REDUCED FOXP1 EXPRESSION AS A CONTRIBUTING FACTOR

IN HUNTINGTON'S DISEASE

by

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This dissertation is dedicated to my ever-supporting parents, Louis Sam Titus and Sarojam, and to my loving wife, Angela Steffi.

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Neurodegenerative diseases affect the quality of life and place huge emotional, physical and economic strain on families and society. Neurodegenerative diseases feature an uncontrolled neuronal apoptosis. There are currently treatments which are aimed at alleviating the symptoms of the disease, but there are no treatments which stop the uncontrolled loss of neurons in the brain. A proper understanding of the underlying mechanisms of neurodegeneration will aid in developing better treatment strategies against the disease. Forkhead proteins play a critical role in regulating the transcriptional profile in cells, and Forkhead box protein P1 (Foxp1), a member of the forkhead family of proteins, is highly expressed in striatum and cortex of the brain. While earlier studies on Foxp1 have been focused on its role in cancer and immunological function, we discovered a neuroprotective role of Foxp1. The focus of my dissertation is on identifying the mechanism of neuroprotection of Foxp1.

Chapter 1 of this dissertation reviews the current literature on mechanisms of neurodegeneration in Huntington's disease (HD) and how aberrant reentry of post mitotic neurons into cell cycle is a recurring theme in many neurodegenerative diseases. I also discuss the expression and

regulation of forkhead proteins in the brain and also discuss the recent findings regarding evidences of cell cycle dysfunction in neurons.

In Chapter 2, I describe the research done on neuroprotective protein, Foxp1, the expression of which is decreased in human HD patients and in mouse models of HD, and overexpressing Foxp1 can protect neurons from mutant Huntingtin toxicity. Foxp1 was also found to be neuroprotective against a wide range of apoptotic stimuli, and the neuroprotection of Foxp1 occurs by the up-regulation of p21Waf1/Cip1, a well-known inhibitor of cell cycle.

In Chapter 3, I summarize my findings on neuroprotection of Foxp1 and discuss the therapeutic potential of repurposing cancer treatment drugs in the treatment of neurodegenerative diseases.

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CHAPTER 1

NEURODEGENERATION IN HUNTINGTON'S DISEASE AND ROLE OF FOX PROTEINS AND CELL CYCLE

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II. ABSTRACT

Apoptosis is a highly regulated mechanism by which cells are triggered to die. During normal brain development about 50% of the neurons that are produced, die by apoptosis. This is to ensure that only the neurons that form well connected circuits survive and others be eliminated. Neuronal apoptosis can be triggered by glutamate, calcium influx, and oxidative stress. Neurodegenerative diseases involve an unregulated and uncontrolled loss of neurons in select brain regions triggered by diverse stimuli. For example, in Alzheimer's disease neurodegeneration is caused by beta amyloid plaques and neurofibrillary tangles of tau leading to neuronal loss in the cerebral cortex and hippocampus, while Parkinson's disease results from depletion of dopamine producing neurons in substantia nigra, as a result of aggregated α-synuclein, leading to neuronal loss in the midbrain, and Huntington's disease results from aggregation of mutant huntingtin protein causing extensive neuronal loss in the striatum and moderate neuronal loss in the cortex. The loss of neurons in these diseases manifests in the form of dementia, cognitive decline and movement disorders, and leads to shorter lifespan. The reason behind the selective loss of certain neuronal populations in each neurodegenerative disease is unclear but may depend on differential gene expression pattern, vulnerability to oxidative stress or other pathophysiological stimuli including excessive neurotransmitter levels. Neurodegenerative diseases can be inherited / familial or sporadic. Huntington's disease is a hereditary neurodegenerative disease which is inherited in an autosomal dominant fashion. The disease is caused by a polyglutamine expansion in the huntingtin gene, which manifests itself as cognitive decline and movement disorders. The involuntary, jerky movements characterize the disease.

Foxp1 is a transcription factor belonging to the Fox family of forkhead proteins which play key roles in embryogenesis and development. The expression profile of Foxp1 is limited to the striatum and the cortex and overlaps the regions of the brain affected by Huntington's disease. Even though the role of Foxp1 has been well studied in the immune system and in brain development, the role of Foxp1 in adult brain in relatively unexplored. Foxp1 is also down regulated during the pre-symptomatic stage of Huntington's disease, suggesting that this is an early event in the progression of Huntington's disease.

