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Role of STIP1 in TDP-43 Mediated Toxicity and Proteinopathy in Neurodegenerative Disorders

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Abstract

A common hallmark of neurodegenerative disorders is the abnormal aggregation of misfolded proteins. Aggregates of phosphorylated TAR-DNA-binding protein-43 (TDP-43) are found in multiple neurodegenerative disorders. The molecular chaperones Hsp70 and Hsp90 have been shown to be key modulators of TDP-43 phosphorylation and expression, however the mechanisms behind how this interaction occur are unclear. Stress-Inducible PhosphoProtein 1 (STIP1) is a critical co-chaperone linking Hsp90 to Hsp70 to modulate chaperone client stability. In this study, we evaluated the potential role of STIP1 in TDP-43 stability and cellular toxicity. We demonstrated that STIP1 interacts with TDP-43 and the deletion of STIP1 leads to decreased TDP-43 protein expression in SN56 cells. In contrast, partial reduction of STIP1 in mice led to an increase in TDP-43 levels. Additionally, the deletion of STIP1 increased TDP-43 cytotoxicity in SN56 cells, whereas TDP-43 toxicity could be ameliorated by overexpressing STIP1 suggesting that STIP1 modulates TDP-43 toxicity and proteinopathy.

Keywords: Neurodegenerative disorders, ALS, TDP-43, STIP1, Hsp70, Hsp90, Molecular chaperones, Co-chaperones, Clients, Protein misfolding

Co-Authorship Statement

All experiments except for the generation of SN56 KO cells were conducted solely by the author.

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Animal Protocol Certificate

eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2016-103; 2016-104

2016-103; 2016-104

AUP Number: 2016-103; 2016-104
AUP Title: a)Elucidating the Function acetylcholine in the central and peripheral nervous system; b)Role of the Cellular Prion Protein in physiological and pathological conditions
Yearly Renewal Date: 03/01/2018

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2016-103; 2016-104 **has been approved, and will be approved for one year following the above review date.**

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

1 Introduction and Literature Review

1.1 Neurodegenerative Disorders

Approximately 747,000 Canadians are currently living with some type of a neurodegenerative disorder (Statistics Canada, 2016). These diseases have a tremendous economic burden and currently cost Canadians approximately $22.7 billion annually and this figure is expected to increase to $150 Billion by the year 2030 (NeuroScience Canada, 2006).

Neurodegenerative disorders are diseases of the central nervous system that involve the progressive deterioration or death of neurons. This is an umbrella term that encompasses a variety of different disorders. The associated symptoms associated with disease progression depend on the particular group of neurons that are being affected (Amador-Ortiz et al., 2007). Alzheimer's disease (AD) is the most common type of neurodegenerative disorder and involves the degeneration of neurons in various parts of the brain (Schmitz and Spreng, 2016). It has been reported that the basal forebrain cholinergic neurons are amongst the first regions to show AD pathology and degeneration (Schmitz and Spreng, 2016). Other brain regions that are also significantly affected include the temporal lobe, neocortex and limbic system (Coleman and Flood, 1989; Price, 1993; Braak and Braak 1995; Delacourt et al., 1999; Nelson et al., 2007). As a result, the clinical manifestations of AD include cognitive impairments, mood and emotional disturbances and loss of appetite (Simic et al., 2009).

One of the main causes of neuronal death in neurodegenerative disorders is protein misfolding. Misfolded proteins tend to aggregate, as large hydrophobic regions can become exposed and facilitate the formation of protein clumps. The formation of protein aggregates is deleterious because the proteins are unable to remain in their native conformational state and thus become incapable of performing their intrinsic functions (Chiti and Dobson, 2006; Walsh and Selkoe, 2007). Moreover, aggregated proteins can sequester other proteins in cellular deposits and there is evidence that certain misfolded proteins become toxic. Misfolded proteins are observed in a number of neurodegenerative disorders such as Parkinson's, Huntington's, Amyotrophic Lateral Sclerosis (ALS), Frontotemporal lobar degeneration (FTLD) and Alzheimer's Disease (Yanker et al., 1989; Koh et al., 1990; Pike et al., 1992). For example, Alpha-synuclein, a protein that has been shown to regulate endocytosis and exocytosis, is misfolded and found in abnormal protein aggregates in Parkinson's disease affected individuals (Spillantini et al., 1998; Kalia and Elang, 2015; Lautenschläger et al., 2017). Individuals with Huntington's disease have neuronal inclusions which consist of misfolded Huntingtin proteins that have numerous glutamine repeats in their protein (Hoffner & Djian, 2002; Green 1993). Aggregates in motor neurons of ALS patients can contain several misfolded proteins such as Superoxidase dismutase (SOD1), TARDP-43 (TDP-43) and Fused in Sarcoma (FUS). SOD1 is responsible for breaking down superoxide radicals, which are toxic to the cells, TDP-43 is primarily involved with RNA processing and FUS is a nuclear regulator of gene expression that binds to DNA/RNA (Li et al., 1998; Neumann et al., 2006; Sharma et al., 2016). Genetically, a pathogenic hexanucleotide repeat expansion in the *C9orf72* gene is responsible for most inherited ALS and FTLD cases (Murphy et al., 2017). This repeat expansion accounts for

1 out of every 10 ALS patients and is the most commonly identified mutation in both of these disorders to date (Murphy et al., 2017).

1.2 Protein Misfolding

In order to prevent the formation of aggregates, proteins must remain properly folded. Specifically, proteins must be folded into precise three-dimensional conformations (Figure 1). This process is aided by molecular chaperones and the cellular quality-control machinery, such as the heat shock protein system, which are discussed in more detail in the following sections (Hartle & Hayer-Hartl, 2009). If these systems are dysfunctional or if client proteins manage to avoid chaperones, protein aggregates may be formed which are most likely to be toxic to cells in which they occur.

Protein misfolding disorders are distinguished by the presence of accumulated abnormal proteins which are associated with cellular dysfunction (Hartle & Hayer-Hartl, 2009). Eukaryotic cells have developed strategies to deal with the continuous bombardment of misfolded proteins that compromise cellular protein homeostasis and endanger cellular viability. These strategies involve a network of molecular chaperones and protein degradation factors that ensure the regulation of proteostasis. This system facilitates misfolded proteins to be either refolded, degraded or delivered to distinct quality control compartments that gather against potentially harmful misfolded species (Chen et al., 2011).

Mammalian cells eradicate misfolded proteins mainly through one of three proteolytic systems. These systems include the ubiquitin-proteasome pathway (UPP),

chaperone mediated autophagy (CMA) and macroautophagy. Most intracellular proteins are degraded by the UPP which is the first line of defense for degrading misfolded proteins (Hedge & Upadhya, 2007; Rogel et al., 2010). The UPP is a selective degradation system in which only substrates tagged with ubiquitin, a 8.5 kDa regulatory protein, are processed and degraded. Misfolded proteins are recognized by molecular chaperones, such as CHIP, and may interact with distinct ubiquitin ligases that promote the transfer of ubiquitin to substrates (Ciechanover and Kwon, 2015). The attachment of ubiquitin to this substrate requires a series of enzymatic reactions. Once near the proteasome, these substrates are untagged by deubiquitinating enzymes (DUBs) and reused for future degradation reactions. They are them unfolded into preliminary polypeptide chains which are cleaved into short peptides as they pass through the narrow chamber of the proteasome (Rogel et al., 2010).

Many cytosolic proteins that survive the UPP can be alternatively degraded by the CMA (Klionsky et al., 2010). The heat shock-cognate protein of 70 kDa (hsc70) recognizes a pentapeptide sequence, of a specific charge, in all substrates destined for the CMA. This substrate is then targeted for the lysosome where it is unfolded by lysosomal membrane proteins so that it can cross the lysosomal membrane (Taylor et al., 2003). Once in the lumen, the substrate undergoes complete degradation. The CMA plays a significant role in prolonged starvation amino acids can be recycled for future protein synthesis reactions, although basal CMA activity can be detected in almost all cells (Cuervo and Wong, 2014).

Some misfolded proteins that bypass both the UPP and the CMA or form aggregates undergo macroautophagy (Feng et al., 2014). These substrates are segregated by double membrane structures called autophagosomes. These autophagosomes then fuse with the lysosome where they are degraded by hydrolases (Ciechanover and Kwon, 2015). Almost

all substrates are successfully degraded by these proteolytic systems. The dysregulation of these systems or post-translational modification, such as hyperphosphorylation, may cause misfolded proteins to escape these system and form cytotoxic inclusion bodies that underlie the pathogenesis of many neurodegenerative disorders (Sikosek and Chan, 2015).

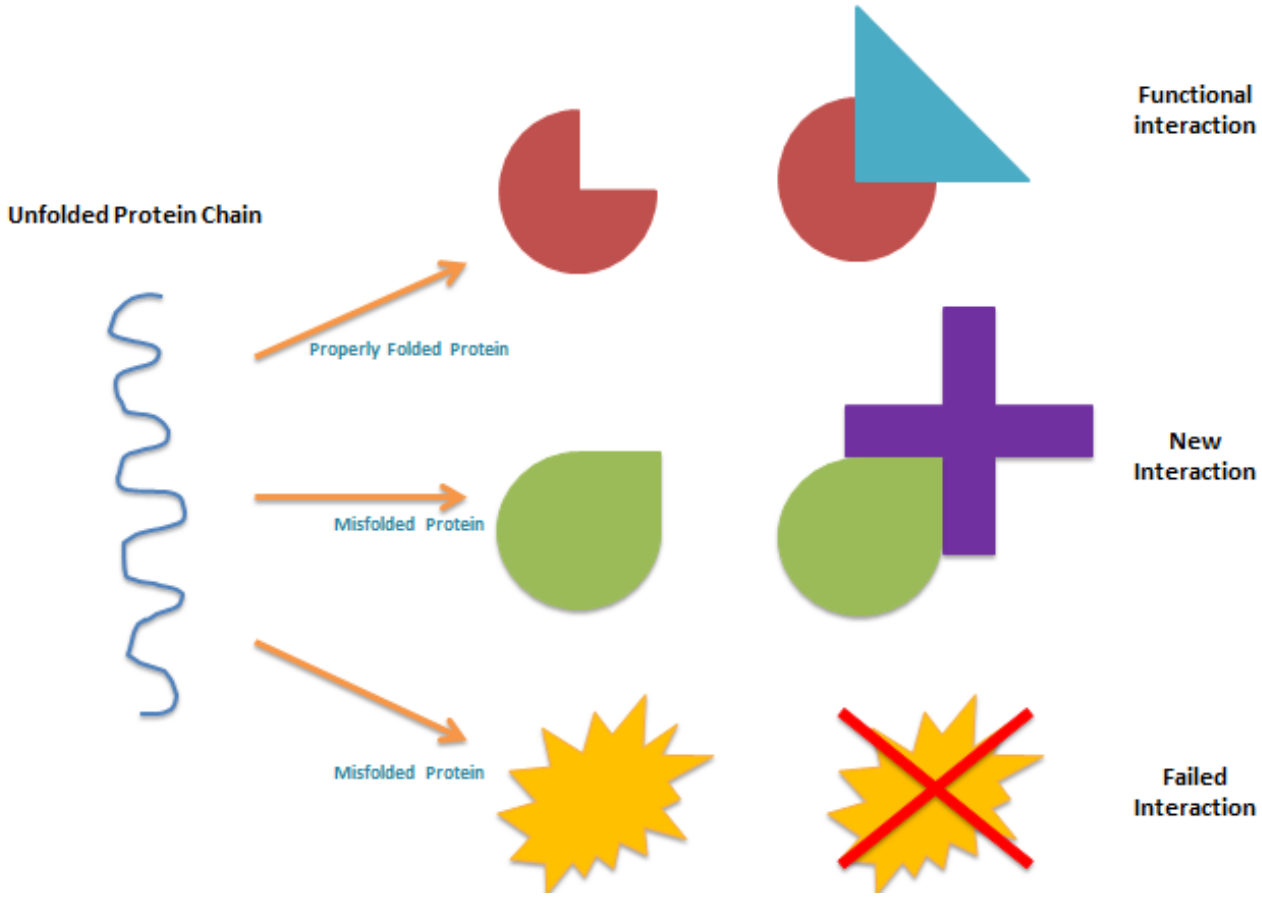


Figure 1: Schematic drawing indicating possible effects of mutations on protein folding and interactions. Mutations can result in a number of interaction alterations. Mutations that have no effect on the structure of functionally folded protein will allow it to interact as intended with its interaction partners. Mutations that result in structural or functional changes may cause the protein to interact with new partners or render it unable to interact with any partner at all (Adapted from Sikosek and Chan, 2015).

1.3 Molecular Chaperones

The term molecular chaperone was first used to describe nucleoplasmin, a nuclear protein that is essential for the reformation of disrupted chromatin and histone interactions in amphibian eggs (Laskey et al., 1978). Chaperones were initially described as only assisting with the assembly of folded protein subunits into oligomeric structures (Ellis, 1978). It was later found that they support the folding of newly synthesized and stress-denatured polypeptide chains (Ellis, 2005 & Hartl, 2004). Proper protein folding is critical for cellular growth, function and survival (Figure 2). Heat shock proteins (Hsps) represent a large proportion of molecular chaperones. Hsps levels are increased in response to high stress conditions such as extreme temperature or oxidative stress (Herczenik & Gebbink, 2008). This response was first observed in *Drosophila* that showed a change in salivary gland transcription rate in response to different incubation temperatures (Ritossa, 1962). Heat shock factor 1 (HSF1) is the primary regulator of heat shock genes. HSF1 is normally located in the cytosol in its inactivated form. Under stress conditions, such as heat and hypoxia, HSF1 becomes activated through trimerization and phosphorylation and migrates to the nucleus (Sun et al., 2000), where it can bind heat-shock element (HSE), found in stress response genes, and upregulate the transcription of heat shock genes leading to an increase in heat shock protein expression (Morimoto, 1998). This heat shock response is critical for the control of cell cycle, protein translation and glucose metabolism (Dai et al., 2007). It is now understood that heat shock proteins are essential for protein quality control and in maintaining homeostatic proteostasis. Hsps are also involved in regulating proteins that have unfolded due to various cellular stressors (Dai et al., 2007).

Heat shock proteins are classified into several families according to their molecular weights: Hsp100s, Hsp90s, Hsp70s, Hsp60s, Hsp40s and small heat shock proteins (sHSPs). These proteins can have either inducible expression or constitutive expression.

Hsp100 functions as a protein disaggregase in bacteria, fungi and plants. It helps solubilize and reactivate proteins that have been misfolded due to severe cellular stress (Glover and Lindquist, 1998; Goloubinoff et al., 1999; Motohashi et al., 1999; Zolkiewski, 1999). Therefore, it has a unique role of providing these organisms with resilience in conditions of tremendous physiological and environmental stress (Sanchez and Lindquist, 1990; Squires et al., 1991; Hong and Vierling, 2000; Queitsch et al., 2000).

Hsp60 is a mitochondrial chaperonin that is responsible for refolding all proteins transported into the mitochondria and it also supports the restoration of any unfolded or misfolded proteins (Hartl, 1996; Ranford and Henderson, 2002; Deocaris et al., 2006).
Hsp40 is an evolutionarily conserved chaperone that is constitutively expressed in most cells. It performs numerous functions such as transcriptional and translational regulation as well as facilitation of protein folding (Fan et al., 2003; Qiu et al., 2006; Daugaard, 2007; Vos et al., 2008). The structure and function of Hsp40 has been extensively researched and reviewed in the literature (Kampinga and Craig, 2010). In brief, most Hsp40 proteins contain a J-domain through which they interact with Hsp70 and act as a co-chaperone to assist in the early stages of protein folding (Figure 2). The proteins that molecular chaperones, such as Hsp70, act upon, fold and process are referred to as client proteins. Hsp40 provides client specificity to Hsp70 by either delivering the client to Hsp70 or by activating Hsp70 when the client is nearby.

Small Hsps were first observed in *Drosophila melanogaster* after a heat shock response (Tissières et al., 1974). sHsps are found in almost all organisms and their expression pattern depends on the tissue and the development stage of the cell (Maaroufi and Tanguay, 2013). Different from other heat shock proteins, sHsps are ATP-independent chaperones (Benesch et al., 2008). sHsps interact directly with their substrates and prevent protein misfolding and aggregation by promoting refolding through the Hsp70 chaperone complex (Bruey et al., 2000). Hsp70 and Hsp90 will be discussed in more detail as they are the focus of this project.

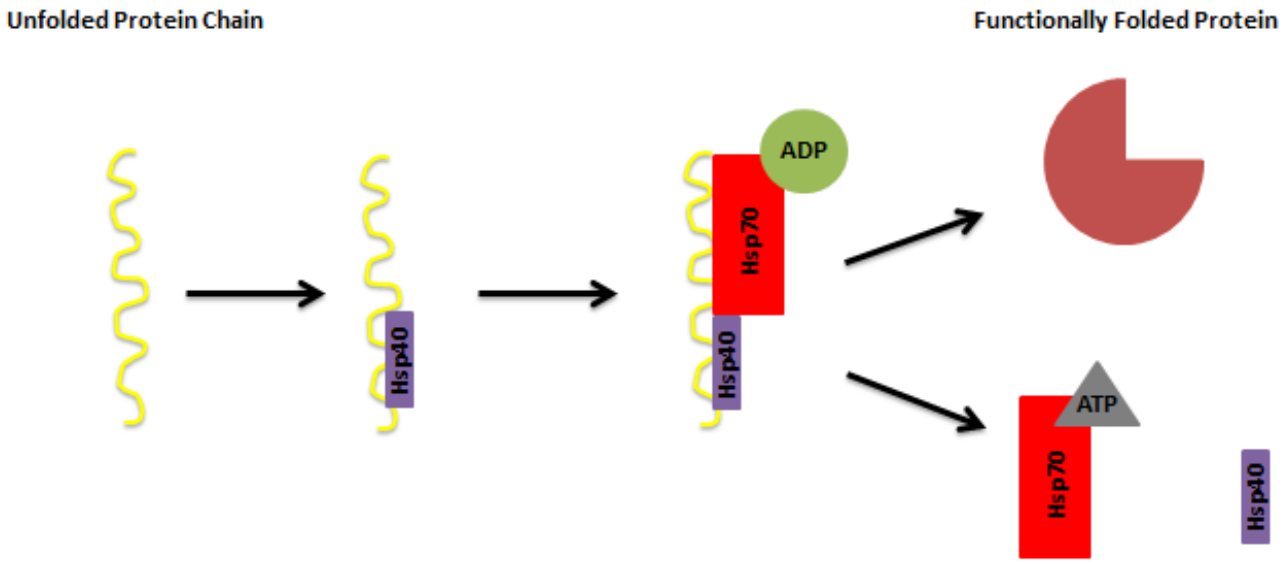


Figure 2: Functioning of molecular chaperones. Molecular chaperones help in folding proteins, refolding misfolded proteins and degrading misfolded proteins which are called clients. Co-chaperones assist chaperones in performing these functions. For example, Hsp40 provides client specificity for Hsp70 by binding to unfolded protein strands and recruiting them to Hsp70 for proper folding. Hsp70-interacting protein (HIP), which is a Hsp70 attenuator, also interacts with this complex to delay substrate release. Client proteins can either be folded properly so they can perform their native functions or be ubiquitinated and destined for degradation (Adapted from Kosmaoglou et al., 2008).

1.4 Hsp70

The abundantly expressed 70-kDa heat shock proteins (Hsp70s) are highly evolutionarily conserved. These ATP-dependent molecular chaperones are critical to cellular protein surveillance networks and are involved with numerous protein folding processes (Meimaridou et al., 2009; Mayer and Bukau, 2005). Hsp70s are known to interact with proteins in their misfolded, unfolded and aggregated states. Interestingly, Hsp70s do not interact with proteins in their correctly folded states (Meimaridou et al., 2009; Mayer and Bukau, 2005). A few Hsp70 proteins have organelle specific localization and tissue-specific expression but their functions are mostly overlapping. The majority of Hsp70 proteins are localized in the cytoplasm, but they can be found in the nucleus as well (Daugaard et al., 2007; Rérole et al., 2011; Brodsky et al., 2006). Hsp70 proteins enhance survival in highly stressful states by protecting cells against the toxicity from denatured and misfolded proteins that may have accumulated. Instead of having true "client" proteins like other Hsps, Hsp70 proteins bind to exposed hydrophobic residues on misfolded and unfolded proteins, forcing them to maintain these states to prevent their aggregation. Hsp70 can directly unfold misfolded proteins via its ATPase activity (Daugaard et al., 2007; Rérole et al., 2011; Brodsky et al., 2006).

Hsp70 proteins have two main binding domains called the nucleotide binding domain (NBD) and the substrate binding domain (SBD). ATP binding and hydrolysis at the NBD domain allows Hsp70 to switch between its low substrate affinity state and high substrate affinity state. When ATP is bound to the NBD of Hsp70, the protein is in a low substrate affinity state where the association and dissociation of the polypeptide with the

SBD occur at fast rates. Upon activation of Hsp70 activity, NBD bound ATP is hydrolyzed and Hsp70 assumes a high substrate affinity state with slow polypeptide association and dissociation rates (Schmid et al., 1994; Pierpaoli et al., 1998; Gässler et al., 2001; Takeda and McKay, 1996). Hsp70 usually interacts with its clients at the early stages of protein folding (Rudiger et al., 1997). It interacts with short hydrophobic sequences that are rich with leucine residues because they are highly exposed during the early stages of protein translation and folding (Hartl and Hayer-Hartl, 2009). This early association of Hsp70 with the substrate prevents aggregate formation. If the substrate cannot be folded properly, co-chaperones aid Hsp70 to promote the degradation of the misfolded protein (Meacham et al., 2001; Petrucelli et al., 2004; Jana et al., 2005; Dickey et al., 2007; Muller et al., 2008).

