Chapter 1

General Introduction

1.1 The Polyglutamine Diseases

The polyglutamine diseases include dentatorubral pallidoluysian atrophy (DRPLA), Huntington's disease (HD), spino and bulbar muscular atrophy (SBMA), and spinocerebellar ataxias (SCA) 1, 2, 3, 6, 7, and 17 (Nakamura et al., 2001; Ross and Poirier, 2004; Zoghbi and Orr, 2000). All of these neurodegenerative disorders are inherited in an autosomal dominant manner with the exception of SBMA, also known as Kennedy's disease, which is X-linked recessive **(Table 1-1)**. These diseases are genetically characterized by an expansion of a polyglutamine encoding and normally polymorphic CAG trinucleotide repeat in the affected gene. Interestingly, the disease genes show no homology to each other outside of the CAG repeat.

These diseases show genetic anticipation, where successive generations of affected families experience earlier age of onset and more rapid disease progression, due to intergenerational repeat instability that is particularly marked in paternal transmissions. Also, disease severity and age of onset are closely correlated with the number of CAG repeats. The length of the CAG repeat is positively correlated with disease severity and inversely correlated with age of onset. However, the clinical phenotype in and between families is often heterogeneous, perhaps due to environmental or genetic modifiers. Also, reduced penetrance has been associated with pathogenic repeat lengths near the disease-specific threshold in certain polyglutamine disorders, including HD (Nance, 1997) and SCA17 (Zuhlke et al., 2003; Oda et al, 2004). Interestingly, when the expansion becomes large and leads to severe, juvenile-onset disease, there is significant overlap in the phenotypes of these disorders. Juvenile onset of SCA1 manifests as the characteristic ataxia and brain stem dysfunction but also some cognitive impairment and dystonic

features. Juvenile-onset HD patients develop dystonia and seizures in addition to the classical phenotype of chorea and dementia seen in adult patients. This common pathology suggests that large polyglutamine repeats can also affect neuronal subtypes that are normally spared when the repeat sizes are in the moderate range. Furthermore, the massive expansion of polyglutamine repeat in the SCA7 gene can affect non-neuronal tissues, like the heart (Zoghbi and Orr, 2000).

The polyglutamine expansion is typically thought to be a gain-of-function mutation, as these disease phenotypes are not recapitulated in mouse models null for the polyglutamine proteins, while knock-in and transgenic animal models are able to accurately mimic clinical phenotypes and neuropathology of the diseases. However, it may be important to understand the normal function of the polyglutamine proteins and establish their role in pathogenesis. The function of disease proteins, SBMA and SCA17, the androgen receptor and TATA-binding protein (TBP), respectively, have been well characterized. Hence, study of how these disease proteins mediate neurodegeneration could provide insight into the pathological mechanisms of other polyglutamine diseases. Despite abundant evidence for the gain of toxic function of polyglutamine proteins, it is likely that there is partial contribution of the loss of function of the wild- type protein to pathogenesis. Notably, combined loss-of-function and gain-of-function effects have been reported in the androgen receptor (Lieberman et al., 2002) and mutant huntingtin (htt) (Cattaneo et al., 2005).

Several pathological features are shared by all nine polyglutamine diseases. They are progressive and late onset, typically striking in midlife, and initially causing neuronal dysfunction, leading to eventual neuronal loss decades after symptoms first appear. The

polyglutamine diseases cause selective neurodegeneration, despite ubiquitous expression of the affected polyglutamine proteins in the brain and other tissues. Finally, translation of the pathogenically expanded polyglutamine tract causes protein misfolding, leading to the formation of neuronal aggregates, which represent a pathological hallmark of all nine diseases. These shared features point to the potential for common mechanisms of molecular pathogenesis in the polyglutamine disorders. Elucidation of the underlying pathological mechanisms of these diseases will facilitate the development of targeted and effective therapeutic strategies to offset polyglutamine induced neurotoxicity and neuropathology.

1.2 Neuropathology of Polyglutamine Disease

Most of the polyglutamine disease proteins are widely expressed in the brain and throughout the body. Although differences in protein context of the polyglutamine proteins may account for distinct neurological phenotypes and neurodegeneration, it remains unclear as to why there is selective degeneration of neurons in polyglutamine disease. One possibility may include the selective accumulation of aggregates or inclusions that could contribute to neuronal vulnerability. These aggregates are most often neuronal, though some aggregates have been seen in peripheral tissues in SCA7 (Jonasson et al., 2002) and SBMA (Li et al., 1998). The inclusions can be localized exclusively to the cytoplasm as in SCA2 (Huynh et al., 1999) and SCA6 (Ishiwaka et al., 1999, 2001), however nuclear and cytoplasmic aggregation are evident in HD (DiFiglia et al., 1997; Gutekunst et al., 1999), DRPLA (Hayashi et al., 1998) and SBMA (Adachi et al., 2005). The presence of nuclear inclusions is age-dependent and correlate with

disease progression. Interestingly, aggregation to some degree is modulated by protein context. For instance, the most common TBP allele in Caucasians encodes for 38 glutamines in the polyQ tract (Reid et al., 2003), but a polyglutamine domain of this length in five of the other eight polyglutamine disease proteins induce aggregation and toxicity. It has been suggested that polyglutamine aggregation kinetics may underlie the correlation between repeat length and age of onset in the polyglutamine diseases (Chen et al., 2002).

Sub-cellular localization of the polyglutamine disease proteins can also affect aggregate formation, suggesting that different environments can foster or deter the formation of aggregates. For example, polyglutamine expanded ataxin-3 is a predominantly cytoplasmic protein (Paulson et al., 1997), but only forms nuclear aggregates *in vivo* (Bichelmeier et al., 2007). However, other polyglutamine disease proteins, such as mutant htt, are capable of forming aggregates inside and outside of the nucleus, and manipulation of its subcellular localization through attachment of nuclear localization signals (NLS) or nuclear export signals (NES) does not affect aggregate formation (Hackam et al., 1999). However, in the case of htt, toxicity and aggregation may be dependent on the cell type and the size or protein context of the mutant htt fragment (Hackam et al., 1999; Peters et al., 1999). Furthermore, targeting the N-terminal 171 amino acid fragment of htt containing 82 glutamines to the nucleus of neurons in transgenic mice, recapitulates the neurological phenotype caused by N-171-82Q htt, suggesting that nuclear accumulation of the polyglutamine containing fragment is sufficient to produce typical disease symptoms (Schilling et al., 2004).

Although nuclear accumulation of mutant polyglutamine proteins is a pathological feature of polyglutamine diseases, the relevance of the formation of aggregates is unclear. Studies suggest that aggregates are not required for neurodegeneration (Klement et al., 1998; Sadou et al., 1998) and they are not necessarily present in brain regions that selectively degenerate. Instead, aggregates are often found in non-affected regions of the brain. In HD, aggregates are found in the cortex (Gutekunst et al, 1999) and in striatal interneurons (Kuemmerle et al., 1999), though the major affected region in HD is the medium spiny neurons of the dorsalstriatum. Similarly, neuropathological evaluation of post-mortem brains from SCA17 patients (Fujigasaki et al., 2001) as well as other polyglutamine diseases has revealed the presence of polyglutamine aggregates in unaffected neurons (Adachi et al., 2005; Yamada et al, 2001).

1.3 Transcriptional Dysregulation in Polyglutamine Disease

Though the role of aggregates in polyglutamine toxicity remains controversial, it is clear that mutant polyglutamine proteins can abnormally interact with transcription factors in the nucleus, leading to transcriptional dysregulation (Cha, 2000; Sugars and Rubinsztein, 2003; Li and Li, 2004). Many of these transcription factors also contain polyglutamine domains or glutamine-rich regions **(Table 1-2)**. Unlike other polyglutamine proteins, TBP is exclusively an intranuclear protein, and is likely mediating pathology through its role in the nucleus. There is now evidence that all but one of the polyglutamine disease proteins can enter the nucleus (either in an unprocessed form or as a polyglutamine containing proteolytic fragment) (Riley and Orr, 2006),

suggesting that transcriptional dysregulation may be a common feature of polyglutamine disorders (Helmlinger et al., 2006). Furthermore, several studies have shown that nuclear localization of mutant polyglutamine proteins leads to neuropathology (Jackson et al., 2003; Klement et al., 1998; Peters et al., 1999; Sadou et al., 1998). However, the precise causes of transcriptional alterations and how they relate to the observed phenotype remain elusive. Based on the observation that certain transcription factors or co-factors, including CBP, TBP, TAF4 (formerly $TAFII_{130}$), and p53 (Huang et al., 1998; Nucifora et al., 2001; Shimohata et al., 2000; Steffan et al., 2000), co-localize with polyglutamine aggregates **(Table 2-2)**, a sequestration model was originally proposed **(Fig. 1-1b)**. Specifically it has been argued that the recruitment and concomitant entrapment of key transcription factors into nuclear inclusions could explain pathogenically relevant changes in gene expressions.

However, it has become increasingly clear that neither neuronal dysfunction (Klement et al., 1998; Saudou et al., 1998) nor transcriptional dysregulation (Hoshino et al., 2004; Obrietan and Hoyt, 2004) are contingent on the presence of polyglutamine aggregates. The sequestration model relies mostly on co-localization data derived from cell models, in which the polyglutamine proteins are overexpressed and rapidly form inclusions. However, the formation of nuclear polyglutamine inclusions in the brain is an age-dependent and slow process. Furthermore, immunohistochemical examination of postmortem brains from HD patients and electron microscopy studies of HD mice have not substantiated the idea that nuclear inclusion recruitment can reduce the level of transcription factors (Dunah et al., 2002;Yu et al., 2002). An alternative explanation to explain transcriptional defects is the possibility that soluble polyglutamine expanded

proteins are sufficient to induce transcriptional dysregulation **(Fig. 1-1c)** (Li et al., 2002; Yu et al., 2002).

Transcriptional dysregulation has been most extensively studied in HD (Cha, 2000). Interaction studies, including co-immunoprecipitation and *in vitro* binding studies have demonstrated that the soluble version of mutant htt can be distinguished from normal htt by a high affinity for certain transcription factors such as TBP and Sp1 (Dunah et al., Li et al., 2002; Shaffar et al., 2004). The aberrant interaction undermines the normal function of the transcription factor, compromising its capacity to bind to its cognate promoter sequence. Given that a variety of genes are dependent on TBP and Sp1-mediated expression, as well as other transcription factors, it is expected that the nuclear accumulation of polyglutamine proteins man have a significant imapct on gene transcription, as a large number of genes are altered in HD mouse brains, and down-regulation is more prominent that up-regulation (Luthi-Carter et al., 2002).

Certain transcription pathways, namely those involving the cAMP response element (CRE)- binding protein (CREB) and Specificity protein-1 (Sp1), have been implicated in the pathogenesis of multiple polyglutamine diseases. Various components of these pathways have been reported to interact with soluble and/or aggregated polyglutamine-expanded proteins **(Table 1-2)**. Reporter assays carried out in cell models of certain polyglutamine disorders indicate that expanded polyglutamine proteins inhibit both CRE-mediated (Shimohata et al., 2002; Nucifora et al., 2001) and Sp1-dependent transcription (Li et al., 2002; Dunah et al., 2002). Overexpression of the co-factor CREB-binding protein (CBP) (Nucifora et al., 2001) can rescue CRE-mediated transcription,

while overexpression of Sp1 and TAF4 can inhibit the effect of mutant htt on Sp1-dependent reporter activity (Dunah et al., 2002).

There is also some evidence to suggest that basal transcriptional dysregulation, possibly facilitated by neuronal-specific and polyglutamine-mediated changes in the histone code, may also be relevant to polyglutamine induced pathology (Hoshino et al., 2004). Strikingly, histone acetylation is disrupted in the presence of mutant polyglutamine proteins (Bodai et al, 2003) and it has been demonstrated that histone deacetylase (HDAC) inhibitors can attenuate polyglutamine- induced toxicity in cell and animal models of polyglutamine disease (Ferrante et al., 2003; Hockly et al., 2003; Steffan et al., 2001). Hypoacetylation of H3 and H4 has been documented in multiple polyglutamine disease models (McCampbell et al., 2001; Steffan et al., 2001) and acetylation of lysine residues on histone H3, K9 and K14, may be necessary for the recruitment of the transcription factor complex, TFIID to promoters (Agalioti et al., 2002). Accordingly, polyglutamine induced histone deacetylation might inhibit this crucial step in the formation of the preinitiation complex at certain RNA polymerase II promoters. It should also be noted that one of the polyglutamine disease proteins, TBP, is a general transcription factor and was recently implicated as the cause of the newest polyglutamine disorder, SCA17 (Nakamura et al., 2001). The study of SCA17 could lend insight into the molecular mechanisms of transcriptional dysregulation in polyglutamine disease.

1.4 Spinocerebellar Ataxia 17

TBP was first found to be associated with nuclear aggregates in several polyglutamine disorders (Perez et al., Uchihara et al., 2001; van Roon-Mom et al., 2002). Subsequently, it was found that TBP itself could cause neurodegeneration when it underwent expansion of the N-terminal polyglutamine tract, leading to a newly discovered spinocerebellar ataxia (Koide et al., 1999; Nakamura et al., 2001), Spinocerebellar Ataxia 17 (SCA17). Given the critical and ubiquitous involvement of TBP in transcription, the neurological phenotype of SCA17 is likely mediated by transcriptional dysregulation. Because TBP is the best characterized in terms of its normal function, SCA17 serves as an ideal model to investigate the effect of polyglutamine expansion on normal protein function and its role in the molecular pathogenesis of polyglutamine disease. Also, SCA17 serves as a model to better understand transcriptional dysregulation, a pathological mechanism involved in the neuropathology of polyglutamine disease.

Expansion of the CAG repeat in TBP was first identified in a single patient presenting with ataxia, prominent cerebellar atrophy, short stature, atypical absence, and pyramidal sign and mental deterioration (Koide et al., 1999). The patient had no family history of neurological disease, but had an expanded CAG repeat in the TBP gene coding for 63 glutamines, far exceeding the range of CAG repeats in normal individuals (25-42 CAGs). The expansion was a *de novo* mutation resulting from paternal transmission of a TBP allele with 39 CAG repeats. The mutation in the polyglutamine tract of TBP can present with different genotypes in different families. The basic structure of the repeat is $(CAG)_3$ $(CAA)_3$ $(CAG)_{7\text{-}11}$ CAA CAG CAA $(CAG)_{9\text{-}22}$ CAA CAG (Gostout et al., 1993). The most frequent occurring expansion of CAG triplets is in the section that can

vary from 9-22 repeats (van Roon-Mom et al., 2005). CAG repeat instability has been seen in both germline and somatic tissues (Zoghbi and Orr, 2000), however, interruption of the CAG tract with synonymous CAA codons confers stability to the repeat, which is resistant to changes in size during meioses (Reid et al., 2003). However, loss or mutation of interupting CAA codons can lead to genomic instability of the repeat. For example, loss of the CAA CAG CAA segment that separates the two polymorphic CAG stretches has been detected in some mutant *TBP* alleles, and is associated with intergenerational repeat instability (Zuhlke et al., 2001; Maltecca et al., 2003; Zuhlke et al., 2005). This phenomenon is also seen in SCA1 and SCA2 where a lack of CAA repeat interruptions results in an instability or expansion of the number of glutamines in the protein (Sobczak and Krzyzosiak, 2004). However, the most common form of mutation in TBP involves pathogenic expansion of the longest homogenous CAG segment without any alteration of repeat structure (van Roon-Mom et al., 2005) protecting these SCA17 pedigrees from intergenerational repeat instability. The mechanism of repeat expansion in polyglutamine proteins is unclear but may involve unequal crossing over during meiosis, or DNA replication, repair or transcription pathways (Bowater and Wells, 2001).

The normal range of the polyglutamine tract in TBP is 25-42 glutamine residues (Gostout et al., 1993), however expansion past this range does not necessarily cause disease. Incomplete or reduced penetrance has been seen in the intermediate disease allele range of 43-48 (Nakamura et al., 2001; Silveira et al., 2002; Rolfs et al., 2003; Oda et al., 2004; Zuhlke et al., 2001, 2003, 2005). Though repeat size is closely correlated with age of onset in other polyglutamine disorders, this is not the case for SCA17. Approximately 45% of the variation in the age of onset in SCA17 can be explained by the

number of CAG/CAA repeats in TBP, whereas close to 70% of the variation in age of onset has been calculated for HD, SCA1, SCA6 and SCA7 (Zoghbi and Orr, 2000). This suggests that there may be genetic modifiers that contribute to age of onset for the disease, as this is the case in SCA3 (van de Warrenburg et al., 2005), which also has a reduced correlation between repeat size and age of onset.

Clinical symptoms of SCA17 typically include ataxia, dystonia, parkinsonism, dementia, psychiatric abnormalities, seizures, and early death (Koide et al., 1999; Nakamura et al., 2001; Toyoshima et al., 2004). The affected area of SCA17 is the cerebellum as evidenced by cerebellar atrophy, pronounced Purkinje cell loss and reactive gliosis in SCA17 patients (Koide et al., 1999; Nakamura et al., 2001; Fujigasaki et al., 2001; Zuhlke et al. 2003; Toyoshima et al., 2004). There is a great deal of clinical heterogeneity in the SCA17 phenotype, which can often resemble the phenotype of HD (Nakamura et al., 2001; Bauer et al., 2004; Toyoshima et al., 2004; Schneider et al., 2006)), suggesting an overlap in phenotype in these two polyglutamine diseases. The prevalence of SCA17 remains low in all populations studied thus far (Sasaki et al., 2003; Alendar et al., 2004; Brusco et al., 2004; Craig et al., 2005; Jiang et al., 2005), likely due to the crucial role TBP plays in cellular function. However, homozygous cases of SCA17 have been identified (Zuhlke et al., 2003; Toyoshima et al., 2004) and an expansion in the polyglutamine tract in both *TBP* alleles does not result in embryonic lethality, indicating that the normal function of the protein during development is not impaired by expanded polyglutamine repeats.

1.5 Structure and Function of TATA-binding Protein

TBP plays an essential part in the initiation of transcription of all three eukaryotic polymerases (Gill and Tjian, 1992). RNA Polymerase II (Pol II) synthesizes predominantly mRNA and there are two groups of transcriptions factors involved in Pol II transcription, those that are essential for transcription of all Pol II genes, and those that are sequence specific for only a small subset of genes. The general transcription factors, which include TBP, form a preinitiation complex with Pol II and bind to the TATA box (Roeder, 1991). Other specific transcription factors upstream of the promoter site are needed for proper transcription levels (Buratowski et al., 1989).

In eukaryotes, the core promoter serves as a platform for the assembly of the transcription preinitiation complex (PIC) that includes TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and RNA polymerase II (Thomas and Chiang, 2006). These protein complexes function collectively to initiate transcription from a given promoter. TBP is part of a larger complex, TFIID, and acts as the DNA binding element of the TFIID complex. TBP binds to the TATA box, which has a consensus sequence of TATAAA (Butler and Kadonaga, 2002). This A-T- rich conserved sequence is approximately 28-34 nucleotides upstream from the start site of transcription. (Sandelin et al., 2007). The formation of this preinitiation complex is dependent on the interaction of TBP at the TATA box (Fire et al., 1984; Peterson et al., 1990). The general transcription factor TFIID is the first to bind to the TATA box and this step is essential for initiating transcription by Pol II (Sharp, 1992; Gostout et al., 1993). DNA binding is followed by the entry of other general transcription factors and RNA Polymerase through either a sequential assembly or a preassembled holoenzyme pathway. Formation of this complex is sufficient for a basal level of transcription. However, for regulated transcription, co-

factors are often required to transmit regulatory signals between gene-specific activators and the general transcription machinery (Davidson, 2003). *In vivo*, TBP is tightly associated with specific transcription factors that direct it to a particular class of promoters. Upon binding of TBP to the TATA box, the DNA is bent and this bending appears to regulate transcription (Tenharmsel and Biggin, 1995) and contribute to the selective binding of TBP with different binding sites (Starr et al., 1995).

TBP is also involved in basal transcription from TATA-less promoters. Transcription from these sites uses the same general transcription factors as promoters with a TATA element, however, it is clear that TBP plays a different role at TATA-less promoters, as it is not the rate limiting step in initiation of transcription (Colgan and Manley, 1992). Besides the upstream promoter site, a downstream basal promoter element has been identified approximately 30 nucleotides downstream of the start site. This element is thought to provide a binding site for TFIID in the absence of a TATA box (Burke and Kadonaga, 1996). TFIID binds this region via one or more of its TBP associated factors (TAFs), and TBP is thought to play a role in positioning the polymerase correctly on the DNA. TAFs are thought to mediate the stable association of TBP with the promoter at TATA-less promoters (Pugh and Tjian, 1991).Though, there is also some evidence suggesting that the DNA binding capability of TBP is dispensable at TATA-less promoters (Martinez et al., 1994), and TFIID may instead act as a TAF.

TAFs are required for transcription and to determine promoter specificity (Hoffman et al., 1990; Peterson et al., 1991; Martinez et al., 1995). It is known based on the structure of several different TFIID complexes, that TBP can form functional protein complexes with different sets of TAFs (Timmers and Sharp, 1991) and that the TAFs are

essential for binding activator proteins (Kobayashi et al., 1995). This allows TBP specificity in initiation of transcription, even though it is a general transcription factor.

TBP is a bipartite structure, consisting of a highly conserved C-terminus and an evolutionary divergent N-terminus. The C-terminus participates in all transcriptional activities, including DNA binding, dimerization, and various transcriptionally relevant protein-protein interactions. The C-terminus consists of 180 residues that are arranged in two direct repeats interrupted by a lysine-rich segment **(Fig. 1-2)**. These repeats allow the C-terminus to fold into a saddle shaped tertiary structure in order to bind DNA. When TBP binds to the TATA box , it distorts the DNA by inserting amino acid side chains between the base pairs, partially unwinding the helix, and doubly kinking it. The distortion is accomplished through a great amount of surface contact between TBP and the DNA. TBP binds to the negatively charged phosphates in the DNA backbone through its positively charged lysine and arginine amino acid residues. Binding of TBP to the minor groove produces a sharp bend in the DNA (Pugh, 2000) that is produced through projection of four bulky phenylalanine residues into the minor groove. As the DNA bends, its contact with TBP increases, thus enhancing the DNA-protein interaction. The strain imposed on the DNA through this interaction initiates melting of the strands. Because this region of DNA is rich in adenine and thymine residues, which base pair through only two hydrogen bonds, the DNA strands are more easily separated. Separation of the two strands exposes the bases and allows RNA polymerase II to begin transcription of the gene. The concave surface of the C-terminus is comprised of 5 β sheets on each side, and interacts with the promoter DNA while specific residues in the alpha helices that cover the convex surface are involved in interactions with general transcription

factors and upstream transcriptional activators (Burley and Roeder, 1996; Davidson, 2003).

The TBP N-terminal region varies greatly between eukaryotes (Hernandez 1993), however, the sequence of this domain is largely conserved among vertebrates (Hernandez 1993; Sumita et al. 1993; Nakashima et al. 1995; Shimada et al. 1999). Conversely, TBP sequences from nonvertebrates lack nonrepetitive sequences resembling the vertebrate N-terminus (Lichtsteiner and Tjian 1993; Muhich et al. 1990).The vertebrate TBP N-terminus can be divided into four subdomains **(Fig. 1-2)**. Two of these domains do not resemble each other or any other known proteins and flank a central glutamine-rich repeat region. Near the junction with the conserved C-terminus is an imperfect repeat region consisting of sequence, $(Pro\text{-}X\text{-}Thr)_n$, where X is generally Met, Ala, or Ile. This repeat exists in most or all metazoan TBPs, and it is thought that it serves as a connector between the N- terminus and the universal functions of the C-terminus (Bondareva and Schmidt, 2003). The N-terminus may function as a signaling domain by which the basal transcription machinery receives specific regulatory signals and that variation within this region represents species specific gene regulation. However, replacement of most of the N-terminus with a FLAG epitope does not result in embryonic lethality (Hobbs et al., 2002) or massive transcriptional dysregulation (Schmidt et al., 2003) as one might predict based on the absence of related sequences from lower eukaryotes.

The function of the N-terminus remains unclear, though this region is thought to modulate the DNA binding activity of the C-terminus. Modulation of DNA binding affects the rate of transcription complex formation and initiation of transcription. The N-terminus of human TBP (hTBP), and in particular the polyglutamine domain, has been

implicated in transcriptional regulation. The relatively long polyglutamine domain can activate transcription, either by itself or in the context of the intact N-terminus, when fused to a heterologous DNA-binding domain (Gerber et al., 1994; Seipel et al., 1993). Furthermore, a monoclonal antibody against the polyglutamine domain of hTBP can block basal transcription from TATA containing promoters *in vitro* (Lescure et al., 1994), suggesting that the N-terminal polyglutamine domain may be an important interface for transcriptionally relevant protein-protein interactions. However, very few proteins are known to interact with either the polyglutamine domain or any other domains of the N-terminal region of TBP (Das and Scovell, 2001; Mittal and Hernandez, 1997), thus making it unclear what role the polyglutamine domain plays in transcriptional regulation.

