TDP-43, we treated SH-SY5Y cells with our peptide cocktails for 24 hours. This timeframe was chosen to allow for sufficient cytoplasmic TDP-43 to accumulate before substantial compensatory changes in isoform expression could take effect. Our data from both subcellular fractionation and immunocytochemical staining showed that, in healthy cells, the inhibition of this protein-protein interaction was not sufficient to induce the cytoplasmic sequestration of TDP-43.
There are a number of potential reasons as to why our peptides were unable to induce TDP-43 mislocalization. First, peptides are rapidly degraded by cellular proteases and degradation systems once they penetrate the cell. It may be that although our peptides induced TDP-43 mislocalization initially, they were no longer effective at the time of assessment (24 hours) due to degradation. However, this scenario is unlikely, as both sequestration and clearance of cytoplasmic TDP-43 would have to occur within 24 hours, a relatively short time frame. It is much more feasible that the constitutive expression of other KPNA isoforms was sufficient to maintain normal subcellular TDP-43 distribution in a compensatory manner. Although our co-immunoprecipitation data showed that, of the KPNA proteins examined, KPNA2 exhibited the highest affinity for TDP-43 under healthy conditions, the affinities of any other isoforms present may be such that their effects become more relevant as the level of cytoplasmic TDP-43 increases.

Another possible reason we observed no mislocalization of TDP-43 in response to our peptide treatments is that we performed our treatments on healthy cells. Under normal physiological conditions, TDP-43 resides primarily within the nucleus. In response to insults, such as oxidative stress, TDP-43 translocates to the cytoplasm and associate with stress granules (Colombrita, Zennaro et al. 2009). It follows that pathological TDP-43 cytoplasmic mislocalization may be the result of deficiencies in the nuclear import system paired with mild cellular insult.
**Figure 1.** Schematic representation of classical nuclear import cycle. 1: Cytoplasmic karyopherin-β (KPNB) binds karyopherin-α (KPNA), allowing KPNA to bind a nuclear-targeted cargo protein (cNLS); this protein complex enters the nucleus via the nuclear pore complex (Nup). 2: Nuclear Ran-GTP binds KPNB, causing dissociation of the protein trimer and release of the cargo protein. 3: Cellular apoptosis susceptibility-RanGTP dimer (CAS-RanGTP) binds and transports KPNA to the cytoplasm while KPNB facilitates its own cytoplasmic translocation; both mechanisms utilize the Nup. 4: Cytoplasmic hydrolysis of RanGTP to RanGDP induces the release of KPNA from CAS-RanGDP, completing the cycle.
**Figure 2:** Karyopherin-α2 interacts with TDP-43 in SH-SY5Y cells. **A:** Western immunoblotting of TDP-43 after co-immunoprecipitation of SH-SY5Y cell lysate utilizing karyopherin-α isoforms 1, 2, 4 and 6 as bait proteins. TDP-43 is pulled-out of SH-SY5Y cell lysate by KPNA2, but not by KPNA1, KPNA4, or KPNA6. **B:** Western immunoblotting against KPNA isoforms. Cell lysates and immunoprecipitation samples from (A) were probed for the presence of individual KPNA isoforms. Strong bands located at 50kDa correspond to either rabbit (KPNA1, KPNA4, KPNA6) or goat (KPNA2) heavy-chain IgG.
**Figure 3:** TDP-43-derived high-density peptide array. 

A: Control membrane, probed with anti-KPNA2 antibody in the absence of KPNA2 protein. 

B: Interaction membrane, probed with anti-KPNA2 antibody after incubation with recombinant KPNA2 protein. Dark spots appearing on (B) that are absent from (A) indicate putative KPNA2-binding regions of TDP-43. 