Neurodegenerative diseases despite having varied causes and manifestations have some common features which include impairment of proteasome and accumulation of misfolded proteins, reduction of mitochondrial activity and accumulation of free radicals leading to oxidative stress and also re-entry into cell cycle. Even though neurons are post mitotic, in several neurodegenerative diseases there is an aberrant reentry into cell cycle, which ultimately leads to apoptosis.

While there are treatments that reduce the symptoms associated with some neurodegenerative diseases, such as Parkinson's disease, there are no such therapies to slow down or stop the relentless loss of neurons in any neurodegenerative disease. Understanding the molecular and cellular underpinnings of neurodegenerative diseases will identify molecules that can be targeted in the development of effective therapies.

III. INTRODUCTION

Neurodegeneration is defined as an uncontrolled loss of neurons from specific brain regions. The neuronal loss manifests itself in loss of memory, movement disorders and cognitive decline. A few treatment strategies exist but they are directed towards ameliorating the symptoms of the disease instead of addressing the neurodegeneration. There are numerous mechanisms that have been implicated in neurodegeneration, like proteostatic homeostasis (Tanaka & Matsuda, 2014), DNA damage (Madabhushi, Pan, & Tsai, 2014), misfolding of proteins, excitotoxicity and mitochondrial dysfunction (Pellegrino & Haynes, 2015). Endoplasmic reticulum (ER) stress has also been a converging pathway in many neurodegenerative disorders (Vidal, Caballero, Couve, & Hetz, 2011).

Polyglutamine disorders

A class of progressive & fatal neurodegenerative diseases are the polyglutamine disorders, all of which are inherited. The common feature of these polyglutamine disorders is the presence of aggregates, which maybe nuclear or cytoplasmic aggregates (Weber, Sowa, Binder, & Hübener, 2014). The association of ubiquitin and heat shock proteins (HSPs) with these aggregates suggests a disturbance of ubiquitin-proteasome system (Chai, Koppenhafer, Bonini, & Paulson, 1999). Also proteolytic cleavage of these mutant proteins, form fragments which contains the polyglutamine repeats. These fragments are a lot more aggregated, and are much more toxic than the full length versions of the proteins (Marsh et al., 2000).

The expansion of glutamine tract in various proteins results in selective degeneration of specific regions of the brain. For example the expansion of CAG in Atrophin 1 (ATN1) causes Dentatorubral pallidoluysian atrophy, which leads to neuronal loss in the basal ganglia, red

nucleus and dendate nucleus. The CAG expansion in the protein Attaxin-1 causes Spinocerebellar Ataxia Type 1 (SCA1), which is characterized by degeneration of the cerebellum. Spinobulbar Muscular Atrophy (SMBA) is caused by polyglutamine expansion in the Androgen receptor gene on the X-chromosome; this disease predominantly affects males and neurodegeneration occurs in the spinal cord and bulbar region (Evert, Wüllner, & Klockgether, 2000). One of the well-studied polyglutamine diseases is the Huntington's disease caused by a polyglutamine expansion in the huntingtin gene.

Huntington's disease

Huntington's disease affects 4-8 individuals per 100,000 of European descent (Harper, 1992). The age of onset of the disease correlates with the number of CAG repeats in the affected individual. Even though the mutant Huntingtin gene is ubiquitously expressed all over the body, the effects are predominantly seen only in the brain. Huntington's disease causes extensive loss of medium spiny neurons in the caudate nucleus and putamen and a moderate loss of neurons in the cortex (Cattaneo et al., 2001). Normal individuals have 10-26 CAG repeats in the huntingtin gene, individuals with 27-39 CAG repeats have reduced penetrance of the disease and individuals with >40 CAG repeats have full penetrance of Huntington's disease (Walker, 2007). Huntington is a large, 348kDa protein which plays a role in many cellular functions. The N-terminus of Huntington, interacts with several proteins and serves as a docking site for protein-protein interactions (S. Li & Li, 2004). Huntington can also translocate into the nucleus where it can regulate transcription (Cattaneo, Zuccato, & Tartari, 2005).