Table 1. Polyglutamine Disease Proteins

Diseases	Normal Repeats	Expanded Repeats	Protein	MW
HD	6-35	36-150	Huntingtin	348
DRPLA	3-36	49-88	Atrophin	124
SCA-1	6-39	40-83	Ataxin-1	87
SCA-2	14-32	33-77	Ataxin-2	90
SCA-3	12-40	55-86	Ataxin-3	42
SCA-6	4-18	21-30	P/Q Ca Channel	large
SCA-7	7-17	38-130	Ataxin-7	95
SBMA	9-36	38-62	AR	99
SCA17	25-42	46-66	TBP	38

Table 2-2. Transcription Factors Known To Bind Polyglutamine Proteins

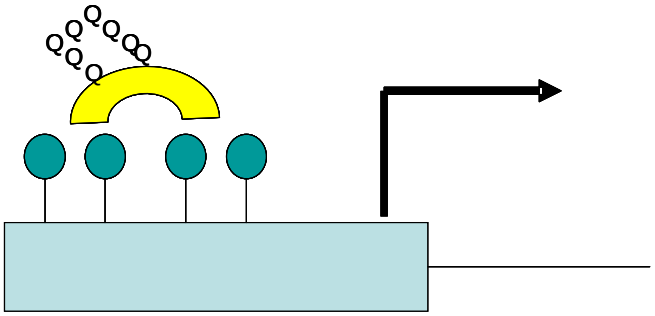
Transcription factor or co-factor	Interacting polyQ disease protein	Co-localizes with polyQ aggregates	Binds soluble polyQ protein	Reference
CA150	htt	+	+	Holbert et al., 2001
CBP	htt, atrophin-1, AR, ataxin-1, ataxin-3, ataxin-7, TBP	+	+	Nucifora et al., 2001; Steffan et al., 200; McCampbell et al., 2000; Chai et al., 2001; Stenoien et al., 2002; Swope et al., 1996; La Spada et al., 2001
CREB	atrophin-1, ataxn-3		+	Shimohata et al., 2000
Crx	ataxin-7		+	Chen et al., 2004
CtBP*	htt		+	Kegel et al., 2002
ETO/MTG8*	atrophin-1		+	Wood et al., 2000
HYP-B	htt		+	Faber et al, 1998
mSin3a*	htt	+		Boutell et al., 1999
NCoR*	htt		+	Boutell et al., 1999
NF-κ B	htt, TBP		+	Takano and Gusella, 2002; Schmitz et al., 1995
p53	htt, TBP	+	+	Steffan et al., 2000; Truant et al., 1993
p300	ataxin-3, TBP		+	Li et al., 2002a; Swope et al., 1996
P/CAF	ataxin-3, htt		+	Steffan et al., 2001; Li et al., 2002a
REST/NRSF*	htt, TBP		+	Zuccato et al., 2003; Murai et al., 2004
SMRT*	ataxin-1	+	+	Tsai et al., 2004
Sp1	htt, TBP, atrophin-1, ataxin-3	+	+	Shimohata et al., 2000; Dunah et al., 2002; Li et al., 2002b; Emili A, 1994
TAF4	htt, atrophin-1, ataxin-2, ataxin-3	+	+	Shimohata et al., 2000; Dunah et al., 2002
TAF10	ataxin-7	+		Yvert et al., 2001
TBP	htt, atrophin-1, ataxin-3	+	+	Huang et al., 1998; Schaffar et al., 2004; Shimohata et al., 2000

* denotes repressor or co-repressor activity
bold font indicates the presence of a polyglutamine or glutamine-rich domain in the transcription factor

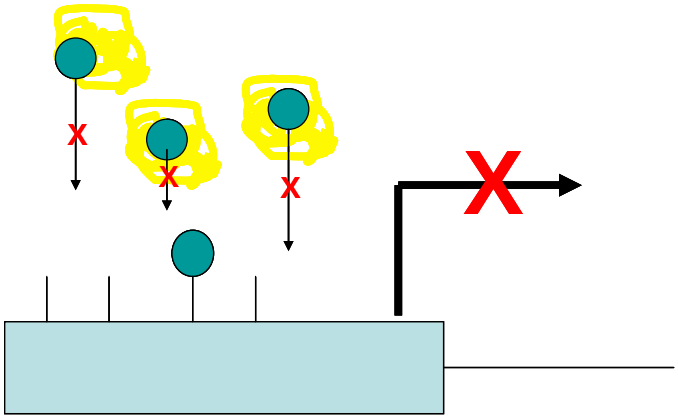
Abbreviations: AR, androgen receptor; CA 150, co-activator 150; CBP, (cAMP-response element binding protein) binding protein; CtBP, C-terminal binding protein; CREB, cAMP-response element binding protein; Crx, cone-rod homeobox containing gene; HYP-B, htt-yeast partner; mSin3a, mammalian Sin3 protein-A; MTG8, myeloid translocation gene on 8q22; NCoR, nuclear receptor co-repressors; NF-κB, nuclear factor – κB; REST/NRSF, repressor element-1 transcription factor/neuron restrictive silencer factor; SMRT, silencing mediator of retinoid and thyroid hormone receptors; Sp1, specificity protein-1; TAF4, TBP- associated factor4; TAF10, TBP- associated factor 10; TBP, TATA-box binding protein

Figure 1-1

A



B



C

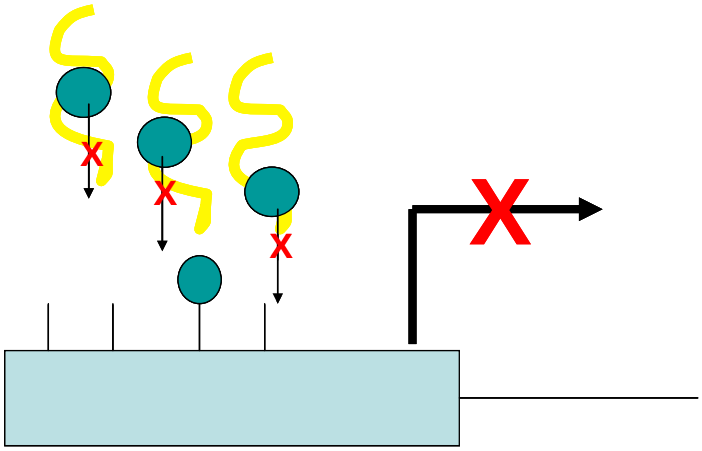


Figure 1-1. Sequestration models of toxicity. (a) Model showing transcription under normal conditions, where the polyglutamine protein (yellow shape) is in the nucleus, but is not inhibiting binding of transcription factors (blue circles) to their cognate promoters, allowing for proper transcription and gene expression. **(b)** Model showing polyglutamine aggregates (yellow shape) sequestering transcription factors (blue circles) away from the cognate promoter sequence, thereby dysregulating transcription from that promoter. **(c)** Model showing soluble polyglutamine expanded protein (yellow shape) sequestering transcription factors (blue circles) away from the cognate promoter sequence, thereby dysregulating transcription from that promoter, in the absence of aggregates.

Figure 1-2

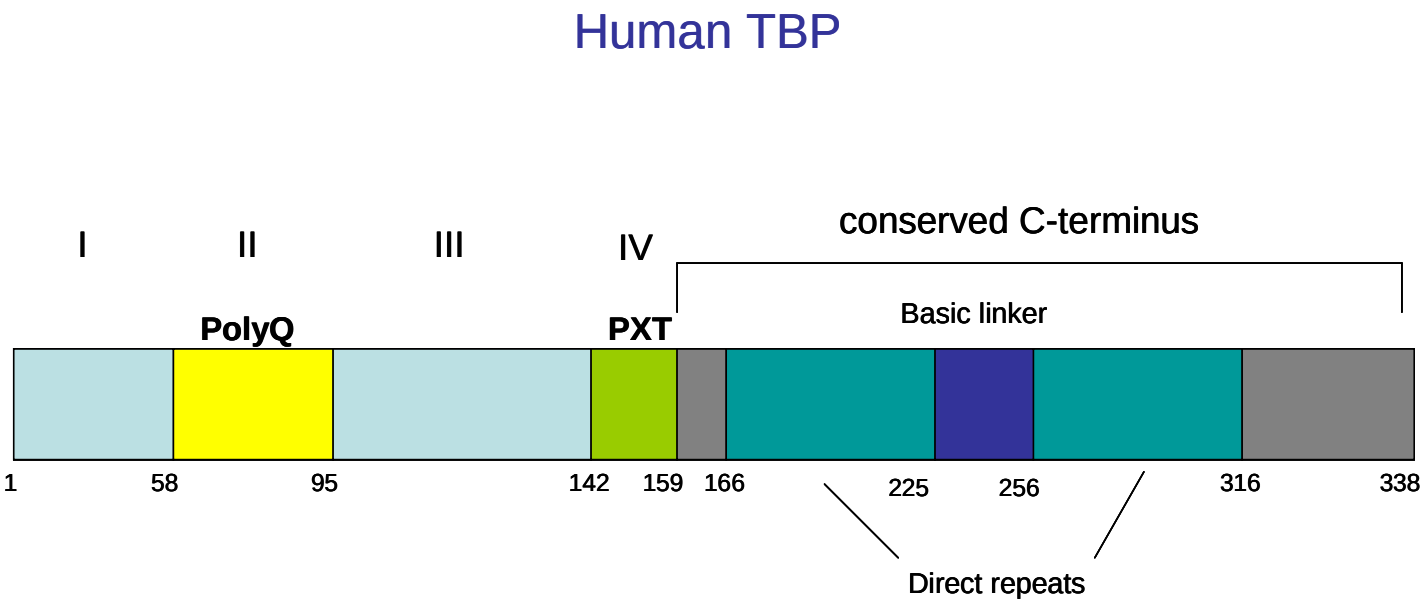

Human TBP
I
II
III
IV
conserved C-terminus
PolyQ
PXT
Basic linker
1
58
95
142
159
166
225
256
316
338
Direct repeats

Figure 1-2. Schematic representation of Human TBP. The primary structure of TBP is shown. Regions I-IV represent the N-terminus, including the polyglutamine domain (II), which is polymorphic and contains 25-42 glutamines in normal individuals, while Region IV comprises the imperfect PXT repeat. Regions I and II are non-conserved and show no homology to each other or any other known protein. The C-terminus includes two direct repeats separated by a basic region. The amino acid numbering is based on an individual with 38 glutamine residues.

Chapter 2

Characterization of a PC12 Cell Model of SCA17

This chapter includes findings and discussions previously published as Friedman MJ, Shah AG, Fang ZH, Ward EG, Warren ST, Li SH, and Li XJ. Polyglutamine domain modulates the TBP-TFIIB interaction: implications for its normal function and neurodegeneration. *Nat. Neurosci.* 10, 1519-28 (2007) and a manuscript currently submitted as Shah AG, Friedman MJ, Roberts M, Li XJ, and Li SH. Transcriptional dysregulation of TrkA associates with neurodegeneration in Spinocerebellar Ataxia 17. *Hum. Mol. Genet.* (2009). Constructs for generation of cell model were designed by Shihua Li and Xiao-Jiang Li. Human HSPB1 construct was cloned by Meyer Friedman. TBP adenoviral vectors were made by Welgen, Inc. All other experiments were carried out by Anjali Shah. Xiao-Jiang Li participated in the writing and editing of the published and submitted manuscript.

2.1 Abstract

Spinocerebellar Ataxia type 17 (SCA17) is one of nine dominantly inherited neurodegenerative diseases caused by a polyglutamine expansion in the affected protein. SCA17 is characterized by an N-terminal polyglutamine expansion in the TATA-binding protein (TBP). To establish a neuronal cell model of SCA17, we used a tet-off system that stably expressed normal (13Q) and pathogenic (105Q) TBP in PC12 cells. Cells expressing stably transfected TBP-105Q showed characteristic signs of neuronal dysfunction, including decreased cell viability and defective neurite outgrowth. These cells also exhibited lower levels of the small heat shock protein HSPB1 as compared to TBP-13Q cells. However, overexpression of HSPB1 partially rescued the defect in neurite outgrowth, increased cell viability and also increased levels of TrkA protein, the major receptor involved in the neurite outgrowth pathway. These findings indicate that the expression of mutant TBP is sufficient to cause neuronal dysfunction and overexpression of HSPB1 can rescue some of this dysfunction.

2.2 Introduction

There are nine inherited neurodegenerative disorders characterized by an expansion of the polyglutamine tract of the affected protein. These proteins have no homology other than the polyglutamine domain and are ubiquitously expressed (Li and Li, 2004). Despite their widespread expression in the brain and body, mutant proteins with expanded polyglutamine domains cause selective neurodegeneration in distinct brain regions in each disease (Orr and Zoghbi, 2007). Although polyglutamine proteins have different structure and function, they induce common pathological changes characterized

by the accumulation of mutant polyglutamine proteins and the resulting pathological mechanisms such as transcriptional dysregulation (Cha 2000; Sugars and Rubinsztein, 2003; Orr and Zoghbi, 2007). Since the protein context can significantly modulate polyglutamine toxicity and confer selective pathology in each polyglutamine disease (Cornett et al., 2006; Wang et al., 2008), it is believed that understanding the normal function of polyglutamine proteins is important for elucidating the pathogenesis of polyglutamine diseases. Indeed, each polyglutamine disease protein has been found to abnormally interact with different proteins to mediate pathological changes (Harjes and Wanker, 2003; Li and Li, 2004; Orr and Zoghbi, 2007). It is likely that the polyglutamine expansion causes dysfunction of the mutant protein resulting in disease pathology, and perhaps the loss of function of the normal protein may also contribute to disease pathology, making polyglutamine expansion both a gain and loss of function mutation.

Numerous animal and cellular models have been developed to study the molecular mechanisms of polyglutamine-induced neuropathology. Transgenic mouse models of polyglutamine disease, such as HD, often show very severe and rapid phenotypes as exhibited in the R6/2 mouse model (Carter et al., 1999). These mice usually display weight loss, motor deficits or abnormalities, and markedly reduced survival. Though these mouse models recapitulate clinical phenotypes seen in patients, it is often difficult to accurately model late onset disorders with such severe phenotypes. Knock-in mice, where the transgene is expressed under the endogenous mouse promoter, allow for more accurate temporal and spatial expression of mutant proteins, though the presentation of characteristic pathological features may be dramatically delayed, limited, or even absent. In both models, the neuropathology can be drawn out, without the

presence of a striking phenotype even at later stages of the disease, suggesting a long course of cellular dysfunction, eventually leading to massive amounts of cell death (Mangiarini et al., 1996; Heintz and Zoghbi, 2000).

Cultured cells are often used to transiently or stably express the mutant proteins, and the type of cell used can vary from non-neuronal cells to primary neurons cultured from transgenic animal models or transfected primary neurons. The pathological hallmarks of these cell models can include both cytoplasmic and intranuclear aggregates and decreased cell viability. The severity of disease in these models is dependent on many factors, such as transgene expression levels, cell type, polyglutamine tract length, and whether the protein is expressed as a full length protein or truncated product. Additionally, the subcellular localization of the mutant protein is also important and can have an impact on the mechanism of disease pathology. For example, mutant htt has been found to associate with the mitochondria in mouse models of HD causing impaired mitochondrial function (Orr et al., 2008).

Neuronal dysfunction occurs in various polyglutamine disease models, such as HD, spinobulbar and muscular atrophy (SBMA), and Spinocerebellar Ataxias 1, 7 and 17 (Abel et al, 2001; Li et al, 1999; Lee Y, 2008; Bowman et al, 2005; Friedman et al, 2007) and recent studies suggest that transcriptional dysregulation may be an important pathological mechanism underlying cellular dysfunction and eventual cell death (Cha et al, 2000; Jiang et al, 2003; Mantamadiotis et al, 2002).

We have chosen to study SCA17 because TBP, unlike the other polyglutamine disease proteins, is very well characterized in terms of both structure and function. Thus, study of mutant TBP may also give us insight into the pathology of other polyglutamine

diseases. In addition, TBP is a nuclear protein and remains nuclear even with the expansion, allowing for investigation whether nuclear expression of this mutant protein could lead to cytoplasmic dysfunction via transcriptional dysregulation. In this study we develop and characterize a PC12 stable cell model of SCA17. We expressed normal and mutant TBP in PC12 cells using a Tet-off system. Mutant cell lines exhibited characteristic signs of neuronal dysfunction, such as decreased cell viability and neurite outgrowth, allowing this cell model to be used for a detailed investigation of the molecular pathogenesis of SCA17.

2.3 Materials and Methods

Plasmids and reagents:

Stably transfected PC12 cell lines were generated by transfecting the Tet-off stable cell line (Clontech) with PBI-EGFP-TBP constructs (TBP-105Q and TBP-13Q) using Lipofectamine 2000 (Invitrogen). TBP containing 13Q or 105Q and EGFP were independently expressed from the CMV promoter in these constructs. Adenovirus expressing GFP and TBP-13Q or 105Q was made by Welgen (Worcester, MA). HSPB1 was cloned as previously described in Friedman et al., 2007. Transfections in HEK 293T cells were done with Lipofectamine (Invitrogen). Antibodies against TBP included N-12 (Santa Cruz). Other antibodies used for these studies were, γ-tubulin (Sigma), TrkA (Upstate Biotechnologies), HSPB1 (C-20, Santa Cruz), and Hoechst 33258 (Molecular Probes).

Cell Culture

Cell lines stably expressing TBP were generated by transfecting a stable Tet-off PC12 cell line (Clonetech) with PBI-EGFP-TBP constructs (TBP-13Q and TBP-105Q). TBP containing 13Q or 105Q and EGFP were independently expressed from a bidirectional minimal CMV promoter in these constructs. Transfected cells were selected with 500 µg/ml G418. Single cell colonies were picked under a fluorescence microscope and expanded to obtain stably transfected cell lines. The neuronal cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. To turn off transgene expression, 100 ng/ml of Doxycycline was added to culture medium for 16 hours.

Cortical neurons were dissected from embryonic day 18 rats and plated on poly-D-lysine-coated plastic culture plates (Corning Costar) in B27- supplemented medium (Invitrogen). At DIV2, neurons were infected with either adenoviral vectors independently expressing GFP and TBP-13Q or GFP and TBP-105Q (Welgen, Inc.) at a 1:1000 dilution of the virus (titer = 1×10^{12}). Cells were fixed and stained with tubulin 24 hours post infection.

Human embryonic kidney (HEK) 293T cells used for transient transfection of HSPB1 and TBP-105Q were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Western blotting

Cultured cells were harvested and homogenized in RIPA buffer [50 mM Tris-HCl, (pH8.0), 150mM NaCl, 1mM EDTA (pH8.0), 1mM EGTA (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and protease inhibitors], sonicated for 10 s before and after 30 min incubation on a rotating apparatus at 4 °C, and clarified at 3000 rpm for 5 min. Fifty micrograms of cell supernatant was used for Western blotting. Protein samples were run on 4–20% Tris-glycine polyacrylamide gels (Invitrogen). Protein was transferred onto a nitrocellulose membrane and then stained in Ponceau S Red, before blocking in 5% milk/1X PBS. Primary antibody was incubated with 3% BSA and secondary antibody was incubated in 5% milk/1X PBS. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) kit.

Cell Viability Assay:

Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Titer 96; Promega), which is based on the conversion of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2-*H*-tetrazolium by mitochondrial dehydrogenase to a formazan product, and measured at an absorbance of 490 nm. Wild- type and stably transfected PC12 cells expressing either TBP-13Q or 105Q were plated in 96-well plates at a density of 10,000 cells/well and maintained overnight in DMEM supplemented with 5% FBS and 10% horse serum. Cells were then changed to serum free medium in the absence or presence of staurosporine (dissolved in DMSO, 50 nM) for 5 hours. After drug treatment, 20 µl of MTS reagent was added to each well. The cells were then incubated for 30–45 min at 37°C in a 5% CO2 incubator. The reaction

was stopped by adding 25 µl of 10% SDS. The plates were read with a microplate reader (SPECTRAmax Plus; Molecular Devices, Palo Alto, CA) at 490 nm. Each data point was obtained using a triplet-well assay. A ratio of untreated and staurosporine treated cells was used to determine cell viability. Cell viability in cells overexpressing HSPB1 was performed by transfecting cells with HSPB1, and then 36 hours after transfection, cells were treated with staurosporine, as described above, for 5 hours, then fixed with 4% paraformaldehyde, and stained with TBP, HSPB1, and Hoechst. The fragmented nuclei of TBP-105Q cells overexpressing HSPB1 were counted and compared to the number of fragmented nuclei in cells only expressing TBP-105Q and not HSPB1.

Neurite Outgrowth Assay:

Wild- type Tet-off and stably transfected PC12 cell lines expressing either TBP-13Q or TBP-105Q (Friedman et al, 2007) were plated into 12-well plates at ~40% confluency. Cells were treated with 100 ng/ml Nerve Growth Factor (NGF) for 48 hours at 37°C in serum free media, then fixed with 4% paraformaldehyde for 10 min, and permeabilized with 0.2% Triton X-100. Cells were blocked with 3% bovine serum albumin in 1X phosphate buffered saline for 30 min. Cells were washed 3X in phosphate buffered saline and then stained with an antibody against α-tubulin (1:5000, Sigma) for 1 hour at room temperature, followed by staining with rhodamine-X conjugated secondary antibody (1:500, Jackson immunoLabs) for 30 min. Hoechst staining was used to visualize nuclei. Fluorescent images were captured with a Zeiss microscope (Axiovert 200 MOT; Carl Zeiss Imaging) and stored in a computer. Quantitative results of neurite outgrowth were

obtained by counting the number of cells that extended neurites twice the length of the cell body against all the cells that were in all the counted images.

Cortical neurons were dissected from embryonic day 18 rats and plated on poly-D-lysine-coated plastic culture plates (Corning Costar) in B27- supplemented medium (Invitrogen). At DIV2, neurons were infected with either adenoviral vectors expressing GFP-TBP-13Q or GFP-TBP-105Q (Welgen, Inc.). After 24 hours, neurites were assessed as described above.

Cells overexpressing HSPB1 were evaluated for neurite outgrowth as described above, except that cells were transfected with HSPB1, stimulated with NGF for 48 hours, then fixed and stained for HSPB1. Neurite extension in PC12 cells transfected with HSPB1 was quantified as described above.

2.4 Results

Generation of PC12 cell model of SCA17

PC12 cells are a cell line derived from a pheochromocytoma of the rat adrenal medulla (Greene and Tischler, 1976). PC12 cells stop dividing and terminally differentiate into cells with neuron- like properties when treated with the neurotrophic factor, nerve growth factor (NGF). This makes PC12 cells a useful model system to study neuronal dysfunction. We generated a tet-off PC12 cell line to express our normal (TBP-13Q) and mutant (TBP-105Q) TBP. The system is comprised of two plasmids, the regulator and response plasmid **(Fig. 2-1)**. The regulator plasmid is under a CMV promoter and produces the tetR/VP16 protein, a transactivator protein that binds the tetracycline responsive element in the response plasmid. The parent cells, or control cells

only stably express this regulator plasmid. The response plasmid is comprised of a bidirectional minimal CMV promoter, from which one direction GFP is expressed, and from the other direction the TBP transgene (13 or 105Q TBP) is expressed. The GFP expression allows for easy visualization of cells expressing transgenic 13Q or 105Q TBP. The CMV promoter region of the response plasmid contains a TRE (tetracycline responsive element) where the tetR/VP16 transactivator protein can bind to stimulate transcription from the minimal CMV promoter. The transgenic TBP cell lines are stably expressing both the regulator and response plasmids. In the presence of tetracycline, the drug binds to the tetR/VP16 protein, blocking it from binding the TRE, and therefore shutting off gene expression. In the absence of tetracycline, the tetR/VP16 transactivator protein is able to bind to the TRE and stimulate transcription from the promoter to induce transgene expression, in this case GFP and TBP-13Q or GFP and TBP-105Q.

Fluorescence microscopy shows GFP fluorescence and western blotting with an antibody to the N-terminal region of TBP, demonstrates expression of our TBP-13Q and TBP-105Q transgenes **(Fig. 2-2).** The control cell line only expresses endogenous levels of TBP and both TBP transgenic cell lines show equal expression of the TBP transgene. Addition of doxycycline (a tetracycline derivative) to the system was able to effectively shut off TBP transgene expression **(Fig. 2-3)**.

Characterization of PC12 Stable Cell Model of SCA17

After establishing a cell model stably expressing normal and mutant TBP, we wanted to characterize the phenotype of these cells and identify any neuronal dysfunction. To determine whether mutant TBP affects the viability of PC12 cells, we

performed a cell viability assay. We treated the stably transfected PC12 cells with the apoptotic stimulator staurosporine and then measured cell viability via an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. TBP-105Q cells are more sensitive to apoptotic stimulation and show a significant decrease in their viability compared to non-transfected PC12 cells and TBP-13Q transfected cells **(Fig. 2-4)**.

Taking advantage of the neuronal properties of PC12 cells, stably transfected PC12 cells expressing TBP-13Q and TBP-105Q were treated with nerve growth factor (NGF), a potent inducer of differentiation and neurite outgrowth in PC12 cells. The cells were then fixed and stained with an antibody against tubulin to visualize neurites. Cells expressing TBP-105Q show defective neurite extension as compared to those cells expressing TBP-13Q after NGF treatment **(Fig. 2-5a)**. Quantifying the number of cells with neurites longer than two cell bodies confirmed shorter neurites in TBP-105Q cells than those of TBP-13Q cells **(Fig. 2-5b)**.