Many members of the Hsp70 family of proteins have overlapping function. In fact, yeast have 6 Hsp70 homologs. Due to the redundancy in their function, the deletion of up to 3 of these homologs allows yeast to survive (Werner-Washburne et al., 1987). Despite being able to survive, the deletion of a few homologs makes the yeast sensitive to stress conditions in which their growth is impaired. The deletion of 4 of the 6 homologs is lethal in yeast suggesting that partial Hsp70 functioning is critical for survival in this organism (Werner-Washburne et al., 1987). Humans have 8 unique Hsp70 gene products (Tavaria et al., 1996). Mice who are deficient in Hsp70-1a and Hsp70-1b are viable and fertile but show high sensitivity to sepsis, cardiac ischemia, genomic instability and radiation (Hampton et al., 2003; Hunt et al., 2004; Singleton and Wischmeyer, 2006). Hsc70 is a member of the Hsp70 family that is constitutively expressed and is critical for mammalian cell development and viability (Florin et al., 2006; Daugaard and Jäättelä, 2005). Hsc70's primary functions involve the promotion of nascent polypeptide chain folding and the

translocation of proteins across membranes (Florin et al., 2004). It influences protein folding by delaying or promoting this process. Hsp70 isoforms also have inducible expression in the brain and liver (Scott et al., 2003). Forced anoxia conditions in the western painted turtle led to a threefold increase in Hsp73 levels in the brain and a sevenfold increase in in the liver.

Therefore, even though proteins from the Hsp70 family are highly homologous with overlapping functions, they can have varying modes of action such as being either constitutive and inducible. Regardless of their differences, these members are critical for proteome stability and for growth and development.

1.5 Hsp90

Heat shock protein 90 (Hsp90) is also an ATP-dependent molecular chaperone that is ubiquitously expressed and has been conserved evolutionarily (Figure 3). It is involved in the later stages of protein folding and only interacts with certain clients (Nathan et al., 1997). Interestingly, Hsp90 interacts with substrates that are partly folded, misfolded and even properly folded (Karagöz and Rüdiger, 2015). Bacteria contain a Hsp90 homologue called HtpG (Buchner, 2010; Shiau et al., 2006; Bardwell and Craig, 1987). Surprisingly, this homologue is not essential for growth as bacteria can survive without it. However, its full functions remain to be investigated (Steeves et al., 2011; Genest et al., 2011). In mammals, Hsp90 has numerous isoforms with different localizations. Two of the major cytosolic isoforms are Hsp90α and Hsp90β (Picard, 2002). Hsp90β is a constitutively expressed isoform while Hsp90α expression is inducible as it is produced in response to a stressor (Sreedhar et al., 2004; Grad et al., 2010). Hsp90β is critical to mammalian

development and its deletion in mice is embryonically lethal (Voss et al., 2000). This is believed to occur because the trophoblast is unable to develop properly (Voss et al., 2000). In contrast, the deletion of Hsp90α does not affect the development in mice. The only major deficit reported in Hsp90α knockout mice was the inability to produce sperm (Grad et al., 2010).

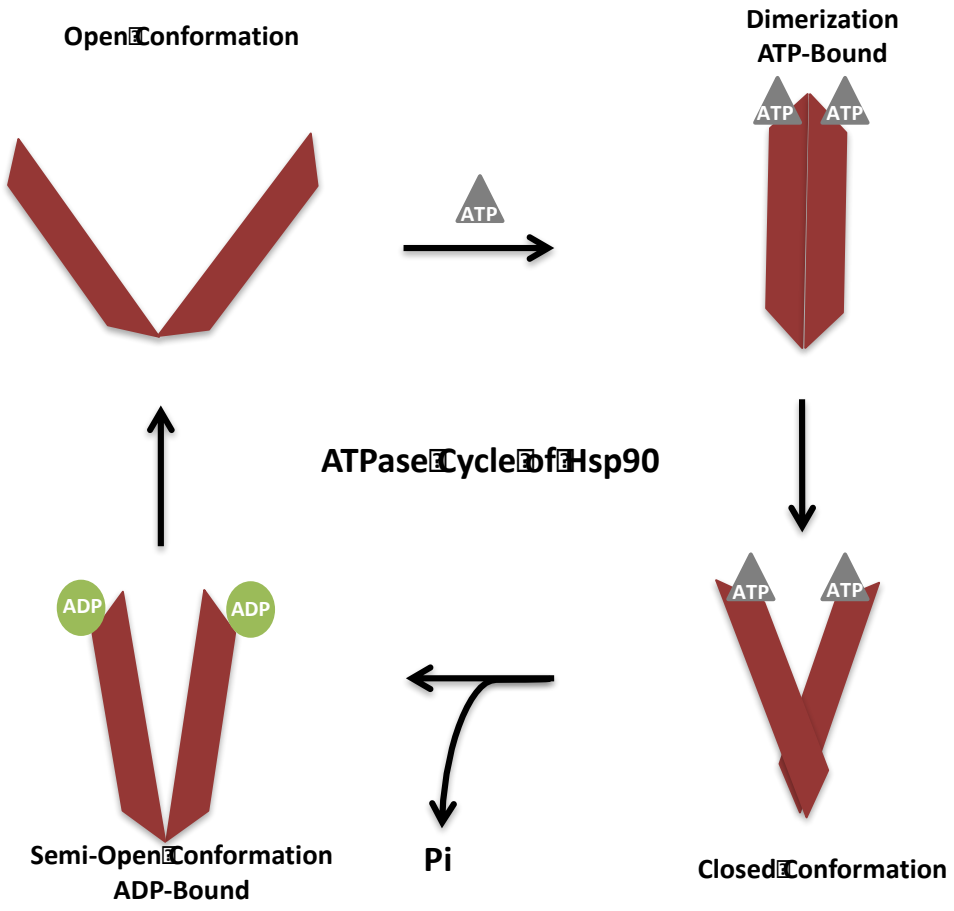


Figure 3: ATPase cycle of Hsp90. Hsp90's conformational transformations are associated with ATP binding and hydrolysis. Initially, when there is no ATP bound, Hsp90 maintains an open conformation. ATP binds the N terminals and induces an Hsp90 conformational change to the closed state. ATP hydrolysis into ADP changes this to a semi-closed conformation. Lastly, ADP release generates an open Hsp90 conformation (Adapted from Lackie et al., 2017).

Hsp90 is a homodimer protein with three main domains which include the N-terminal domain, the middle domain and the C-terminal domain (Ali et al., 2006). This structure is almost completely conserved from bacteria to mammals (Dutta and Inouye, 2000). The middle domain is responsible for ATP-hydrolysis that provides the energy for Hsp90 cycle. This domain is also where many clients and co-chaperones bind (Meyer et al., 2004). The N-terminal domain is responsible for nucleotide binding while the C-terminal domain is needed for proper dimerization of the Hsp90 molecular chaperone (Pearl and Prodromou, 2006; Taipale et al., 2010). Identifying Hsp90's client binding domains is challenging because of the difficulty associated with analyzing its unique homodimer structure using current structural biology methods. Previous studies have proposed that there is a client binding site in each of its structural domains but the correct structure representation remains elusive (Harris et al., 2004; Meyer et al., 2003; Prodromou et al., 1997).

Hsp90 has been shown to control the stability of some client proteins (Karagöz and Rüdiger, 2015). Hsp90 has an extended substrate binding interface with several hydrophobic regions that demonstrates specificity for substrates with exposed hydrophobic regions. This binding specificity ensures that substrates bind to Hsp70 early on in the folding pathway and that downstream folding intermediates bind Hsp90. This control of substrate influx from Hsp70 is how Hsp90 controls the stability of select clients. For example, Hsp90 binds and regulates tau stability with the help of several co-chaperones Shelton et al., 2017).

As a pivotal protein for growth and development, Hsp90 partners up with numerous co-chaperones and regulates their activity and influence their conformational

rearrangement and vice versa. These co-chaperones are functionally important because they take advantage of Hsp90 conformational plasticity by binding to it and allowing it to adapt to different client proteins (Street et al., 2011; Retzlaff et al., 2010). Several co-chaperones and clients have been shown to regulate Hsp90 by regulating its conformational changes and ATPase activity. One well-characterized Hsp90 co-chaperone is the Carboxy-terminus of the Heat shock cognate 70-Interacting Protein (CHIP). CHIP is a ubiquitin ligase that tags Hsp90 client proteins for degradation through the proteasome (Dickey et al, 2007; Dickey and Ash et al., 2006). An example of a target protein that CHIP interacts with is tau. Tau is a microtubule stabilizing protein that has been shown to form aggregates in the brain of Alzheimer's and Parkinson's patients (Dickey and Yue et al., 2006). Mice that have CHIP knocked out have greater tau accumulation (Dickey and Yue et al., 2006). It is speculated that increasing CHIP expression could reduce tau levels and potentially alleviate symptoms of disorders associated with Tau proteinopathy (Dickey and Yue et al., 2006).

FK506 binding protein 51 kDa (FKBP51) is another Hsp90 co-chaperone that is associated with clients such as glucocorticoid receptors (GR) and Tau. FKBP51 is a cis-trans isomerase that interacts with Hsp90 to ensure proper protein folding in response to proteotoxic stress (Smith et al., 2000; Rein, 2016). Hsp90 interacts with FKBP51 to regulate hormone signalling, chaperone stress response and GR signalling through several feedback loops (Riggs et al., 2003; Takeuchi et al., 2015). FKBP51 expression is increased in the aging brain as well as in Alzheimer's patients and its levels have been shown to correlate with the amount of Tau oligomers present in cells (Peattie et al., 1992). FKBP51 binds Hsp90 and increases neurotoxic tau oligomer production by making tau more

susceptible to phosphorylation. Protein phosphatase 5 (PP5) performs the opposite function by dephosphorylating pathological tau and restoring its homeostatic form. Like FKBP51, Cyclophillin A (CypA) and Pin1 are also Hsp90 co-chaperones which are propyl isomerases that assist chaperones in refolding misfolded proteins (Fischer et al., 1984; 1989; Lang et al., 1987; Schmid, 1993; Schmid et al., 1993; Wang and Heitman, 2005; Yeh et al., 2006).

In order to appreciate the diverse functional outreach of Hsp90, it is worth mentioning some of it's significant clients. G protein-coupled receptor kinases (GRK2), which are amongst the most common Hsp90 clients, are protein kinases that modulate G-protein coupled receptors by phosphorylation. Hsp90 plays a vital role in the stabilization and maturation of various GRK family members (Luo and Benovic, 2003). Glucocorticoid receptors are another well-established Hsp90 client that are critical for several biological functions (Kirschke et al., 2014). Their primary function, when glucocorticoids are bound, is the regulation of gene transcription.

Lastly, in addition to its intracellular function, Hsp90 has been shown to have extracellular roles as well (Eustace and Jay, 2004). The best studied extracellular role for Hsp90 is in innate and adaptive immunity where it is involved in antigen processing (Basu and Srivastava, 2000). It increases the invasiveness of tumours by interacting with metalloproteinase-2. Hsp90 has also been shown to be secreted from vascular smooth muscle cells in response to oxidative stress (Liao et al., 2000). Since extracellular Hsp90 can be detected on the surface of tumour cells and has been shown to regulate their invasiveness, cancer therapies have the potential to target extracellular Hsp90 and exploit its function and reduce tumour invasiveness and toxicity (Wong and Jay, 2016).

1.6 Stress-inducible Phosphoprotein 1

Stress-inducible phosphoprotein 1 (STIP1/STIP1) also known as HOP (Heat-shock organizing Protein) is an important co-chaperone needed for the transfer of clients between Hsp70 and Hsp90 (Johnson, Schumacher et al., 1998, Lee, Graf et al., 2012, Rohl, Wengler et al., 2015b, Schmid, Lagleder et al., 2012b). STIP1 was first identified in yeast as a protein that is upregulated in response to cellular stress (Nicolet and Craig, 1989). It has three major tetratricorticopeptide repeat domains called TPR1, TRP2A and TPR2B and two domains that are rich in aspartate and proline named DP1 and DP2 as seen in Figure 6 (Schmid et al., 2012b;). TPR1 and TPR2B interact with Hsp70 and TPR2A interacts with Hsp90 (Figure 4) (Scheufler et al., 2000; Rohl et al., 2015b). The protein ispredominantly located in the cytoplasm, however, as STIP1 has a nulear localization signal (NLS), it can be found also in the nucleus. Nuclear shuttling is initiated by the phosphorylation of different STIP1 sites by casein kinase II (CKII) and cell division cycle kinase (CDCII) (Longshaw et al., 2004). Interestingly, STIP1 localizes to the nucleus in astrocytes when exposed to gamma-radiation suggesting that it plays a role in astrocyte's stress response (Soares et al., 2013).

In addition to its intracellular function as a molecular co-chaperone, STIP1 also has extracellular cytokine-like activity (Lime et al., 2007; Arantes et al., 2009; Hajj et al., 2013). Majority of the literature on the function of extracellular STIP1 focus on its interaction with the cellular prion protein (PrP^C), a cell surface protein that scaffolds signalling complexes (Linden et al., 2008). Extracellular STIP1 can be secreted by exosomes from astrocytes in order to interact with PrP^C and exert a variety cellular effects

such as influencing cellular growth and survival (Hajj et al., 2013; Beraldo et al., 2013). This interaction has protective effects as it reduced anisomycin (a protein sysnthesis inibitor) induced-apoptosis (Chiarini et al., 2002). Similarly, extracellular STIP1 also protected cultured neurons against protein synthesis inhibitors (Lopes et al., 2005). Recombinant STIP1 added to hippocampal cell cultures elicits neuritogenesis due to its interaction with PrP^C) at the neuronal surface in PrP^C expressing cells but not in PrP^C null cells (Lopes et al., 2005). This interaction activates protein kinase A and ERK1/2 which resuts in neuroprotection as well as increased neuritogenesis (Caetano et al., 2008). Extracellular STIP1 also interacts with PrP^C and alpha7 nicotinic acetylcholine receptor complex to transduce extracellular signals into the intracellular environement (Beraldo et al., 2010). Lastly, the interaction between STIP1 and PrP^C also provides neuronal protection against toxic stimuli such as Aβ oligomers (Ostapchenko et al., 2013).

The deletion of the STIP1 homolog (CeHOP) in *Caenorhabditis elegans* is not lethal but the worms are more susceptible to stress and present deficits in sexual development (Gaiser et al., 2009; Song et al., 2009). Elimination of STIP1 is also nonlethal in yeasts (Nicolet and Craig, 1989). On the other hand, STIP1 deletion is lethal in mice, leading to death by 10.5 days of embryonic development (Beraldo et al., 2013). This suggests that STIP1 is critical at early stages of mammalian development. Deletion of STIP1 down regulates the levels of various Hsp90 clients suggesting that it plays a vital role in maintaining chaperone network homeostasis (Beraldo et al., 2013).

In yeast, STIP1 has been shown to modulate toxicity of protein aggregates, being the TPR1 domain important for this activity (Wolfe et al., 2013). STIP1 has also been suggested to modulate mutant tau and Aβ toxicity (Ambegaokar et al., 2011; Brehme et al.,

2014; Maciejewski et al., 2016). Therefore, STIP1 also plays a role in modifying protein aggregates and mediating their toxicity (Maciejewski et al., 2016).

Figure 4: Schematic of STIP1 structure and its regulation by Hsp90. Intracellularly, STIP1 functions as a molecular co-chaperone. STIP1 has three main tetratricopeptide repeat domains (TPR1, TPR2A and TRP2B). It is able to bind to various chaperones (Hsp70 and Hsp90) and co-chaperones to perform its functions. It binds to Hsp70 using the TPR1 and TPR2B domains while it binds to Hsp90 using the TPR2A domain.

1.7 The Role of Molecular Chaperones in Neurodegenerative Disorders

Hsp90 is able to refold numerous non-native proteins within the cell, preventing the formation of toxic aggregates (Freeman and Morimoto, 1996; Mayer and Bukau, 1999). One pathological non-native protein that Hsp90 in unable to refold is misfolded Tau because of its post-translational modifications (Nakai et al., 1993; Neve et al., 1988). Tau is a microtubule stabilizing protein whose pathological form is present in neurodegenerative disorders such as AD and PD (Nakai et al., 1993; Neve et al., 1988). Tau is predominantly located in neurons of the central nervous system where is promotes tubulin assembly into microtubules (Avila et al., 2002). In a number of diseases, tau becomes hyperphosphorylated and forms aggregates as observed for example in AD (Rabindran et al., 1993). Hsp90 functions to preserve tau in order to optimize microtubule kinetics but is unable terminate this function when tau becomes aberrant (Karagoz et al., 2014). Therefore, Hsp90 continues to preserve and enhances tau even when it is in its pathological state which promotes neurodegeneration (Sarge et al., 1991).

Molecular chaperones also play a major role in the processing and clearance of Aβ, a major component of amyloid plaques which are a prominent hallmark of AD. Aβ peptides are produced from the cleavage of amyloid precursor protein (APP), a transmembrane glycoprotein with unknown functioning (O'Brien and Wong, 2011). Cleavage of this protein can occur through one of two pathways. The first pathway involves the cleavage of APP primarily by alpha secretase followed by gamma secretase processing which yield non-

amyloidogenic products. The second pathway, which yield amyloidogenic Aβ products, involve APP cleavage by beta secretase followed by gamma secretase. The difference in the site of actions of alpha and beta secretases is what yields these variations in cleavage products with varying fates (O'Brien and Wong, 2011). Many of the genetic mutations that are implicated in AD occur in the APP gene and cluster around the gamma secretase cleavage site (Shen and Kelleher, 2007). Interestingly, stress in the form of heat shock increases transcription of the beta-APP gene suggesting that the Hsp molecular chaperone system is involved with Aβ metabolism (Dewji and Do, 1996). In fact, a heat shock element was found in the promotor region of the APP gene (Dewji and Do, 1996). Furthermore, Hsp90 inhibitors have been shown to be protective against Aβ-induced toxicity (Ansar et al., 2007). Aβ induced cognitive impairments were also rescued by inhibiting Hsp90 in mice while having no effect on Aβ plaque load (Chen et al., 2014; Ho et al., 2013). Thus, more studies need to be conducted in order to parse out the mechanisms by which Hsp90 modulates the toxic effects of tau and Aβ in different neurodegenerative disorders.

The chaperone machinery also regulates CMA (Cuervo and Dice, 2000). Heat stress is a potent stimulator of the heat shock response and exerts a complex effect on autophagy. Heat stress triggers autophagy in various cell types such as human hepatoma cells (Han et al., 2013). Studies have shown that the deletion of the HSF1 gene increases basal autophagy levels (Dokladny et al., 2013). Additionally, stress conditions that promote the formation of unfolded proteins upregulate the heat response and autophpagy. Treatment of cells with proteosomal inhibitors induces mitochondrial dysregulation and ER stress which triggers an upregulation of Hsp70 and induces autophagy (Selimovic et al., 2012). In addition to clearing misfolded substrates, various other cellular proteins need to constantly recycled in

order to modulate intracellular processes. Hsp70 recognizes the substrate and brings it to the lysosome where it undergoes complete degradation in the lysosomal matrix (Chiang et al., 1989). CMA has a great impact on neurodegenerative disorders (Arias and Cuervo, 2011; Koga and Cuervo, 2011, Orenstein and Cuervo, 2010). One of the defining characteristics of these diseases is the failure of the CMA to properly dispose of misfolded proteins.

Parkinson's disease (PD) is a typical example of a neurodegenerative disorder that is affected by the CMA (Cuervo et al., 2004; Orenstein et al., 2013; Mak et al., 2010; Vogiatzi et al., 2008). PD is characterized by the selective loss of dopaminergic neurons and the presence of misfolded α-synuclein and leucine-rich repeat kinase 2 (LRRK2) that form toxic aggregates called Lewy bodies (Cuervo et al., 2004; Orenstein et al., 2013; Mak et al., 2010; Vogiatzi et al., 2008). Sequence analysis revealed that both of these proteins have CMA-targeting motifs which suggest that the CMA play a major in the proper clearance of these proteins (Cuervo et al., 2004; Orenstein et al., 2013). Hsp72 confers cellular protection against apoptosis by inducing autophagy in mesothelial cells following injury in rats (Shu et al., 2011). Another study inhibited HSF-1 protein expression using siRNA and overexpressed Hsp70 using adenovirus and observed that HSF1 silencing enhanced starvation induced autophagy while Hsp70 upregulation blocked this enhancement (Dokladny et al., 2015).

The huntingtin protein (HTT) is a protein that is misfolded and forms pathological aggregates which cause selective neuronal loss in HD (Roos, 2010; Gusella and MacDonald, 1998; Ramaswamy et al., 2007). The HTT protein has several glutamine (polyQ) repeats which influence HTT toxicity (Jana et al., 2000). In fact, the number of

polyQ repeats correlates with the amount of HTT deposition (Jana et al., 2000; Muchowski et al., 2000). Remarkably, Hsp70 can prevent the toxicity that is normally caused by misfolded protein in animal models of HD (Ravagnan et al., 2001; Gurbuxani et al., 2003). Increased levels of Hsp40, Hsp60, Hsp70 and Hsp100 all have been shown to attenuate polyQ induced protein aggregation and slow disease progression. It has been proposed that molecular chaperones promote formation of spherical and annular HTT oligomer, leading to the formation of less toxic fibrillary and amorphous aggregation (Ravagnan et al., 2001; Gurbuxani et al., 2003).

Amyotrophic lateral sclerosis (ALS) is another neurodegenerative disorder that is affected by the chaperone machinery. ALS is a progressive neurodegenerative disorder that manifests in adults and is characterized by motor neuron dysfunction (Banks et al, 2008). This is a fatal disorder whose symptomology includes paralysis and respiratory failure followed by death. Many ALS patients also suffer from frontal and temporal lobe neuronal death which is called fronto-temporal lobar degeneration (FTLD) (Banks et al, 2008). TAR DNA-binding protein (TDP-43) is a major component in misfolded aggregates that are toxic and contribute to neurodegeneration in these disorders (Neumann et al., 2006). Hsp40 and Hsp70 protein levels and availability regulates TDP-43 aggregation and are important targets for therapeutic interventions (Udan-Johns et al., 2014). Numerous other proteins are misfolded and found in aggregates in ALS disease and are discussed in greater detail in section 1.1. TDP-43 pathology is of particular interest because it is observed in multiple neurodegenerative disorders, including Alzheimer's, and therefore deciphering the cellular mechanism by which TDP-43 stability is regulated has the potential to provide essential directions for new therapeutic approaches.