The mechanism of toxicity in Huntington's disease has long been debated on, whether it is the loss of function of the normal huntingtin gene or a toxic gain of function by the mutant

huntingtin. The loss of function theory is based on the evidence that huntingtin plays a crucial role in maintaining the levels of brain-derived neurotrophic factor (BDNF), a trophic factor required for the survival of striatal and cortical neurons (Zuccato et al., 2001). Huntingtin was also then shown to transcriptionally regulate BDNF levels by binding to the BDNF promoter and mutant huntingtin could not activate the BDNF production (Zuccato et al., 2003). Huntingtin interacts with Huntingtin-Associated-Protein-1(HAP-1) and mediates the transport of BDNF vesicles, this molecular machinery cannot be performed by mutant huntingtin (Gauthier et al., 2004). Thus mutant huntingtin suppresses both the production and transport of BDNF. It has also been shown that BDNF overexpression can reduce the disease phenotype in YAC128 mice suggesting a therapeutic potential (Xie, Hayden, & Xu, 2010). Also supporting the loss of function mode of pathogenesis is the finding that mice hemizygous for the huntingtin gene display postnatal degeneration (Dragatsis, Levine, & Zeitlin, 2000).

The mutant huntingtin protein contributes to ER stress, interferes with axonal transport of mitochondria and associates with the mitochondrial membrane and impairs the electron transport chain, resulting in depletion of cellular ATP and increased reactive oxygen species. (Mochel & Haller, 2011). Mutant huntingtin can also disrupt the ubiquitin protease system (UPS) and sequesters the UPS components in inclusion bodies. This leads to an accumulation of polyubiquitinated proteins in the brains of Huntington patients. Proteosomal activator PA28gamma, has been shown to reduce the toxicity of mutant huntingtin in striatal neurons (Seo, Sonntag, Kim, Cattaneo, & Isacson, 2007).

Neuroprotective functions of wild type huntingtin can also be due to protein-protein interactions. Wild type Huntingtin binds with a high affinity to HDAC3, a neurotoxic protein but the mutant

huntingtin has a low interaction with HDAC3 (Bardai et al., 2013). The release of HDAC3 facilitates its binding with HDAC1 and activates the transcriptional machinery leading to cell death (Bardai, Price, Zaayman, Wang, & D'Mello, 2012). Huntingtin also plays an important role in differentiation of neural stem cells, and deleting huntingtin causes differentiation of these stem cells preferentially into $GFAP^+$ astrocytes rather than $MAP2^+$ neurons (Conforti et al., 2013). While wild-type huntingtin exists in the cytoplasm and does not form aggregates, mutant huntingtin forms inclusion bodies in the nucleus and in the perinuclear region (DiFiglia et al., 1997). While the initial hypothesis was that these protein aggregates might be the causative mechanism of the neurodegeneration, later studies have shown that the huntingtin activates apoptosis machinery in the nucleus but there is no correlation between cell death and the presence of aggregates (Saudou, Finkbeiner, Devys, & Greenberg, 1998). In fact, the neuronal apoptosis correlated with the expression level of mutant huntingtin and the number of polyglutamine repeats, but cell death can occur without the formation of aggregates (Arrasate, Mitra, Schweitzer, Segal, & Finkbeiner, 2004). Inclusion bodies are now being studied as a cellular mechanism for coping with the mutant protein. The presence of inclusion bodies is associated with neuronal survival and these inclusion bodies might have neuroprotective functions (Kuemmerle et al., 1999).

Models of Huntington's disease

There are several mouse models of Huntington's disease. One of the widely used models of HD is the R6/2 mouse model, in which transgenic mice overexpress the exon -1 of Huntingtin and have polyglutamine repeats ranging from 115-150 (Mangiarini et al., 1996). Another model, N171-82Q mice are the transgenic mice, expressing the N-terminal 171 aminoacids of

Huntingtin with 82 polyglutamine repeats (Schilling et al., 1999). The N-terminal mouse models of HD featured rapid progression of neurodegeneration and were based on the fact that the Huntington protein is cleaved in the cell and these fragments are neurotoxic (Ehrnhoefer, Butland, Pouladi, & Hayden, 2009). Also, cleaved Huntington fragments and truncated Huntington have different cellular localizations (Warby et al., 2008). Also, the truncated forms of Huntington don't mimic the protein-protein interactions which occur with the full length form. The full length Huntington mouse models like the YAC128 mice (Slow et al., 2003) and BACHD (Gray et al., 2008) mice have a slower neurodegenerative phenotype characterized by loss of neurons in the striatum and the cortex along with cognitive decline and motor deficits. The most representative mouse model of HD is the knock-in mouse model (Hdh-KI $^{Q150/Q150}$), where the polyglutamine expansion is introduced into the mouse gene, and the length of the polyglutamine tract correlates with the severity of the phenotype (Heng, Tallaksen-Greene, Detloff, & Albin, 2007).