To ensure that the defect in neurite outgrowth was not related to the stable transfection of TBP-105Q, we examined if mutant TBP, encoded by an adenoviral vector, also affects neurite outgrowth of cultured primary cortical neurons. We infected cultured embryonic rat brain cortical neurons with a TBP-13Q or TBP-105Q expressing adenoviral vector. Staining of infected neurons with an antibody to tubulin allowed us to visualize neurites of neurons expressing the TBP adenovirus, which also co-expresses GFP to reflect TBP expression **(Fig. 2-6a)**. Neurons with long neurites were then counted using the method described above. The results show that much fewer neurons expressing TBP-105Q display long neurites **(Fig. 2-6b)**. Thus, examination of stably transfected

PC12 cells and cultured primary neurons revealed that mutant TBP can inhibit neurite outgrowth, a characteristic feature of neuronal dysfunction.

Next we examined the expression of HSPB1, a small heat shock protein that was previously found to be down-regulated in a microarray analysis of SCA17 mouse brains (Friedman et al., 2007). HSPB1 is essential for axonal and neuronal integrity (Evgrafov et al., 2004; Williams et al., 2005) and is protective against various neuronal insults *in vivo* (Akbar et al., 2003; Sharp et al., 2006). Mutations in this protein can also lead to various different neuropathies (Gooch et al, 2008; Dierick et al, 2008; Rohkamm et al., 2007), suggesting an important role for HSPB1 in neuronal function. Consistent with the down-regulation seen in the microarray study, western blot analysis of PC12 cell lines that stably expressed TBP-105Q showed lower expression of HSPB1 as compared to control cells and cells expressing TBP13Q **(Fig. 2-7a)**. Because HSBP1 has been shown to be involved in neurite outgrowth (Williams et al., 2005; Hong et al., 2009; Read, 2009), we tested whether neurite outgrowth defects in TBP-105Q PC12 cells could be rescued by overexpression of exogenous HSPB1. Importantly, overexpression of HSPB1 significantly increased neurite extension in TBP-105Q PC12 cells **(Figure 2-7b)**. Neurons with long neurites were counted using the method previously described. The results show that TBP-105Q neurons expressing HSPB1 display longer neurites as compared to those cells without exogenous HSPB1 **(Fig. 2-7b, lower panel)**.

To further clarify the significance of HSPB1 in neurite outgrowth we wanted to determine its effect on the high affinity NGF receptor, TrkA. Other cell models of polyglutamine disease, such as Huntington's disease have shown decreased protein levels of TrkA (Li et al., 1999; Suzuki and Koike, 2005; Song et al., 2002) in cells expressing

the mutant polyglutamine protein. We tested whether overexpression of HSPB1 could increase levels of TrkA in PC12 cells expressing TBP-105Q. Interestingly, western blot analysis showed an increase in TrkA levels when exogenous HSPB1 was overexpressed **(Fig. 2-8)**. These findings are consistent with a prominent role for HSPB1 in neurite outgrowth and neuronal function.

Furthermore, by overexpressing HSBP1 in TBP-105Q cells, we were also able to rescue the decreased cell viability phenotype **(Fig. 2-9)**. TBP-105Q cells overexpressing HSPB1 showed less cells with fragmented nuclei, a sign of apoptosis, as compared to cells only expressing TBP-105Q. This finding indicates that HSPB1 not only plays a role in neurite outgrowth but also displays a protective function in cells even in the presence of polyglutamine expanded TBP.

It is well known that chaperone and heat shock proteins can be protective to the cell (Perrin et al, 2007; Chang et al, 2005), however these protective effects are often thought to be mediated through modulation of aggregate formation (Outeiro et al, 2006; Liberek et al., 2008; Fujimoto et al., 2005). To determine if the protection conferred by HSPB1 involved this mechanism, we co-transfected HSPB1 with TBP-105Q in HEK293T cells **(Fig. 2-10)**. Surprisingly, overexpression of HSPB1 actually caused a modest increase in aggregation as compared to TBP-105Q cells transfected with empty vector, suggesting that the protection conferred by HSPB1 does not involve modulation of mutant TBP misfolding.

2.5 Discussion

The goal of this study was to develop a cell line that accurately models some SCA17 pathology. The phenotype of our PC12 stable cell model of SCA17 was similar to neuronal dysfunction seen in other polyQ disease cell models (Lee et al., 2008; Li et al., 1999), which are characterized by decreased cell viability and defective neurite outgrowth. These data are also able to recapitulate some of the neurological phenotype seen in SCA17 transgenic mice (Friedman et al., 2007).

However, the presence of aggregates was not seen in PC12 cells stably expressing TBP-105Q. This is likely due to the fact that the expression levels of the TBP-105Q transgene are not high enough to induce aggregate formation. Consistently, transient transfection studies in HEK293T cells, which generally have much higher expression levels of the transgene, showed aggregation of TBP in cells expressing TBP-105Q **(Fig. 2-10)**. Thus, cytotoxicity can be caused by mutant polyglutamine expanded proteins in the absence of polyglutamine aggregates.

The role of aggregates formed of the mutant polyglutamine proteins is unclear and controversial (Sisodia, 1998). Nuclear inclusions have been found in cortical and striatal neurons in HD mouse models (Wang et al., 2008) and in Purkinje cells in SCA1 mouse models (Clark and Orr, 2000). These inclusions are typically found in the affected region of the brain and not in unaffected regions, and the presence of the polyglutamine tract alone is sufficient to cause aggregation, independent of the surrounding protein context (Ordway et al., 1997; Marsh et al., 2000). The presence of nuclear inclusions in vulnerable neurons and their association with the clinical and pathological phenotypes of these disorders have led to the view that these aggregates are toxic, and therefore pathogenic (Kitamura et al., 2006; Vacher et al., 2006). However, many studies have

shown (Klement et al., 1998, reviewed in Michalik and Van Broeckhoven, 2003) that aggregates are not required for polyglutamine associated toxicity and the presence of nuclear inclusions is in no way predictive of neurodegeneration and death (Saudou et al., 1998). Instead, the neuropathology of these diseases may be dependent on the native interaction of these proteins in soluble complexes. For example, the overexpression of wild- type Ataxin-1 in a knock- in SCA1 mouse model suppresses neuropathology by decreasing incorporation of mutant ataxin-1 in native complexes (Bowman et al., 2007). Consistent with this idea, the PC12 cell model of SCA17 was able to cause neuronal dysfunction and pathology in the presence of mutant TBP, and in the absence of nuclear inclusions. Since polyglutamine aggregates are also able to sequester TFIIB in SCA17 mouse brains (Friedman et al., 2007), it is likely that polyglutamine-induced toxicity is dependent on and modulated by protein context.

Previous studies have shown down-regulation of HSPB1 in the cerebellum of SCA17 mice (Friedman et al., 2007), suggesting a neuronal function for this small heat shock protein. Similarly, we found decreased HSPB1 protein levels in our PC12 cell model stably expressing TBP-105Q. Generally, the presence of misfolded or aggregated proteins induce stress responses and up-regulate molecular chaperones and heat shock proteins (Kalmar and Greensmith, 2009; Dodge et al., 2005; Chang et al., 2005), however there is an opposite effect in SCA17 mice and PC12 cell model of SCA17. Many of the small heat shock protein genes contain TATA boxes (Farkas et al., 2000; Tonkiss and Calderwood, 2005) and their activation is regulated by the transcription factor heat shock factor 1 (HSF1) (Trinklein, et al., 2004). Interestingly, HSF1 and TBP are found to interact *in vitro* (Yuan et al., 2000), suggesting that the polyglutamine expansion in TBP

could render an aberrant interaction with HSF1 and negatively affect the ability of HSF1 to activate expression of HSBP1. Alternatively, mutant TBP can reduce HSPB1 expression by abnormally interacting with other transcription factors, such as Sp1, that mediate HSPB1 transcription. Interestingly, homozygous deletion of *Hsf1* in mice causes progressive changes in the brain that are typical of neurodegenerative disease, including demyelination of axons and gliosis (Homma et al., 2007).

Many heat shock proteins such as Hsp40 and Hsp70, act as molecular chaperones to reduce polyglutamine associated aggregation in the mutant proteins (Perrin et al., 2007). The protection of these heat shock proteins against polyglutamine-induced toxicity was also found to be independent of polyglutamine aggregation (Zhou et al., 2001). Similarly, limited chaperone activity has been demonstrated *in vivo* for HSPB1 (Carra et al., 2005; Bryantsev et al., 2007), instead HSPB1 exerts its neuroprotective effect not through its chaperone activity but by limiting the production of reactive oxygen species and regulating apoptosis through effects on caspase activation (Wyttenbach et al., 2002). Similarly, overexpression of HSPB1 in cells expressing TBP-105Q led to an increase in cell viability, but failed to reduce TBP aggregation.

Overexpression of exogenous HSPB1 led to partial rescue of neurite extension, an increase in TrkA levels, the primary receptor involved in NGF signaling, and increased cell viability in cells expressing TBP-105Q. It is clear that HSPB1 can influence neuronal function and viability (Lachtman, 2005). For example, suppressing HSPB1 expression with small interfering RNA (siRNA) inhibits neurite outgrowth while the introduction of exogenous HSPB1 has the opposite effect in cultured neurons (Williams et al., 2006). Similarly, siRNA against HSPB1 correlates with increased apoptosis (Dodge et al.,

2005). Moreover, down-regulation of HSPB1 (Chang et al., 2005; Tsai et al., 2005) and the protective effect conferred by its overexpression (Wyttenbach et al., 2002) have been reported in cell models of polyglutamine diseases.

Although we were able to partially rescue the defect in neurite outgrowth and increase cell viability in TBP-105Q cells overexpressing HSPB1, this effect remains to be seen *in vivo*. Overexpression of HSPB1 in animal models of HD and ALS (Zourlidou et al., 2007; Krishnan et al., 2008) showed no amelioration in phenotype and neuropathology, though HSPB1 was found to be protective in acute models of disease such as ischemia (Stetler et al., 2008) indicating the diverse effects of HSPB1 on acute versus chronic models of disease.

Expression of HSPB1 is primarily glial although it is expressed in some neuronal populations and is developmentally regulated in peripheral dorsal root ganglion (DRG) neurons, which may play a role in the decreased vulnerability of these neurons to heat shock or trophic factor withdrawal (Mearow, et al., 2002). This possibility may explain the decreased cell viability in cells expressing mutant TBP, as these cells also show decreased levels of HSPB1. More importantly, missense mutations in *HSPB1* have been implicated in two related peripheral neuropathies, Charcot-Marie-Tooth disease type2 (CMT-2) and distal hereditary motor neuropathy (HMN) (Evgrafov et al., 2004; Dierick et al., 2005). These diseases feature axonal degeneration, suggesting that HSPB1 is important in maintaining axonal integrity. Furthermore, HSPB1 co-localizes with actin and tubulin in lamellepodia, filopodia, focal contacts and mature neurites and growth cones in neurons (Williams et al., 2005) and inhibition of HSPB1 results in an atypical growth pattern that may be attributable to an effect of HSPB1 on the stability of the actin

cytoskeleton, suggesting that HSPB1 has multiple function to promote neuritogenesis and subsequent neurite extension. Consistent with this role, overexpression of HSPB1 was able to increase levels of TrkA, the high affinity NGF receptor. Activation of TrkA signaling leads to changes in neuronal differentiation and induces neurite outgrowth making it a viable molecular candidate through which SCA17 pathology may be mediated.

Figure 2-1

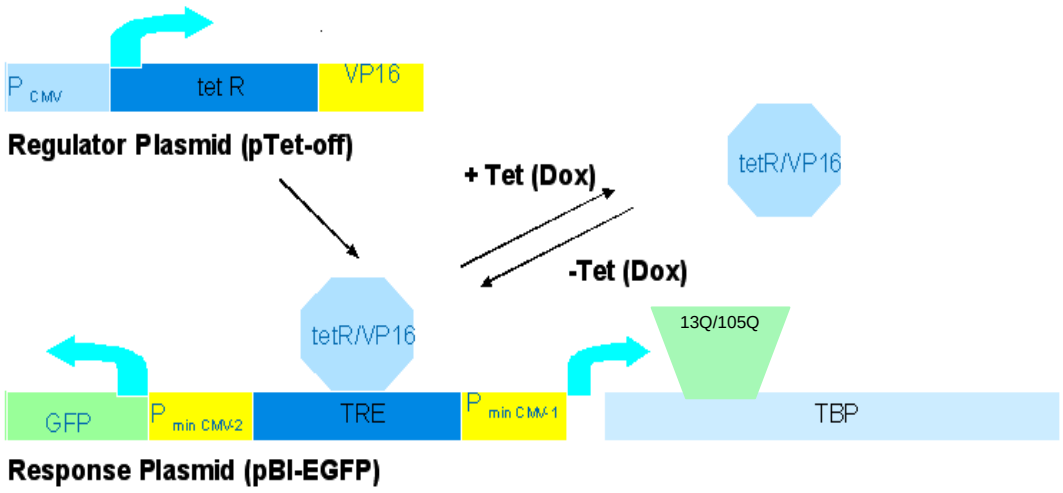
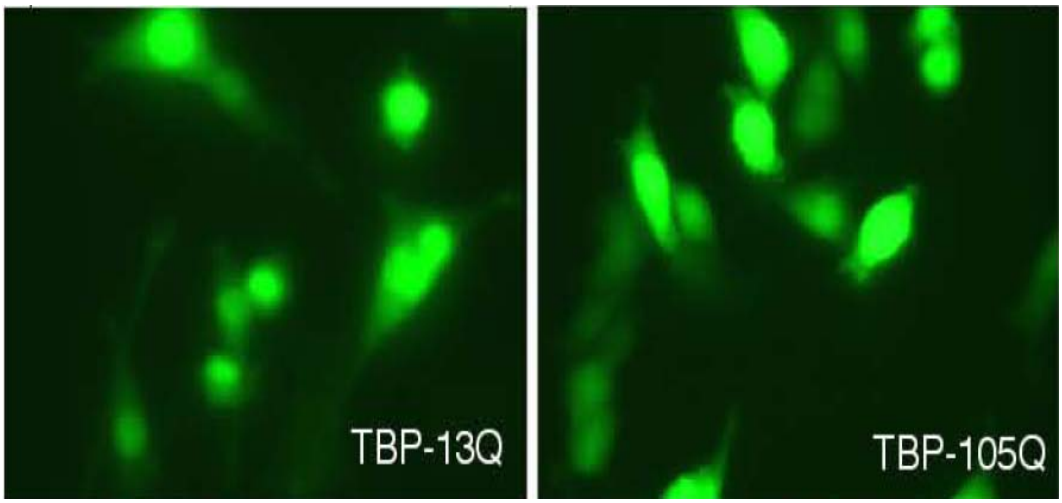

P CMV
tet R
VP16
Regulator Plasmid (pTet-off)
+ Tet (Dox)
-Tet (Dox)
tetR/VP16
tetR/VP16
13Q/105Q
GFP
P min CMV-2
TRE
P min CMV-1
TBP
Response Plasmid (pBI-EGFP)

Figure 2-1 Schematic representation of Tet-off PC12 stable cell line. This system is comprised of two plasmids, the regulator and response plasmid. The regulator plasmid is under a CMV promoter and produces the tetR/VP16 tetracycline responsive element binding protein. The parent cells, or control cells only express this regulator plasmid. The response plasmid is comprised of a bidirectional minimal CMV promoter, from which one direction GFP is expressed, and from the other direction the TBP transgene (13 or 105Q TBP) is expressed. In this promoter region is a TRE (tetracycline responsive element) where the tetR/VP16 protein can bind. The transgenic TBP cell lines are stably expressing both the regulator and response plasmids. In the absence of tetracycline, the tetR/VP16 protein is able to bind to the TRE and stimulate transcription from the promoter to induce transgene expression. In the presence of tetracycline, the drug binds to the tetR/VP16 protein, blocking it from binding the TRE, and therefore shutting off gene expression.

Figure 2-2

A



B

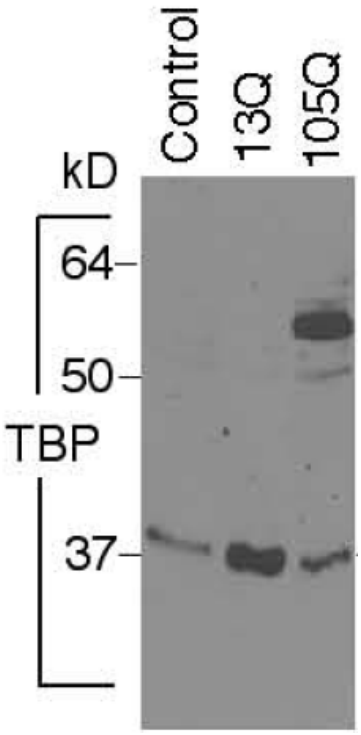


Figure 2-2 Expression of TBP in PC12 stable cell model of SCA17. (a) Fluorescent images of GFP expression in the stable cell lines expressing TBP-13Q or TBP-105Q. **(b)** Western blot demonstrating expression of stably transfected TBPs in TBP-13Q and TBP-105Q PC12 cells. Control PC12 cells only express endogenous TBP, shown at the 37 kDa molecular weight marker. Both transgenic cell lines show equal expression level of transfected TBP.

Figure 2-3

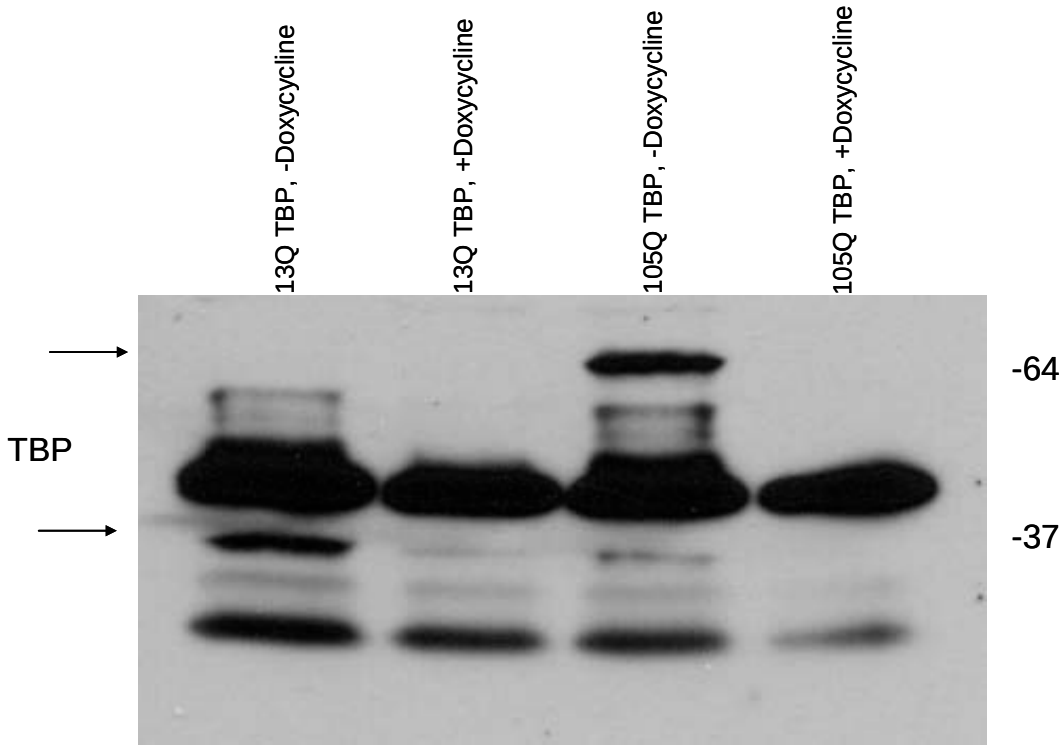

13Q TBP, -Doxycycline
13Q TBP, +Doxycycline
105Q TBP, -Doxycycline
105Q TBP, +Doxycycline
TBP
-64
-37

Figure 2-3 Doxycline is able to diminish transgene expression in Tet-off PC12 stable cell line. A western blot demonstrating expression of the 13Q and 105Q transgenes in the absence or presence of doxycycline (a derivative of tetracycline). In the absence of doxycline, there is robust expression of the transgene, as indicated by the arrows. In the presence of doxycycline, the expression of the TBP transgene is diminished.

Figure 2-4

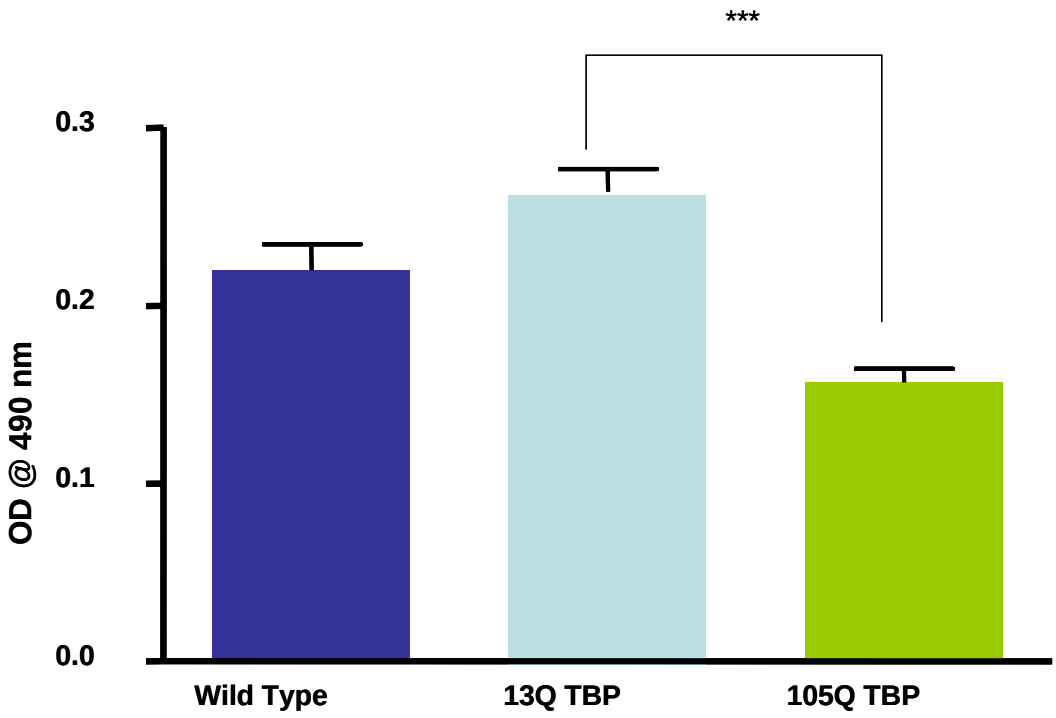


Figure 2-4 TBP-105Q cells show decreased cell viability. Control and stably transfected PC12 cell lines were treated with staurosporine (50 μM, 5 hours) and then assessed for cell viability using the MTT assay. *** $P<0.001$ compared with TBP-13Q cells.