1.8 TARDP43 (TDP-43)

TDP-43 is a widely-expressed protein whose primary role includes interaction with DNA and RNA and modulating RNA expression (Figure 4). This protein has two RNA recognizing motifs and a prion-like C-terminal domain that makes it aggregation-prone (Ou et al., 1995). It also contains a nuclear localization sequence and a nuclear export signal (Winton et al., 2008). Therefore, this protein is predominantly localized in the nucleus but it is capable of nucleocytoplasmic shuttling. TDP-43 is also involved with RNA splicing and regulating the stability of a large number of mRNA transcripts (Gregory et al., 2004 Xu, 2012). TDP-43 is important for resilience in conditions of cellular stress where it sequesters mRNAs that are needed for survival into aggregates (King et al., 2012). TDP-43 was identified as a major component of cytoplasmic inclusion found in motor neurons of ALS patients and in cortical regions of FTLD patients (Neumann et al 2006). Neurodegenerative disorders with TDP-43 cytoplasmic depositions are known as “TDP-43 Proteinopathies”.

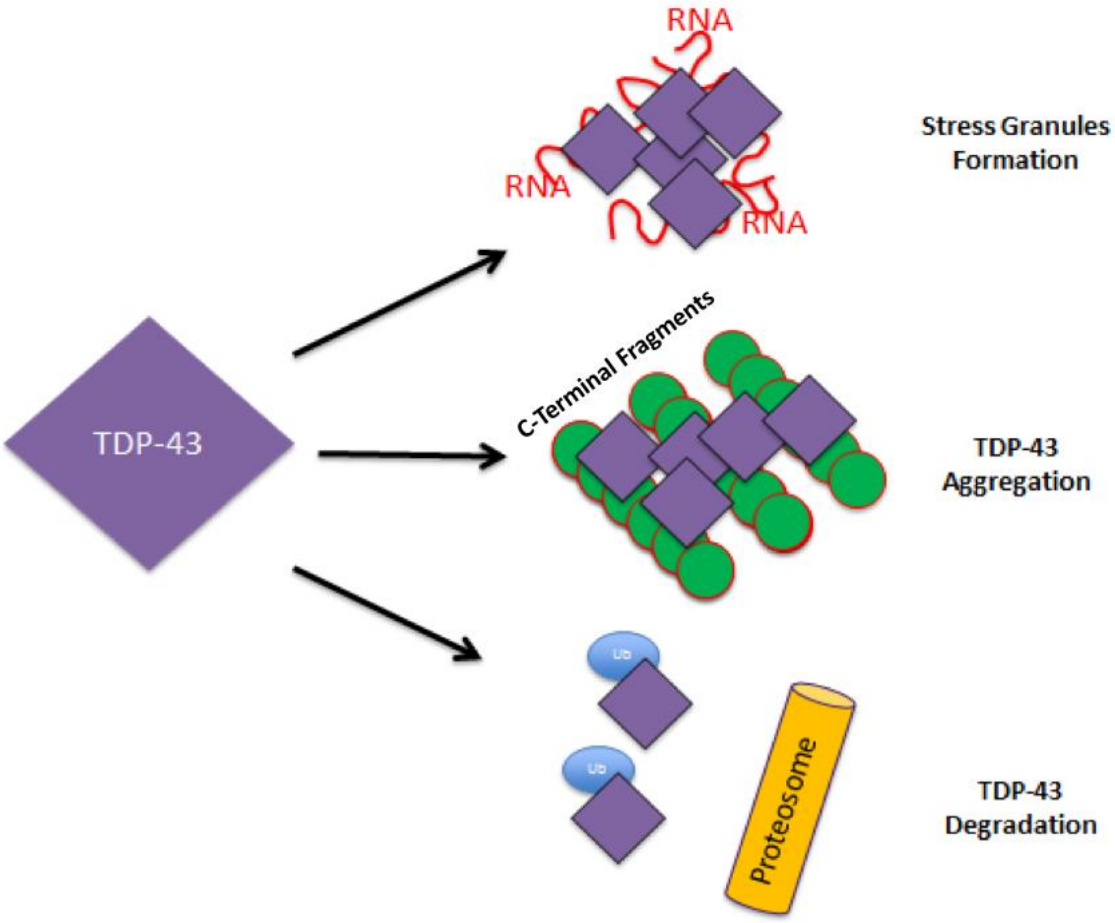


Figure 5: Mechanisms involved in TDP-43 pathology. TDP-43 functions predominantly within the nucleus and modulates gene expression, spliceosome functioning, mRNA transport and stabilization. It also has to ability to localize to the cytoplasm where it can associate with RNA strands and form stress granules, it can interact with other pathological proteins and form cytoplasmic aggregates or it can be degraded through one of the numerous cellular clearance pathways (Adapted from Janssens and Broeckhoven, 2013).

There are numerous upstream genetic and physiological stressors that have been implicated in TDP-43 proteinopathies, but these disorders seem to be irreversible in nature. Even though TDP-43 mutations have been found in ALS and FTLD patients, these forms of the disease are rare (Scotter et al., 2015). The majority of TDP-43 gene mutations that are implicated in stress granule formation are located in its prion-like C-terminal domain (Sreedharan, 2008; Van Deerlin, 2008; Rutherford, 2008). Almost all cases of ALS, as well as tau- negative frontotemporal dementia (FTD), show the deposition of TDP-43-positive protein inclusions suggesting a common pathogenic pathway amongst various neurodegenerative disorders (Scotter et al., 2015). When these aggregates are studied thoroughly, it is observed that both native and mutated TDP-43 accumulates into insoluble cytoplasmic aggregates in ALS (Van Deerlin, 2008; Rutherford, 2008). Mutated TDP-43 is unable to process RNA and it loses the ability to undergo axonal transport even before it forms pathological aggregates (Alami et al., 2014; Lie-Yesucevitz et al., 2014; Arnold et al., 2013). This result is further supported by the observation that mutated TDP-43 is unable to rescue the loss of function in motor neurons with knocked out TDP-43, suggesting a potential role of genetics in TDP-43 Proteinopathies (Alami et al., 2014; Lie-Yesucevitz et al., 2014; Arnold et al., 2013). Intrinsically disordered proteins, such as TDP-43 and FUS, have the tendency to mediate and form membraneless organelles through liquid-liquid phase separation (LLPS; Li et al., 2013). These membraneless organelles can have numerous functions such as ribonucleoprotein assembly (Uversky, 2017).

The majority of ALS has unknown causes and inheritance only account for a small fraction of all cases (Scotter et al., 2015). Therefore, physiological and environmental factors are thought to play a major role in the development of such disorders (Scotter et al.,

2015). Numerous factors, such as osmotic stress, oxidative stress and endoplasmic reticulum stress have been proposed to initiate the redistribution of TDP-43 from the nucleus to the cytoplasm, where they are included in cytoplasmic stress granules (Dewey et al., 2010; Colombrita et al., 2009; Barmada et al., 2014). This early mislocalization tends to be reversible unless the cells are under prolonged stress conditions which may seed the stress granules into an irreversible form (Wang et al., 2012; Udans-Johns et al., 2014; King et al., 2012). In neurodegenerative disorders, these stress granules are post-translationally modified and co-localize with TDP-43 aggregates in ALS spinal neurons (Liu Yesucevitz et al., 2010; McDonald et la., 2011). Together, these diverse environmental stressors are believed to promote irreversible TDP-43 changes that are commonly observed in neurodegenerative disorders (figure 5). The exact mechanism by which TDP-43 toxicty occurs is not currently known. Nontheless, TDP-43 is cleaved by caspase-9 before it is ubiquintintated, phosphorylated and mislocalized to the cytoplasm. Therefore caspase dependent apoptosis is one suggested mechanism for this toxicity. (Liu Yesucevitz et al., 2010; McDonald et la., 2011).

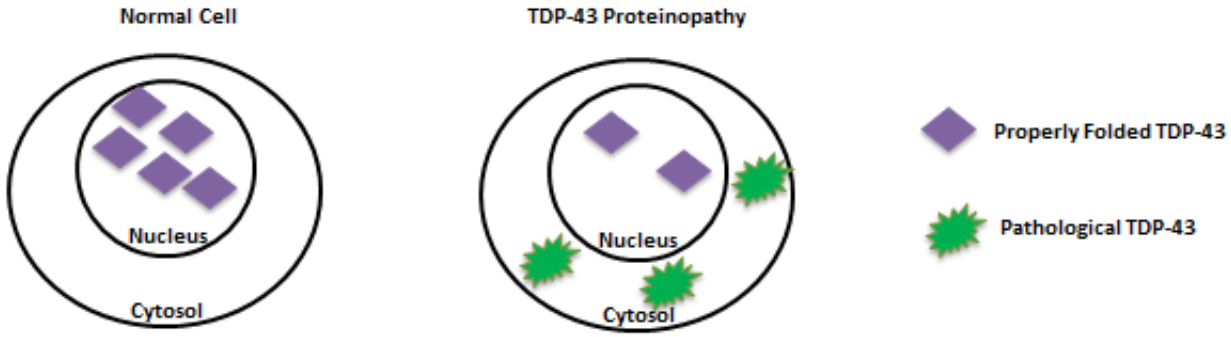


Figure 6: TDP-43 proteinopathy. In normal cells, TDP-43 is primarily localized within the nucleus. In some diseases TDP-43 forms stress granules in the cytoplasm. This proteinopathy is observed in affected neurons of ALS, FTLD and AD patients (Adapted from Neumann et al., 2006).

Pharmacological upregulation of the heat shock response in *Drosophila* has shown that small heat shock proteins (sHsp) enhance TDP-43 solubility and that Hsp104 can potentiate TDP-43 refolding (Gregory et al., 2012; Torrente et al., 2016). Additionally, co-immunoprecipitation studies in human neuroblastoma cells demonstrated that TDP-43 interacts with Hsp70 and Hsp90 (Zhang et al., 2010). Down-regulation of either Hsp70 or Hsp90 was shown to correlate with an increase in TDP-43 levels and phosphorylation state, indicating a potential role of heat shock proteins in TDP-43 phosphorylation and aggregation (Zhang et al., 2010). In fact, Chang et al. (2013) have shown that the dysregulation of the heat shock molecular chaperone system influence TDP-43 aggregation and degradation. Preliminary yeast studies in Dr. Duenwald's lab (The University of Western Ontario) show that Hsp90 inhibitors exacerbate TDP-43 toxicity. They also showed that the deletion of Stress-Inducible Phosphoprotein 1 (STIP1), a co-chaperone that links Hsp70 and Hsp90, also exacerbates TDP-43 toxicity and aggregation in yeast. These results suggest a novel role for STIP1, a modulator of the molecular chaperone network, in regulating TDP-43 toxicity and stability.

1.9 Rationale, Study Aim, and Hypothesis

Although few treatment options exist, there are no current cures for ALS, FTLD and AD. TDP-43 proteinopathy is a common pathological feature in all of these disorders. Misfolded TDP-43 accumulates in the cytoplasm where it forms toxic aggregates that are implicated in neurodegeneration. The inhibition of key molecular chaperones, such as Hsp90, induces TDP-43 upregulation and aggregation suggesting that it is a special client of Hsp90 (Zhang et al., 2010). The role STIP1 plays in TDP-43 mislocalization and aggregation in neurodegenerative disorders, if any, is unknown. The exact role these proteins play in regulating TDP-43 stability and pathology is also undetermined. **Therefore, we hypothesize that the deletion of STIP1 leads to an increase TDP-43 toxicity and proteinopathy.**

Study Aims:

1) To determine whether TDP-43 interacts with STIP1 and if STIP1 modulates the interaction of TDP-43 with other heat shock proteins.

2) To determine if STIP1 regulates TDP-43 and Hsp90 client levels

3) To evaluate the effect of STIP1 in TDP-43 induced cellular toxicity.

2 Materials and Methods

2.1 Animals

The *STIP1*$^{\Delta TPR1}$ mouse line (Lackie et al., preprint) expresses and hypomorphic *STIP1* lacking the TPR1 domain. This provides a model that can be used to study the function of the TPR1 domain and allow a better understanding of *Stip1* in mammals. The effect of increased and decreased *STIP1* was studied using the *STIP1*^{TGA} and *STIP1*$^{-/+}$ mouse lines respectively (Beraldo et al., 2013, Beraldo et al., 2015). *STIP1*$^{-/-}$ mouse embryos (discussed in more detail in section 2.4) were also used, as described by Beraldo et al. (2013). All of the mouse lines were in the C57BL/6J background. The 5xFAD mouse line (Oakley et al., 2006), obtained from the Jackson Laboratory (Bar Harbor, Maine), is an Alzheimer's Disease AD mouse model that rapidly develops cerebral β-amyloid 42 (Oakley et al., 2006). 5xFAD mice were crossed with *STIP1*^{TGA} mice to obtain the *STIP1*$^{TGA;5xFAD}$ (Lackie et al., manuscript in preparation) which is an AD mouse model with a fivefold increase in *STIP1* expression.

2.2 Ethics

All animals used in this research were handled and maintained by the University of Western Ontario Animal Care and Veterinarian Services. All procedures were in accordance with approved animal use protocols at the University of Western Ontario **(2016-103; 2016-104)** following guidelines outlined by the Canadian Council of Animal Care.

2.3 Mouse Embryonic Fibroblasts

STIP1$^{\Delta TPR1}$ Mouse Embryonic Fibroblasts (MEFs) were provided by Rachel Lackie (Western University). *STIP1*$^{\Delta TPR1}$ MEFs were obtained from *STIP1*$^{\Delta TPR1}$ mice as previously described (Beraldo et al., 2013, Lackie et al., preprint, Migliorini et al., 2002). MEFs were initially frozen in liquid nitrogen. When first thawed, they were plated in thawing media (DMEM supplemented with 20% FBS (Fetal Bovine Serum-a serum supplement for cell cultures), 1% L-Glutamine, 1% penicillin & streptomycin) in T75 flasks. After 2 days, fibroblasts were washed with D-PBS and then incubated in DMEM supplemented with 10% FBS, 1% L-Glutamine, 1% penicillin & streptomycin (replaced every 3 days). Once cell confluence reached 80%, fibroblasts were transferred to a new T75 flask at a 40% confluence while the rest were discarded. Growing fibroblasts were always maintained at 37^0C and 5% carbon dioxide. All experiments were conducted at cell passage 6 (P6).

2.4 Mouse Embryo Isolation

STIP1$^{-/-}$ mouse embryos were developed and collected as previously described (Beraldo et al., 2013). Briefly, heterozygous (*STI*$^{+/-}$) females were super-ovulated and mated with heterozygous males. Super-ovulation was induced by injecting female mice with 5 IU of pregnant mare serum. 48 hours after, females were further injected with 5 IU human chorionic gonadotropin. Immediately after this injection, female mice were mated

with male mice overnight; the males were removed the following day. Embryos were collected and genotyped at successive time intervals.

2.5 Cell Cultures

Human Embryonic Kidney (HEK293 cells were purchased from American Type Culture Collection, Manassus, VA, USA) cells and Mouse Septal Neuronal cells (SN56 cells were generous provided by Bruce Wainer, Emory University) were thawed using thawing media and grown in complete media (described above). Media was replaced every 2 days or as needed. Cells were grown in T75 flasks and transferred to new flasks once a confluence of 80% was reached. All experiments were conducted between P5 and P10. Cells were placed in the following physiological conditions for 24 hours to assess cellular viability and TDP-43 localization: 1 mM Azetidine-2-carboxylic acid (AZC; Sigma-Aldrich, Cat# A0760-100MG), 20 uM MG132 (Sigma-Aldrich, Cat# M7449-200), 10 uM Radicicol (Cayman Chemicals, Cat# 13089), 10 uM Thapsigargin (Sigma-Aldrich, Cat# 67526-95-8). The 0.5 mM sodium arsenite treatment was for 30 mins (Sigma-Aldrich, Cat# S7400-100G).

2.6 CRISPR/Cas9 generation of STIP1 HEK293 KO Cells and STIP1 SN56 KO Cells

STIP1 knockouts of HEK293 and SN56 cells were generated as previously described (Lackie et al., preprint). STIP1 expression was eliminated using CRISPR-Cas 9

(Clustered Regularly Interspaced Short Palindromic Repeats) editing system. CRISPR/Cas 9 is an acquired immunity system that was initially discovered in bacteria (Barrangou et al., 2007). In this system, bacterial Cas9 enzymes (endonuclease enzymes that cleave phosphodiester bonds between nucleic acid monomers) process invading foreign DNA by targeting and cleaving the DNA sequences at specific sites (Zhang et al., 2014). The resulting DNA fragments are then integrated into the host genome's highly conserved CRISPR sequence which generates host immunity. This CRISPR sequence becomes the spacers that act as transcriptional templates that produce crRNA which assist Cas9 in detecting future viruses and invading genetic material. Researchers take advantage of this genomic integration system by designing special Cas9 enzymes with synthetic guide RNA sequences that can target specific DNA sequences. Once the Cas9 enzyme binds the DNA sequence of choice, a double stranded DNA break is initiated (Ma et al., 2014). These breaks initiate DNA repair processes. These repairs processes can be manipulated by inserting donor repair template sequences of choice to induce the desired genetic modifications (Zhang et al., 2014).

In our study, the Optimized CRISPR Design Software (http://crispr.mit.edu/) was used to design guide RNAs for the mouse STIP1 gene to transfect SN56 cells (STIP1 Top 1: 5'CACCGGTAGTCTCCTTTCTTGGCGT 3' and STIP1 Bottom 1 5'AAACACGCCAAGAAAGGAGACTACC 3') and the human STIP1 gene to transfect HEK293 cells (STIP1 Top 1: 5' CACCGGCATCGATGTTACCCACGCTC 3' and STIP1 Bottom 1 5' AAACGAGCGTGGGTAACATCGATGCC 3'). The guide RNAs were phosphorylated, annealed and cloned at the BbSI enzyme restriction site into the Addgene vector (px330 modified vector). After this construct was sequenced, Lipofectamine 2000

(Invitrogen) was used to transfect both SN56 and HEK293 cells (the corresponding guide was desi. Clones were isolated using serial dilution in 96-well plates. Isolated clones were separated until colonies were established. These colonies were then plated in 6-well plates in duplicates. One duplicate was maintained while the other one was lysed for immunoblot analysis. Several clones with decreased STIP1 levels were obtained but only clones with complete elimination of STIP1 expression were expanded and used to investigate the role of STIP1 in TDP-43 mediated toxicity and pathology.

2.7 SN56 Cellular Differentiation

SN56 cells were differentiated as previously described (Blusztajn et al., 1992. Differentiation was induced by incubating SN56 cells in complete median containing 1mM of dbcAMP (Sigma-Aldrich), a cAMP analog, for 48 hours.

2.8 Western Blotting

Western blotting was conducted as described by (Beraldo et al., 2013, Guzman, De Jaeger et al., 2011). Protein from MEFs, brain tissues, mouse embryos, HEK293 cells and SN56 cells were extracted using RIPA buffer (50mM Tris, 150mM Sodium Chloride, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, pH 8.0), with added protease inhibitors (Protease Inhibitor Cocktail Set 3-EMD Mllimore, Burlington, MA) and phosphatase inhibitors (Phosphotase Inhibitor Cocktail Set 5-EMD Mllimore, Burlington, MA), on ice for 15 minutes. Protein extracts were centrifuged at 12 000 x g at 4^0C for 20 minutes and supernatant was collected and quantified using the BioRad protein assay

method as described by the manufacturer. 5-30 ug of proteins were loaded onto Bolt 4-12% Bis-Tris gradient pre-cast gels (Invitrogen, Cat#NW00100BOX).

For subcellular fractionation studies, the Nuclear and Cytoplasmic Extraction Kit (ThermoFisher Scientific, Cat# 7833) was used. In short, the RIPA buffer was replaced with the cytoplasmic extraction and nuclear extraction reagents. After cells were collected as described above, the cytoplasmic reagent 1 was added and the lysate was vortexed for 15 seconds and then incubated on ice for 10 minutes. Ice cold cytoplasmic reagent 2 was then added and this mixture was vortexed for 5 seconds, placed on ice for 1 minute and then vortexed again for 5 seconds. It was then centrifuged at max speed (15 g) for 5 minutes and the supernatant, which contains the cytoplasmic protein extracts, was extracted and stored in the -4^0 freezer (or placed on ice). The nuclear extraction reagent was added to the remaining pellet (which contains nuclear proteins) from the centrifugation described above; which was vortexed for 15 seconds and then placed on ice for 10 minutes, repeatedly 5 times. The supernatant obtained contains the nuclear proteins and was stored for later analysis. The following antibodies were used for immunoblotting: anti-STIP1 (1:5000, in-house antibody generated by Bethyl Laboratories Montgomery, USA) (Beraldo et al., 2013), anti-Hsp90 (1:1000, Cat# 4877, Rabbit mAb, Cell Signalling), anti-Hsp70 (1:1000, Cat# ab2787, Abcam), anti-CHIP (1:1000, Cat# 2080, Rabbit mAb, Cell Signalling), anti-Glucocorticoid Receptor (1:1000, Cat# 3660, Rabbit mAb, Cell Signalling), anti-GRK2 (1:1000 Cat# 3982, Rabbit pAb, Cell Signalling) anti-FKBP51 (1:1000, ab2901, Rabbit pAb, Abcam), anti-CypA (1:2000, Cat# ab126738), anti-Pin1 (1:250, Cat# sc-15340, Rabbit pAb, Santa Cruz), anti-TDP-43 full length (1:1000, Cat# 10782-2-AP, Proteintech), anti-TDP-43 C-terminal (1:1000, Cat# 12892-1-AP, Proteintech), anti-NeuN (1:5000, Cat#

ab104225, abcam), anti-Tubulin (1: 10000, Cat# 2020-TUB, PhosphoSolutions), anti-KI67 (1:1000, Cat# ab15580, abcam).