C: Full-length human TDP-43 schematic showing known clinical mutations associated with ALS pathogenesis. Six peptides (p1-p6) were derived from KPNA2-binding regions of TDP-43 as indicated, with their respective amino acids in brackets. Five residues, whose mutations are thought to be associated with ALS, are encompassed by peptide 6 (p6) and are displayed on the lower right corner.
**Figure 4:** Peptides interfere with the interaction between KPNA2 and TDP-43. SH-SY5Y cells were treated with a specific peptide cocktail, at a concentration of either 15uM or 40uM, for four hours before harvesting for co-immunoprecipitation: scrambled peptide (pS), all six peptides (pALL), or peptides 2, 5 and 6 (p256). TDP-43 immunostaining was quantified and normalized to total TDP-43 content within the lysate of each sample. Each sample was then normalized to TDP-43 staining observed in the IgG control. [★ compared with PD, ♦ compared with pS-15; student’s t-test. Error bars represent mean ± SEM from n = 3]
**Figure 5:** Subcellular localization of TDP-43 following 24-hour *in vitro* peptide treatment and nuclear fractionation. SH-SY5Y cells were treated for 24 hours with a 15uM peptide cocktail comprised of either a scrambled control peptide (pS), all six peptides (pALL), or peptides 2, 5, and 6 (p256). **A:** Cytoplasmic (C) and nuclear (N) fractions were resolved via SDS-PAGE and Western immunoblotted for TDP-43, LMNB1 and HSP-90. **B:** The ratio of mean cytoplasmic to nuclear TDP-43 staining was plotted for each peptide treatment. Error bars represent the mean ± SEM from n = 3.
Figure 6: Immunocytochemical analysis of the subcellular distribution of TDP-43 following 24-hour *in vitro* peptide treatment. Four combinations of interference peptides, and one scrambled control peptide, were utilized on SH-SY5Y cells at a final concentration of either 15uM (A) or 40uM (B): scrambled control peptide (pS), all six peptides (pALL), peptides 2, 5, and 6 (p256), peptides 2 and 6 (p26) and peptide 6 (p6). The ratio of mean cytoplasmic to nuclear TDP-43 staining was found to be unaffected by the interference peptides when compared to the scrambled control peptide. Error bars represent the mean ± SEM from n = 3.
3: Conclusions and Future Directions

In this study, we set out to determine if blocking the specific interaction between TDP-43 and its nuclear import proteins is sufficient to induce TDP-43 cytoplasmic mislocalization, as seen in diseases such as FTLD-U and ALS. The following is a summary of what we found:

1) Through the use of co-immunoprecipitation, we found karyopherin-α2 to exhibit the highest binding affinity for TDP-43 of the KPNA isoforms examined in SH-SY5Y cells.

2) We identified six putative KPNA2-binding sites on TDP-43, utilizing a high-density peptide array, and developed six short interference peptides from these regions.

3) The specific interaction between KPNA2 and TDP-43 was impaired in vitro using low concentrations (15uM) of all six interference peptides as assessed by Co-IP. At higher concentrations (40uM) the peptides did not appear to interfere with the formation of the KPNA2-TDP-43 protein complex.

4) Blocking the interaction between TDP-43 and KPNA2 for 24 hours in vitro was not sufficient to induce TDP-43 cytoplasmic mislocalization in healthy SH-SY5Y cells.

Our investigation of the relationship between impaired TDP-43 nuclear import and TDP-43 proteinopathy was performed on healthy SH-SY5Y cells. A previous study by Columbrita et al. (2009) found that TDP-43 is retained primarily in the nucleus under normal cellular conditions; only in the face of cellular insult does it
migrate to the cytoplasm and accumulate within stress granules. TDP-43 proteinopathies, such as ALS and FTLD-U, typically do not present until later in life, despite the fact that any associated mutations would have been present from birth. It follows that the pathogenesis of TDP-43 proteinopathies may require an underlying genetic component coupled with the progressive accumulation of cellular insults over one's lifetime. For future studies, it may be more biologically relevant to study the effects of nuclear import impairment alongside a cellular stressor, such as oxidative stress. We plan to utilize our interference peptides on SH-SY5Y cells that have been subjected to sodium arsenite, which is an established oxidative stressor (Colombrita, Zennaro et al. 2009), and investigate the effects on the subcellular localization of TDP-43.

As it is likely that multiple KPNA isoforms are involved in the nuclear targeting of TDP-43, in future studies we will use high-density peptide arrays to investigate the specific regions of TDP-43 that interact with each individual KPNA isoform. By comparing these putative interaction regions, we aim to identify common sites on TDP-43 that bind to all KPNA isoforms. From these regions, we aim to develop interference peptides that specifically target the interaction between TDP-43 and its nuclear importers, and use these peptides to further investigate the role of nuclear import impairment in the pathogenesis of TDP-43 proteinopathy.
Bibliography


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