Figure 2-5

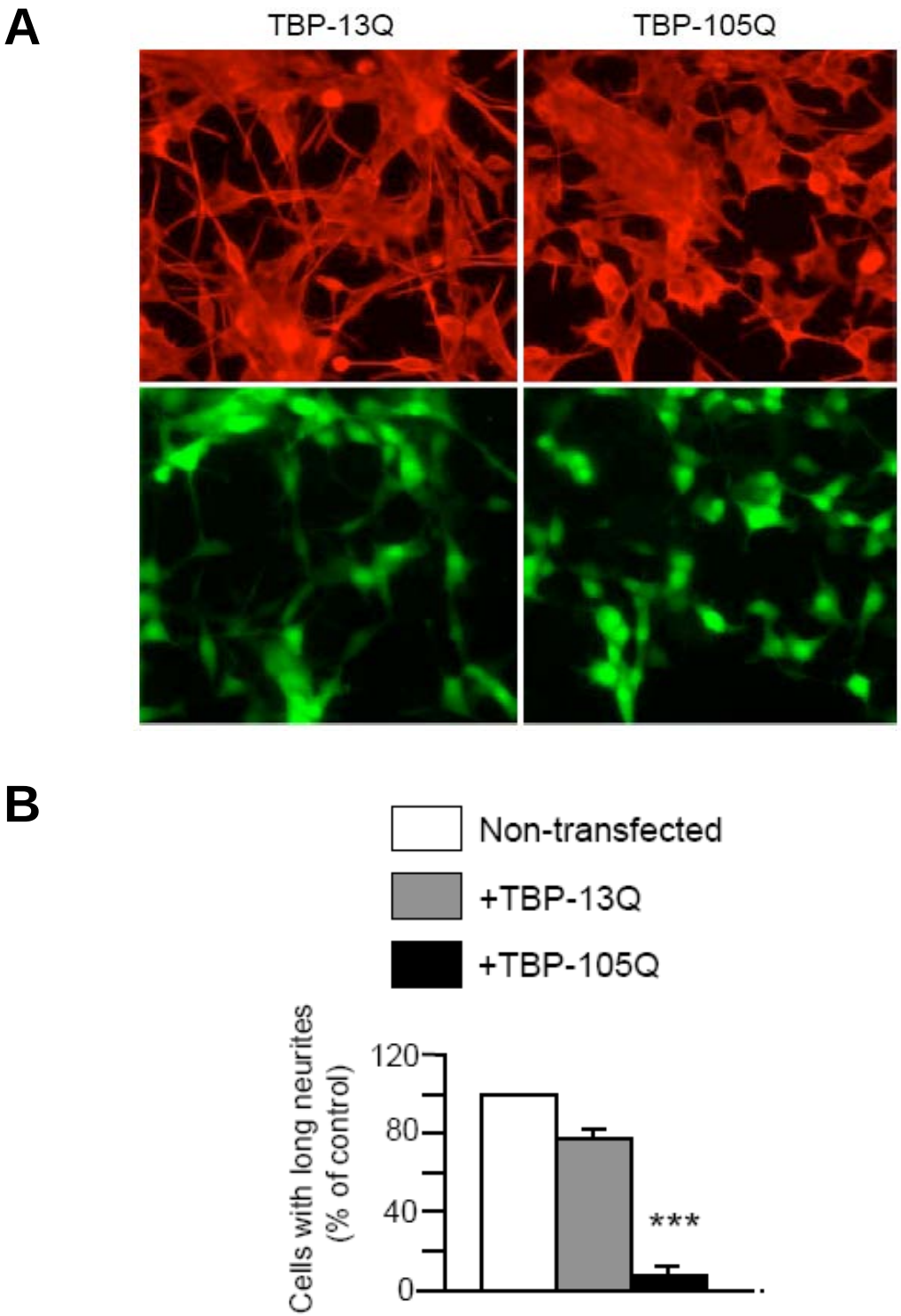
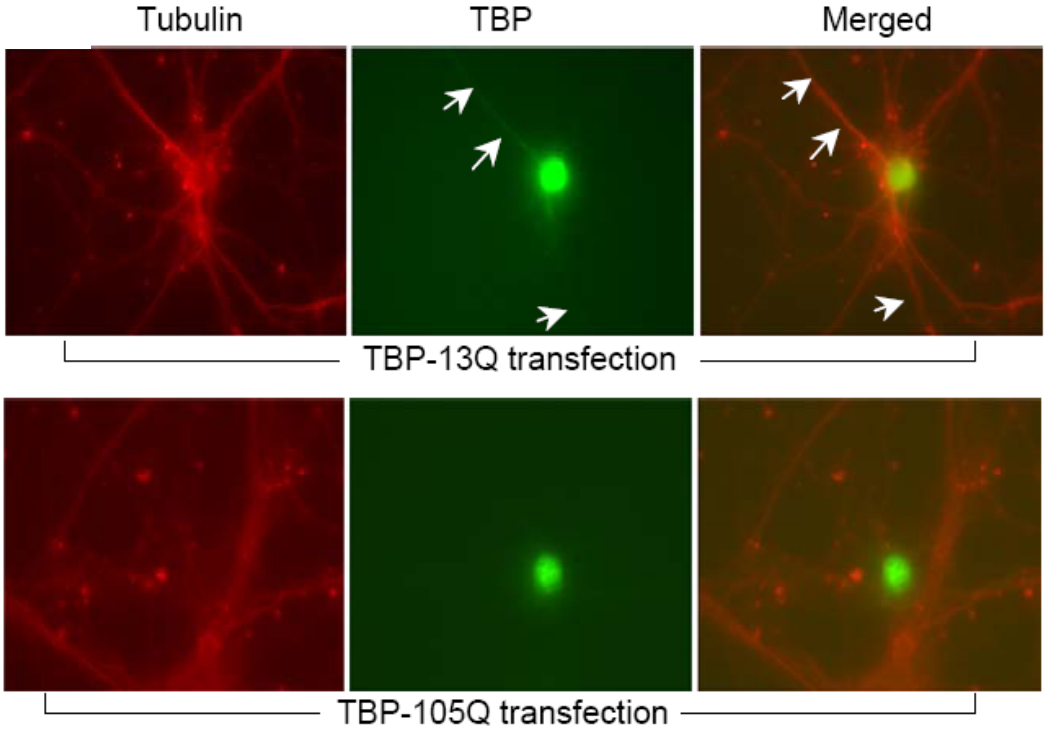

A
TBP-13Q
TBP-105Q
B
Non-transfected
+TBP-13Q
+TBP-105Q
Cells with long neurites
(% of control)
120
80
40
0
***

Figure 2-5. TBP-105Q cells show defective neurite outgrowth. **(a)** TBP stably transfected PC12 stable cell lines were stimulated with NGF (100 ng/ml, 48 h). Upper panels are images showing tubulin labeling. Lower panels are images showing GFP that reflects the expression of transfected TBP. **(b)** Percent of cells with long neurites (twice the length of the cell body) after treatment with NGF shows cells expressing TBP-105Q exhibit defective neurite outgrowth. *** $P < 0.001$.

Figure 2-6

A


Tubulin
TBP
Merged
TBP-13Q transfection
TBP-105Q transfection

B

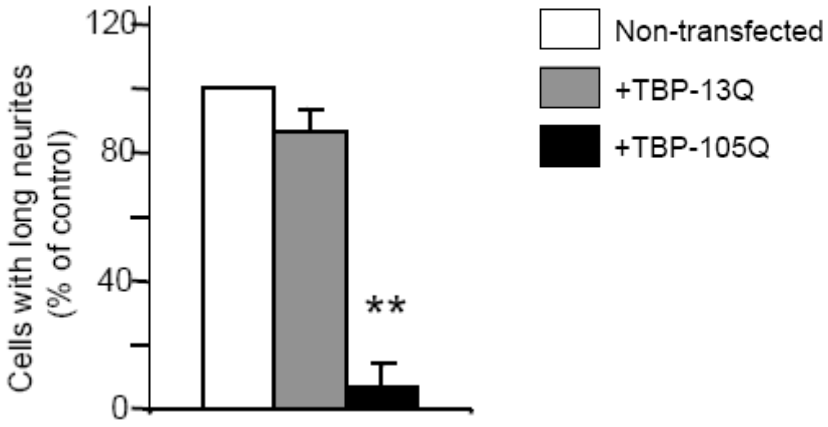
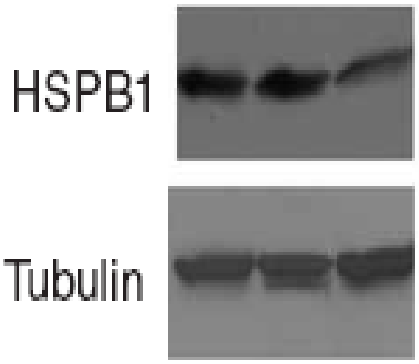

120
80
40
0
Cells with long neurites
(% of control)
**
Non-transfected
+TBP-13Q
+TBP-105Q

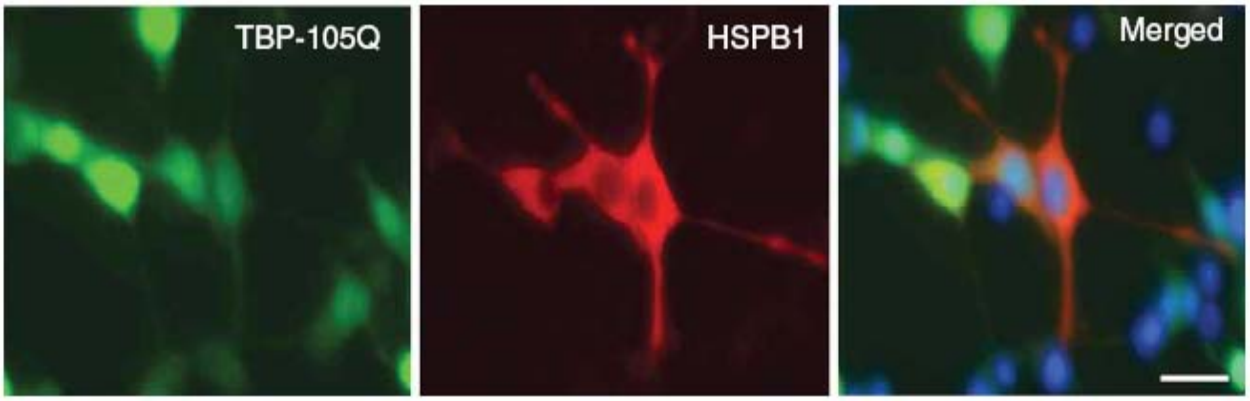
Figure 2-6 Cortical neurons infected with TBP-105Q adenovirus show decreased neurite outgrowth. (a) Cultured primary rat cortical neurons were infected with TBP-13Q or TBP-105Q adenovirus. The infected cells were stained with anti-tubulin to reveal neurites (arrows). (**b**) The quantification of neurite length demonstrates that TBP-105Q containing cells show a significant decrease in neurite extension relative to that of non-infected and TBP-13Q infected neurons. ** $P<0.01$.

Figure 2-7

A


HSPB1
Tubulin

B


TBP-105Q
HSPB1
Merged

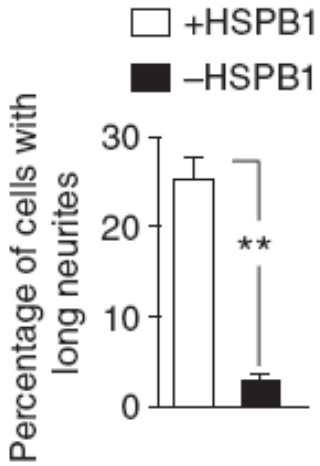

+HSPB1
–HSPB1
Percentage of cells with long neurites
30
20
10
0
**

Figure 2-7 Rescue of neurite outgrowth defect in PC12 stable cell model of SCA17 by overexpression of HSPB1. (a) Western blot demonstrating lower HSPB1 expression in TBP-105Q PC12 cells. **(b)** Immunofluorescent images demonstrating that overexpression of HSPB1 (red) enhanced neurite outgrowth in TBP-105Q PC12 cells (green) as compared to non-HSPB1 transfected cells. The bottom panel shows the percentage of cells with long neurites in the presence or absence of transfected HSPB1. **P < 0.01.

Figure 2-8

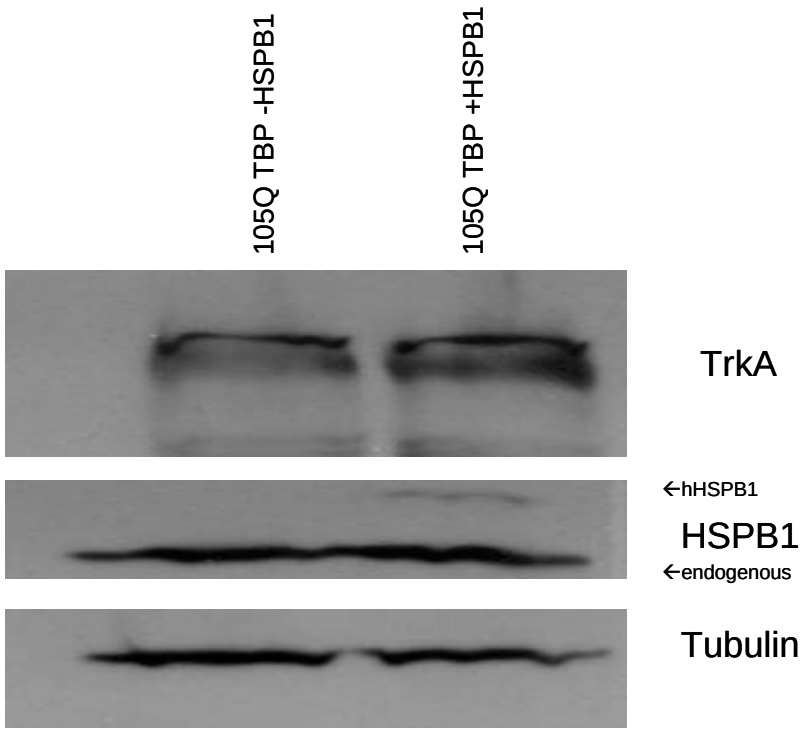

105Q TBP -HSPB1
105Q TBP +HSPB1
TrkA
←hHSPB1
HSPB1
←endogenous
Tubulin

Figure 2-8. Overexpression of HSPB1 rescues TrkA levels in 105Q-TBP PC12 cells. Western blot demonstrating TBP-105Q cells have increased levels of TrkA protein in the presence of transfected HSPB1 (upper panel) as compared to non-HSPB1 transfected TBP-105Q cells. Expression of endogenous and transfected HSPB1 is also presented (middle panel).

Figure 2-9

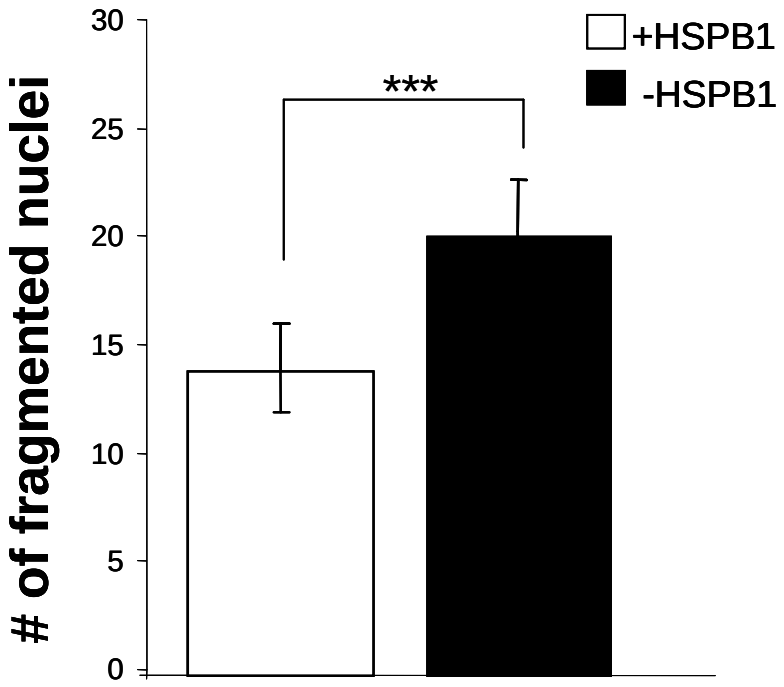


Figure 2-9 Overexpression of HSPB1 shows partial rescue of cell death in PC12 cell model of SCA17. Quantification of number of cells showing fragmented nuclei, in the presence or absence of HSPB1 overexpression in TBP-105Q PC12 cells is shown. TBP-105Q cells overexpressing HSPB1 show a significant decrease in the number of fragmented nuclei as compared to cells not expressing HSPB1. Cells were quantified by counting the number of cells with fragmented nuclei within the total number of cells in a given field of view. Data is presented as percent of total number of cells. n=4, with 50 fields counted per experiment, with an average of 20 cells per field. ***P< 0.001.

Figure 2-10

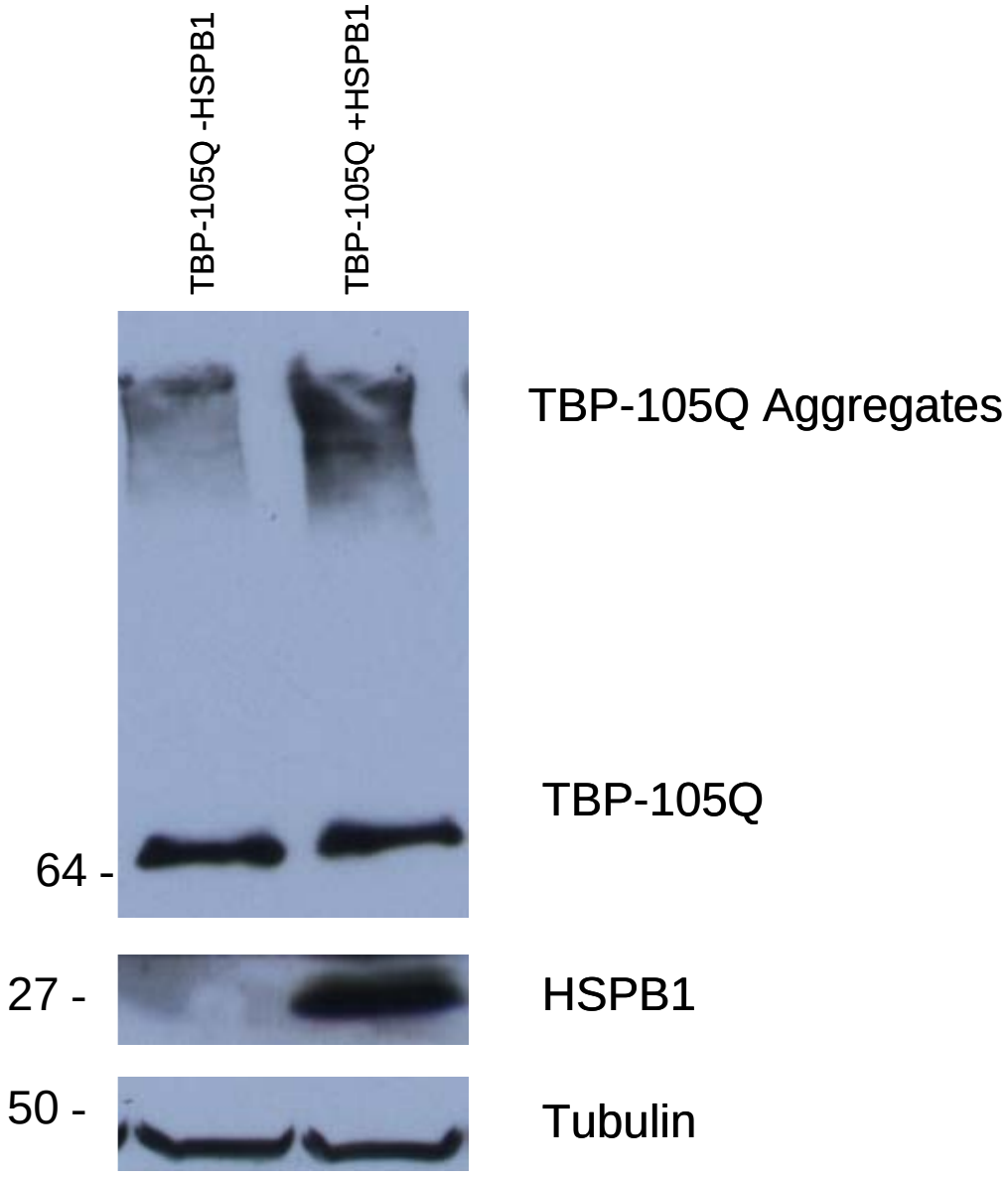

TBP-105Q -HSPB1
TBP-105Q +HSPB1
TBP-105Q Aggregates
TBP-105Q
64 -
27 -
HSPB1
50 -
Tubulin

Figure 2-10 HSPB1 overexpression does not reduce aggregation of TBP-105Q in HEK293T cells. Western blot showing HSPB1 overexpression does not reduce aggregation of polyglutamine expanded TBP but instead increases aggregation in these cells. The blot was sequentially probed with tubulin, TBP, and HSPB1 antibodies.

Chapter 3

Decreased TrkA in Cellular and Mouse Models of SCA17

This chapter includes findings and discussions from a manuscript currently submitted as Shah AG, Friedman MJ, Roberts M, Li XJ, and Li SH. Transcriptional dysregulation of TrkA associates with neurodegeneration in Spinocerebellar Ataxia 17. *Hum. Mol. Genet.* (2009). Constructs for generation of mouse and cell models were designed by Shihua Li and Xiao-Jiang Li. SCA17 mice were generated and characterized by Meyer Friedman. TBP adenoviral vectors were made by Welgen, Inc. All other experiments were carried out by Anjali Shah. Xiao-Jiang Li participated in the writing and editing of the submitted manuscript.

3.1 Abstract

The NGF signaling pathway is the primary signaling cascade involved in neurite outgrowth and differentiation, both of which are important components of neuronal function and survival. The activation of this pathway occurs upon binding of nerve growth factor to receptors TrkA, the high affinity NGF receptor and $p75^{NTR}$, the low affinity NGF receptor. We used a PC12 cell model and mouse model of SCA17 to investigate dysfunction of the NGF signaling pathway. In the cell model, mutant TBP caused specific down-regulation of TrkA and phospho-Erk, both of which play a very important role in neurite outgrowth signaling. In the mouse model, cerebellar levels of TrkA were reduced in transgenic mice expressing TBP-71Q, and this decrease occurred prior to Purkinje cell degeneration. These findings implicate decreased levels of TrkA as a possible causative mechanism in SCA17 neuropathology.

3.2 Introduction

Trk receptors are a family of three receptor tyrosine kinases, each of which can be activated by one or more of the four neurotrophins—nerve growth factor (NGF), which binds TrkA, brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT4) which bind TrkB, and neurotrophin 3 (NT3) which binds TrkC (Lei and Parada, 2006). Neurotrophins are largely derived from target tissues innervated by Trk expressing neurons. Signaling through these receptors regulates cell survival, proliferation, the fate of neural precursors, axon and dendrite growth and patterning, and the expression and activity of functionally important proteins, such as ion channels and neurotransmitter receptors (Huang and Reichardt, 2001,2003). Each neurotrophin supports distinct

neuronal populations in the central and peripheral nervous systems. The main targets of NGF are sympathetic neurons as well as subpopulations of sensory and central neurons.

The cytoplasmic domain of TrkA receptors contain several sites of tyrosine phosphorylation that recruit intermediates in intracellular signaling cascades. As a result, TrkA receptor signaling activates several small G proteins as well as pathways regulated by MAP kinase/ERK and PI 3-kinase (Vaudry et al., 2002). The G protein Ras and one of its downstream effector cascades, the Erk (extracellular- regulated kinase) cascade is an extremely well conserved signaling cascade, found in species ranging from yeast to mammals (Klesse and Parada, 1999). The Ras/Erk cascade has been demonstrated to be both necessary and sufficient for the differentiation of PC12 cells induced by NGF stimulation (Klesse et al., 1999; Burry, 2001). However, TrkA receptor signaling not only induces neuronal differentiation and neurite outgrowth but also neuronal cell survival. While the Ras/Erk cascade is not required for NGF induced survival of PC12 cells, activity of PI 3- kinase is necessary (Klesse et al., 1999; Yao and Cooper, 1995). Activated PI 3- kinase catalyzes the formation of lipid second messengers, which then activate the serine threonine kinase Akt to promote cell survival in neurons and PC12 cells (Ashcroft et al., 1999). These findings indicate that NGF acts through at least two distinct signaling cascades to promote its neuronal differentiation and neuronal survival effects on PC12 cells.

$p75^{NTR}$ binds all neurotrophins with similar affinities (Chao, 1994) and regulates cellular processes through interactions between the cytoplasmic domain of $p75^{NTR}$ and effector molecules (Mukai et al., 2003), though it lacks any intrinsic catalytic activity. This receptor has multiple functions in the nervous system where it is expressed widely

during the developmental stages of life, though its expression decreases dramatically by adulthood. However, expression of $p75^{NTR}$ can often increase in pathological states related to neural cell death (Chen et al., 2009).

$p75^{NTR}$ has been proposed to be involved in retrograde transport of neurotrophins (Johnson et al, 1987), ligand discrimination (Clary et al.,1994; Benedetti et al., 1993), and ligand dependent protection from, as well as induction of, apoptosis (Van der Zee et al., 1996; Frade et al., 1996). Either by interacting with Trk receptors or via the independent binding of neurotrophin, $p75^{NTR}$ can induce neurite outgrowth and cellular survival or cell apoptosis through several complex signal transduction pathways. Most of these signaling pathways remain to be elucidated.

It has been suggested that both receptors are required for NGF high affinity binding, although TrkA appears to be sufficient for transducing most of the biological effects of NGF (Lewin et al., 1996). Mice homozygous for null mutations in genes encoding neurotrophins and cognate Trks display striking similarities in their phenotypes, demonstrating that *in vivo* the neurotrophins mediate, to a large extent, their effects through activation of Trk receptors. Mutant animals display severe abnormalities in the nervous system, and most die shortly after birth, underlining the functional importance of these molecules during development. The $p75^{NTR}$ null mutant animals, on the other hand, display a less severe phenotype, with $p75^{NTR}$ null mice living to several months of age but with reduced fertility (Lee et al., 1992). Some evidence suggests that p75 could play a modulatory role on TrkA activation and that NGF binding to $p75^{NTR}$ enhances responsiveness to this ligand, particularly when NGF is present at limiting concentrations (Ryden et al, 1997). Absence of $p75^{NTR}$ or blocking of NGF binding to $p75^{NTR}$ reduces

the responsiveness of several TrkA-mediated effects, including tyrosine autophosphorylation and cell differentiation (Verdi et al., 1994). However, other studies suggest enhanced neurite outgrowth in $p75^{NTR}$ null neurons, demonstrating that TrkA and Erk signaling in sympathetic neurons were increased when $p75^{NTR}$ function is disrupted (Hannila et al., 2004). These findings suggest a modulatory role for $p75^{NTR}$ in NGF-mediated signaling and this modulation may have opposing effects on different NGF signaling cascades.

We previously saw a defect in neurite outgrowth in cells expressing TBP-105Q, suggesting that dysfunction of the NGF signaling pathway may occur in these cells. As both $p75^{NTR}$ and TrkA receptors are involved in some capacity in neurite outgrowth and cell survival, it will be important to identify any possible dysfunction in these receptors. In the present study we show decreased transcript levels of TrkA, but not $p75^{NTR}$, in the presence of TBP-105Q. Furthermore, we show decreased levels of TrkA protein in various cell models of SCA17, as well as in the cerebellum of SCA17 mice. Importantly, this decrease in TrkA occurs prior to cerebellar degeneration. These results provide a molecular link through which polyglutamine expanded may be mediating neuronal dysfunction in SCA17.