2.9 Immunofluorescence

Immunofluorescence was performed on both brain sections and cell cultures using the same protocol described by Beraldo et al. (2013) and Lackie et al. (Preprint). Briefly, mouse brains were extracted after transcardial perfusion and fixed in 4% paraformaldehyde (PFA) for 48 hours. Fixed half brains were sliced in 20 uM sections using the Leica VT1000s. Brain sections and tissues were stored in PBS + 0.02% sodium azide. Brain sections were permeabilized (PBS-T for 2x8 minute intervals), blocked (PBS with 3% BSA, 2% normal goat serium and 0.2% Triton-X for 60 minutes) and then placed in primary antibody solution (PBS with 0.2% Triton-X, 1% normal goat serum) overnight on the rocker at 4^0C. The following day, sections were washed 3 times then placed in secondary antibody solution. Sections were washed and cover slips were sealed for analysis.

Cell cultures were plated on poly-lysine covered glass coverslips and then fixed in 4% PFA. They permeabilized (PBS with 0.05% Triton-X for 5 minutes), blocked (PBS with 5% BSA 1 hour) and incubated in primary antibody solution (PBS with 0.1% Triton-X, 0.1% BSA) overnight on the rocker at 4^0C. They were washed 3 times and then placed in secondary antibody solution (same as primary antibody solution). The coverslips were then washed three times and sealed with DAPI FLuoroshield (GeneTex, Cat# GTX30920) for analysis.

2.10 Quantitative RT-PCR

Quantitative RT-PCR was performed on cell cultures as described previously (Beraldo et al., 2013, Beraldo et al, 2015, Lackie et al., Preprint). In brief, cells were plated in 6-well plates. When 80% confluence was reached they were homogenized in TRIzol and RNA was isolated using the Aurum^{TM} Total RNA Fatty and Fibrous Tissue Kit (BIORAD, Cat# 732-6830) according to the manufacturer's instructions. RT-PCR was used to generate cDNA according to protocol (Applied Biosystems, Cat# 4368813). qPCR was performed using SYBR Green on a Bio-Rad CFX96 thermocycler.

2.11 Proliferation

Cellular proliferation was measured using KI67, as previously described (Gerdes et al., 1983). KI67 is a nuclear protein that is present in proliferating cells but absent in resting cells and it can be quantified using immunofluorescence. A secondary mouse 488 antibody was used to visualize KI67 positive cells (green). Percent of total cells that are proliferating was calculated by divided the number of KI67 positive cells by the total number of cells present. Quantification was performed using the ImageJ software.

2.12 Transfection with GFP Vectors

Cells and fibroblasts were transfected using the Lipofectamine 3000 reagent (ThermoFisher Scientific, Cat# L3000015). In short, the Lipofectamine 3000 regent was first combined with Opti-MEM and DNA (5 ug per well in 6 well plate) and was then diluted in Opti-MEM and P3000 reagent. These two solutions were combined and diluted

for 15 minutes at room temperature after which they were added to the cell media in which the cells were plated. Transfection efficiency was calculated using the Evos Imaging system. All experiments had a minimum of 80% transfection efficiency. For the vectors that were not tagged with GFP (HA-Empty and HA-STIP1), co-transfection with a GFP-Empty vector was used to calculate transfection efficiency. Cells were incubated at 37^0C for 1-4 days prior to analysis. Media was changed after the first 12 hours of transfection. The following DNA vectors were used: GFP-EMPTY and GFP-TDP-43 were obtained from Dr. Michael Strong (The University of Western Ontario), HA-EMPTY and HA-STIP1 were developed by Dr. Marliene Lopes (University of Sao Paulo).

2.13 Co-Immunoprecipitation

Co-immunoprecipitation was performed as described previously (Beraldo et al., 2010). In short, HEK293 cells were transfected with HA-EMPTY, as a control, and HA-STIP1 using Lipofectamine 3000. Two days after transfection, cells were lysed using immunoprecipitation buffer (IP buffer-20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% Sodium Deoxycholate, pH 7.5) and incubated in Sepharose beads (abcam, Cat# ab193256) for pre-clearance. Anti-HA agarose beads (ThermoFisher, Cat# 26181) were then added to the cell lysates which rotated in the orbital over night at 4^0C. The next day, the lysates were centrifuged, the supernatant was discarded. 4x SDS sample buffer (40% Glycerol, 240 mM Tris/Hcl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol) was added to the beads which were then heated at 95^0C for 5 minutes, centrifuged and then immunoblotted with antibodies against the proteins in question. SN56 cells were used to immunoprecipitae endogenous TDP-43. SN56 cell

lysates, which were pre-cleared with Sepharose beads, were incubated in rabbit polyclonal anti-TDP-43 antibody overnight (Proteintech, Cat# 10782-2-AP). In the control condition the SN56 cell lysates were incubated with rabbit polyclonal anti-GFP antibody (Abcam, Cat#ab6556) antibodies after pre-clearance on the orbital shaker overnight at 4^0C. The lysates were then incubated in Protein A agarose beads for 4 hours on the orbital shaker at 4^0C. Lysates were centrifuged boiled and immunoblotted as described above.

2.14 Cellular death and Viability Assays

The Live/Dead^{TM} Viability/Cytotoxicity Kit (ThermoFisher Scientific, Cat# L3224) was used to measure cellular toxicity/viability as previously described (Beralo et al., 2013). Ethidium homodimer-1 (dead cell marker) and calcein AM (live cells marker) were added to Dulbecco's phosphate-bufferred saline (D-PBS) at concentrations of 4 uM and 2 uM, and vortexed. 150 uL of the combined Live/Dead^{TM} assay reagents were added to the surface of coverslips so that the cells were covered in solution. These cells were incubated in the dark for 45 minutes after which they were mounted on coverslips and analyzed under the Invitrogen Evos FL Auto 2 cell imaging system (Thermofisher, Cat# AMAFD2000 , Light cubes-DAPI, Texas Red, GFP) . Live cells produced intense uniform green fluorescence (494 nm – 517 nm) whereas dead cells produced a bright red fluorescence (528 nm – 617 nm). Percent of dead cells were counted by dividing the number of dead cells by the total number of cells. Staurosporin (Invitrogen, Burlingotn, ON) was used as a positive control to induce cellular death.

The CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, Cat# G7050) was also used to measure cellular viability. Cells were first plated onto a 96-well plates

with 100 μL of complete media for 24hs. After treatment, 100 μL of the CellTiter-Glo® reagent was added to each well (equal to the amount of media already in the well). Empty wells (no cells or media) were used as a control. This 96-well plate was shaken and placed on a rocker for 2 minutes in darkness to induce cell lysis. The plate was then incubated in darkness at room temperature for 10 minutes to allow for the luminescent signal to stabilize. Luminesence was recorded using a multi-well plater reader. Cellular viability was calculated by normalizing luminescence values to those of WT cells and calculated as percentages.

2.15 Statistical Analysis

Statistical analysis for all Western blots, toxicity assays, viability assays were performed on GraphPad Prism 5 software (Version 5.0 for Windows, La Jolla California USA). All results were expressed as mean +/- standard error (SEM) and analyzed using unpaired t-tests, one-way ANOVA or two-way ANOVA (when comparing control and experimental conditions in the various immunoblots, immunofluorescence, and toxicity assays). The Tukey's post-hoc test was performed using GraphPad Prism. Significance levels are indicated using asterisks, where **** is $p<0.0001$, *** is $p<0.001$, ** is $p<0.01$, * is $p<0.05$.

3 Results

3.1 STIP1 interaction with TDP-43

Zhang et al. (2010) observed that TDP-43 interacts with both Hsp70 and Hsp90. They also showed that silencing Hsp70 and Hsp90 affects TDP-43 protein levels and phosphorylation. In order to determine whether or not STIP1 is involved in modulating TDP-43 toxicity and proteinopathy, we first tested if STIP1 interacts with TDP-43. We performed a co-immunoprecipitation (CO-IP) experiment in HEK293 cells by transfecting them with HA-Empty (control) or HA-STIP1. Figure 7 shows a representative blot of the CO-IP. This experiment demonstrated that the anti-HA antibody specifically precipitated HA-STIP1 (63 kDa band) and co-immunoprecipitated endogenous TDP-43. This experiment was repeated three times.

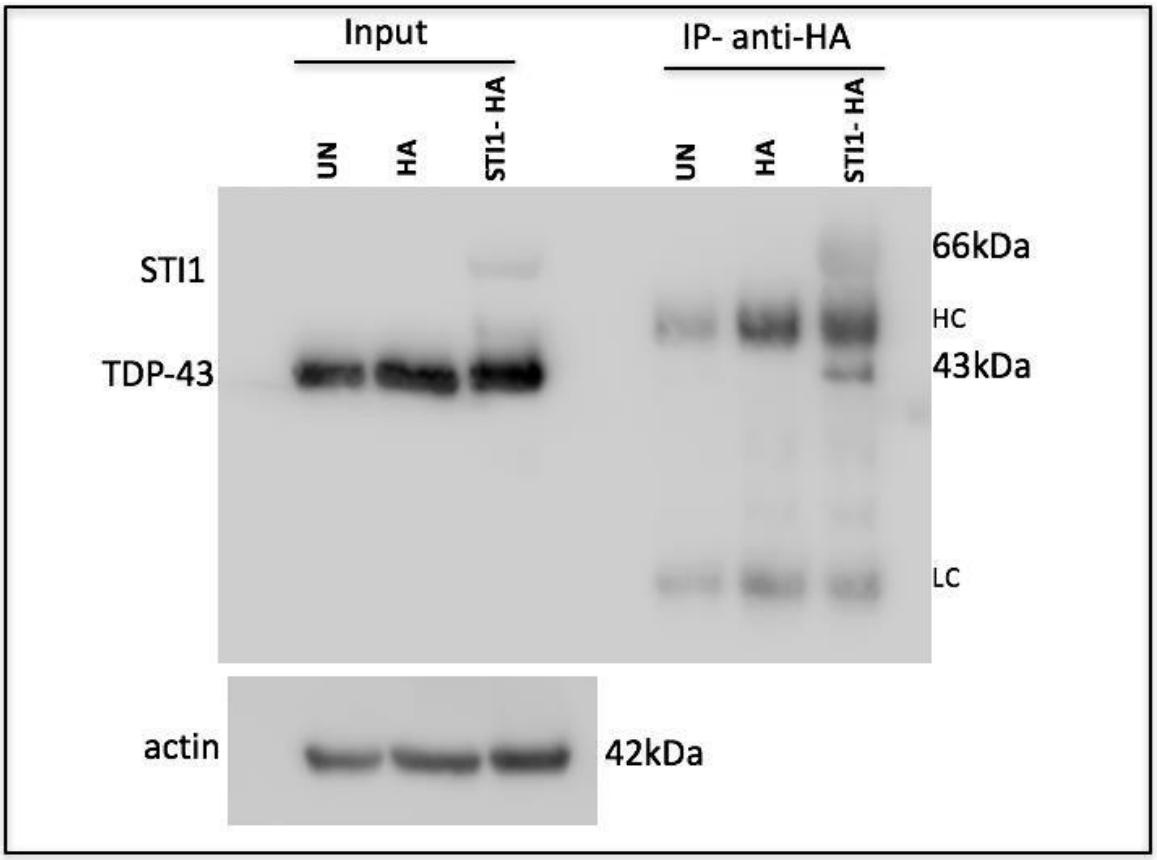


Figure 7: TDP-43 co-immunoprecipitates with STIP1 in HEK293 Cells. HEK293 cells were transfected with HA-STIP1, HA-empty vector or non-transfected (un) and then Anti-HA agarose beads were used to precipitate HA-STIP1. Co-IP proteins were resolved by SDS-PAGE, transferred to a membrane and blotted with an antibody against TDP-43 or HA. HC and LC are heavy and light chain of antibodies respectively. Note that endogenous TDP-43 co-IPs with STIP1. Representative blot out of 3 experiments.

3.2 TDP-43 interacts with Hsp70 and Hsp90 in the absence of STIP1

Although we have shown that STIP1 interacts with endogenous TDP-43 (Figure 7) it is unclear what role STIP1 plays in TDP-43 folding and homeostasis. STIP1 has been shown to serve as a scaffold to bridge Hsp90 to other proteins, including PIWI, a protein involved in the metabolism of non-coding RNA (Gangaraju et al., 2011). Therefore, we tested whether STIP1 has a critical scaffolding function to bridge Hsp90 and TDP-43. We used neuronal SN56 cells to determine whether KO of STIP1 affects the interaction between TDP-43 and heat shock proteins. Figure 8 is a representative blot of the SN56 clones that were generated using CRISPR-CAS9 with varying levels of STIP1. Clone 8 has no STIP1 protein expression. Next, CO-IP analysis were performed in both WT SN56 cells and STIP1-KO SN56 cells (clone 8). Figure 9 is a representative blot for these CO-IPs. Anti-TDP-43 precipitated endogenous TDP-43 which co-immunoprecipitated with Hsp70 and Hsp90 in both WT and STIP-KO SN56 cells. Therefore, TDP-43 continues to interact with Hsp70 and Hsp90 in the absence of STIP1 in SN56 cells. When quantified, the amount of Hsp90 immunoprecipitated is not significantly different between WT and STIP-KO cells. This CO-IPs was repeated three times.

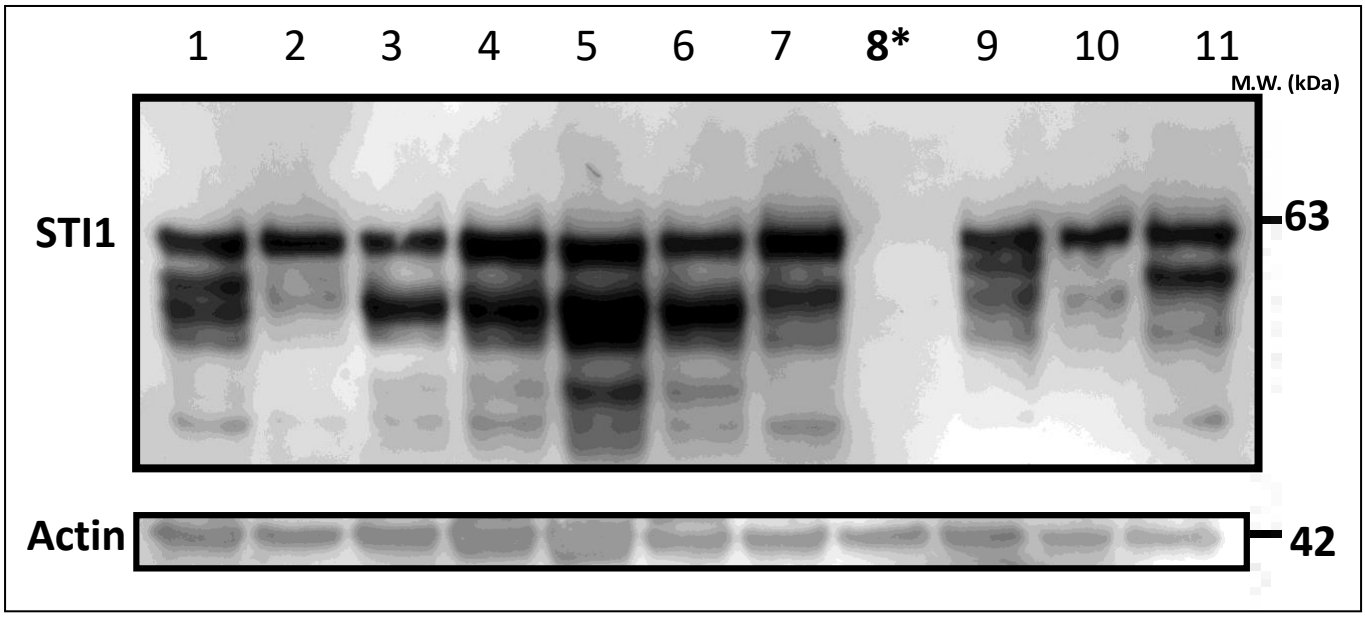


Figure 8: Generation of STIP1-KO SN56 cells using CRISPR/CAS9. The CRISPR CAS9 genomic editing system was used to eliminate STIP1 protein expression in SN56 cells. After transfection and seeding, we were able isolate 11 clones, expressing varying levels of STIP1, using serial dilution. Proteins from these 11 clones were resolved using SDS-PAGE, transferred to a membrane and blotted with an antibody against STIP1 and actin (loading control). Note that clone #8 is a complete STIP1 knockout. This is the clone that was expanded and used for the remainder of the experiments.

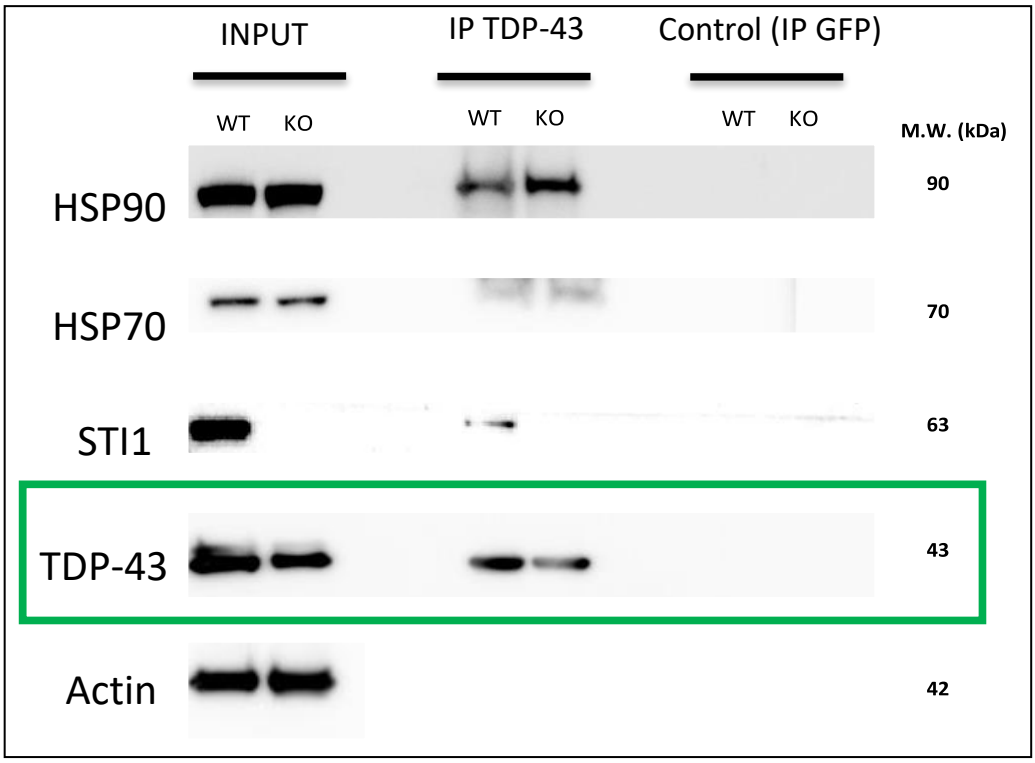


Figure 9: Co-immunoprecipitation of Hsp90, Hsp70 and STIP1 with endogenous TDP-43 in SN56 Cells. Input represents total cell extracts used for immunoprecipitation. IP TDP-43 lanes represent endogenous TDP-43 immunoprecipitated extracts. Control (IP GFP) lanes represent extracts that were immunoprecipitated with antibody anti-GFP. For each of the three conditions both STIP1-WT (WT) and STIP1-KO SN56 cells (KO) were used. Protein A agarose beads were used to precipitate out endogenous TDP-43. Co-IP proteins were resolved by SDS-PAGE, transferred to a membrane and blotted with antibodies against TDP-43, STIP1, Hsp70, Hsp90, GFP and actin (loading control). HC and LC are heavy and light chain of antibodies respectively. Note that endogenous TDP-43 specifically co-IPs with Hsp70 and Hsp90 both in the presence and absence of STIP1. It also co-IPs with STIP1 when it is present. Representative blot out of 3 experiments

3.3 Hsp90 client protein levels and TDP-43 in cells lacking STIP1.

Beraldo et al. (2013) showed that STIP1 is critical for mammalian development and mice lacking STIP1 are unable to survive embryonic development (E10.5). We also observed that in a mouse models with reduced STIP1, numerous Hsp90 client protein levels are decreased by 50% (Lackie et al., preprint). We used non-neuronal HEK293 cells and neuronal SN56 cells to test whether KO of STIP1 in these cells affects client protein stability. HEK293 STIP1-KO cells were generated by Dr. Marilene Lopes using a similar approach as for SN56 cells (Figure 10A). We compared the levels of Hsp90 clients, co-chaperones and TDP-43 in these cells. FKBP51, Cyclophillin A (CypA), Pin1 and CypA are Hsp90 co-chaperones which function as propyl isomerases that assist chaperones in refolding misfolded proteins. Glucocorticoid Receptors are classical Hsp90 clients that play a role in modulating development and metabolism via gene regulation. We first studied client protein stability in HEK293 cells (Figure 10). Figure 10A shows a representative blot and panels B-E present the quantification of Western blots. In HEK293 cells the expression of the Hsp90 co-chaperones and clients was unchanged when STIP1 was eliminated.

Next, we studied client protein stability in neuronal SN56 cells (Figure 11). Figure 11A shows a representative blot and panels B-F present the quantification of Western blots from this cell line. Hsp90 clients and co-chaperones were reduced by approximately 50% when STIP1 was eliminated in SN56 cells. Figure 11G shows a representative blot of Hsp90 client and co-chaperone protein levels when STIP1 was reintroduced in STIP1-KO

SN56 cells. Panels H-I present quantification of this rescue experiments. In SN56 cells, reintroducing STIP1 re-established baseline Hsp90 client and co-chaperone levels.