3.3 Materials and Methods

Plasmids and Reagents

SCA17 mice were generated (Friedman et al., 2007) and maintained in the animal facility at Emory University under specific pathogen-free conditions in accordance with

institutional guidelines of The Animal Care and Use Committee at Emory University. Mouse and human TBP cDNA constructs and plasmids were described in Friedman et al., 2007. Stably transfected TBP PC12 cell lines were generated by transfecting the Tet-Off stable cell line (Clontech) with PBI-EGFP-TBP constructs (TBP-105Q and TBP-13Q) using Lipofectamine 2000 (17). TBP-13Q or -105Q and EGFP were independently expressed from a bidirectional CMV promoter in these PC12 cells. Antibodies against TBP included N-12 (Santa Cruz) and 1TBP18 (QED). Other antibodies used for these studies were, tubulin (Sigma), phospho-Erk and phospho-Akt (Cell Signaling), Calbindin (Chemicon), and TrkA (Upstate Biotechnologies). Adenovirus expressing GFP and TBP-13Q or 105Q was made by Welgen (Worcester, MA).

Gene Expression:

Total RNA were purified from wild-type PC12, TBP-13Q, and TBP-105Q cells. The RNA were reverse transcribed and used to examine gene expression. Primers used for PCR reaction were 5'- tgtggaagtgggggatgacg-3' (sense) and 5'-gcactcagcaagaaagacct-3' (antisense) for TrKA; 5'-ccacattccgacgactgatg-3' (sense) and 5'-ccaagaatgagcgcactaac-3' (antisense) for p75; and 5'-acgacccttcattgacctc-3' (sense) and 5'-gggggctaagcagttggtgg-3' (antisense) for GAPDH. Reverse transcription (RT)-PCR was performed as described previously (Li et al., 1998b). First-strand cDNA (Superscript, Invitrogen) was generated from RNA of cultured PC12 cells. PCR conditions were:

1. 96°C for 3 min
2. 96°C for 45s
3. 60°C for 1 min

4. 72°C for 2 min

Repeat steps 2-4 34X

5. 72°C for 5 min

PCR products were electrophoresed on a 1% agarose gel.

Quantitative-PCR was performed using total RNA extracted from cerebella of wild-type, TBP-13Q or TBP-71Q SCA17 mice. Gene expression of TrkA and GAPDH were assayed using Taqman probes and reagents (Applied Biosystems). All quantitative PCR experiments were done on the Eppendorf realplex 4 machine. First strand cDNA was generated using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems).

Western Blotting

Brain tissue samples and cultured cells were harvested and homogenized in RIPA buffer [50 mM Tris-HCl (pH8.0), 150mM NaCl, 1mM EDTA (pH8.0), 1mM EGTA (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100, with protease inhibitors (Sigma)], sonicated for 10 s before and after 30 min incubation on a rotating apparatus at 4 °C, and clarified at 3000 rpm for 5 min. Two hundred micrograms of the brain supernatant and fifty micrograms of cell supernatant were used for Western blotting. Protein samples were run on 4–20% Tris-glycine polyacrylamide gels (Invitrogen). Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) kit.

Generation of SCA17 transgenic mice

TBP cDNAs were inserted into the XhoI cloning site of an expression vector that utilizes the murine prion promoter (Schilling et al., 1999a-b). Constructs, which encoded TBP-13Q, TBP-71Q and TBP-105Q under the control of the prion promoter, were microinjected into the male pronucleus of fertilized oocytes from FVB mice at the Emory Transgenic Facility. Founder mice were identified by PCR analysis of tail DNA using primers that flanked the TBP CAG/CAA repeat: (forward 5'-cca cag cct att cag aac acc-3') and (reverse 5'-aga agc tgg tgt ggc agg agt gat-3'). Positive founders were backcrossed onto the FVB strain background.

Immunofluorescence:

The cerebellum of 71Q-TBP transgenic and littermate control mice were rapidly isolated and sectioned (10 μm) with a cryostat at –20°C. The brain sections were placed on gelatin-precoated precleaned glass slides. Sections were fixed in 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.4% Triton X-100 in PBS for 30 min, blocked with 5% normal goat serum (NGS) in PBS for 1 hr, and incubated with primary antibodies in 2% NGS and PBS overnight at 4°C in a moisture chamber. After several washes, the sections were incubated with secondary antibodies conjugated with either Alexa Fluora 488 (Invitrogen) or rhodamine Red (Jackson ImmunoResearch). Hoechst was used to label the nuclei. Fluorescent images were acquired on a Zeiss microscope (Axiovert 200 MOT; Carl Zeiss Imaging) equipped with a digital camera (Hamamatsu Orca-100) and Openlab software (Improvision Inc). 10X, 40X and 63X objectives were used for image acquisition.

3.4 Results

Neurite extension of PC12 cells is largely regulated by the neurotrophin, nerve growth factor (NGF), which acts on receptors $p75^{NTR}$ and TrkA. We established a defective neurite outgrowth phenotype in a PC12 cell model of SCA17 and wanted to investigate molecular dysfunction in this pathway. It has been shown that other HD cell models (Li et al., 1999, Song et al., 2002) also have defective neurite outgrowth as well as decreased protein and mRNA levels in both NGF receptors, TrkA and $p75^{NTR}$, which is the first step towards impaired signaling. We used semi-quantitative RT-PCR analysis to identify altered expression of $p75^{NTR}$ or TrkA in a PC12 cell model of SCA17. The same cDNA materials were also analyzed for GAPDH transcripts, which served as an internal control. Interestingly, and contrary to previous studies, this assay shows that TrkA, but not $p75^{NTR}$, transcript is lower in TBP-105Q PC12 cells as compared to TBP-13Q cells **(Fig. 3-1a)**, suggesting that polyglutamine expansion in TBP can selectively inhibit the transcription of TrkA. The ratio of TrkA or p75 to GAPDH transcripts confirms the ability of TBP-105Q to selectively inhibit gene expression of TrkA and not $p75^{NTR}$ **(Fig. 3-1b)**. Since neurite outgrowth is more dependent on the activation of TrkA than $p75^{NTR}$ (Bai and Kusiak, 1997; Vaudry et al., 2002; Hisata et al., 2007; Isada et al., 2007), the reduced TrkA expression is consistent with the decrease in neurite outgrowth seen in neuronal cells that express mutant TBP.

Based on the above result, we focused on the effect of mutant TBP on TrkA. As protein levels do not necessarily correlate with transcript levels because of different half-lives, we performed western blot analysis to examine TrkA at the protein level. Interestingly, the result showed that TrkA is indeed decreased in TBP-105Q cells as

compared to non-transfected PC12 cells and TBP-13Q cells **(Fig. 3-2a)**. Quantification of the ratio of TrkA to tubulin confirms the decreased level of TrkA in TBP-105Q cells **(Fig. 3-2b).** To exclude the possibility that decreased TrkA resulted from stably transfected mutant TBP, we infected normal PC12 cells with adenoviral vectors encoding TBP-13Q or TBP-105Q for 48 hours. PC12 cells expressing transgenic TBP-105Q also show a decreased level of TrkA compared with cells overexpressing TBP-13Q **(Fig. 3-3)**. These finding suggest there is a clear down regulation of TrkA in the presence of mutant TBP, at both the transcript and the protein level.

Following NGF binding, the TrkA receptor rapidly becomes phosphorylated on its tyrosine residues, and the tyrosine kinase domain is activated (Klein et al., 1991). The TrkA cytoplasmic domain also serves as an anchor for binding these downstream signaling molecules, such as Ras and PI-3 kinase (Schlessinger and Ullrich, 1992). These proteins couple TrkA to several intracellular signaling pathways like the extracellular signal-regulated kinases (ERK) pathway (Kaplan and Miller, 1997) and the PI3K/AKT pathway (Ma et al., 2009).

Activation of ERK is followed by activation of transcription factors and induction of immediate-early genes. NGF-treated PC12 cells cease proliferating, exhibit somatic hypertrophy, acquire neurites, differentiate and show a dependence on NGF for survival in serum-free medium (Reichardt, 2006; Huang and Reichardt, 2003). Activation of the PI3K/AKT pathway leads to the phosphorylation of Akt and subsequently many of its pro-apoptotic substrates, IKK, Bad, and Caspase-9, making them inactive, and thus promoting a cell survival response (Eggert et al., 2000).

TrkA activation increases the phosphorylation of both Erk and Akt. Phospho-Erk stimulates neurite outgrowth and is more important for neuronal differentiation than Akt phosphorylation (Bai and Kusiak, 1997; Vaudry et al., 2002; Liu et al., 2001; Zentrich et al., 2002), which is involved in neuronal survival. Consistent with this view, western blot analysis showed that Erks phosphorylation is decreased in TBP-105Q cells as compared to TBP-13Q cells **(Fig. 3-4)**. Surprisingly, there is no significant difference in the phosphorylation of Akt between the different cell lines even though the cells expressing mutant TBP show decreased cell viability. These findings suggest a specific down regulation of the Erk pathway by mutant TBP.

We have previously shown that Purkinje cells degenerate in SCA17 mouse brains (Friedman et al., 2007). Degeneration of Purkinje cells can be detected by examination of their distinct cell bodies and dendrites in the cerebellum. A decrease in the number of calbindin-positive cerebellar neurons, widely considered to reflect the degeneration of Purkinje cells, was evident in the SCA17 transgenic mouse lines. There was also loss or disruption of calbindin-positive neurites in the cerebellar molecular cell layer of mutant TBP mice. There have been few studies to show TrkA expression in Purkinje cells, however it is known that TrkA expression decreases in an age-dependent manner (Marinelli et al., 1999) and is decreased in Alzheimer's disease brains (Hock et al., 1998 and Ginsberg et al., 2006). Based on the Purkinje cell degeneration seen in SCA17 mice, we performed immunofluorescent staining of the cerebellum of five month old, symptomatic SCA17 mice with an antibody to TrkA. Interestingly, we saw decreased expression of TrkA in the Purkinje cell layer of the cerebellum **(Fig. 3-5)** in the TBP-71Q mice as compared to the wild-type mice. However, it was difficult to know whether this

decrease in TrkA expression was due to loss of Purkinje cells or decreased expression of TrkA within the Purkinje cells. To better clarify this question, we performed immunofluorescent staining of these mouse brains under the same conditions as above and controlled for identical exposure parameters. Looking at these fluorescent images under higher magnification, it was clear that TrkA expression levels were decreased in the existing Purkinje cells of the TBP-71Q as compared to the wild type cerebellar sections **(Fig. 3-6a)**. Additionally, western blot analysis of cerebellar tissues showed decreased levels of calbindin, a Purkinje cell specific marker, in the 71Q-TBP animals as compared to the wild type mice **(Fig. 3-6b)**, suggesting not only loss of TrkA expression in existing Purkinje cells but also a loss of Purkinje cells in the TBP-71Q SCA17 mice. Similarly, western blot analysis **(Fig. 3-7a)** and quantitative PCR analysis **(Fig. 3-7b)** of cerebellar tissues showed decreased levels of TrkA protein and mRNA, respectively, in 71Q-TBP SCA17 mice as compared to wild type or TBP-13Q animals.

Although decreased expression of TrkA in the Purkinje cells of SCA17 mice was an important finding, it did not address whether loss of TrkA expression is a consequence of degeneration or if it precedes degeneration and could contribute to the neuropathology seen in the disease. To investigate the contribution of TrkA to neuropathology, we examined neurodegeneration and TrkA levels in young 3-6 week old, asymptomatic SCA17 mice. At this age, SCA17 mice did not develop obvious neurological symptoms (Freidman et al., 2007). Similarly, we did not observe obvious degeneration of Purkinje cells in these SCA17 mice, as staining of Purkinje cells with an antibody against calbindin, a cellular marker for Purkinje cells, did not reveal any significant differences between wild type control and SCA17 mouse brains **(Fig. 3-8a)**. Both high and low

magnification showed morphologically intact Purkinje cells with proper extension of Purkinje cell dendrites into the molecular layer of the cerebellum. Western blot analysis did not show any alteration of calbindin levels in the cerebellar tissues of SCA17 mice at 3 weeks of age, suggesting that the purkinje cell layer is comprised of equal numbers of Purkinje cells **(Fig. 3-8b)**. Interestingly, western blot analysis of TrkA shows that TrkA is decreased in the cerebellar tissues from two SCA17 mouse lines (71Q-16 and 71Q-27) **(Fig. 3-9a)**. To confirm this decrease, we performed quantitative RT-PCR, and revealed decreased levels of TrkA transcript in SCA17 mice at 3 weeks of age **(Fig. 3-9b)**. These findings demonstrate that TrkA is reduced in the cerebellum of SCA17 mice prior to obvious neurological symptoms and Purkinje cell degeneration and may ultimately contribute to SCA17 disease pathology.

3.5 Discussion

Decreased TrkA seen in mutant TBP-containing PC12 cells may largely explain the defective neurite outgrowth in these cells. It is known that neurite outgrowth of PC12 cells is triggered by NGF via its activation of membrane receptors $p75^{NTR}$ and TrkA. RT-PCR analysis showed a specific decrease in TrkA transcripts over $p75^{NTR}$ transcripts in cells expressing mutant TBP **(Fig. 3-1a,b)**. Though both receptors are involved in neurite outgrowth, TrkA is the high affinity receptor and $p75^{NTR}$ is the low affinity receptor for nerve growth factor. TrkA's sole ligand is NGF, and this receptor-ligand interaction is the most important to stimulate neuronal differentiation and neurite outgrowth.

TrkA is expressed in various brain regions including the cerebellum (Sobreviela et al., 1994; Muragaki et al., 1995; Torres et al., 1995; Dohrman et al., 1997; Riva-Depaty et

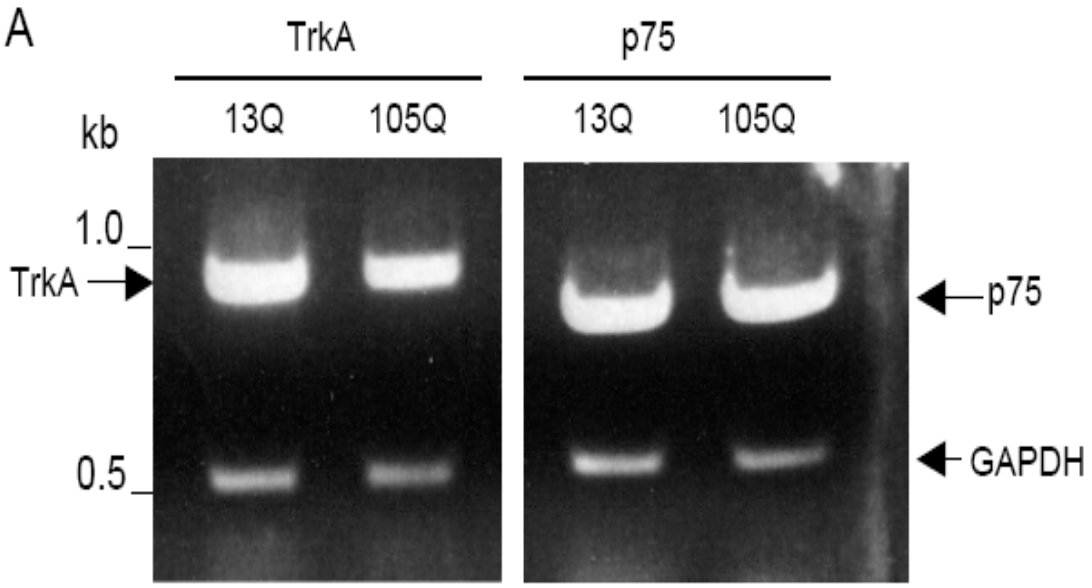
al., 1998). It is critical for neuronal survival and viability, and its decrease was observed in Alzheimer's disease (Boissiere et al., 1997; Hock et al., 1998; Dubus et al., 2000; Savaskan et al., 2000). We found that TrkA levels are decreased in a PC12 cell model of SCA17 and transgenic SCA17 mouse cerebella and more importantly, this decrease precedes the degeneration of Purkinje cells in SCA17 mouse cerebella, suggesting a causal role of the decreased TrkA level in the degeneration of Purkinje cells.

Furthermore, only the ERK signaling component was affected by the decrease in TrkA, the activation of the Akt signaling component remained unaffected. It is somewhat unexpected that the Akt signaling pathway would not be affected by the dysregulation of TrkA, as it is involved in cell survival and there is decreased cell viability in the PC12 cell model of SCA17 and increased caspase activity in SCA17 mice (Friedman et al., 2007). Perhaps, Akt may function normally in the presence of mutant TBP because cell death is being mediated by some other mechanism, rather than through the inactivation of the Akt signaling component. Alternatively, NGF signaling may be causing the inactivation of Akt in this specific signaling cascade, but other pathways may compensate to act on Akt and keep its activation stable. Akt can be phosphorylated by the PI-3 kinase family of enzymes, however, PI-3 kinase is not only activated by tyrosine receptor kinases, but also by G-protein receptors (Murga et al., 1998). Proper activation of a G-protein receptor, would lead to activation of PI-3 kinase, which could then lead to activation of Akt, all while bypassing the dysfunctional NGF signaling pathway. Additionally, Akt can be activated by other kinases, such as protein kinase A (PKA) (Robinson-White and Stratakis, 2002).

As discussed previously, HSB1 plays a protective role in neurons and can protect cells from apoptosis (Mearow et al., 2002; Rane et al., 2003). The decrease that we see in

HSPB1 could lead to the decrease in cell viability that we see, even though the activation of Akt cell is unaffected. Alternatively, perhaps the Akt signaling component of TrkA is dysregulated, but the dysfunction is occurring further down stream of the activation of Akt. Because we were using Akt as our read out for dysfunction in this pathway, we could have missed a more down stream target that is responsible for the decreased cell viability that we see in the cell and mouse model of SCA17. Again, HSPB1 may play a role in this. Studies have shown that Akt may act as a kinase that phosphorylates HSPB1 to activate its pro-survival role (O'Shaughnessy et al., 2007), but if there is less HSPB1 to be phosphorylated, there would, concomitantly, be a decrease in the amount of activated HSPB1 that could carry out the protective function, thus leading to a loss of protection and increased cell death.

Figure 3-1


A
TrkA
p75
kb
13Q
105Q
13Q
105Q
1.0
TrkA
0.5
p75
GAPDH

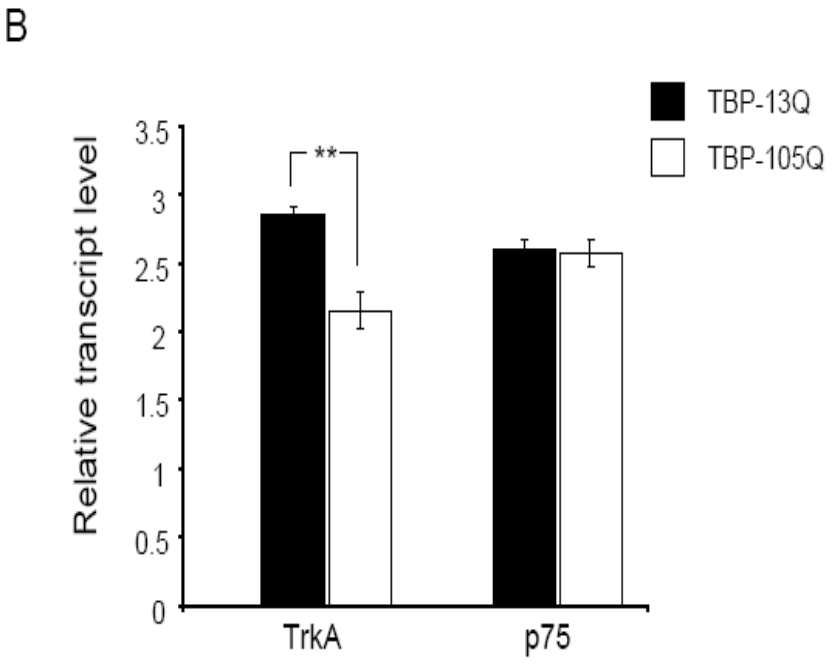
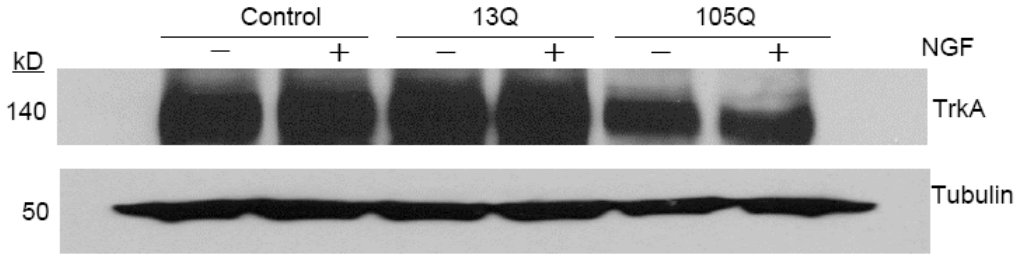

B
TBP-13Q
TBP-105Q
Relative transcript level
3.5
3
2.5
2
1.5
1
0.5
0
**
TrkA
p75

Figure 3-1 Decreased level of TrkA transcripts in stably TBP-105Q transfected PC12 cells. (**a**) RT-PCR analysis of TrkA and p75 ^{NTR} transcripts in PC12 cells stably transfected with TBP-13Q or TBP-105Q. For the internal control, the primers for GAPDH were included in the PCR. (**b**) The ratio of TrkA or p75 ^{NTR} transcripts to GAPDH transcript. TBP-105Q cells show a lower level of TrkA as compared to TBP-13Q cells. ** $P<0.01$.

Figure 3-2


Control
13Q
105Q
kD
−
+
−
+
−
+
NGF
140
TrkA
50
Tubulin

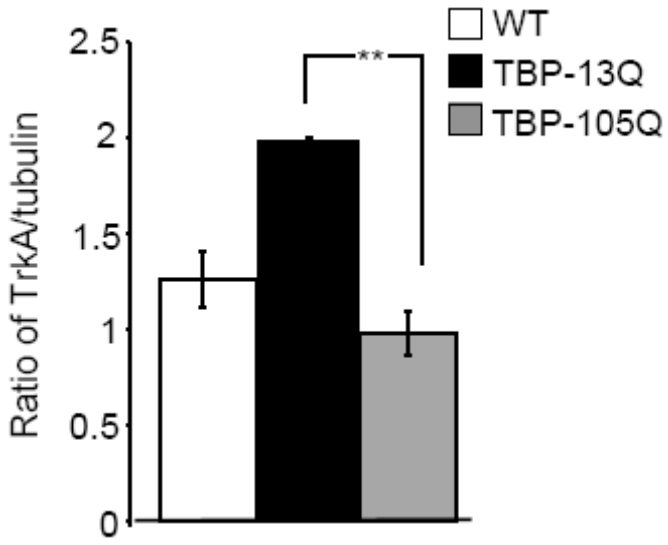

WT
TBP-13Q
TBP-105Q
**
Ratio of TrkA/tubulin
2.5
2
1.5
1
0.5
0

Figure 3-2 Decreased protein levels of TrkA in TBP-105Q PC12 cells. Representative western blot of stably transfected PC12 cell lines treated with (+) or without (–) NGF (100 ng/ml, 15 min). Cells expressing TBP-105Q show decreased levels of TrkA regardless of NGF stimulation (upper panel). Ratios of TrkA to tubulin for the results in the upper panel also verify the decreased level of TrkA in TBP-105Q cells (lower panel). ** $P<0.01$.

Figure 3-3

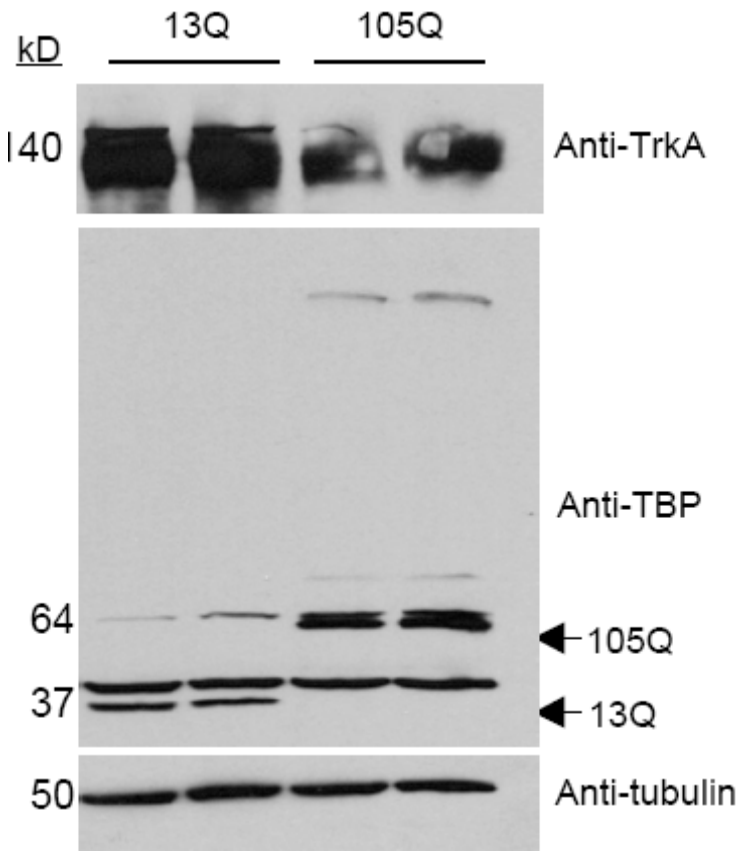

kD
13Q
105Q
140
Anti-TrkA
Anti-TBP
64
105Q
37
13Q
50
Anti-tubulin

Figure 3-3 Decreased levels of TrkA in PC12WT cells infected with TBP-105Q adenovirus. Representative western blot of PC12WT cells infected with TBP-13Q or 105Q adenovirus. Cells infected with TBP-105Q virus show decreased levels of TrkA protein. The same samples were probed with antibodies to TrkA (upper panel), TBP (middle panel) and tubulin (lower panel).