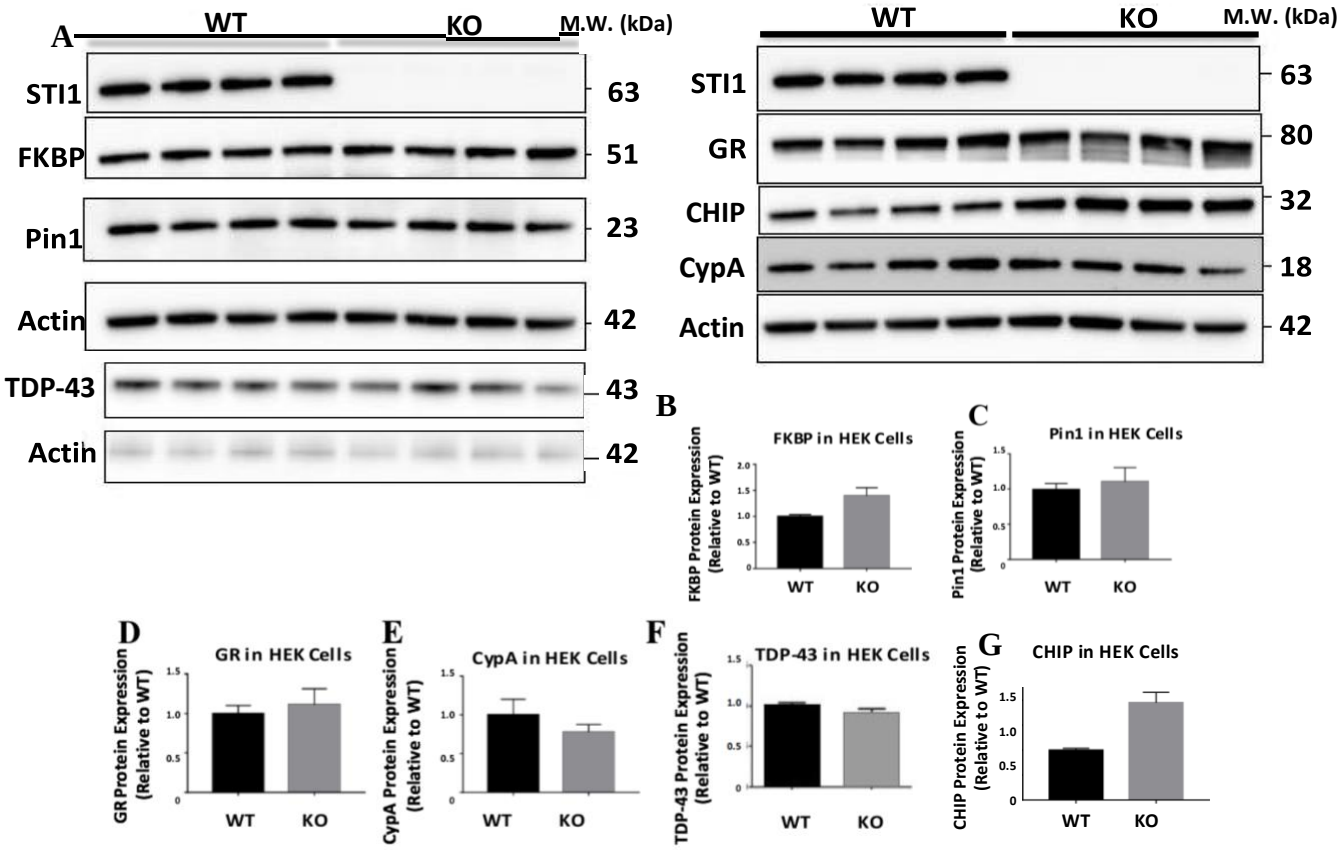


Figure 10: HSP90 client proteins and co-chaperones levels are unchanged in STIP-KO HEK293 Cells. A) CRISPR CAS 9 was used to eliminate STIP1 expression in HEK293 cells. Independent cell cultures were grown and their proteins were resolved by SDS-PAGE and blotted with antibodies against FKBP51, Pin1, TDP-43, Glucocorticoid Receptors, CHIP, CypA and Actin (loading control). Left 4 columns are WT HEK293 cells while the right 4 columns are STIP1-KO HEK293 cells. **B-F)** Quantification of protein levels (FKBP, Pin1, GR, CypA and TDP-43) between WT and STIP1-KO HEK293 cells. T-test analysis suggest that there is no difference in any of the Hsp90 co-chaperones and clients when STIP1 is deleted. Data are Mean ± S.E.M. ($p>0.05$). Results from 8 independent cultures (n=8). The experiment was repeated twice, each time with 4 independent cultures.

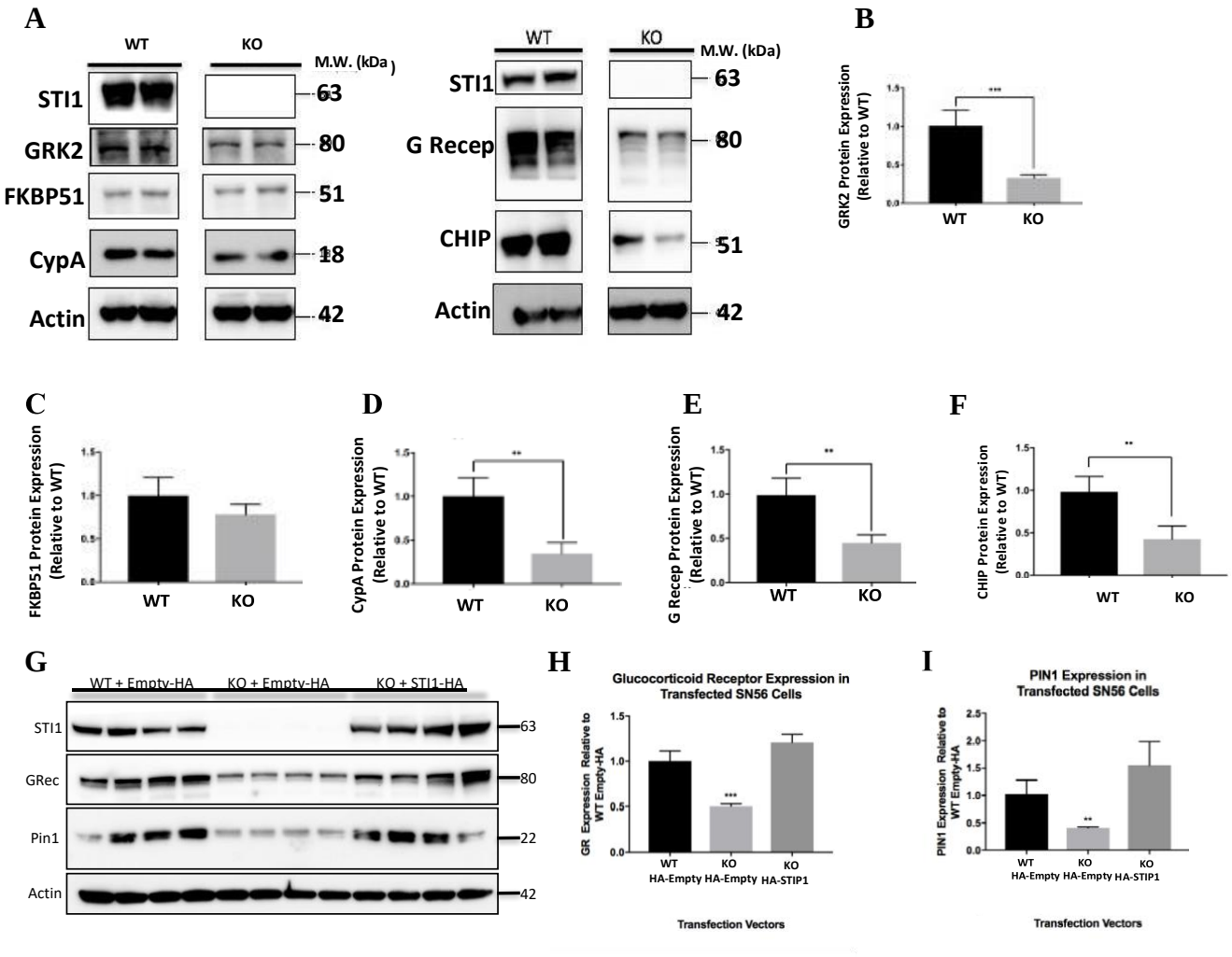


Figure 11: HSP90 client proteins and co-chaperones levels are decreased in STIP-KO SN56 Cells but these levels are rescued when STIP is introduced. A) WT and STIP1-KO SN56 cells were grown (n=4) and their proteins were resolved by SDS-PAGE and blotted with antibodies against GRK2, STIP1, FKBP51, CypA, GR, CHIP, and Actin (loading control). **B-F)** Quantification of protein levels (GRK2, FKBP51, CypA, G Recep and CHIP, between WT and STIP-KO SN56 cells. GRK2 and CypA were reduced by approximately 60% in the STIP1-KO cells while GR and CHIP were reduced by 60%.

Similarly, FKBP51 had a tendency to be reduce as well (p=0.08). Data are Mean ± S.E.M. *p<0.05, **p<0.01, *** p<0.0001. **G)** WT SN56 and STIP1-KO cells were transfected with HA-Empty vectors and STIP1-KO SN56 cells were also transfected by HA-STIP1 (n=4). **H-I)** Quantification of GR and Pin1 protein levels in all 3 transfection conditions. Both GR and Pin1 protein levels increased to those in WT SN56 cells (one-way ANOVA). Data are Mean ± S.E.M. *p<0.05, **p<0.01, *** p<0.0001. Results from 12 independent cultures (n=12). The experiment was repeated thrice, each time with 4 independent cultures.

3.4 STIP1 deletion promotes a reduction in TDP-43 protein levels in both undifferentiated and differentiated SN56 cells

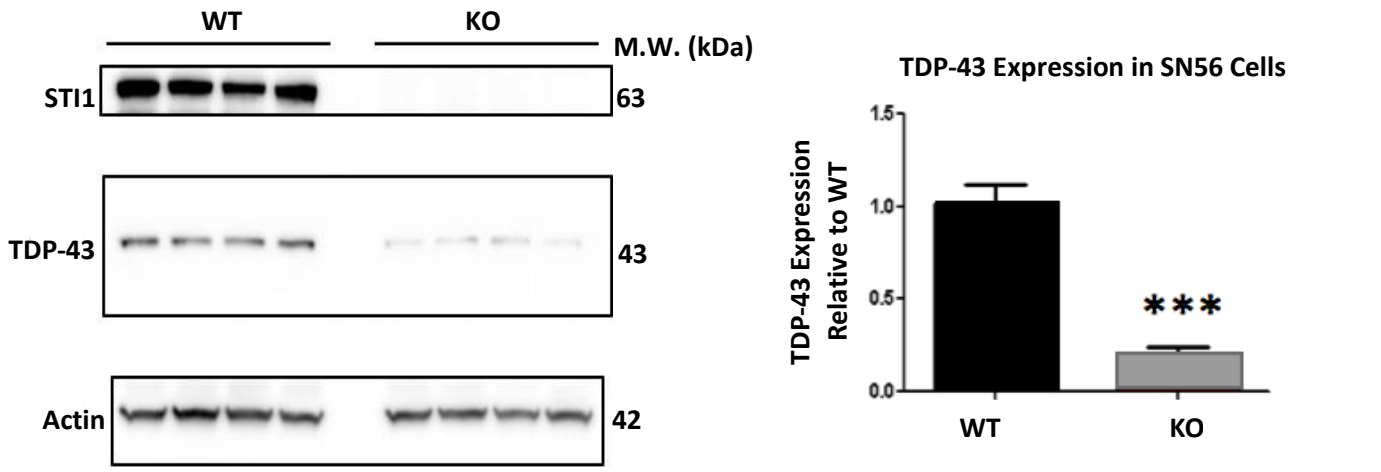
We observed that classical Hsp90 clients are reduced by 50% or more when STIP1 is deleted in SN56 cells. If TDP-43 is indeed an Hsp90 client, then we expect to see similar patterns of decreased protein expression and a rescue when STIP1 is introduced into these cells. First we compared TDP-43 levels between WT SN56 cells and STIP-KO SN56 cells. Figure 12A shows a representative blot and its quantification. TDP-43 levels are reduced by approximately 70% in STIP1-KO cells. Next we expressed STIP1 in STIP1-KO SN56 cells. Figure 11B is a representative blot of this rescue experiment along with its quantification. Re-expression of STIP1 restored baseline TDP-43 levels.

SN56 cellular differentiation (by cAMP treatment) induces arborisation and acetylcholine synthesis and release (Blusztajn et al., 1992). To test whether differentiation of SN56 cells affects TDP-43, we induced cellular differentiation in both WT and STIP1-KO SN56 cells. Figure 12C is a representative blot and quantification of this differentiation experiment. We observed that TDP-43 levels were indistinguishable between undifferentiated and differentiated WT SN56 and between undifferentiated and differentiated STIP1-KO cells.

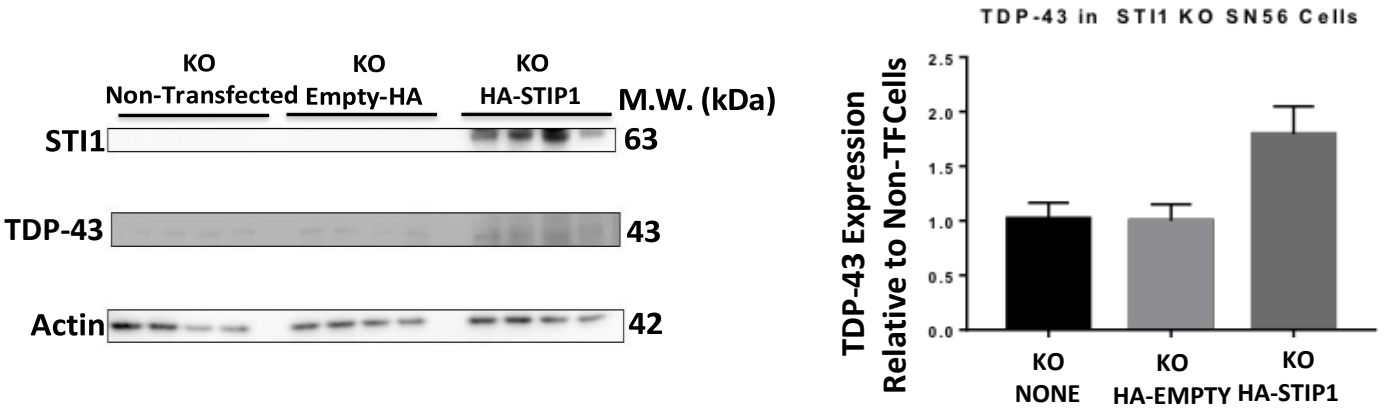
In order to see whether the reduction in TDP-43 that is observed in STIP1-KO SN56 cells is occurring in the nuclear or cytoplasmic compartment we performed a fractional protein extraction. Figure 12D is the representative blot and quantification of this experiment. We observed that there was a decrease in nuclear TDP-43 in the STIP1-KO SN56 cells and that there was no significant TDP-43 detected in the cytoplasm of both cell

types. Lastly, we used q-PCR to determine whether this change in TDP-43 protein levels was due to changes in TDP-43 mRNA expression (Figure 12E). There was no difference in TDP-43 mRNA expression levels between STIP1-WT SN56 and in STIP1- KO SN56 cells.

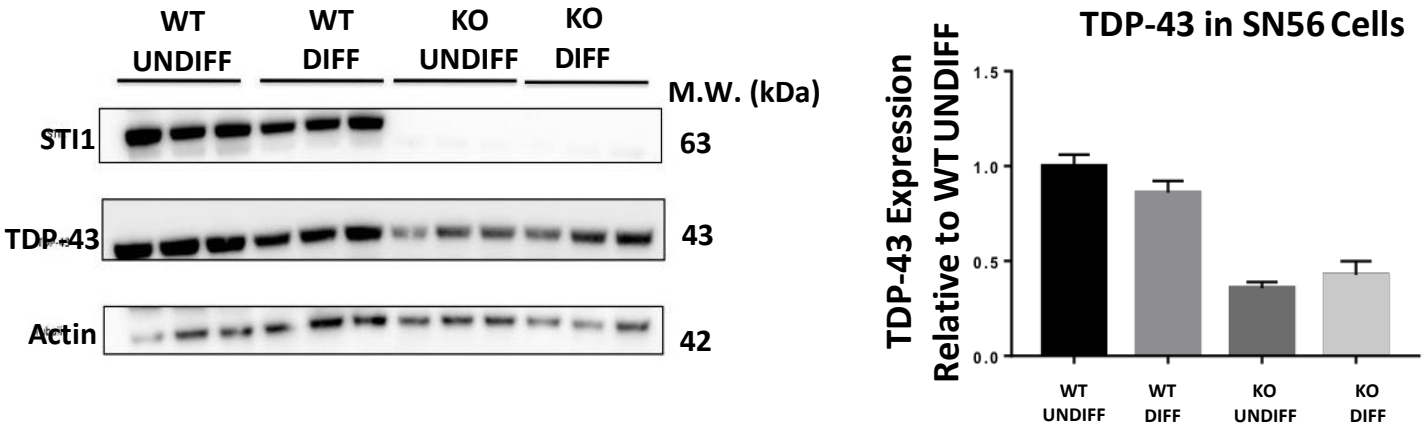
A


WT
KO
M.W. (kDa)
STI1
63
TDP-43
43
Actin
42
TDP-43 Expression in SN56 Cells
TDP-43 Expression Relative to WT
1.5
1.0
0.5
0.0
***
WT
KO

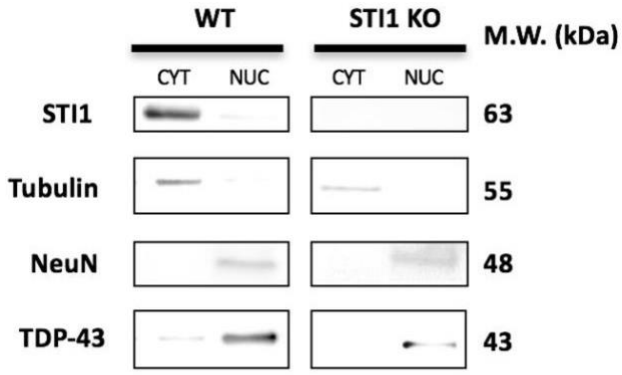
B

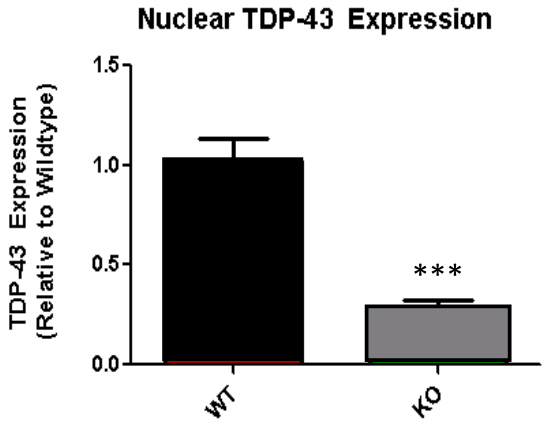

KO Non-Transfected
KO Empty-HA
KO HA-STIP1
M.W. (kDa)
STI1
63
TDP-43
43
Actin
42
TDP-43 in STI1 KO SN56 Cells
TDP-43 Expression Relative to Non-TFCells
2.5
2.0
1.5
1.0
0.5
0.0
KO NONE
KO HA-EMPTY
KO HA-STIP1

C


WT UNDIFF
WT DIFF
KO UNDIFF
KO DIFF
M.W. (kDa)
STI1
63
TDP-43
43
Actin
42
TDP-43 in SN56 Cells
TDP-43 Expression Relative to WT UNDIFF
1.5
1.0
0.5
0.0
WT UNDIFF
WT DIFF
KO UNDIFF
KO DIFF

D


WT
STI1 KO
M.W. (kDa)
CYT
NUC
CYT
NUC
STI1
63
Tubulin
55
NeuN
48
TDP-43
43


Nuclear TDP-43 Expression
TDP-43 Expression
(Relative to Wildtype)
1.5
1.0
0.5
0.0
***
WT
KO

E

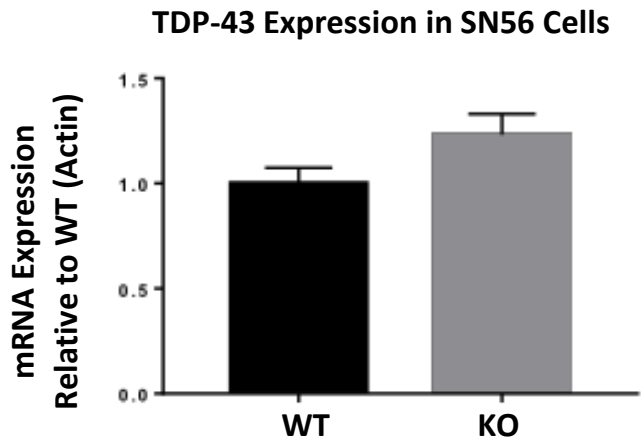
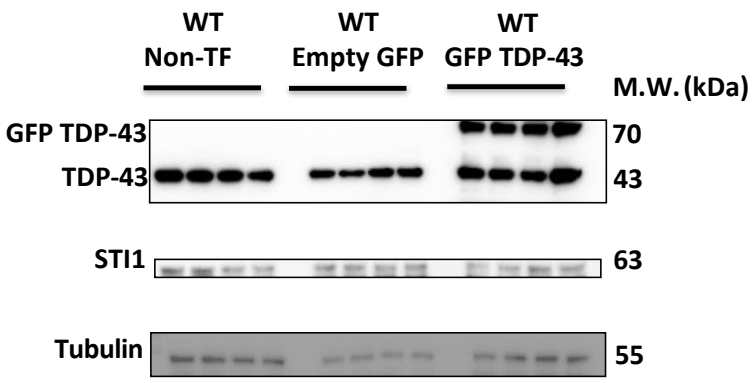

TDP-43 Expression in SN56 Cells
mRNA Expression
Relative to WT (Actin)
1.5
1.0
0.5
0.0
WT
KO

Figure 12: TDP-43 protein and mRNA expression in SN56 cells. A) Left 4 columns in this blot represent WT SN56 cells and the right 4 represent STIP1-KO SN56 cells. T-test statistical analysis suggest that there is a reduction in TDP-43 STIP1-KO SN56 which is statistically significant. This is a representative blot out of 3 experiments, each of which consisted of 4 independent cultures (n=4) **B)** KO SN56 were transfected with STIP1 showing a rescue in TDP-43 levels which is also statistically significant (one-way ANOVA) n=4 **C)** SN56 cells were differentiation by incubation in cAMP for 48 in complete cell media. Both WT and KO SN56 cells were differentiated and TDP-43 protein levels were compared. No difference was observed (in TDP-43 protein levels) between undifferentiated and differentiated SN56 cells (one-way ANOVA) 3 independent cultures grown for each of the conditions (n=3). **D)** Fractional protein extraction was performed in WT and KO SN56 cells. Tubulin was that nuclear loading control while NeuN was the cytoplasmic loading control. STIP1 was localized in the cytoplasm while TDP-43 was predominantly localized in the nucleus, as was expected. There was a decrease in nuclear TDP-43 and there was no significant cytoplasmic TDP-43 (one-way ANOVA) **E)** Q-PCR was performed and TDP-43 mRNA level was compared between WT and KO cells. Actin was used as a reference gene (T-test, $p=0.11$). Actin was also the loading control for the western blots (except for the fractional WB) n=4, $p=0.11$. Data are Mean ± S.E.M. *$p<0.05$, **$p<0.01$, *** $p<0.0001$. Results from 12 independent cultures (n=12). The experiment was repeated thrice, each time with 4 independent cultures.