Figure 3-4

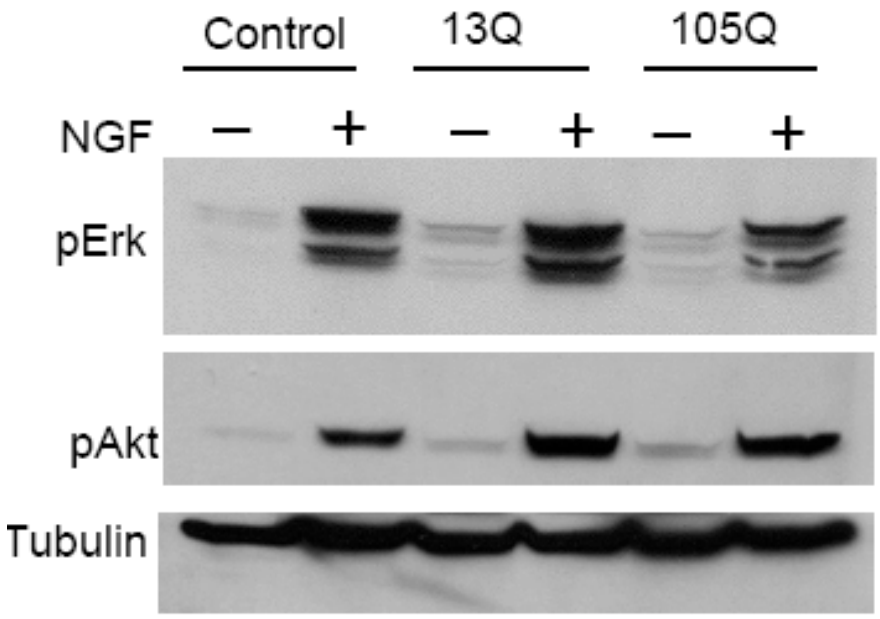


Figure 3-4 Decreased levels of pERK in PC12 cell model of SCA17. Stably transfected PC12 cells were subjected to western blotting with antibodies to phosphorylated Erk (pErk) or Akt (pAkt) after stimulation with NGF (100 n/ml, 15 min). NGF drastically increases the signal of pErk in control and TBP-13Q cells but this increase is reduced in TBP-105Q cells. There is no significant difference in pAkt levels between the three cell lines.

Figure 3-5

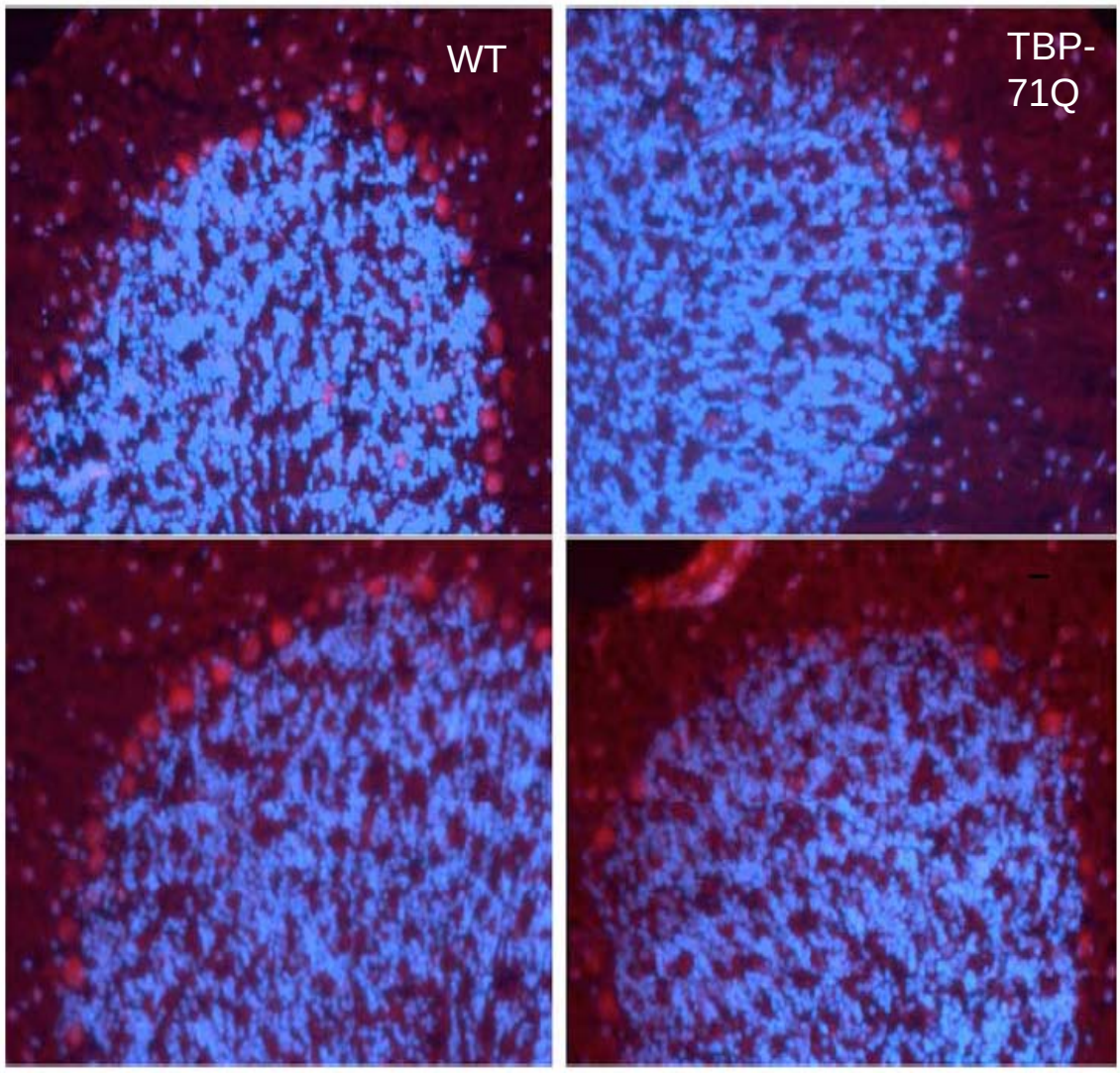
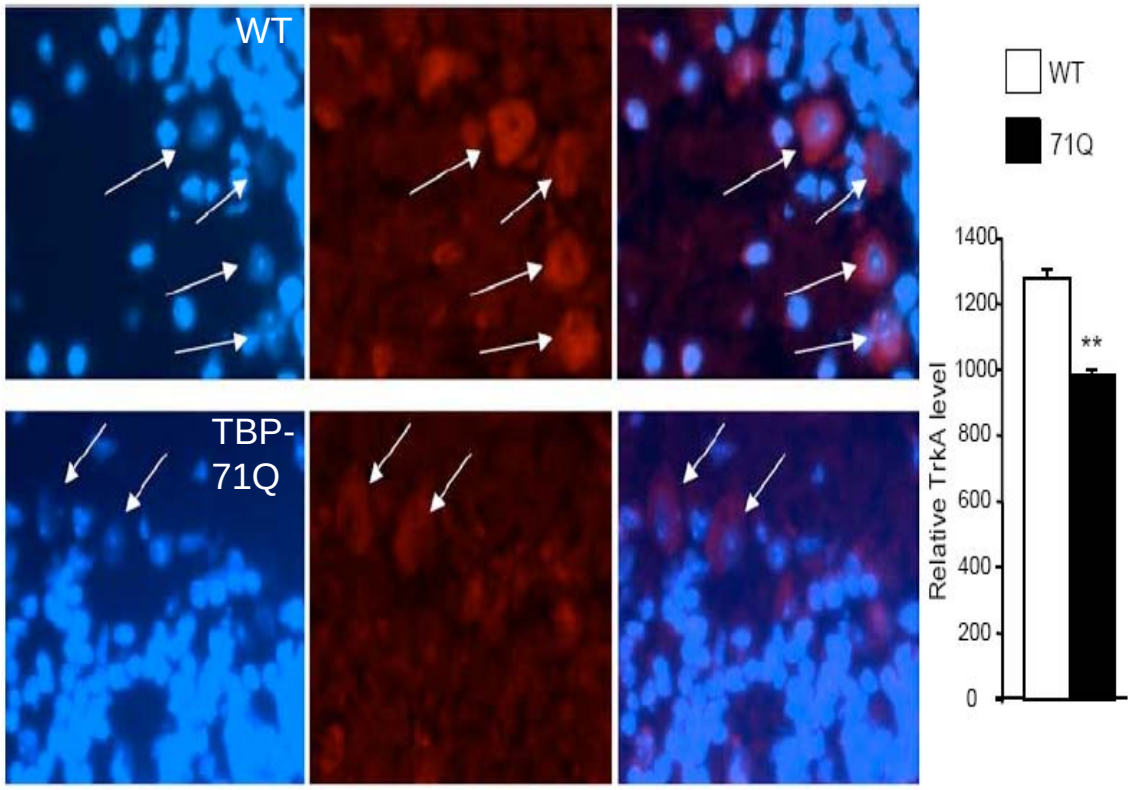


Figure 3-5 Decreased levels of TrkA in Purkinje cells of TBP-71Q SCA17 symptomatic mice. Immunoflourescent images of representative cerebellar sections (10 uM) from 5 month old TBP-71Q mice show decreased TrkA staining (red) as compared to control mice, particularly in the Purkinje cell layer.

Figure 3-6

A


WT
TBP-71Q
WT
71Q
Relative TrkA level
1400
1200
1000
800
600
400
200
0
**

B

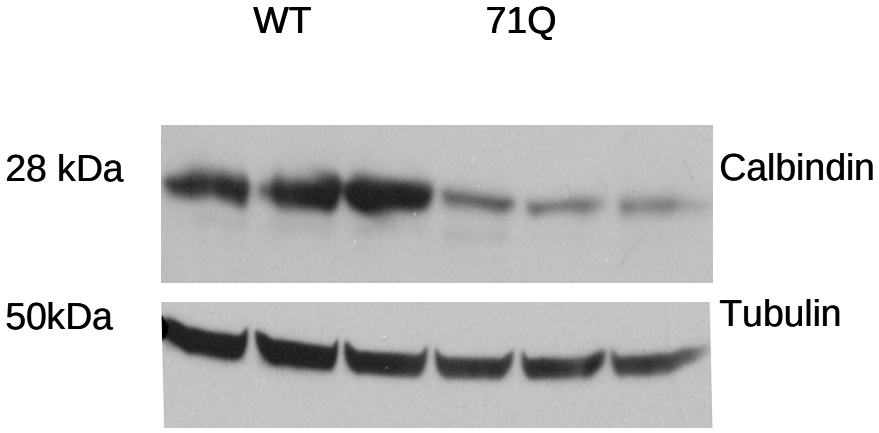
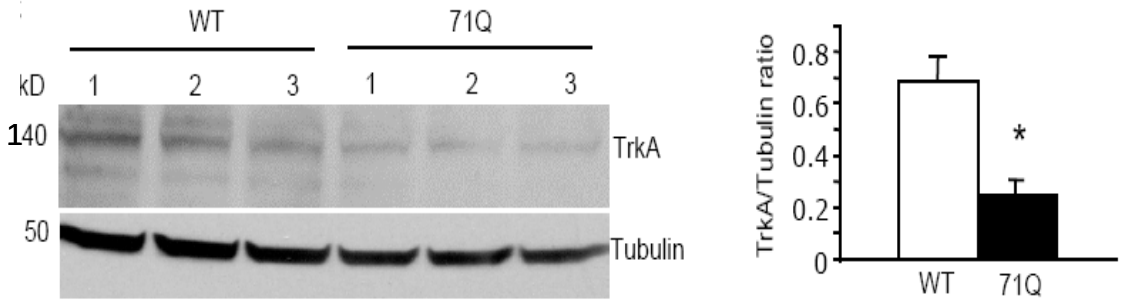

WT
71Q
28 kDa
Calbindin
50kDa
Tubulin

Figure 3-6 Degeneration of Purkinje cells in symptomatic TBP-71Q SCA17 mice. (a) High magnification (63X) micrographs showing TrkA-containing Purkinje cells (arrows). The TrkA red fluorescence signals were quantified (right panel), also showing decreased TrkA in TBP-71Q cerebellum as compared to control cerebellar sections. ** $P<0.01$. **(b)** Western blot showing decreased calbindin levels inTBP-71Q SCA17 mice as compared to control mice.

Figure 3-7

A


WT
71Q
kD
1
2
3
1
2
3
140
50
TrkA
Tubulin
TrkA/Tubulin ratio
0.8
0.6
0.4
0.2
0
*
WT
71Q

B

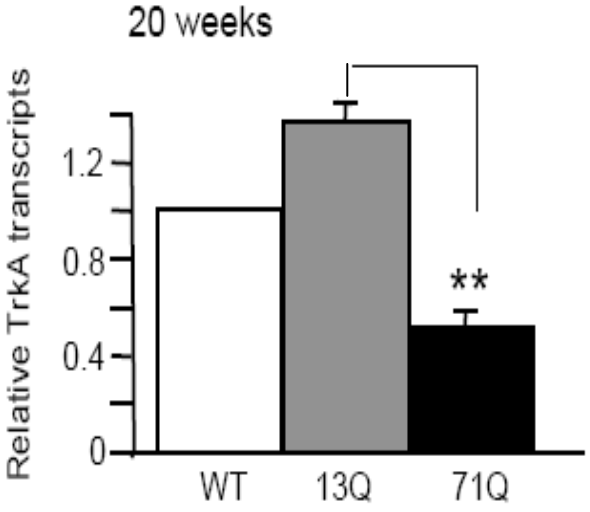
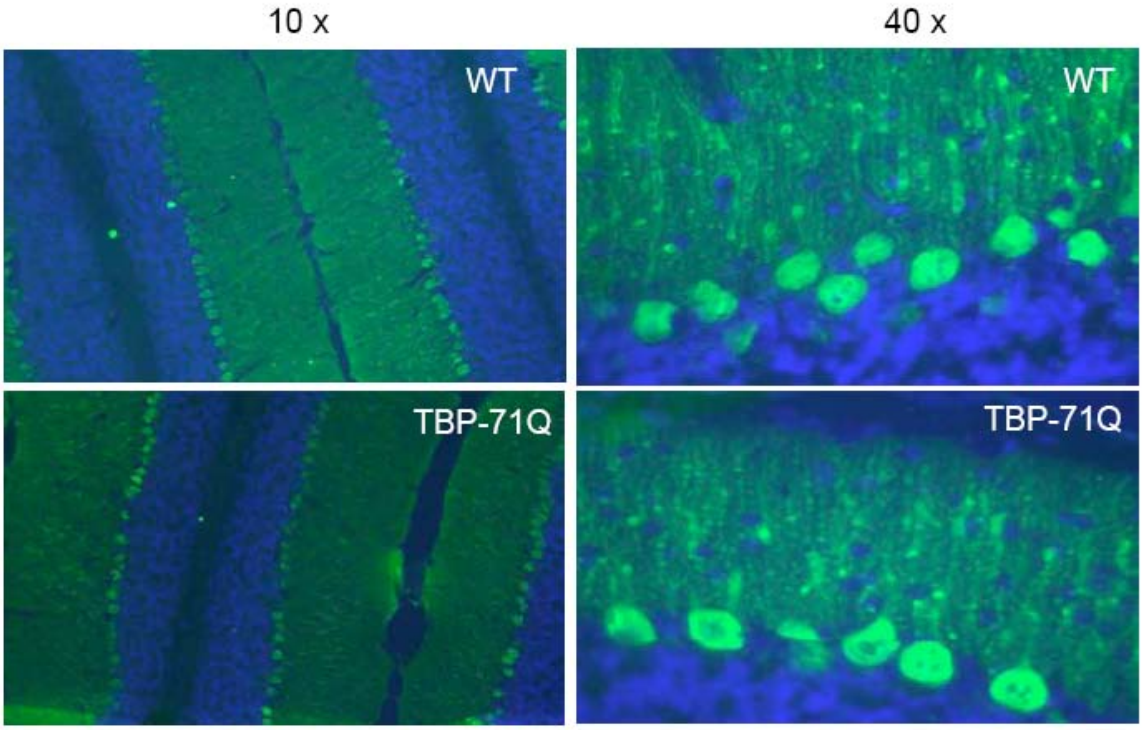

20 weeks
Relative TrkA transcripts
1.2
0.8
0.4
0
**
WT
13Q
71Q

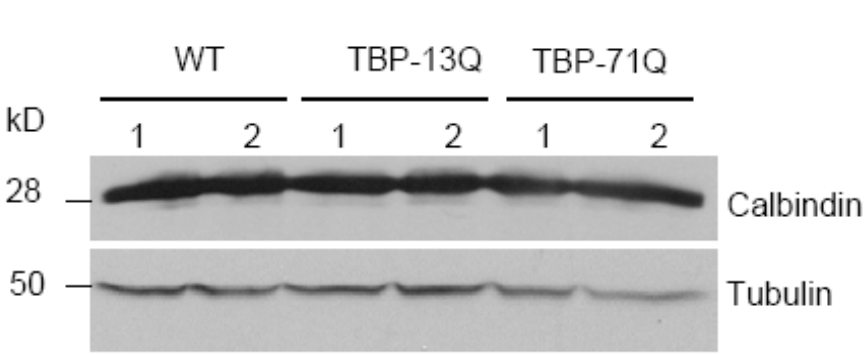
Figure 3-7 Decreased levels of TrkA protein and transcripts in TBP-71Q SCA17 mice. (a) Representative western blot of cerebellar extracts of wild-type (WT) and TBP-71Q transgenic mice at 20 weeks of age. TrkA levels are decreased in the TBP-71Q cerebellum as compared to the wild-type cerebellum. The ratio of TrkA to tubulin is also presented (right panel). * $P<0.05$. **(b)** Quantitative RT-PCR analysis of TrkA transcripts in these mice reveal decreased levels of TrkA in TBP-71Q cerebella as compared to wild-type and TBP-13Q mice. ** $P<0.01$.

Figure 3-8

A


10 x
40 x
WT
WT
TBP-71Q
TBP-71Q

B


WT
TBP-13Q
TBP-71Q
kD
1
2
1
2
1
2
28
50
Calbindin
Tubulin

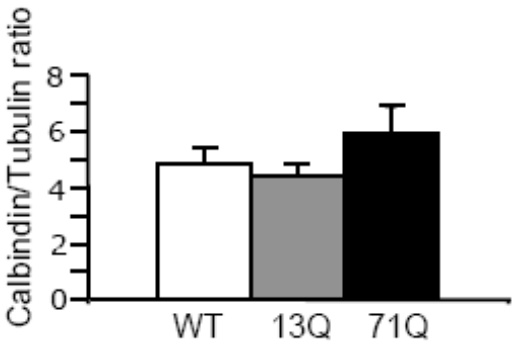
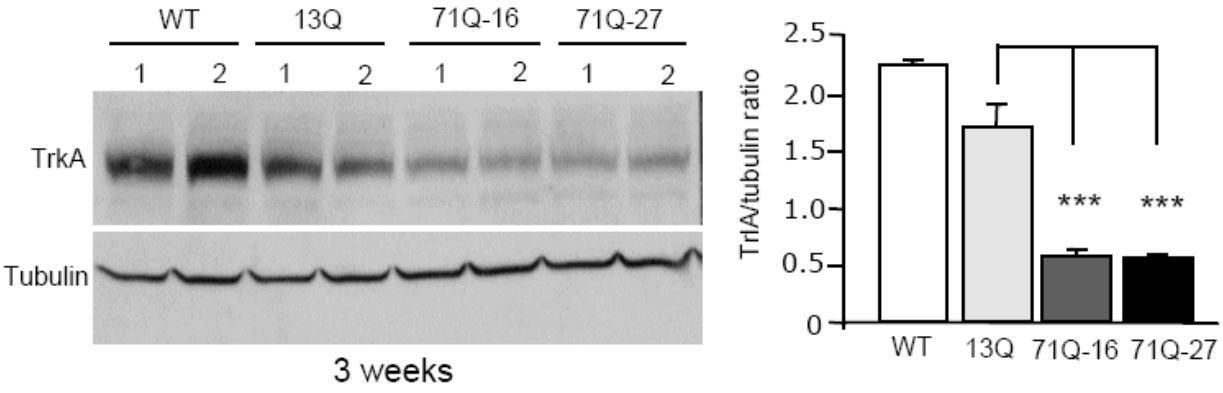

Calbindin/Tubulin ratio
8
6
4
2
0
WT
13Q
71Q

Figure 3-8 Asymptomatic TBP-71Q SCA17 mice show a lack of degeneration of Purkinje cells. (a) The cerebellum of TBP-71Q and wild-type mice at 3 weeks of age were labeled with an antibody to calbindin (green) and nuclear Hoechst staining (blue). Low (10X, left panel) and high (40X, right panel) magnification immunofluorescent images do not show any significant difference in calbindin staining of the Purkinje cell layer. **(b)** Western blotting of the cerebellar tissues of wild- type (WT), TBP-13Q, and TBP-71Q mice. Two mice in each group were examined for the expression of calbindin. The ratio of calbindin to tubulin is also presented (right panel).

Figure 3-9

A


WT
13Q
71Q-16
71Q-27
1
2
1
2
1
2
1
2
TrkA
Tubulin
3 weeks
TrkA/tubulin ratio
2.5
2.0
1.5
1.0
0.5
0
***
***
WT
13Q
71Q-16
71Q-27

B

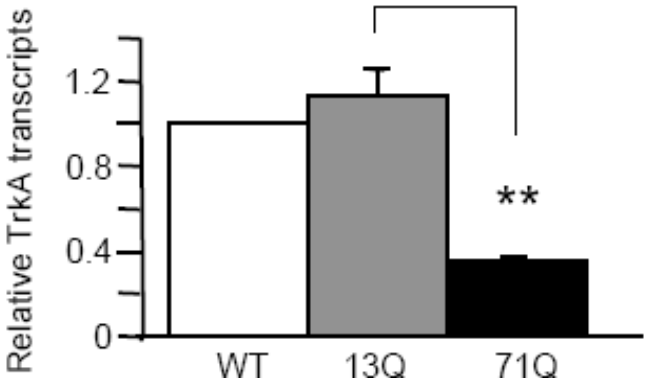

Relative TrkA transcripts
1.2
0.8
0.4
0
**
WT
13Q
71Q

Figure 3-9 Young asymptomatic TBP-71Q SCA17 mice show decreased levels of TrkA. (a) Representative western blot of cerebellar extracts of 3-week-old wild- type (WT), TBP-13Q, and TBP-71Q mice. The samples were probed with antibodies to TrkA and tubulin. The ratios of TrkA to tubulin are also presented (right panel). *** $P<0.001$. **(b)** Quantitative RT-PCR analysis of TrkA transcripts in these mice reveal decreased levels of TrkA in TBP-71Q cerebella as compared to wild- type and TBP-13Q mice. ** $P<0.01$.

Chapter 4

Transcriptional Dysregulation of TrkA

This chapter includes findings and discussions from a manuscript currently submitted as Shah AG, Friedman MJ, Roberts M, Li XJ, and Li SH. Transcriptional dysregulation of TrkA associates with neurodegeneration in Spinocerebellar Ataxia 17. *Hum. Mol. Genet.* (2009). Constructs for generation of mouse and cell models were designed by Shihua Li and Xiao-Jiang Li. TBP-Sp1 binding studies, making of fusion proteins, and cloning of TBP constructs were performed by Meyer Friedman. All other experiments were carried out by Anjali Shah. Xiao-Jiang Li participated in the writing and editing of the submitted manuscript.

4.1 Abstract

TBP is a general transcription factor that is required at the promoter of genes regulated by all three nuclear RNA polymerases. However, despite functional consequences due to the polyglutamine expansion in TBP, there is a lack of global transcriptional dysregulation. Abnormal interactions of mutant TBP with other proteins may underlie the molecular pathogenesis of SCA17. Using our neuronal cell model of SCA17 we show that mutant TBP inhibits transcriptional activity from the TrkA promoter, binds more Sp1, and reduces the occupancy of Sp1 at the TrkA promoter. These findings suggest that the transcriptional down-regulation of TrkA is caused by an aberrant interaction between Sp1 and mutant TBP, ultimately contributing to SCA17 pathogenesis.

4.1 Introduction

Transcription of DNA into mRNA is one of the most highly regulated processes in the cell. Transcriptional regulation depends on a complex molecular machine consisting of over one hundred components. Many of these proteins are transcription factors, and their interacting domain consists of polyglutamine tracts or glutamine- rich regions (Frieman and Tjian, 2002). Association of glutamine- rich proteins represents a class of protein-protein interactions that enable transcription factors to signal one another about regulating the expression of specific genes. However, the presence of polyglutamine expanded mutant proteins could involve deregulation of specific transcriptional programs in brain neurons. For example, there is specific interaction between the glutamine- rich activation domains of Sp1 and the glutamine- rich subunit of

TFIID called TAF4 (Saluja et al., 1998) and this interaction was found to be disrupted in the presence of mutant huntingtin, leading to dysregulation of Sp1-mediated gene expression (Dunah et al., 2002).