3.5 Increasing TDP-43 levels in SN56 cells does not alter STIP1 levels

Since TDP-43 interacts with STIP1 and the elimination of STIP1 levels triggers a decrease in TDP-43 protein levels, TDP-43 is most likely an Hsp90 and STIP1 client. Changes in STIP1 has a strong influence on TDP-43 proteins levels. TDP-43 is a modulator of RNA expression and alternative splicing) and can regulate multiple RNA targets. We then tested if increasing TDP-43 expression affects STIP1 levels. In order to do this, we transfected SN56 cells with GFP-WT-TDP-43 vectors and GFP-Empty (control) vectors. Figure 13A is a representative blot of this experiment and Figure 13B is its quantification. Increasing TDP-43 protein levels in SN56 does not alter STIP1 levels.

A



B

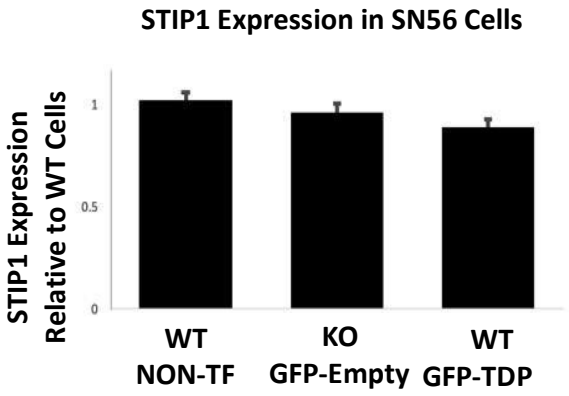
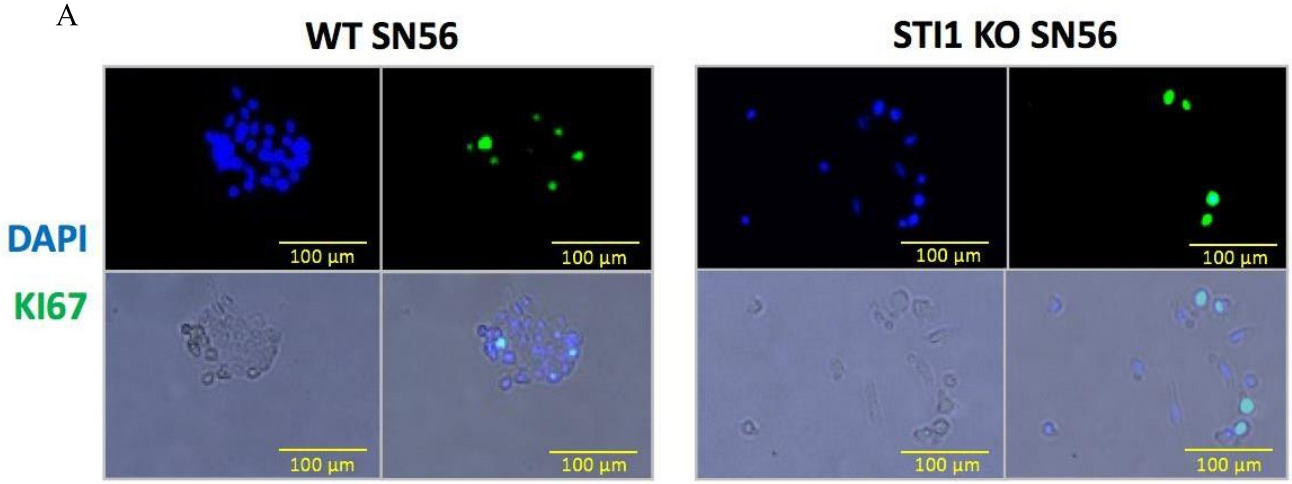


Figure 13: STIP1 protein expression in cells with exogenous TDP-43. A) Left four columns represent WT SN56 cells that were not transfected, middle four represent wells that were transfected with GFP-Empty vectors while right four were WT SN56 cells transfected with GFP-WT TDP-43. Tubulin was used as a loading control. Note that the molecular weight for GFP-WT-TDP-43 is 70 kDa. **B)** Statistical analysis (one-way ANOVA) suggest that there is no statistical difference in STIP1 levels between the three conditions ($p=0.1$). Results from four independent cultures ($n=4$). The experiment was performed once.

3.6 STIP1 elimination does not alter proliferation levels in SN56 cells

It has been previously shown that STIP1 is critical for cellular proliferation in mammalian cells during development (Beraldo et al., 2013). STIP1 deletion induces a decrease in cellular proliferation in mouse embryonic fibroblasts (MEFs). In order to evaluate whether STIP1 plays a role in cellular proliferation in neuronal SN56 cells, we used an anti-KI-67 antibody (a protein only detected during cellular proliferation). Figure 15A is a representative image of this immunofluorescence experiment. Figure 15B is the quantification of SN56 with KI-67 positivity. We observed that there was no significant statistical difference in KI-67 immunoreactivity in WT and STIP1-KO SN56 cells, suggesting there are no alterations in proliferation in neuronal SN56 cells.

A



B

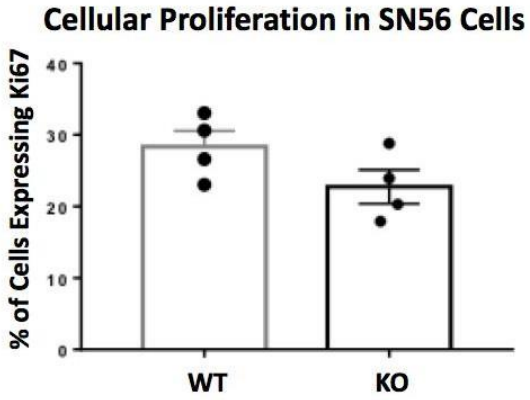


Figure 14: Proliferation rates in WT and KO SN56 cells. A) DAPI (blue) was used as a nuclear marker while KI-67 (green) was used as a proliferation marker. Transmitted light images allow for a visualization of what the cells look like. **B)** Statistical analysis (T-test) claim that there is no significant difference in cellular proliferation rates between WT and STIP1-KO SN56 cells (p=0.084). This experiment was performed once n=4 (4 independent cultures were grown for each cell type and 5 images were taken of each independent culture at random).

3.7 Cellular Toxicity and Viability in SN56 cells

The deletion STIP1 in mice embryos has shown to cause embryonic lethality where these embryos do not survive past E10.5 (Beraldo et al., 2013). This suggests that STIP1 is critical for mammalian cell survival during development. In order to test whether STIP1 KO would affect viability in neuronal SN56 cells, we measured cytotoxicity using a Live/Dead assay kit. We tested also if increased expression of TDP-43, which is generally toxic in cells (Wils et al., 2010), and it is observed in sporadic ALS cases, is affected by STIP1. Figure 16A is the quantification of the proportion of dead cells in SN56 WT and STIP1-KO cells in the presence of empty vectors, overexpressed WT TDP-43 and ALS-linked mutated A315T TDP-43. Sequencing of the TDP-43 in motor neuron disease (MND) patients revealed that the missense Ala-315-The (A315T) mutation was present in al MND patients but absent in all health control patients (Gitcho et al., 2008). This suggested a direct link between TDP-43 function and neurodegeneration. Mice that express this mutant form of TDP-43 also develop the fatal disease phenotypic characteristic of ALS and FTLD patients (Wegorzewska et al., 2009). This included the accumulation of toxic ubiquitinated protein in the frontal cortex and spinal motor neurons which is followed by neuronal death.

The deletion of STIP1 in SN56 cells resulted in the death of 40% of the total number of cells, which is four-fold greater than the percent of cells that are dead in their WT counterparts (10%). Overexpressing WT TDP-43 in both WT and STIP-KO cells also led to a significant increase in the percentage of cell that are dead in both cell lines (40% and 70% of cells were dead in WT and STIP1-KO cells respectively). Expressing mutant

A315T TDP-43 caused cellular death to peak at 70% in both WT and STIP1-KO SN56 cells. Staurosporine was used as a positive control for cellular death.

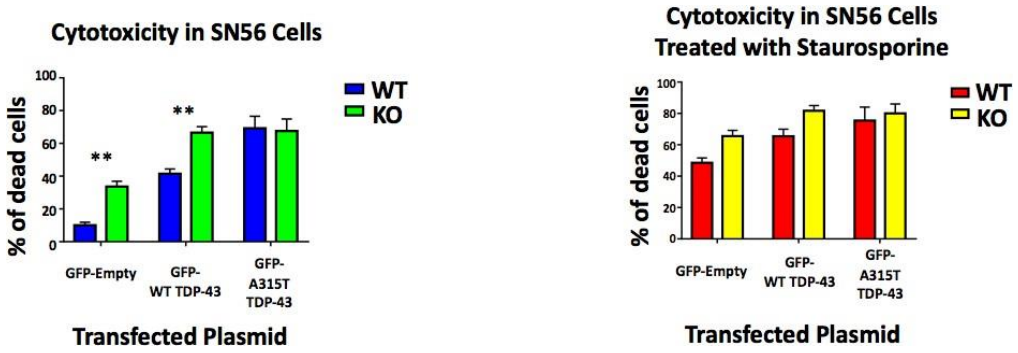
Next we measured cellular viability in the same conditions using ATP quantification (CellTiter-Glo® Luminescent Cell Viability Assay Kit). Figure 16B shows the quantification for the cellular viability relative to control cells (WT SN56 cells). The deletion of STIP1 led to a 50% decrease in cellular viability when compared to their WT counterpart. Overexpressing WT-TDP-43 in both WT and STIP1-KO cells led to a further 50% reduction in cellular viability in both cell lines (overexpressing WT TDP-43 in WT SN56 and STIP1-KO cells led to only 50% and 25% cellular viability, respectively, when compared to control). In attempt to rescue cellular viability in these cells, we overexpressed STIP1. Figure 16C is the quantification of these viability rescue experiments. Interestingly, overexpressing STIP1 and WT TDP-43 results in greater cellular viability than by only overexpressing WT TDP-43. This increase was significantly different only in WT SN56 cells, but not in STIP1-KO cells.

Lastly, we tested how various cellular stresses that are known to induce protein misfolding and aggregation into stress granules formation affect cellular viability in STIP1-KO cells when compared to WT cells (Figure 15D). The following stressors were used; Azetidine-2-carboxylic acid (AZC), MG132, radicicol and Thapsigargin. AZC is a proline homolog that incorporates into the TDP-43 polypeptide sequence, in place of proline, and promotes irreversible TDP-43 misfolding and toxicity (Dasuri et al., 2011). MG132 is a reversible inhibitor of the UPP (Homma et al., 2015). MG132 treatment in cell cultures prevents the proper degradation of misfolded proteins and leads to TDP-43 aggregation (Homma et al., 2015). Radicicol is an Hsp90 inhibitor that disturbs the proteostasis network

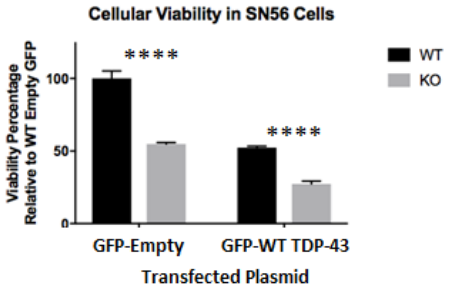
which in turn alters the stability of various Hsp90 protein leading to the accumulation of misfolded proteins (Choi et al., 2015). Lastly, Thapsigargin is an endoplasmic reticulum stressor that leads to increased ubiquitination and misfolded protein aggregation (Llieva et al., 2007). ER stress, accompanied by increased in ER chaperone is present in the spinal cord of ALS patients (Llieva et al., 2007).

Almost all stressor significantly reduced cellular viability in WT SN56 cells, with the exception of MG132. As observed above, deletion of STIP1 reduces cellular viability by 50% before any stressors are applied. Only Thapsigargin treatment reduced cellular viability in STIP1-KO cells significant more than only STIP1 deletion.

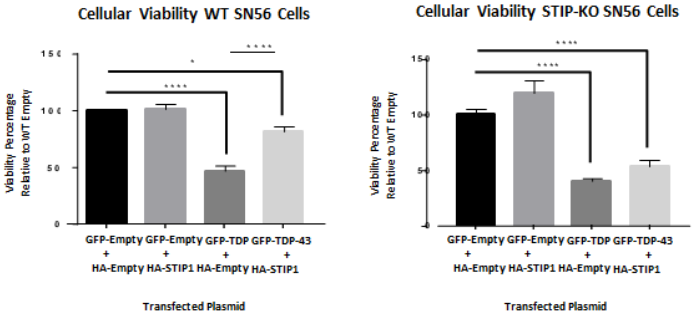
A


Cytotoxicity in SN56 Cells
% of dead cells
WT
KO
GFP-Empty
GFP-WT TDP-43
GFP-A315T TDP-43
Transfected Plasmid
Cytotoxicity in SN56 Cells Treated with Staurosporine
% of dead cells
WT
KO
GFP-Empty
GFP-WT TDP-43
GFP-A315T TDP-43
Transfected Plasmid

B


Cellular Viability in SN56 Cells
Viability Percentage Relative to WT Empty GFP
WT
KO
GFP-Empty
GFP-WT TDP-43
Transfected Plasmid

C


Cellular Viability WT SN56 Cells
Viability Percentage Relative to WT Empty
GFP-Empty + HA-Empty
GFP-Empty + HA-STIP1
GFP-TDP + HA-Empty
GFP-TDP-43 + HA-STIP1
Transfected Plasmid
Cellular Viability STIP-KO SN56 Cells
Viability Percentage Relative to WT Empty
GFP-Empty + HA-Empty
GFP-Empty + HA-STIP1
GFP-TDP + HA-Empty
GFP-TDP-43 + HA-STIP1
Transfected Plasmid

D

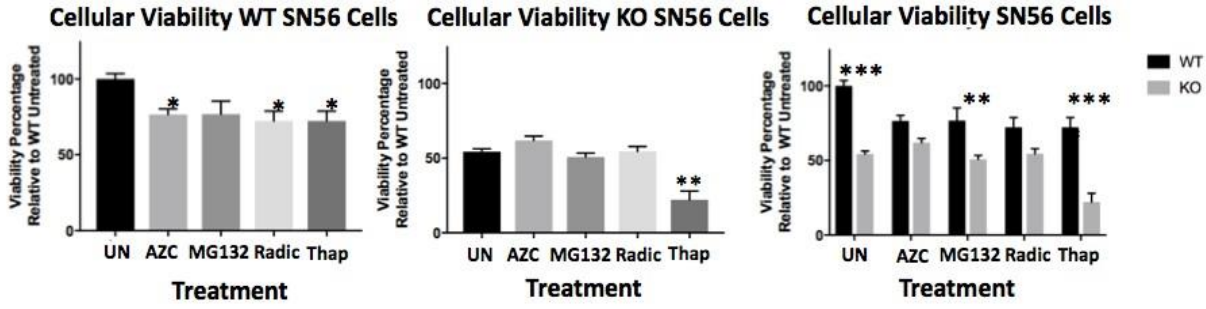
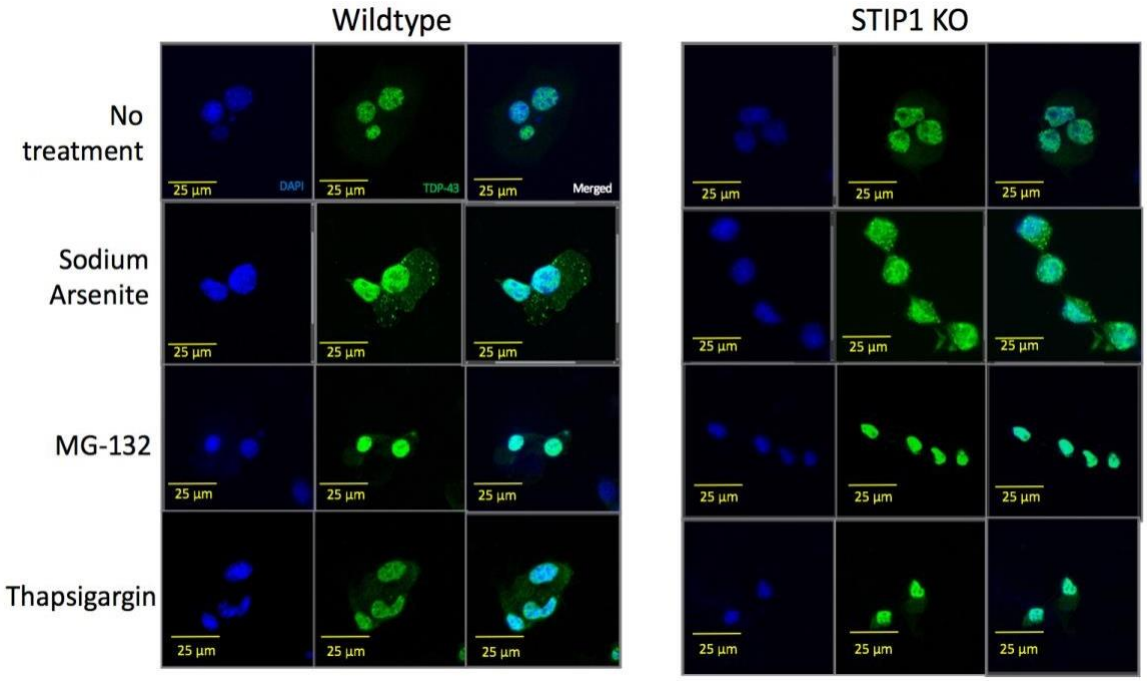

Cellular Viability WT SN56 Cells
Viability Percentage Relative to WT Untreated
UN AZC MG132 Radic Thap
Treatment
Cellular Viability KO SN56 Cells
Viability Percentage Relative to WT Untreated
UN AZC MG132 Radic Thap
Treatment
Cellular Viability SN56 Cells
Viability Percentage Relative to WT Untreated
WT
KO
UN AZC MG132 Radic Thap
Treatment

Figure 15: Cellular toxicity and viability in SN56 cells. A) SN56 WT and KO cells were transfected with GFP-Empty, GFP-WT-TDP-43 and GFP-A315T-TDP-43 mutated TDP-43. Live/Dead assay was performed to measure cellular toxicity while Staurosporine was used as a positive control. STIP1-KO cells are 50% more cytotoxic than WT cells and transfection with GFP-WT-TDP-43 adds to this cytotoxicity (two-way-ANOVA). **B)** Cellular viability was measured in SN56 cells using ATP quantification after they were transfected with HA-Empty, HA-STIP1, GFP-Empty and GFP-WT-TDP-43 vectors. STIP1-KO cells were less viable than their WT counter parts and GFP-TDP-43 transfection reduced viability by another 50% (two-way ANOVA). **C)** Overexpressing STIP1 alone had no effect on cellular viability in either cell line. Overexpressing WT TDP-43 reduced cellular viability by 50% in both cell lines. Over expressing both STIP1 and WT TDP-43 rescued cellular viability significantly in only WT SN56 cells (two-way ANOVA) **D)** In WT SN56 cells, treatment with AZC, Radicicol and Thapsigargin significantly reduced cellular viability. In STIP1-KO cells, only Thapsigargin further reduced cellular viability (in addition to the reduction caused by STIP1 deletion). Only Thapsigargin treatment was able to further reduce cellular viability in STIP1-KO cells. (one-way ANOVA). Data are Mean ± S.E.M. *$p<0.05$, **$p<0.01$, *** $p<0.0001$. This experiment was performed once, n=4 (4 independent cultures grown for each cell lines in each treatment condition).

3.8 STIP1 KO SN56 Cells are more susceptible to TDP-43 proteinopathy caused by oxidative stress

Since STIP1-KO cells had a 50% reduction in CHIP levels, which is an Hsp90 co-chaperone critical in the UPP mediated degradation of misfolded proteins, we predicted these cells to be less efficient in clearing misfolded proteins aggregates. The following cellular stress conditions were applied to these cells; sodium arsenite (oxidative stress), MG132 and Thapsigargin. Figure 16A shows neither untreated WT or untreated STIP1-KO SN56 cells displayed cytoplasmic TDP-43 aggregates. Only sodium arsenite treatment was stressful enough to elicit TDP-43 proteinopathy in SN56 cells (both WT and STIP1-KO. Figure 16B displays the quantification of the percentage of cells that are positive for TDP-43 proteinopathy. STIP1 deletion made cells more susceptible to oxidative stress – mediated TDP-43 proteinopathy resulting in a greater percentage of stress granule positive cells than compared to WT SN56 cells. This experiment was performed once, n=4 (4 independent cultures grown for each cell lines in each treatment condition).