Transcriptional dysregulation is one of many theories put forward as a pathological mechanism to explain how polyglutamine expanded proteins can cause cellular dysfunction (Sakahira et al., 2002). In this view, neurotoxicity originates from the ability of the polyglutamine proteins to induce the co-aggregation of itself with other proteins essential for cell viability, among them several transcription components which possess non-pathogenic polyglutamine repeats (Sugars and Rubinsztein, 2003). These factors include, TBP, the transcriptional co-activator CREB binding protein (CBP), Sp1 and others. These transcription factors have been reported to co-localize with the aggregates of disease related polyglutamine proteins (McCampbell et al., 2000; Perez et al., 1998), and this may result in their sequestration and loss of function (Chai et al., 2002; Kim et al., 2002; Rajan et al., 2001).

The mechanism by which transcriptional dysregulation occurs is far from clear. Perhaps the increased polyglutamine stretch could confer a gain of function property resulting in the direct binding of the protein to DNA, disrupting the normal pattern of transcription. Or, the mutant protein may bind aberrantly to specific transcription factors forming inactive transcriptional complexes. Along this same line, the mutant protein may form complexes with transcriptional repressor proteins, aberrantly derepressing normally silent genes. Or, the mutant protein may sequester transcription factors through aberrant interactions, depleting the levels of required factors within the cell (Cha 2000). It is unclear which of these mechanisms actually cause transcriptional dysregulation, but it is

clear that dysfunction is mediated by aberrant interactions of the mutant protein with either native or novel binding partners.

Transcriptional dysregulation has been most extensively studied in HD (Cha et al., 2000). The huntingtin (htt) protein is a large protein and in its normal state is mainly cytoplasmic. The function of normal htt still remains elusive but htt is believed to function in neuronal trafficking (Caviston and Holzbaur, 2009). However, the polyglutamine expansion leads to improper processing of the protein leading to several different sized N-terminal fragments that can then enter the nucleus, accumulate and induce toxicity (Cornett et al., 2005). In general, mutant htt has been reported to have a negative effect on Sp1- and CREB- dependent transcriptional pathways (Li et al., 2002; Dunah et al, 2002; Jiang et al, 2002; Sugars and Rubinsztein, 2003). The aggregate sequestration model, in which these or other transcription factors are recruited into nuclear mutant htt aggregates, was initially proposed based on co-localization data. However, *in vivo* studies in HD mouse models have not substantiated these results (Dunah et al., 2002; Yu et al., 2002), and more importantly have indicated that htt aggregation does not correlate with transcriptional dysregulation (Obrietan et al., 2004). Instead, there is compelling evidence to show that aberrant interactions of various transcription factors such as TBP and SP1, with soluble mutant htt is sufficient to sequester these proteins away from their cognate promoter sequences (Dunah et al., 2002; Li et al., 2002; Schaffar et al., 2004) **(Figure 1-1c)**.

Even though TBP is a general transcription factor, there is not global transcriptional dysregulation in the presence of mutant TBP. However, the expanded polyglutamine tract can aberrantly interact with transcriptionally relevant proteins to

cause transcriptional dysregulation of specific genes involved in neuronal function (Friedman et al., 2007). The absence of aggregates in the PC12 cell model of SCA17 makes it unlikely that transcriptional dysregulation is occurring through sequestration of transcription factors into mutant TBP aggregates, though we cannot exclude the possibility of aberrant interactions of transcription factors and soluble mutant TBP. Here we show that cells expressing TBP-105Q have defective transcription at the TrkA promoter and that this promoter is regulated by Sp1. Furthermore, we observed increased binding of Sp1 to soluble mutant TBP, leading to decreased occupancy of Sp1 at the TrkA promoter of cells expressing TBP-105Q, presumably leading to decreased transcription of the TrkA promoter and contributing to SCA17 pathogenesis.

4.2. Materials and Methods

Plasmids and Reagents

SCA17 mice were generated, and mouse and human TBPs were cloned as previously described, (Friedman et al, 2007). Stably transfected PC12 cell lines were generated by transfecting the Tet-off stable cell line (Clontech) with PBI-EGFP-TBP constructs (TBP-105Q and TBP-13Q) using Lipofectamine 2000. TBP containing 13Q or 105Q and EGFP were independently expressed from the CMV promoter in these constructs. Sp1-HA fusion protein was made as previously described (Li et al., 2002), as were pRK-TBP, GST-Sp1 and His-TBPs (Freidman et al, 2007). Antibodies against TBP included 1TBP18 (QED). Other antibodies used for these studies were, tubulin (Sigma), and Sp1 (Upstate Biotechnologies). The human TrkA promoter was cloned from genomic DNA using the following primers: sense 5’- ATCAAAGCTTCACCTCCGAGGCGTTC-

3’ and antisense 5’- GAATTCTCTGTGCGCTCCCAGCTGC-3’. A PCR fragment was cloned into the DsRed 2.1 vector (Clontech) using *HindIII* and *EcoR1* restriction sites. The resulting plasmid was sequenced to confirm the presence of the TrKA promoter sequence.

Reporter Assays

For experiments with transiently transfected TBP, HEK293T cells were co-transfected with the reporter vector, Sp1, TBP-31Q, TBP-105Q or a mock vector. Transfections were done in triplicate in 12-well plates using Lipofectamine (Invitrogen). The cells were harvested in 500 µl of cold 1X PBS 48 h after transfection, and 50 µl of resuspended cells was combined with 50 µl of 1X PBS in a 96-well black bottom plate. Samples from each co-transfection were plated in duplicate, and red fluorescence was detected with a FLOUstar Galaxy microplate reader (BMG LABTECH, Offenburg, Germany). For experiments using the stably transfected PC12 cell lines expressing TBP-13Q or 105Q, the reporter or empty vector was transfected. Transfections were done in duplicate in 12-well plates using Lipofectamine 2000. Fluorescence images were visualized on the Zeiss microscope (Axiovert 200 MOT; Carl Zeiss Imaging), and fluorescence levels were quantitated using OpenLab Software (Improvision Inc).

Cerebellar Granule Cell Culture:

Cerebella were dissected from postnatal day 7-9 transgenic TBP-105Q SCA17 mice or wild-type littermates and placed in cold 1X Hanks’ balanced salt solution (HBSS) containing 0.3%BSA and 20 mM HEPES. After removing the meninges,

cerebella were minced and the tissue was digested by incubating at 37°C for 15 min in the presence of 0.025 mg/ml trypsin and 0.025 mg/ml DNase with occasional inversion of the tube. Digestion was stopped by the addition of 3 volumes of neurobasal A medium (Invitrogen) supplemented with 2% B27, 0.5 mM glutamine, 25 μM glutamate, KCl (final concentration 25 mM), and 1% penicillin/streptomycin (Smith et al., 2004). Samples were centrifuged at 1500 rpm for 5 min, and cell pellets were resuspended in 1 ml of medium prior to filtering and subsequent plating. At two days in vitro (DIV), 10 μM cytosine arabinoside was added to inhibit glial proliferation. Half of the medium in each well was replaced with fresh medium lacking glutamate every third day. After culturing for 12 days, cerebellar granule cells were prepared for immunofluorescence staining as previously described (Friedman et al, 2007). Cells were incubated with anti-Sp1 (Upstate Biotechnologies) and anti-1TBP18 (QED) and examined for co-localization of Sp1 with TBP-105Q aggregates.

Protein Interaction Studies:

His6-tagged TBPs and GST-Sp1 were generated by subcloning cDNAs from pRK5 into pET28a and pGEX-4T-1 vectors, respectively. Recombinant proteins were expressed in Escherichia coli BL21 (DE3) by induction with 1 mM isopropyl-D-thiogalactopyranoside for 1.5 hours at room temperature. Hexhistidine TBP's were purified by Ni-nitrilotriacetic acid (NTA)- agarose chromatography as described previously (Li et al., 2002b). For *in vitro* pull-down assay, 5 μg of GST or GST-Sp1 attached to glutathione beads was incubated with His-TBPs (13Q or 71Q) in 400 μl of 1X phosphate buffered saline (PBS) containing 0.5% NP-40 for 2 hours at 4°C. Beads were

precipitated and washed twice with 1 ml of binding buffer. Input in western blot was 25% of purified TBPs used for pull down.

Chromatin Immunoprecipitation:

ChIP assays with semi-quantitative PCR were performed as described previously (Friedman et al., 2007). PC12 TBP-13Q and 105Q cells were transfected with Sp1-HA, and collected 48 hours after transfection. For each IP, 850 μg of pre-cleared whole cell lysate and 5 μg of anti-Sp1 (Millipore, Upstate Biotechnologies) or no antibody was used. Semi-quantitative PCR, using *PCNA* and *TrkA* primers in separate reactions, was performed on DNA recovered from IP samples and inputs (10% pre-cleared lysate). IP without any antibody and PCR without template DNA served as negative controls. Promoter sequences were acquired using the UCSC genome browser, and primers were designed so that amplicons were inclusive of putative Sp1 sites. The size of the amplicons for *PCNA* and *TrkA* were 282 bp and 183 bp, respectively. Primer sequences and PCR cycling parameters are as follows:
rat *PCNA*: sense 5'-TGGCTTTCATTTCCGTGGC -3' and antisense 5'-AGTCACCTGCGCCCGCAAC-3,
rat *TrkA*: sense 5'-ACATGTGAAGCAATCTGTGGCAG-3' and antisense 5'-CGGGGCGGTGTTAAAGACTAGCC-3'. PCR conditions were as follows:

1. 96° for 3 minutes
2. 96° for 45 seconds
3. 63° for 45 seconds
4. 72° for 1 minute

Repeat steps 2-4 44X

5. 72° for 5 minutes

PCR products were electrophoresed on a 1.8% agarose gel.

Western Blotting

Cultured cells were stimulated with NGF for 48h, then harvested and homogenized in RIPA buffer [50 mM Tris-HCl (pH8.0), 150mM NaCl, 1mM EDTA (pH8.0), 1mM EGTA (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100, with protease inhibitors (Sigma)], sonicated for 10 s before and after 30 min incubation on a rotating apparatus at 4 °C, and clarified at 3000 rpm for 5 min. Fifty micrograms of cell supernatant was used for Western blotting. Protein samples were run on 4–20% Tris-glycine polyacrylamide gels (Invitrogen). Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) kit.

4.4 Results

We were able to develop an animal and cell model of SCA17 that accurately recapitulated neurodegenerative phenotypes seen in SCA17 patients as well as other polyglutamine diseases, including neuritic defects and decreased cell viability. We were also able to identify the dysfunction of a molecule, TrkA, which plays a major role in neuron differentiation, neurite outgrowth and cell survival. However, it is unclear how decreased levels of TrkA can contribute to SCA17 pathology. Previously, we showed that not only was TrkA down-regulated at the protein level, but this down- regulation was transcriptionally mediated, as we saw less TrkA transcript in our PC12 cells stably

expressing TBP-105Q **(Fig. 3-1a,b)** and mice expressing TBP-71Q **(Fig. 3-7b, 3-9b)**. Furthermore, in SCA17 mice, this decrease in TrkA was seen before any obvious neurological phenotypes were seen.

To determine if the decrease in TrkA transcript level was due to decreased transcription from the TrkA promoter and not some other mechanism, such as decreased stability of the transcript, we performed a reporter assay to measure transcription from the TrkA promoter. We generated a reporter construct by attaching the TrkA promoter region to the DsRed reporter gene **(Fig. 4-1a)**, such that transcription from that promoter could be measured by the level of red fluorescence. The reporter was transfected into the PC12 cells stably expressing TBP-13Q or TBP-105Q. Not surprisingly, cells expressing TBP-105Q showed a significant decrease in transcription from the TrkA promoter **(Fig. 4-1b)**. We wanted to further confirm this defect in transcription and be sure that the decrease in transcription was not due to an artifact of the stable expression of mutant TBP in these cells. We repeated the experiment in HEK293T cells, and transfected the reporter alone plus empty vector, the reporter with TBP-13Q, and the reporter with TBP-105Q. However, in this experiment we were unable to detect any transcription from the promoter as there was no detectable red fluorescence signal (data not shown). Given that the promoter of TrkA contains Sp1 binding sites (Sacristán et al., 1999), which are present in our reporter construct, we overexpressed Sp1 to act as a co-activator to stimulate transcription in these HEK 293T cells. Cells were transiently co-transfected with Sp1, the reporter and empty vector; or Sp1, the reporter and TBP-13Q; or Sp1, the reporter and TBP-105Q **(Fig. 4-2a)**. Although overexpressing TBP-13Q and TBP-105Q decreased levels of transcription, we did see a greater reduction in TrkA promoter activity

in cells expressing TBP-105Q than in cells expressing TBP-13Q **(Fig. 4-2b)**. This result confirms a polyglutamine repeat dependent defect in transcription from the TrkA promoter. This defect is not due to different expression levels of transfected proteins, as western blot analysis showed equal expression of all transfected constructs.

After confirming a transcriptional defect of TrkA in the presence of polyglutamine expanded TBP, we sought to determine a mechanism for this transcriptional down regulation. We previously showed that mutant TBP aberrantly interacts with the transcription factor TFIIB, which was also sequestered into TBP aggregates, and had decreased promoter occupancy at various genes of interest (Friedman, et al., 2007). Since Sp1 is found to interact with TBP (Torigoe et al., 2003; Emilli et al., 2004) and other polyglutamine-containing proteins (Li et al., 2002; Dunah et al., 2002; Goold et al., 2007), we examined the distribution of nuclear Sp1 in cerebellar granule neurons of SCA17 mice expressing TBP-105Q. We did not observe co-localization of Sp1 with nuclear TBP inclusions **(Fig. 4-3)**, suggesting that aggregated TBP may not be able to interact with Sp1. Though we did not see co-localization of Sp1 with TBP aggregates, we wanted to determine if there was an increased interaction of Sp1 with soluble mutant TBP. To provide biochemical evidence of an aberrant interaction between soluble mutant TBP and Sp1, we conducted *in vitro* binding experiments with His-tagged TBPs and a GST-Sp1 fusion protein. Interestingly, GST-Sp1 pulled down more TBP-71Q than TBP-31Q **(Fig. 4-4)**. Densitometric analysis of the ratio of pulldown to input verified that mutant TBP (TBP-71Q) binds roughly 40% more Sp1 than TBP-31Q in this *in vitro* assay. This data indicates that polyQ expansion in soluble mutant TBP enhances the binding of TBP to Sp1.

The aberrant interaction of soluble mutant TBP with Sp1 could have functional consequences for Sp1 and lead to transcriptional dysregulation as seen in other polyglutamine diseases (Cornett et al., 2006; Chen-Plotkin et al., 2006; Zhai et al., 2005; Dunah et al., 2002; Riley and Orr, 2006). Similar to the TBP/TFIIB interaction, perhaps the interaction of soluble mutant TBP with Sp1 decreased the presence of Sp1 at the TrkA promoter, causing decreased transcription of TrkA and the resulting disease pathology. To test this possibility we used PC12 cells stably expressing TBP-13Q or TBP-105Q to perform a chromatin immunoprecipitaton assay or ChIP assay. Cells were transiently transfected with Sp1, treated with NGF for 48 hours, then cross-linked. Sp1-DNA complexes were immunoprecipitated with an anti-Sp1 antibody. The precipitates were subjected to PCR with primers for the *TrkA* or *PCNA* promoter sequences. The *PCNA* promoter served as a positive control because it is a known gene regulated by Sp1 and was shown not to be dysregulated in the presence of mutant TBP (Friedman et al., 2007). The TrkA primers were designed to flank three Sp1 binding sites on the *TrkA* promoter. While there is no significant difference in the association of Sp1 with the PCNA promoter when comparing the TBP-13Q and TBP-105Q cells, we observed a decrease in the association of Sp1 with the TrkA promoter in cells expressing TBP-105Q **(Fig. 4-5a).** The ratios of PCR products from the precipitates to those from the inputs confirmed a decreased association of Sp1 with the TrkA promoter in cells expressing TBP-105Q. Western blot analysis also shows equal expression levels of transfected Sp1 in the two cell lines **(Fig. 4-5b)**.

4.5 Discussion

The C-terminus of TBP is well characterized and is known to involve its DNA binding capability, however the function of the N-terminus of TBP has only been partially elucidated (Mittal and Hernandez, 1997; Hobbs et al., 2002), and in particular, the function of the polyglutamine domain is largely unclear. The sequence of the N-terminus is highly conserved among vertebrates (Bondareva and Schmidt, 2003), but the polyglutamine region is highly polymorphic and is generally absent in lower eukaryotes including nematode (Lichtsteiner and Tjian, 1993) and yeast (Schmidt et al., 1989; Hahn et al., 1989; Cavallini et al;, 1989). The length of the polyglutamine tract also varies among vertebrates, suggesting that this polyglutamine domain may specifically and differentially modulate TBP function in vertebrates.

Since transcription factors with polyglutamine domains interact with each other via this domain (Frieman and Tjian, 2002), it seems likely that the polyglutamine expanded domain in mutant TBP could cause impairment among these interactions. The impairment of these interactions is unlikely to cause global alterations in gene expression, but they may affect the gene expression of a small subset of genes. Consistent with this idea, replacement of the N-terminus of mouse TBP including the polyglutamine tract with a FLAG epitope does not result in broad transcriptional dysregulation, nor does it abrogate TBP function (Schmidt et al., 2003). Similarly, microarray analysis of SCA17 mice showed a specific subset of genes to be altered, but global alterations were not seen (Friedman et al., 2007).

Our data indicates that polyglutamine expansion in TBP alters the expression of specific Sp1-mediated genes, in particular, the presence of mutant TBP inhibits transcriptional activity at the TrkA promoter by aberrantly interacting with Sp1 and titrating it away from its cognate promoter. Importantly, this interaction did not support the

sequestration model of toxicity (Chai et al., 2002), as Sp1 did not co-localize with TBP aggregates, but instead interacted more with soluble mutant TBP, suggesting that recruitment into aggregates is dependent on the context of interacting proteins.

It is generally accepted that overexpression of normal TBP (as part of the larger TFIID complex) is stimulatory for TATA-containing promoters and the level of activation is inversely related to the strength of the promoter sequence, however, exogenous TBP does not stimulate TATA-less promoters in cell culture, and in some cases can even reduce transcription from these promoters (Colgan and Manley, 1992), suggesting that the functional contribution of TBP is not identical at TATA-containing promoters and TATA-lacking promoters. Furthermore, it has been demonstrated that TATA-binding activity of TBP is dispensable for transcriptional initiation from TATA-less promoters (Martinez et al., 1994). This finding indicates that the DNA binding activity does not play a role at TATA-less promoters, and that it may not be the limiting factor in transcription initiation. Rather, it may act as a cofactor that is part of a much larger complex of transcription factors or transcription associated factors to induce transcription from a TATA-less promoter.

Consistently, previous *in vitro* studies demonstrated that transcription from a TATA-less promoter composed of the initiator elements from the murine terminal deoxynucleotidyltransferasc (TdT) gene (Smalc and Baltimore 1989) and the GC boxes constituting Spl binding sites from the SV40 early promoter (Dynan and Tjian 1983) requires the TFIID fraction to stimulate transcription but purified TFIID is not sufficient (Pugh and Tjian 1990; Smale et al. 1990). The inhibition of TATA-less promoter activity in cell culture by overexpression of TFIID suggests that a factor able to interact with

TFIID is limiting for TATA-less promoter expression. This is demonstrated in experiments where overexpression of TFIID resulted in substantial reductions of Spl-mediated activation of the synthetic TATA-lacking promoter GI (Colgan and Manley, 1992). The simplest explanation for these findings is that overexpressed TFIID can squelch expression from the TATA-lacking promoters by sequestering a limiting factor (Gill and Ptashne, 1988).

Similarly, in our reporter experiments we found that HEK293T cells transiently transfected with TBP-13Q or TBP-105Q and the TrkA reporter construct showed decreased transcription from the TrkA promoter, as compared to cells transfected with the reporter alone. Importantly, the TrkA promoter is TATA-less and has several Sp1 binding sites. These findings are in line with previous studies and indicate that the overexpression of TBP could be squelching expression from the TrkA promoter by sequestering an important co-factor needed for transcription, in this case, Sp1. Furthermore, in HEK293T cells transfected with TBP-13Q and TBP-105Q with the reporter construct, there was no detectable transcriptional activity, however, when Sp1 is over expressed, robust fluorescence is seen in the cells expressing just Sp1 and the reporter, indicating that Sp1 may be the limiting factor in transcription initiation from the TrkA promoter. This is further corroborated by the decreased transcription levels seen in the cells overexpressing TBP-13Q and TBP-105Q, suggesting that overexpression of TBP is sequestering Sp1. Importantly, there is a greater decrease in transcription from the TrkA promoter occuring in cells expressing TBP-105Q than in cells expressing TBP-13Q, also suggesting that the expanded polyglutamine enhances the interaction of TBP with Sp1 to reduce Sp1's association with the TrkA promoter.

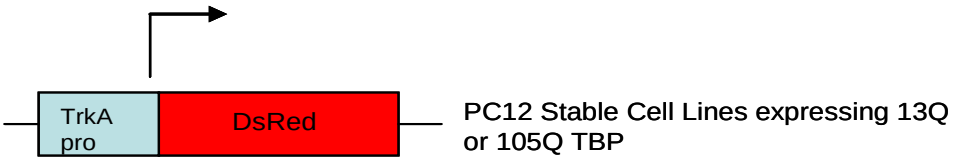
The TrkA promoter has several putative Sp1 binding sites which are conserved between mouse and human (Chang et al, 1998; Sacristan et al., 1999), suggesting that its transcription is regulated by the activity of Sp1. Though it has not been shown that Sp1 directly binds to the promoter of TrkA, it has been immunoprecipitated from DNA-protein complexes at the TrkA promoter (Sacristan et al., 1999) indicating at the very least an indirect association with the promoter via a much larger transcription machinery complex. We see less TrkA reporter activity in HEK293 cells in the absence of Sp1 but it has been shown that TrkA reporter constructs show more transcriptional activity in neuronal cell lines than HEK293 cells (Sacristan et al., 1999), perhaps due to increased levels of co-activators in these neuronal cell lines needed to stimulate transcription of a gene important in neuronal function. Alternatively, the transcription start site may be differentially regulated in a cell type-specific manner, as evidenced by several different transcription initiation start sites in the human TrkA promoter (Chang et al., 1998). These findings suggest that different transcriptional regulation in a cell type specific manner may lend to the selective neurodegeneration of specific cell types seen in polyglutamine diseases.

It is clear that early gene expression changes can precede neuropathology as seen in our study as well as in SCA1 mouse models (Lin et al, 2000; Zabel et al., 2009). Though, the specific transcriptional dysregulation of TrkA is unlikely to be the only pathway involved in the molecular pathogenesis of SCA17. As seen in microarray analysis of SCA17 mice, other genes were down-regulated and altered expression of certain genes important for neuronal function may also contribute to primary events leading to neuropathology. For example, transcript levels of the small heat shock protein

HSPB1 was found to be down-regulated in the cerebellum of SCA17 mice. Interestingly, the *HSPB1* promoter contains a TATA box and 2 Sp1 binding sites (Gaestel et al, 1993), which suggests the involvement of both TBP and Sp1 in transcription initiation at the *Hspb1* locus. Importantly, it has been shown that Sp1 binds the *HSPB1* promoter and is able to activate transcription (Oesterreich, 1996). Furthermore, aberrant interactions between Sp1 and mutant htt lead to decreased transcription at the HSPB1 promoter (Cornett et al., 2006). Perhaps the increased association of Sp1 with soluble mutant TBP could account for the decreased levels of HSPB1 protein seen in the PC12 cell model of SCA17 expressing TBP-105Q. Alternatively, decreased binding of polyglutamine expanded TBP to DNA (Friedman et al., 2007, 2008) could lead to decreased transcription initiation from the *Hspb1* promoter.

Figure 4-1

A


TrkA
pro
DsRed
PC12 Stable Cell Lines expressing 13Q
or 105Q TBP

B

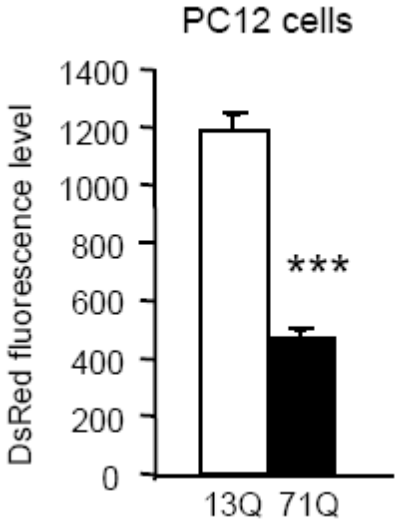
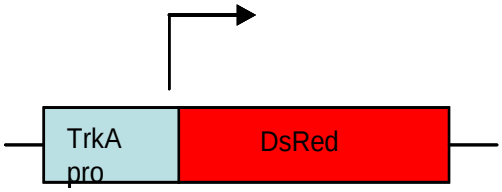

PC12 cells
DsRed fluorescence level
1400
1200
1000
800
600
400
200
0
***
13Q
71Q

Figure 4-1 Decreased transcription from the TrkA promoter in a PC12 cell model of SCA17. (a) Schematic representation of the reporter construct used to transfect cells for the reporter assay. **(b)** PC12 cells stably expressing TBP-105Q show decreased TrkA promoter activity as quantified by the level of red fluorescence. ** $P < 0.01$.