A



B

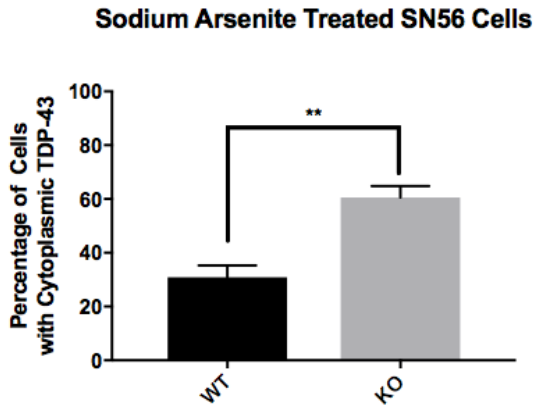


Figure 16: TDP-43 localization in WT and KO SN56 cells under various conditions. Cells were placed under 4 conditions; control, sodium arsenite (0.5 mM for 30 mins), MG-132 (20 uM for 24 hours) and Thapsigargin (10 uM for 24 hours). DAPI (blue) was used as a nuclear marker. TDP-43 (green) was predominantly nuclear except for the sodium

arsenite treated condition which induced sever TDP-43 mislocalization and aggregation. **B)** There was no cytoplasmic TDP-43 in WT or STIP-KO SN56 cells. Sodium arsenite treated caused twice as many STIP1-KO cells to have TDP-43 positive stress granules formed when compared to WT treated cells. This experiment was performed once, n=4 (4 independent cultures grown for each cell lines in each treatment condition).

3.9 Hsp90 Client and TDP-43 protein levels are reduced in STIP1$^{-/-}$ mouse Embryos

If TDP-43 is truly a Hsp90 and STIP1 client, its levels should be affected also in STIP1-null mice, which also show reduced levels of client proteins (Beraldo et al., 2013). Therefore, we tested TDP-43 levels in these embryos to determine whether the relationship that we observed in SN56 cells was upheld in a more biologically complex model (mouse embryo). Figure 17A-B are representative blots. Figure 17C compares TDP-43 mRNA expression between these embryos (qPCR). TDP-43, GRK2, GR and CHIP levels are decreased in STIP1-KO embryos. TDP-43 mRNA expression is unchanged in STIP1$^{+/-}$ KO mouse embryos but decreased by 50% in STIP1$^{-/-}$ KO mouse embryos, suggesting that during development TDP-43 message can be also regulated (only 2 embryos were used and this experiment was performed once).

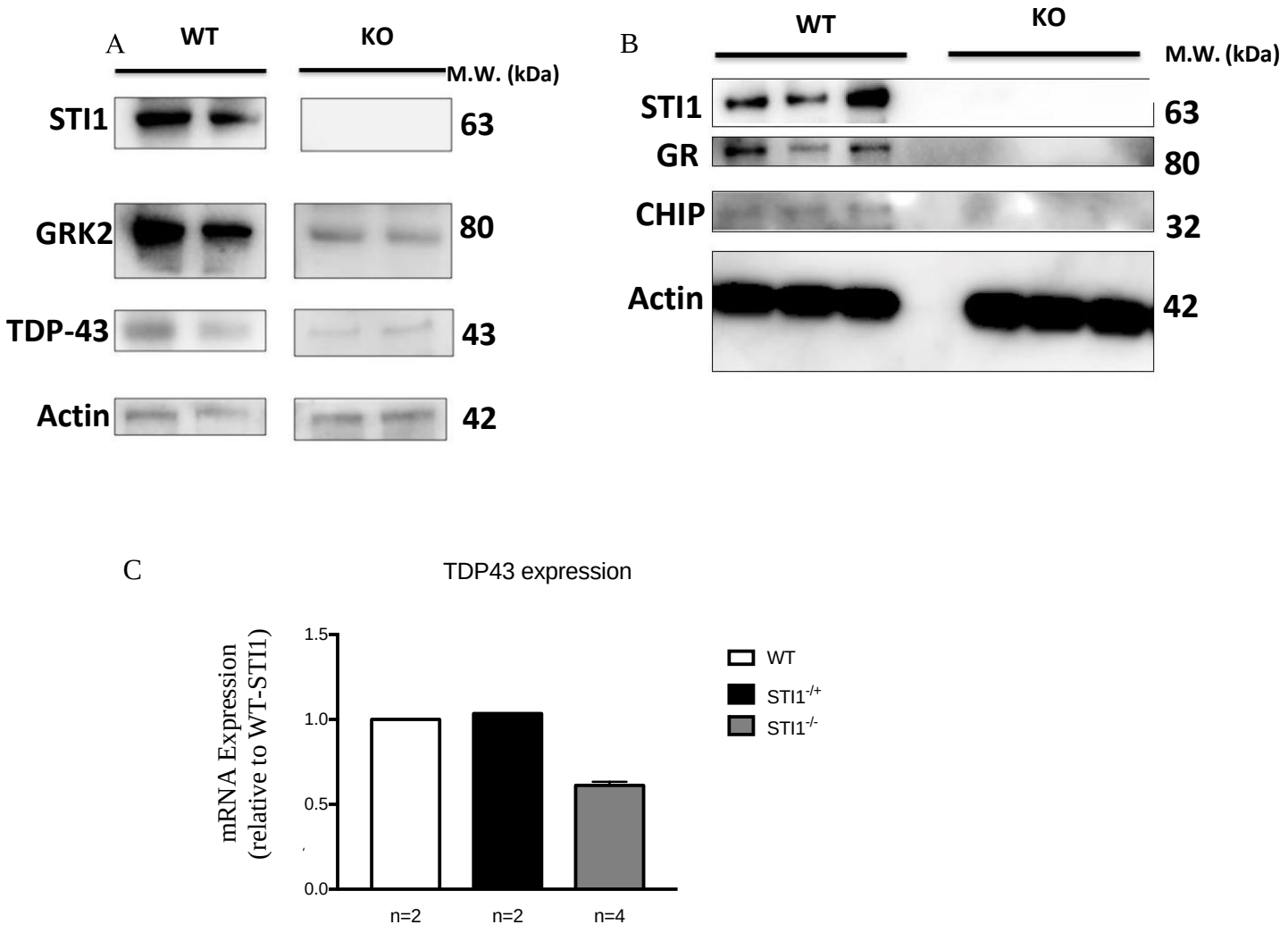


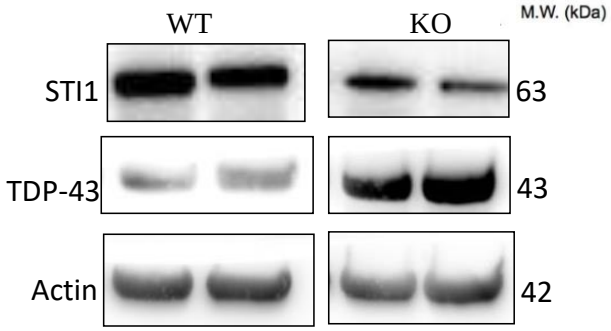
Figure 17: Hsp client and co-chaperone protein expression in mice embryos. A) 2 western blots were performed with 2 and 3 embryos ran on each gel respectively. Protein extracts were collected previously and frozen at -80ºC. In the first gel, GRK2 (Hsp90 client) and TDP-43 were both decreased significantly in the STIP1-KO embryos. n=2 (2 independent embryos of each genotype) **B)** GR (Hsp90 client) and CHIP (Hsp90 co-chaperone) were also significantly reduced in the STIP1-KO embryos. n=3. **C)** TDP-43

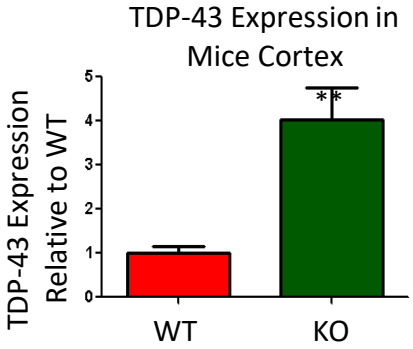
mRNA expression is reduced in STIP1$^{-/-}$ KO mouse embryos but unchanged in STIP1$^{+/-}$ KO mouse embryos.

3.10 TDP-43 Protein levels are increased in Mouse cortices and Mouse Embryonic Fibroblasts with Reduced STIP1 Levels

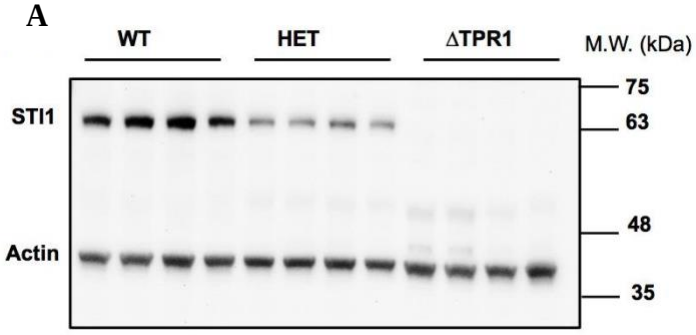
We observed that the elimination of STIP1 in SN56 cells and in mice embryos correlates with a reduction in TDP-43 protein levels (Figure 12 and 14 respectively). However, previous experiments suggested that knockdown of Hsp90 and Hsp70 induced increased TDP-43 expression and processing (Zhang et al., 2010). It is conceivable that the chaperone system may affect differently certain client proteins depending on the levels of STIP1 and functional chaperone activity. Therefore, we investigated TDP-43 levels in tissue from mice that are heterozygous for STIP1 ($STIP1^{+/-}$) and expressing a STIP1 hypomorphic allele.

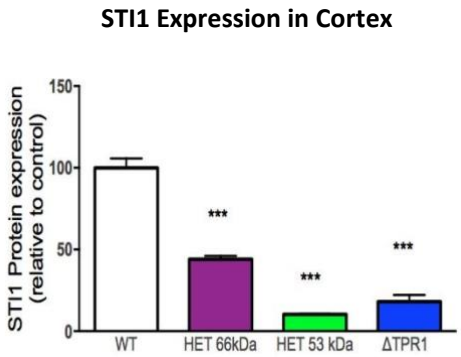
Surprisingly, tissue from $STIP1^{+/-}$ mice had a four-fold increase in TDP-43 expression (Figure 18A). The STIP1ΔTPR1, previously characterized by our lab, had a mutated 53 kDa STIP1 (lacking the ΔTPR1 domain) and a reduction in STIP1 levels (Figure 17B; Lackie et al., preprint). The $\Delta TPR1^{+/-}$ had a 50% reduction in STIP1 levels while the $\Delta TPR1^{-/-}$ had an 80% reduction in STIP1 levels (Lackie et al., preprint). Figure 17C is a representative blot and quantification comparing TDP-43 levels in WT and $\Delta TPR1^{-/-}$ mouse models. Immunoblotting revealed a 1.5-fold increase in TDP-43 protein levels in these $\Delta TPR1^{-/-}$ mice when compared to their wildtype counterparts. Mouse Embryonic fibroblasts (MEFs), collected from these ΔTPR1STIP1 also showed a similar increase (two-fold) in TDP-43 levels (Figure 17D).


WT
KO
M.W. (kDa)
STI1
63
TDP-43
43
Actin
42

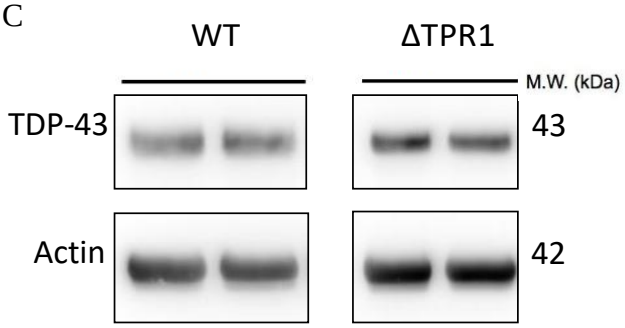

TDP-43 Expression in Mice Cortex
TDP-43 Expression Relative to WT
**
WT
KO

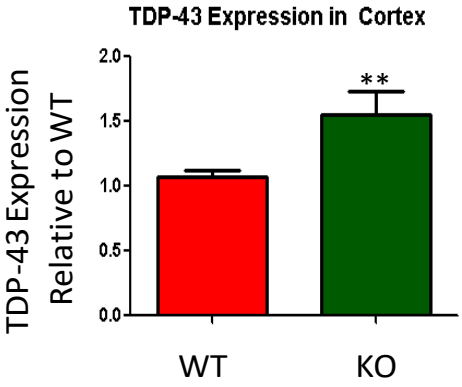
A


WT
HET
ΔTPR1
M.W. (kDa)
STI1
75
63
Actin
48
35

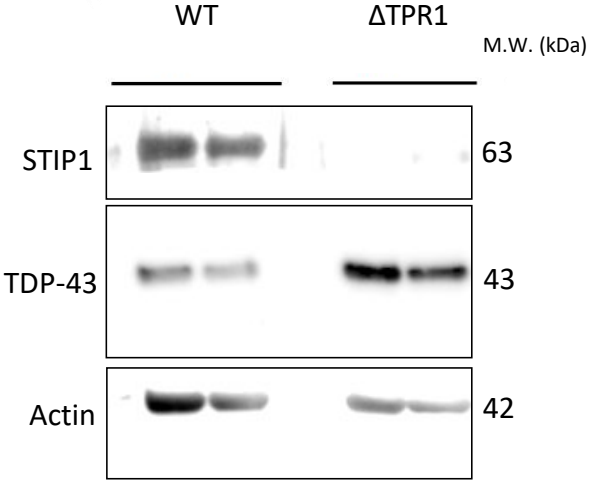

STI1 Expression in Cortex
STI1 Protein expression (relative to control)
***
***
***
WT
HET 66kDa
HET 53 kDa
ΔTPR1

C


WT
ΔTPR1
M.W. (kDa)
TDP-43
43
Actin
42


TDP-43 Expression in Cortex
TDP-43 Expression Relative to WT
**
WT
KO

D


WT
ΔTPR1
M.W. (kDa)
STIP1
63
TDP-43
43
Actin
42

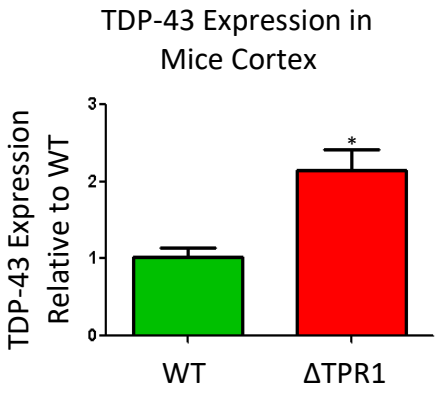

TDP-43 Expression in Mice Cortex
TDP-43 Expression Relative to WT
*
WT
ΔTPR1

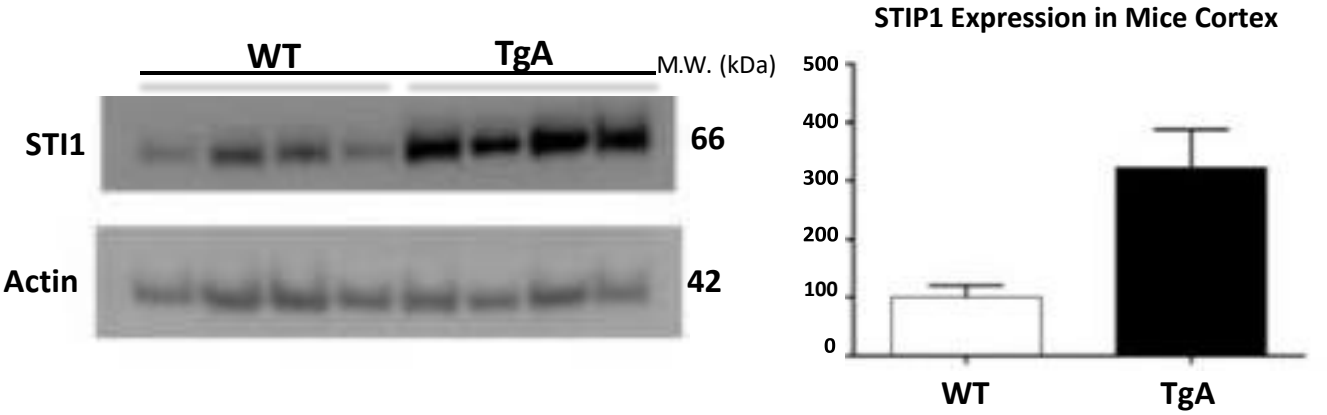
Figure 18: Levels of STIP1, and TDP-43 in mice cortices that reduced STIP1 A) Mice that were heterozygous for STIP1 knockout had a 4-fold increase in TDP-43 protein levels (T-test). **B)** Mice that were heterozygous for ΔTPR1 KO had a 50% reduction in STIP1 levels while those that were homozygous KO for ΔTPR1 had a 80% reduction in STIP1 levels (one-way ANOVA). **C)** Mice that were homozygous ΔTPR1-KO had a 1.5-fold increase in TDP-43 levels (t-test). **D)** MEFs from the homozygous ΔTPR1-KO had a 2-fold increase in TDP-43 protein levels. Data are Mean ± S.E.M. *$p<0.05$, **$p<0.01$, *** $p<0.0001$. 4 independent fibroblast cultures were grown for each genotype (n=4).

3.11 TDP-43 Levels are unaltered in the 5xFAD mouse of Alzheimer's Disease, mice with increased STIP1 levels and in 5xFAD mice with increased STIP1 Levels

Post-mortem analysis of brain tissue from AD patients shows an increase in TDP-43 protein levels and the formation of cytoplasmic TDP-43 positive stress granules in various brain regions (Nelson et al., 2007; Wilhite et al., 2017). We wanted to see whether this pattern of increased TDP-43 protein expression was also observed in the 5xFAD mouse model of AD (model with early and severe amyloid pathology) and what effect would be overexpressing STIP1 have on TDP-43 protein levels in this model. Our lab has previously characterized the STIP1TgA mice model (Figure 18A) which has a three-fold increase in STIP1 levels when compared to WT mice (Beraldo et al., 2013). The 5xFAD mice were crossed with STIP1TgA mice to obtain TgAFAD mice (AD mice model with three-fold increase in STIP1.

The 5xFAD mice model did not display the same increase in TDP-43 protein levels that was observed in AD patients (Figure 18B). TDP-43 protein levels in the STIP1TgA and the TgAFAD mice also did not differ significantly from their WT counterparts.

A



B

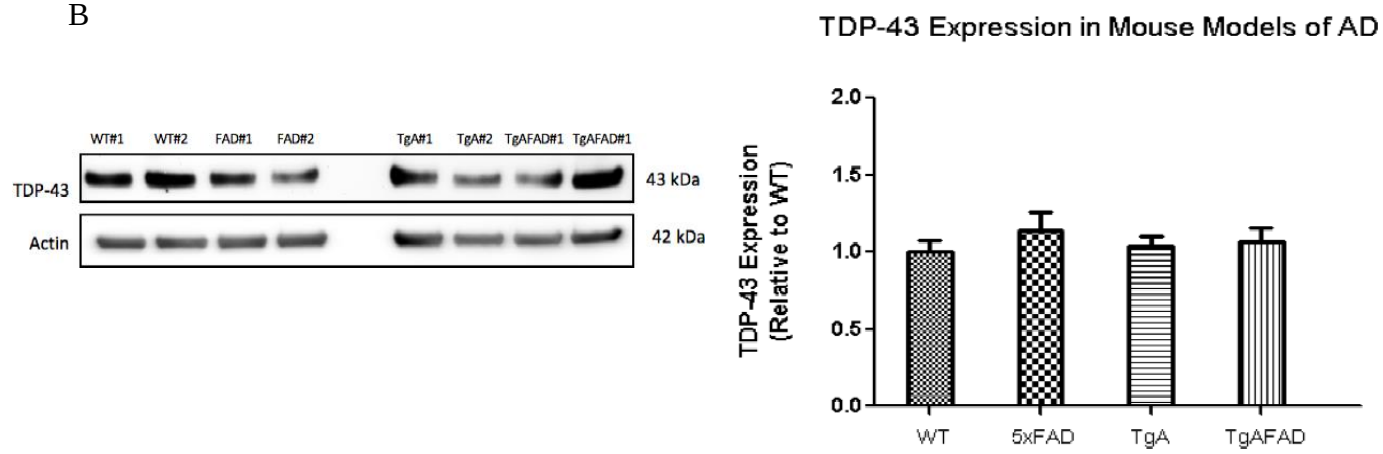


Figure 19: STIP1 and TDP-43 protein expression in the brains of various mouse models. A) STIP1 quantification in the STIP1TgA mice showed a 4-fold increase in STIP1 (T-test). **B)** Mice with increased STIP1 (STIP1TgA), AD mouse models (5XFAD) andAD mouse models with increased STIP1 (TgAFAD) had no changes in TDP-43 protein levels when compared to their wildtype counterparts. Representative blot out of 2 experiments, 4 mice from each genotype were used (n=4).

3.12 TDP-43 Localization in Nuclear in the frontal lobe of TPR1 Mice

Lastly, we tested TDP-43 localization in STIP1ΔTPR1 mice, a model with decreased STIP1 protein levels and increased TDP-43 levels. Figure 20A are representative immunofluorescence images from layer 1 of the frontal cortex of these mice models. Figure 20B is the quantification of TDP-43 localization in these brain section. Figure 20C is the quantification of TDP-43 immunoreactivity (relative to NeuN), in these brain sections. There was no difference in TDP-43 localization (all TDP-43 was nuclear) and immunoreactivity between WT mice and STIP1ΔTPR1 mice.