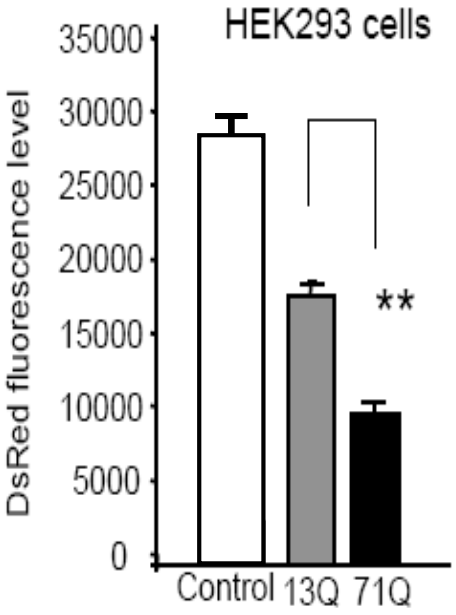
Figure 4-2

A



+ Sp1, + 13Q or 105Q TBP in HEK293T Cells

B



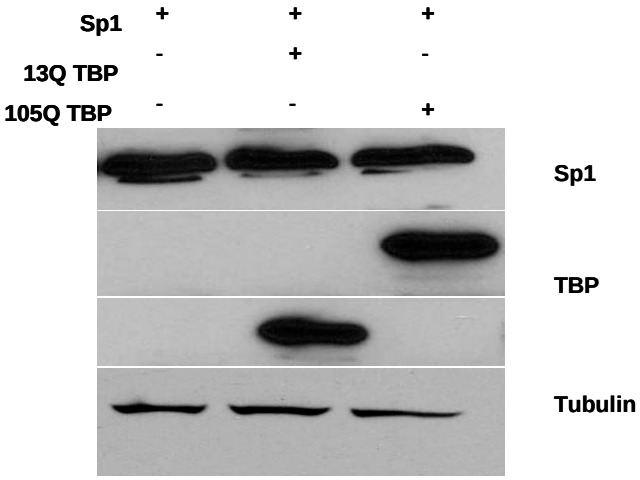


Figure 4-2 Overexpression of Sp1 can stimulate transcription from the TrkA promoter in HEK 293T cells. (a) Schematic representation of the reporter construct used to transfect cells for the reporter assay. (b) Reporter activity is only seen in the presence of Sp1 and cells transfected with TBP-105Q show decreased transcription from the TrkA promoter as quantified by the level of red fluorescence. Equal expression level of transfected Sp1, TBP-13Q, and TBP-105Q is also shown (right panel). ** $P < 0.01$.

Figure 4-3

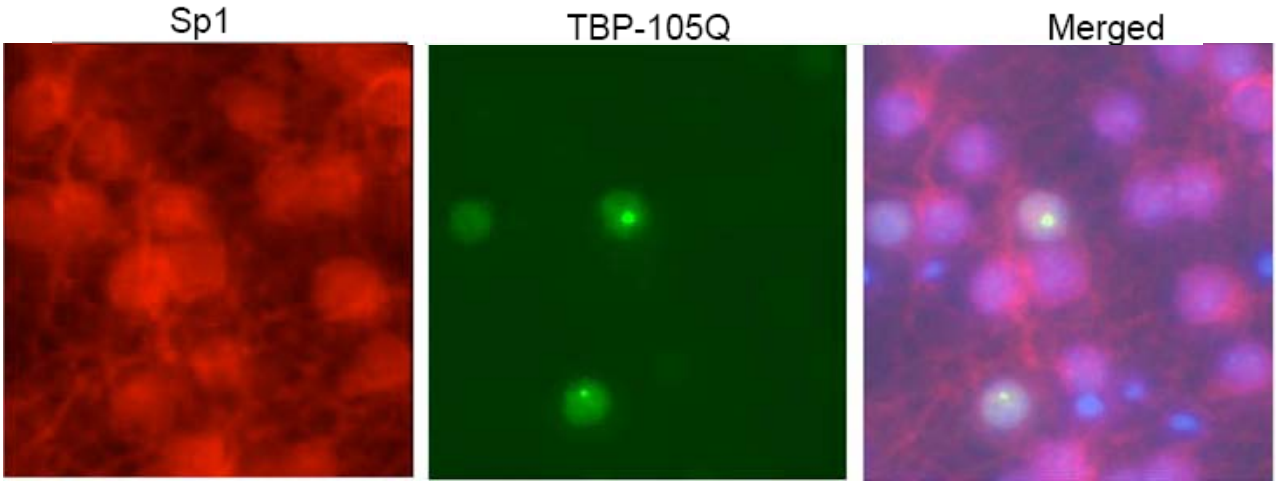


Figure 4-3 Sp1 does not co-localize with aggregates in TBP-105Q mice.
Immunofluorescent images of cultured cerebellar granular neurons stained with Sp1 (red) and TBP aggregates (green) show a lack of co-localization between Sp1 and TBP aggregates.

Figure 4-4

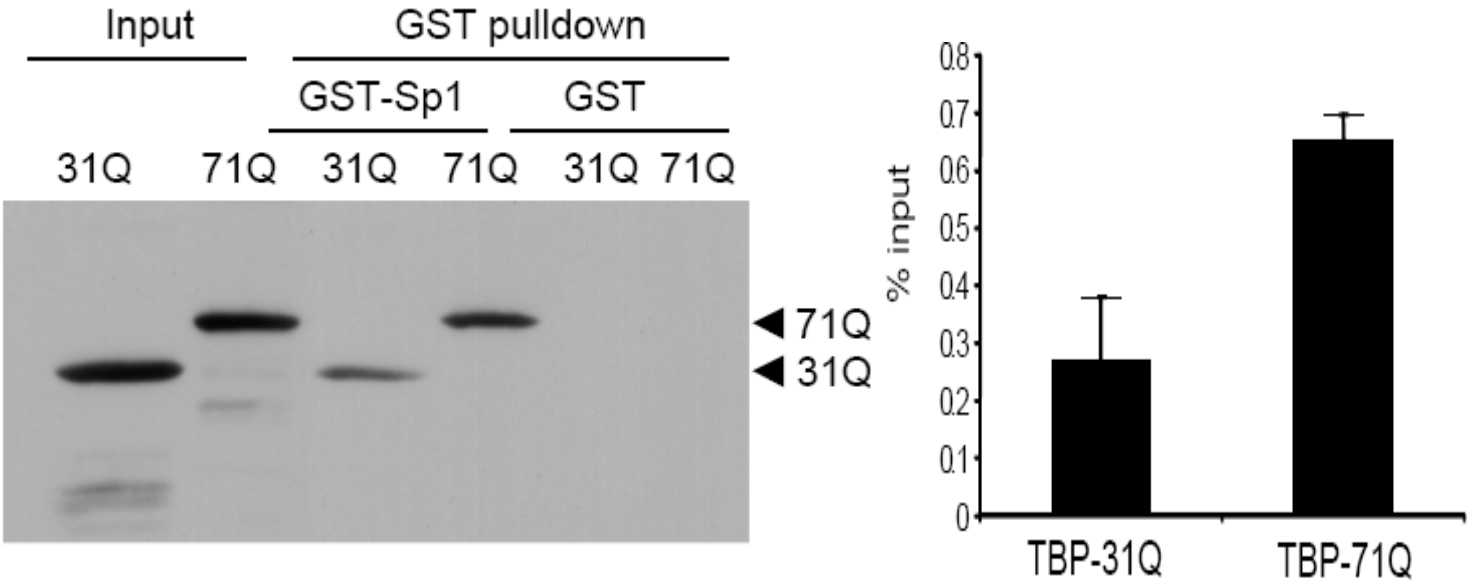
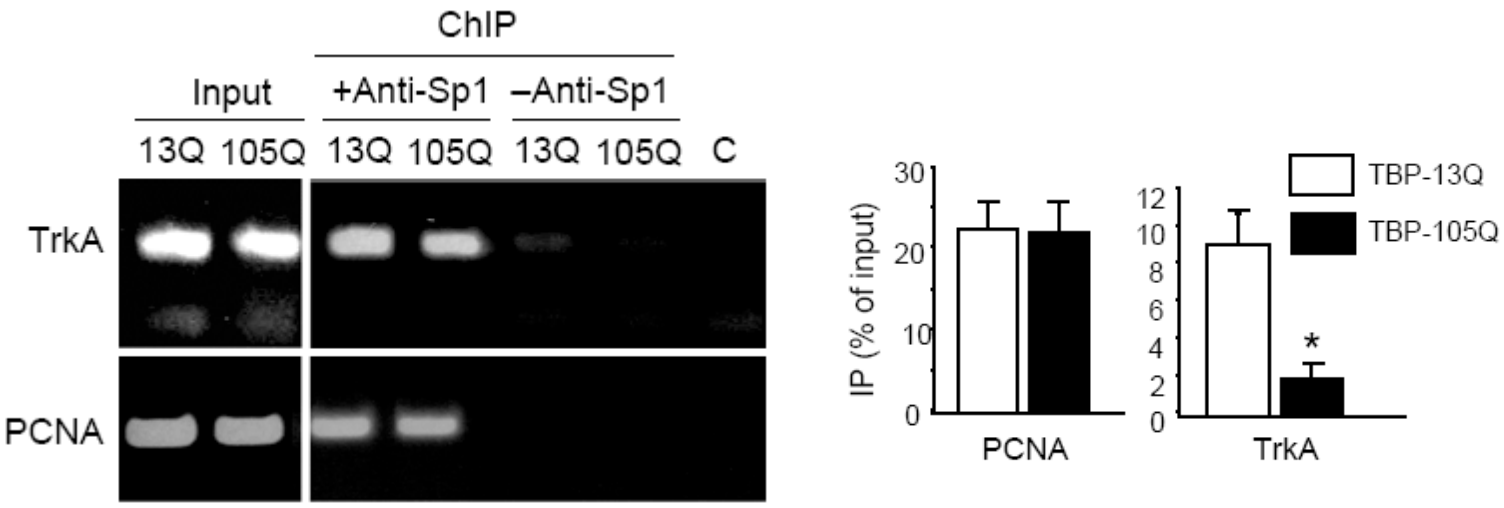

Input
GST pulldown
GST-Sp1
GST
31Q
71Q
31Q
71Q
31Q
71Q
71Q
31Q
% input
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
TBP-31Q
TBP-71Q

Figure 4-4 Increased binding of soluble TBP to Sp1. GST-Sp1 *in vitro* pull down assay shows the increased interaction of Sp1 with soluble TBP-71Q as compared to TBP-31Q. Densitometric analysis is also presented (right panel).

Figure 4-5

A


ChIP
Input
+Anti-Sp1
–Anti-Sp1
13Q 105Q 13Q 105Q 13Q 105Q C
TrkA
PCNA
IP (% of input)
30
20
10
0
PCNA
12
10
8
6
4
2
0
TrkA
*
TBP-13Q
TBP-105Q

B

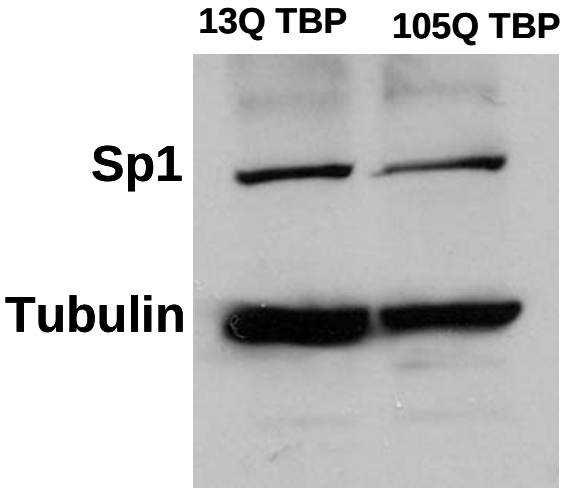

13Q TBP
105Q TBP
Sp1
Tubulin

Figure 4-5 Lower occupancy of Sp1 at the TrkA promoter in a PC12 cell model of SCA17. (a) Stably transfected PC12 cells expressing TBP-13Q or TBP-105Q were transfected with Sp1 and used for ChIP assays to examine the association of Sp1 with the TrkA promoter. There is decreased association of Sp1 with the *TrkA* promoter in TBP-105Q cells as compared to that in TBP-13Q cells. The same precipitates were also used to examine Sp1 association with the *PCNA* promoter, which served as a control. C: no template control. The percent of PCR products from the immunoprecipitates (IP) as compared to the inputs are also presented. * $P<0.05$. **(b)** Western blot demonstrating equal expression of transfected Sp1 is also presented.

Chapter 5

Conclusions and Future Directions

5.1 Summary

In summary, we were able to establish a PC12 cell model of SCA17 that stably expressed TBP-13Q (non-pathogenic) and TBP-105Q (pathogenic). Cells expressing TBP-105Q showed characteristic signs of neuronal dysfunction including decreased cell viability and defective neurite outgrowth. Neuritic defects were also seen in primary cortical neurons infected with a TBP-105Q expressing adenovirus. PC12 cells also showed decreased levels of the small heat shock protein HSPB1, which has been implicated in neurite outgrowth, the maintenance of axonal integrity, and cell survival. Overexpression of HSPB1 in cells expressing TBP-105Q led to increased neurite outgrowth and cell viability. Additionally, TBP-105Q cells overexpressing HSPB1 showed increased levels of TrkA, the high affinity NGF receptor. However, rescue of these phenotypes was mediated independent of any effects HSPB1 has on TBP misfolding and aggregation.

Next we showed that PC12 cells stably expressing TBP-105Q, showed a specific decrease in TrkA transcript level, compared to the low affinity NGF receptor, $p75^{NTR}$. These cells also showed decreased protein levels of TrkA, which was also confirmed in PC12 wild-type cells infected with a TBP-105Q expressing adenovirus. Further characterization of the NGF signaling pathway showed decreased levels of phospho-Erk, suggesting dysfunction of the Erk signaling component, which is involved in neurite outgrowth and neuronal differentiation. However, we did not see a decrease in phospho-Akt levels, the other component in TrkA signaling, which is involved in cell survival. Furthermore, we found decreased protein and mRNA levels of TrkA in cerebellar tissues of young, asymptomatic mice as well as older, symptomatic mice. However, Purkinje cell loss

was only seen in the older late stage of disease mice, suggesting that the down-regulation of TrkA occurs prior to any obvious neurological phenotype and neuropathology.

We next examined the mechanism by which TrkA was being down-regulated. Transcription from the TrkA promoter was decreased in PC12 cells stably expressing TBP-105Q. In HEK293T cells, there was no transcription from the reporter construct in the presence of overexpressed TBP-13Q and 105Q. Reporter activity was only seen if Sp1 was overexpressed, suggesting that the reporter activity is mediated by Sp1, and Sp1 may be binding to the TrkA promoter to stimulate transcription. Importantly, HEK293T cells expressing TBP-105Q also showed a greater decrease in transcription from the TrkA promoter than in cells expressing TBP-13Q. We examined co-localization of Sp1 and mutant TBP aggregates in primary cerebellar neurons expressing TBP-105Q but did not see any co-localization of Sp1 with the aggregates. However, Sp1 showed more interaction with soluble TBP-71Q, suggesting that this aberrant interaction could inhibit Sp1-mediated gene transcription.

Finally we investigated the effect that the aberrant interaction of Sp1 and soluble mutant TBP had on the TrkA promoter. Using chromatin immunoprecipitation we were able to show decreased association of Sp1 with the TrkA promoter, suggesting that the interaction of Sp1 with mutant TBP removes Sp1 from its cognate promoter. This can lead to decreased transcription of TrkA and dysregulation of the NGF signaling pathway, contributing to SCA17 neuropathology. These results provide evidence for transcriptional dysregulation as a mechanism underlying SCA17 molecular pathogenesis.

5.2 Remaining Questions and Future Directions

Based on our previous results, we can conclude that transcriptional dysregulation not only underlies the molecular pathogenesis of SCA17 but likely many of the other polyglutamine disorders. Current animal and cell models of SCA17 have allowed for detailed investigation of the pathogenic pathways mediated by polyglutamine expanded TBP. From these models we have learned of the transcriptional dysregulation of two genes involved in neuronal function and survival, HSPB1 and TrkA. However, various transgenic and knock-in mouse models of SCA17 may be necessary to further study the molecular pathogenesis of the disease.

Selective neurodegeneration in SCA17

A unifying theme among the polyglutamine disorders is the question of how completely unrelated proteins that are ubiquitously expressed, can cause selective neurodegeneration in the presence of a polyglutamine expanded protein (Orr and Zoghbi, 2007). In combination with an earlier finding that mutant TBP does not elicit global alteration of gene transcription (Friedman et al., 2007), the current findings suggest that the abnormal association of mutant TBP with transcription factors selectively alters the expression of genes that are important for neuronal function or viability. It is also likely that processing of polyglutamine proteins and the interaction of polyglutamine proteins and their binding partners may be cell type specific contributing to selective neurodegeneration (Gatchel and Zoghbi, 2005). Additionally, transcriptional regulation may be cell type-specific and can be influenced by tissue specific activators or co-activators (Roeder, 2005). For example, our data showed transcription of the TrkA reporter construct in PC12 cells, however, in HEK293T cells, transcription of the TrkA reporter construct only occurred in

the presence of exogenous Sp1, suggesting that expression of TrkA is cell-type specific but the factors regulating expression of TrkA are also cell type specific. Another possibility is that mutant TBP differentially accumulates in a cell-type specific manner, leading to selective neuropathology. Previous studies found that protein context influences the interaction of Sp1 with huntingtin (Cornett et al., 2006). Similarly, accumulation of a specific degradation products or fragments of mutant TBP may bind Sp1 with different affinities and can contribute to selective neuropathology. Further examination of cell type specific expression and regulation of important factors involved in polyglutamine pathogenesis may help elucidate the mechanisms underlying selective neurodegeneration and other polyglutamine diseases.

Transcriptional dysregulation

Our current findings show that transcriptional dysregulation is at least one mechanism underlying the molecular pathogenesis of SCA17. However, an interesting question is why the abnormal interactions of TBP with Sp1 or other transcription factors only selectively changes the expression of certain genes. Sp1 is ubiquitously expressed in a variety of mammalian cells and binds the GC boxes of DNA to mediate expression of a large number of genes. TBP is an essential transcription factor for the assembly of transcription factor complex at different promoters. Soluble TBP containing an expanded polyQ domain is also able to stimulate promoter transcription (Reid et al., 2003; Freidman et al., 2008), though it can abnormally bind other transcription factors. Thus, the outcome of effects of mutant TBP on Sp1-mediated transcriptional activity is largely dependent on the association of TBP with different transcription factors and regulation by other

transcriptional factors of the interactions between Sp1 and the GC rich binding sites (Courey et al., 1988; Pascal and Tjian, 1991; Emili et al., 1994; Segal et al., 1999). This bears some analogy to the finding that mutant huntingtin selectively reduces the expression of certain Sp1-dependent genes despite its abnormal binding to Sp1 (Chen-Plotkin et al., 2006). The promoter of TrkA contains the Sp1 binding sites but lacks TATA box binding site (Sacristán et al., 1999), and the activity of this promoter is also regulated by Sp3 (Lambiase et al., 2005). It is possible that the context of the transcriptional factor complex and promoter sequences plays an important role in the selective effects of mutant TBP on gene expression.

Previous studies have shown that mutant TBP abnormally binds TFIIB, an important transcription factor, and reduces the expression of HSPB1, a chaperone protein that is critical for neuronal survival and axonal integrity (Friedman et al., 2007). It is evident that polyQ expansion can alter protein-protein interactions to affect multiple targets or functions in cells. For example, mutant ataxin-1 abnormally interacts with several different proteins to cause neuropathology (Lin et al., 200; Tsuda et al., 2005; Lam et al., 2006; Serra et al., 2006; Lim et al., 2008). Similarly, mutant huntingtin binds abnormally to Sp1, CBP, TAFII130, and other transcription factors to affect different transcriptional functions (Dunah et al, 2002; Li and Li 2004; Zhai et al., 2005). It is well known that TBP binds Sp1 (Emili et al., 1994; Segal et al., 1999; Torigoe et al., 2003) and that Sp1 mediates transcription of many TATA-less genes (Emami et al., 1998; Wierstra 2008). Unlike TFIIB that can be sequestered into nuclear TBP inclusions (Friedman et al., 2007; 2008), Sp1 binds soluble mutant TBP and is not co-localized with nuclear TBP inclusions. This phenomenon is similar to the increased interaction of soluble mutant huntingtin to Sp1 (Li

et al., 2002; Dunah et al., 2002). Interestingly, HD and SCA17 share similar neurological symptoms and neuropathology (van Roon-Mom et al., 2005; Stevanin and Brice 2008). Like mutant htt (Chen-Plotkin et al., 2006), polyQ-expanded TBP could reduce Sp1 association with certain promoters via its increased interaction with Sp1. In support of this idea, we found that mutant TBP decreases Sp1 occupancy of the TrkA promoter, which can account for the reduced TrkA expression. Identification of the abnormal interaction of mutant TBP with Sp1 also suggests the potential role of this abnormal interaction in the decreased HSPB1 level found in a previous study, as the promoter of HSPB1 contains Sp1 binding sites (Frohi et al., 1993; Gasterl et al 1993; Oesterreich et al., 1996). Chromatin immunoprecipitation experiments, with antibodies against TBP or Sp1 would demonstrate occupancy of either of these transcription factors at the HSPB1 promoter. Decreased occupancy of the transcription factors in the presence of mutant TBP could account for the decreased levels of HSBP1 seen in the PC12 cell model of SCA17.

ChIP on chip studies, in which chromatin immunoprecipitates of cerebellar samples from SCA17 transgenic mouse and wild-type littermates are evaluated by microarray analysis, should provide insight into polyglutamine-dependent changes in TBP occupancy across the genome. Also, comparing SCA17 mice expressing soluble mutant TBP-71Q with mice expressing aggregated TBP-105Q could reveal a different profile of promoter occupancy, allowing the effect of TBP aggregation to be evaluated at specific promoters of interest.

Contribution of TrkA to SCA17 pathology

Our studies indicate the transcriptional dysregulation of TrkA before any obvious neurological phenotype or neuropathology, suggesting a contribution of TrkA dysregulation to disease pathology. However, it will be important to address the relative contribution of TrkA deficit to SCA17 pathology. Examination of postmortem brains of the limited number of SCA17 patients has revealed neurodegeneration in different brain regions including the cortex, striatum, and cerebellum in which pronounced Purkinje cell loss is evident (Koide et al., 1999; Nakamura et al., 2001; Fujigasaki et al., 2001; Zuhlke et al. 2003; Toyoshima et al., 2004). In transgenic SCA17 mice, there is also obvious Purkinje cell degeneration (Friedman et al., 2007). TrkA is expressed in Purkinje cells and is critical for neuronal function and viability (Muragaki et al., 1995; Torres et al., 1995; Dohrman et al., 1997). Unlike other Trk receptors such as TrkB and TrkC, TrkA is more restricted in certain types of neurons in adult brains (Muragaki et al., 1995; Numakawa et al., 1999). Since the promoters of TrkB and TrkC have not been found to contain the Sp1 binding sites (Sacristán et al., 1999; Lei and Parada, 2007), it is possible that the Sp1 binding sites in TrkA contributes to the more selective expression of TrkA in certain types of neurons. Accordingly, the decreased expression of TrkA by the abnormal TBP-Sp1 interaction could more profoundly affect the viability and function of Purkinje cells in the cerebellum.

It would be useful to look at expression patterns of TrkA in various brain regions, to see which areas have the highest levels. Perhaps the cells in these areas that are most vulnerable to loss of TrkA play an important role in SCA17 neuropathology. Additionally, TrkA rescue experiments would help elucidate what role loss of TrkA is playing in SCA17 pathology. Alternatively, siRNA experiments knocking down levels of TrkA, should recapitulate the phenotype we see in the TBP-105Q cells, further confirming an important

role for TrkA in SCA17. A more *in vivo* approach to elucidate the role of TrkA pathology would be to cross a transgenic mouse overexpressing TrkA with an SCA17 mouse expressing mutant TBP. Amelioration in phenotype and resulting neuropathology would establish TrkA as an important modifier of SCA17 pathogenesis as well as a valuable therapeutic target.

5.3 Conclusions

SCA17 is an extremely rare disorder; however, there may be common mechanisms of pathogenesis among the polyglutamine disorders allowing the study of this disease to have broad implications. By investigating cellular and mouse models of SCA17, we observed a decrease in TrkA expression in both these models and this decrease is specifically associated with the expression of mutant TBP. We have also provided evidence for the transcriptional dysregulation of TrkA by mutant TBP, as analysis of transcript levels and promoter activity of TrkA are consistently inhibited in the presence of mutant TBP. In addition, mutant TBP binds more Sp1 and reduces the association of Sp1 with the TrkA promoter. The findings in our study support the idea that altered protein function by polyglutamine expansion can lead to neuropathology in polyglutamine diseases (Lim et al., 2008). Since the function of TBP has been well-characterized, further characterization of the effects of polyglutamine expansion on the function of TBP will provide valuable information and insight regarding mechanisms of molecular pathogenesis in SCA17 and other polyglutamine disorders.

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