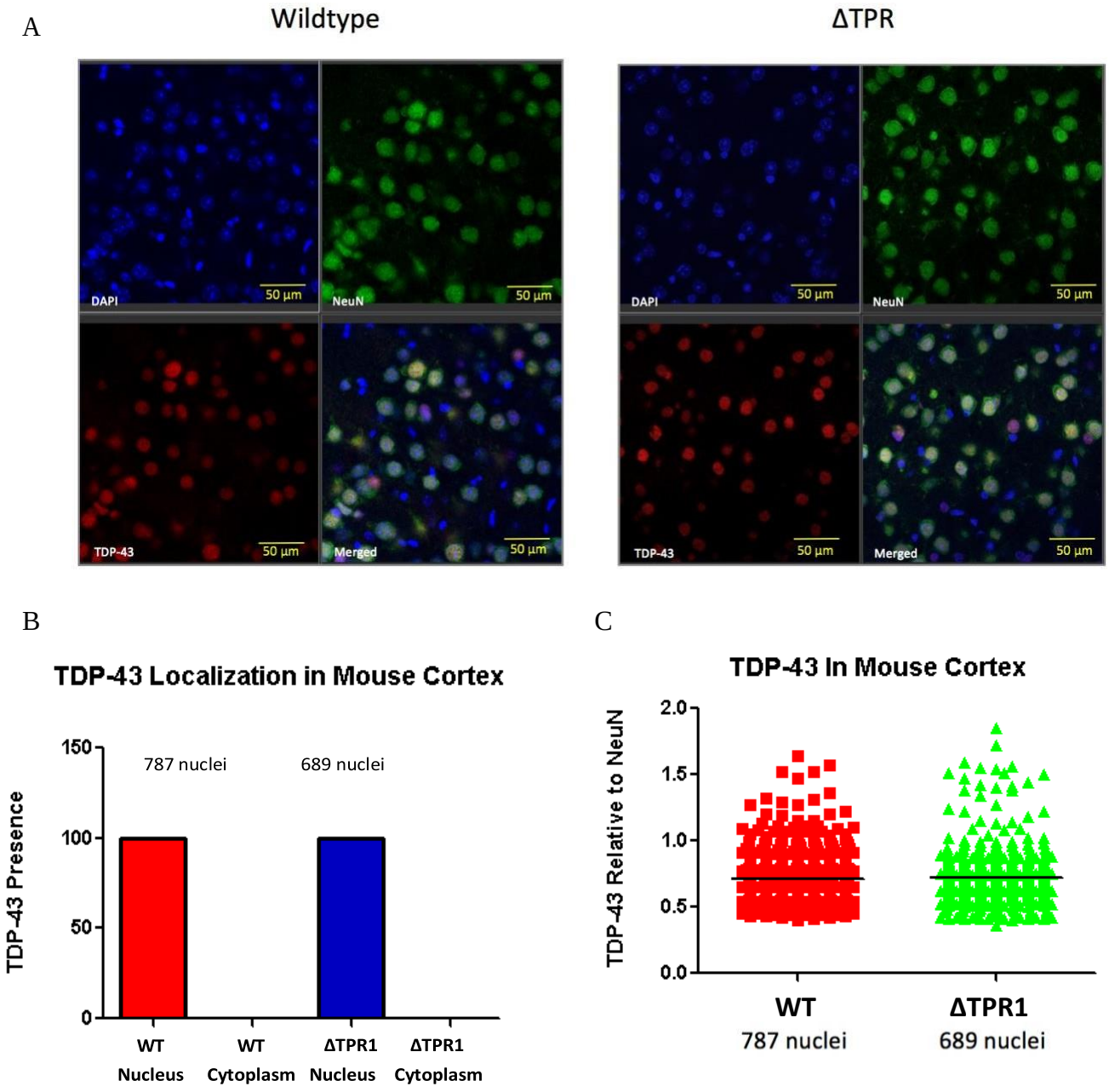


Figure 20: TDP-43 localization and intensity in WT and STIP1ΔTPR1 mice A) DAPI (blue) was used as a nuclear marker, TDP-43 (green) was the protein of interest and NeuN (red) was a neuronal nuclear marker. We collected 4 sections from 4 mice of each genotype (n=4). We took 5 random images from each section and quantified using ImageJ. **B)** Number of nuclei with nuclear and cytoplasmic TDP-43.

4 Discussion

Neurodegenerative disorders are of enormous economic and social burden globally. Current treatment options focus on treating symptoms since no cure exists. TDP-43 misfolding, hyperphosphorylation and aggregation is a pathological hallmark present in disorders such as ALS and FTLD and is believed to play a significant role in neurodegeneration (Neumann et al., 2006). Heat shock proteins have been shown to interact with proteins such as Tau and Aβ and have been identified as potential targets for treatment of neurodegenerative diseases (Karagoz et al., 2014; Wilhelmus et al., 2007). Recent studies showed that Hsp70 and Hsp90 also interact with TDP-43 and modulate its phosphorylation and protein levels (Zhang et al., 2010), however, the molecular mechanisms and the role of these interactions are unknown. Preliminary yeast studies have suggested that STIP1, a molecular co-chaperone for Hsp 70 and Hsp90, plays an important role in TDP-43 toxicity and proteinopathy. Here we demonstrated that STIP1 does indeed interact with TDP-43 and modulate its protein levels both in neuronal cell cultures and in mouse models. STIP1 is also critical for neuronal survival and viability and it seems to mitigate toxicity of overexpressed TDP-43 (Figure 7).

4.1 STIP1 is critical for mammalian cell survival

Although STIP1 is not needed for the survival of *C. Elegans,* its deletion leads to a compromised heat stress response, deficits in sexual development and a reduced life span (Gaiser et al., 2009; Song et al., 2009). Similarly, the elimination of STIP1 is not lethal in yeast but it does lead to impairments in cellular growth in stressful conditions (Flom et al., 2007; Nicolet & Craig, 1989). Interestingly, the deletion of STIP1 is lethal in mice as they are unable to survive past E10.5 (Beraldo et al., 2013). Embryonic lethality was rescued by transgenic expression of the STIP1 gene. MEFs with eliminated STIP1 were also unable to survive in culture. Furthermore, astrocytes with STIP1 haplo-insufficiency were less likely to survive after irradiation (Soares et al., 2013). Here we showed that STIP1 is critical for mouse neuronal survival and its deletion leads to a four-fold increase in the percentage of cells that are dead. Therefore, although STIP1 is not critical for *C. elegans* and yeast survival, it appears to be essential for development and cellular resilience in higher organisms.

Additionally, Beraldo et al. (2013) were able to rescue embryonic lethality in *STIP1*$^{-/-}$ mice using transgenic STIP1 expression. Similarly, when we expressed STIP1 in STIP1-KO SN56 cells, there was a tendency for cellular viability to increase. This experiment was performed once and the increase was statistically insignificant. One possible explanation is that the deletion of STIP1 in SN56 cells is so toxic that the amount of STIP1 DNA that we transfected these cells with was insufficient to significantly improve cellular viability when compared to their wild type counter parts. Therefore, future studies should aim to increase the amount of STIP1 DNA transfected into STIP1-KO SN56 cells and also treating these cultures with recombinant STIP1 and to determine if this can rescue cellular viability significantly.

4.2 TDP-43 as an Hsp90 client

Hsp90 is involved with a number of cellular functions including cell cycle progression and transcriptional regulation (Karagöz & Rüdiger, 2015). Hsp90 influences these physiological events by correctly folding, activating and transporting client proteins that are critical for these processes. STIP1 has also been shown to be a scaffold protein that brings various interaction partners together, such as glycogen synthase kinase-3 beta (GSK3β) with lysine-specific demethylase (LSD1) for cellular proliferation (Tsai et al., 2018) and Hsp90 with Piwi for developmental robustness (Gangaraju et al., 2011).

Histone LSD1 is a major epigenetic regulator that catalyzes the demethylation of the lysine 4 and lysine 9 residues on histone 3 (Shi et al., 2004). LSD1 phosphorylation has been shown to activate gene expression (Nam et al., 2014). In fact, its phosphorylation induces the acquisition of metastatic qualities in breast cancers (Feng et al., 2016). GSK3β is a serine/threonine kinase that is involved in regulating various cellular signaling pathways (Wang & Roach, 1994). Tsai et al. (2018) showed that GSK3β mediates LSD1 phosphorylation and that the STIP1-Hsp90 complex acts as a molecular scaffold that brings GSK3β and LSD1 into close proximity for their interaction to occur thus influencing cellular proliferation. Additionally, STIP1 has been shown to influence development robustness, which is the maintenance of morphological features despite genetic and environmental change, through its scaffolding function (Gangaraju et al., 2011; Rutherford & Lindquist, 1998). Piwi, which is a protein implicated in epigenetic regulation, is involved with transcriptional silencing and heterochromatin formation suggesting that it is a major buffer against phenotypical changes (Rutherford & Lindquist, 1998; Yin & Lin, 2013).

Gangaraju et al. (2011) report that STIP1 functions as a scaffold to mediate the interaction Hsp90 and Piwi thus influencing developmental robustness. They showed that through post-translational modifications, such as phosphorylation, Hsp90 regulates the interaction between Piwi and Piwi-interacting RNA (piRNA) influencing genetic expression.

Using similar co-immunoprecipitation studies, we showed that STIP1 interacts with TDP-43, Hsp90 and Hsp70. TDP-43 is a DNA and RNA interacting protein that influences genetic expression (Ou et al., 1995). Hsp90 has been shown to interact with TDP-43 previously and has also been found to influence its phosphorylation and full length expression (Zhang et al., 2010). We hypothesize that STIP1 influences TDP-43 stability by scaffolding the interaction between Hsp90 and TDP-43. Surprisingly, we also observed that STIP1 is not critical for this interaction to occur as TDP-43 continues to interact with Hsp90 when STIP1 is deleted. Even though STIP1 is not needed for the interaction between Hsp90 and TDP-43 to occur, STIP1 still plays a critical role in TDP-43 stability as its deletion lowers TDP-43 protein levels. Although STIP1 is unlikely to be a scaffold for this interaction, the deletion of STIP1 may make the TDP-43 Hsp90/Hsp70 complex dysfunctional leading to the changes in stability, cellular viability and survival.

Additionally, STIP1 acts as a critical regulatory node for the chaperone machinery that is required for the recruitment and stabilization Hsp90 client proteins (Beraldo et al., 2013; Lacke et al., preprint). It has been shown maintain the stability of cell growth factors and pathways in cancer cells (Tsai et al., 2016). Our results indicate that TDP-43 is also an Hsp90 client. Beraldo et al. (2013) observed that various Hsp90 clients were reduced in $STIP^{-/-}$ mice embryos. We also observed that GRK2 and glucocorticoid receptors, which are classical Hsp90 clients, were reduced by 50% when STIP1 was deleted in SN56 cells.

Expressing STIP1 in these STIP1-KO cells allowed us to rescue Hsp90 client levels. We observed the same pattern in TDP-43 protein expression. TDP-43 protein levels were reduced by 70% in STIP1-KO cells but this decrease was rescued when we expressed STIP1 in this cell line. We also observed that TDP-43 protein levels were reduced in STIP$^{-/-}$ mice embryos, similar to the observation of Beraldo et al. (2013) for other classical Hsp90 client proteins. Therefore, we hypothesize that TDP-43 is a Hsp90 client protein whose stability is regulated STIP1.

We also report that TDP-43 is a unique Hsp90 client as its pattern of protein expression in mice models of reduced STIP1 differ from those of classical Hsp90 clients. Lackie et al. (preprint) characterized the STIP1ΔTPR1-KO mice which has a 50% in STIP1 level in the ΔTPR1$^{+/-}$ knockout and an 80% reduction in the ΔTPR1$^{-/-}$ knockout, which presented a mutated form of STIP1. They observed that Hsp90 clients were very sensitive to this reduction in STIP1 protein levels. For example, GR was reduced in both ΔTPR1$^{+/-}$ and ΔTPR1$^{-/-}$. We observed the opposite effect of reduced STIP1 on TDP-43 in the same ΔTPR1$^{-/-}$ mouse model. TDP-43 expression was increased 1.5-fold in the ΔTPR1$^{-/-}$ when compared to its wildtype counterpart. These results suggest that TDP-43 may have a direct interaction with STIP1 which may regulate other aspects of its physiology. Similarly, mice that were heterozygous for the knockout of STIP1 had a four-fold increase in TDP-43 protein expression. These mice models may be having different effects on TDP-43 stability because they have reduced or mutated forms of STIP1 which may be compensated for by the chaperone network and genetic machinery, whereas the SN56 cells are complete knockouts of STIP1.

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4.3 STIP1 modulates TDP-43 toxicity and proteinopathy

The overexpression of the TDP-43 homolog in yeast has been shown to be toxic (Johnson et al., 2008). In rats, overexpression of human TDP-43 in the substantia nigra, using adeno associated virus vector (AAV) leads to neuronal death (Tatom et al., 2009). Interestingly, the amount of WT TDP-43 has a dose-dependent neurodegeneration effect on cortical and spinal neurons in mice (Wils et al., 2010). This dose-dependent effect was accompanied by cytoplasmic aggregates of ubiquitinated and phosphorylated TDP-43 species which are both characteristic of ALS and FTLD patients, suggesting that overexpression may overwhelm the chaperone system. Overexpression of WT TDP-43 has also been shown to induce neuronal death in various other mouse brain regions such as the forebrain (Igaz et al., 2011). These results suggest autoregulation pathways are critical in maintaining optimal TDP-43 levels and preventing neuronal death (Ling et al., 2013). Overexpression of WT TDP-43 led to apoptosis in mouse cortical progenitor cells and immature neurons (Vogt et al., 2018). In fact, the inactivation of one copy of TDP-43 in mice has no effect on mRNA or protein levels (Kraemer et al., 2010; Sephton et al., 2010). It has also been observed that TDP-43 dependent splicing of TDP-43 mRNA plays a role in mediating this autoregulation (Avendano-Vazquez et al., 2012).

Consistent with previous studies, we observed that overexpressing WT TDP-43 is toxic in mouse neuronal cell cultures. In fact, WT TDP-43 overexpression leads to a four-fold increase in the percentage of dead and a 50% reduction in cellular viability in SN56 cells. These data suggests that the maintenance of optimal TDP-43 levels is essential for cellular survival and development in both lower level and high level organisms. In addition

to autoregulation, we report that the chaperone network plays a role in maintaining TDP-43 levels. In neuronal cells that were overexpressing WT-TDP-43, the deletion of STIP1 led to a 1.5-fold increase in percentage of cells and a 50% reduction in cellular viability. Interestingly, overexpressing both WT-TDP-43 and STIP1 rescued cellular viability, which was diminished in neurons that were overexpressing only WT-TDP-43. These results suggest that STIP1 can modulate TDP-43 toxicity. Future experiments should aim to determine the mechanism by which this modulation occurs.

A common hallmark in various neurodegenerative disorders is the pathological accumulation of cytoplasmic TDP-43 (Chen-Plotkin et al., 2010; Lagier-Tourenne et al., 2010). This suggests that TDP-43 proteinopathy may contribute to the neuronal death observed in these disorders. It is unclear what causes this proteinopathy to occur. Colombrita et al. (2009) showed that oxidative stress causes TDP-43 to accumulate into cytoplasmic stress granules. Prior to this study, most researchers focused genetics and causative mutations as the causes of TDP-43 proteinopathy. Colombrita et al. (2009) proposed that environmental insults are also able induce the pathological protein aggregation observed in neurodegenerative disorders. We also observed that treating neuronal cells with sodium arsenite induced translocation of TDP-43 into cytoplasmic stress granules.

Various cellular proteins have been shown to influence TDP-43 degradation and stability through the UPP. It was recently observed that poly(A)-binding protein nuclear 1 (PABPN1), a nuclear protein involved in the post-transcriptional processing of mRNA, is potential suppressor of TDP-43 proteinopathy and toxicity (Chour et al., 2015). It was observed that overexpressing PARPN1 promotes the degradation of cytoplasmic TDP-43

present in stress granules (Chou et al., 2015). Overexpressing PARPN1 decreased cellular death while PARPN1 knockdown increased cellular dead in mouse neurons displaying TDP-43 proteinopathy (Chou et al., 2015). Lastly, they showed that PARPN1 induces misfolded TDP-43 degradation through the UPP., although the mechanism by which this occurs is unclear.

Similarly, the chaperone network recognizes misfolded proteins and facilitates their degradation through the UPP (Ciechanover & Kwon, 2015). In particular, CHIP recognizes these proteins and promotes their ubiquitination by interacting with ubiquitin ligases (Ciechanover & Kwon, 2015). We showed that the deletion of STIP1 results in a 50% reduction in CHIP protein levels. This decreased level of CHIP is expected to prevent the effective detection and degradation of misfolded proteins in STIP1-KO SN56 cells. As predicted, SN56 cells with eliminated TDP-43, and decreased CHIP levels, had a greater proportion of cells displaying TDP-43 proteinopathy in response to oxidative stress, when compared to their WT counterparts. Therefore, the deletion of STIP1 leads to less efficient degradation of misfolded protein through the UPP. Future experiments should aim to determine if overexpressing STIP1 can rescue TDP-43 proteinopathy in STIP1-KO cells, as does PARPN1.

4.4 TDP-43 Localization

The majority of ALS, FTLD and severe AD cases display TDP-43 pathology suggesting that these disorders have related origins (Mackenzie, 2007; Chen-Plotkin et

al., 2010). Various mouse models of these disorders replicate the expected pathology (Shan et al., 2009; Caccamo et al., 2010). The 3xTg AD mouse model displays tau, Aβ, and TDP-43 pathology (Oddo et al., 2003; Caccamo et al., 2010). In particular, it had increased full length TDP-43 and TDP-35 (cleaved C-terminal fragment) expression alongside increased misfolded cytoplasmic TDP-43 localization (Caccamo et al., 2010). Since the chaperone network aids in degrading misfolded proteins, overexpressing STIP1 in mouse models of TDP-43 proteinopathy may lead to the development of less severe TDP-43 proteinopathy as we observed in SN56 cells.

We crossed the 5xFAD mouse model of AD with the STIP1TgA, a mouse model with increased STIP1, to obtain 5xFADTgA which has both AD pathology and increased STIP1 protein expression. This is the first time this mouse model has been tested for TDP-43 pathology. Surprisingly, we did not find increase TDP-43 or TDP-35 protein levels in the 5xFAd as reported for the 3xTg models. This is most likely because the 5xFAD model only displays severe amyloid pathology while the 3xTg model displays both amyloid and tau pathology. This suggests that TDP-43 pathology may be linked to tau pathology since they appear to co-exist in mice models of AD. As expected, neither 5xFAD nor the 5xFADTgA mouse models had differing levels of TDP-43, when compared to control mice.

Since ΔTPR1 mice had a 40% reduction in CHIP (Lackie et al., preprint), a Hsp90 co-chaperone involved in chaperone-mediated protein degradation, and a 1.5-fold increase in TDP-43 protein expression, we expected to see TDP-43 mislocalization in the cortices of these mice. Surprisingly, the ΔTPR1 mouse model displayed no TDP-43 mislocalization, although TDP-43 levels seem to be increased.

5 Conclusion

Dysregulation of the chaperone network has been suggested to play a major role in neurodegenerative disorders. Being a master controller of the chaperone network, STIP1 plays a critical role in cellular survival and in maintaining the stability of various Hsp90 client and co-chaperone proteins. We suggest that TDP-43 is a unique Hsp90 client whose stability can be modulated by STIP1. Increased STIP1 levels can also mitigate TDP-43 toxicity making it a potential target treatments of neurodegenerative disorders. Future studies should evaluate these proposals in mouse models. Overall, our findings propose novel mechanisms by which TDP-43 pathology may develop which is a significant step towards developing effective treatments of neurodegenerative disorders.

5.1 Limitations and Future Directions

5.1.1 Hsp Clients

We performed our SN56 cell CO-IPs studies numerous times be precipitating our endogenous TDP-43. In order to confirm that TDP-43 is a STIP1 and Hsp90 client, we must try to reverse this immunoprecipitation by pulling down its interaction partners (Hsp70 and Hsp90). Additionally, our findings involving the *STIP1*$^{-/-}$ embryos were obtained in only 2 mice. Future experiments need embryos and need to be repeated multiple time. This is critical to understand how the TDP-43-STIP1 interaction varies at different

stages in the developmental cycle. Experiments could also be performed using antibodies against phosphorylated TDP-43. Immunoblots and immunohistochemistry studies need to be conducted with a positive control (eg. sodium arsenite treatment) to truly ensure that there is mislocalized or phosphorylated TDP-43 in STIP1-KO SN56 cells. Additionally, it will be a more sensitive method for detecting granules in the stress condition studies. Currently, we used a C-terminal specific TDP-43 antibody that detects both full length and cleaved TDP-43 species.

5.1.2 Rescue of Cellular Toxicity and Viability

We optimized our transfection conditions for 100 ng of DNA (per well in 96-well plate) as recommended by the Lipofectamine protocol. This amount of DNA was sufficient to rescue cellular viability in WT SN56 cells that were overexpressing WT TDP-43 but not in STIP1-KO cells that were also overexpressing WT TDP-43. Additionally, expressing STIP1 in STIP1-KO SN56 cells led to a tendency for cellular viability to increase. Future experiments need to increase the amount of DNA being transfected and see if this can rescue cellular viability. Changing the amount of DNA will require the optimization of Lipofectamine reagents. These cultures can also be treated with recombinant STIP1 in attempt to rescue viability. Extracellular STIP1 has shown to serve a protective function against protein synthesis inhibitors in cultures neurons (Lopes et al., 2005).

5.1.3 Mislocalization studies

The majority of the literature on TDP-43 focuses on proteinopathy and aggregation. Even though we see differences in protein levels, we would like to look at TDP-43 phosphorylation, and mislocalization. A interesting future experiment would be to use sodium arsenite to stress cells and then use fractional protein extractions in order to confirm that knockouts are indeed more sensitive to STIP1 deletion and proteinopathy. Additionally, we only measured the percent of cells with mislocalized TDP-43. Other measures that can be employed are cytoplasmic and nuclear intensity measures, stress granule size, co-localization measures and ubiquitination measures. These measures are much more sensitive and would elucidate the intricacies of this TDP-43-STIP1 interaction.

TDP-43 mouse models need to be used to better understand TDP-43 stability and to expand our findings to the organismal level. TDP-43 localization should be studied using immunofluorescence, immunohistochemistry and fractional protein extraction studies. Members of the molecular chaperone network should also be studied in these mouse models to determine the role they play in TDP-43 proteinopathy. These mutated TDP-43 models can be crossed with mice overexpressing and under-expressing STIP1 to see how this interaction is affected. Behavioural studies can also be conducted to determine if the neuropsychological symptoms observed in patients with neurodegenerative diseases are also present in mice and if they can be extinguished by breeding mice with increased STIP1.

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