Huntington disease models have also been generated in *Drosophila melanogaster*, where the expression of mutant Huntington in the retinal neurons is driven by a GMR promoter / GMR-GAL4 system (Jackson, 2008; Tamura et al., 2011), which results in a disorganized retina with reduced number of rhabdomeres. The behavior and motor deficits are observed in flies when Huntington is expressed using a pan-neuronal Elav-GAL4 driver (Gonzales & Yin, 2010; Wolfgang et al., 2005).

Transcriptional dysregulation by Huntingtin

One of the main challenges in identifying the mechanism of neurodegeneration is that mutant huntingtin interferes with many transcriptional mechanisms (Seredenina & Luthi-Carter, 2012).

RNA seq analysis from HD patient brains has revealed that about 5480 genes (about 19% of the total genes) show a differential expression in Huntington's disease (Labadorf et al., 2015). Mutant huntingtin has also been found to cause extensive changes in DNA methylation which might account for the wide spread changes in transcription profile (Ng et al., 2013). It has also been discovered that normal huntingtin plays a vital role in regulation of miRNA mediated transcriptional silencing, while mutant huntingtin reduced the silencing of genes by miRNA by associating with the processing bodies (Savas et al., 2008). Huntingtin can also bind to gene promoters, intronic and intergenic sequences and alter the DNA structure, and the polyglutamine expansion increases the DNA binding causing extensive changes in gene expression (Benn et al., 2008). Several transcription factors have also been found to be bound and sequestered by the huntingtin inclusion bodies (A. Kazantsev, E. Preisinger, A. Dranovsky, D. Goldgaber, & D. Housman, 1999).

Despite these extensive changes in gene expression profile, studies have been performed comparing the expression changes in various mouse models of HD and in human HD patients and scientists have compiled a list of 150 genes that were dysregulated in Huntington's disease (Seredenina & Luthi-Carter, 2012). Among these genes is a transcription factor, Forkhead box protein P1 (Foxp1). Previous work in our lab has been focused on another member of the forkhead family, Foxg1 which was shown to be neuroprotective against various apoptotic stimuli (Dastidar, Landrieu, & D'Mello, 2011). The expression of Foxp1 matched the regions of neurodegeneration in Huntington's disease (Ferland, Cherry, Preware, Morrisey, & Walsh, 2003) and Foxp1 was found be associated with the huntingtin aggregates in R6/1 mice (Tang et al., 2012).

Fox proteins

Fox family of proteins comprises of 17 sub-groups having about 41 genes in humans (Myatt & Lam, 2007). Even though all these genes share the evolutionarily conserved forkhead domain, they perform various functions like embryo morphogenesis, cell cycle progression, cellular differentiation, metabolism and can also regulate survival and apoptotic pathways in the cell (Lam, Brosens, Gomes, & Koo, 2013). The forkhead proteins can dimerize and using the 110 amino acid forkhead domain they can bind to DNA forming a winged-helix structure (Kaufmann & Knöchel, 1996). The interplay between the Fox proteins can be due to two mechanisms. The Fox proteins can homo and hetero dimerize with other members resulting in varying transcriptional profiles. The Fox proteins can also bind to the same DNA sequence creating competition and can also either up regulate or down regulate the transcriptional target (Karadedou et al., 2012). Thus the relative levels of Fox proteins can significantly alter the gene expression profile.

Expression of Foxp proteins in the brain

In the brain, Foxp proteins show a varied pattern of expression and the expression also changes during embryogenesis. This patterning of expression of Foxp proteins highlights the key roles they play in modulating the development and differentiation of neuronal populations of the brain. Foxp1 is expressed in layer 3-5 of the cerebral cortex while Foxp2 is expressed only in layer 6 and in contrast Foxp4 is expressed all over the cortex. Foxp1 is not expressed in the cerebellum where Foxp2 and Foxp4 are expressed highly (Takahashi, Liu, Hirokawa, & Takahashi, 2008). In the hippocampus Foxp1 expression is restricted to the CA1 region whereas Foxp4 is expressed in CA3-CA1 while Foxp2 expression is completely absent in the hippocampus. In the striatum,

Foxp1, Foxp2 and Foxp4 are highly expressed during embryogenesis but the expression of Foxp4 declines postnatally (Ferland et al., 2003).

Humans with mutations in the Foxp1 gene have delayed vocabulary development, below average IQ, and abnormally enlarged ventricles (Pariani, Spencer, Graham, & Rimoin, 2009). Foxp1 has also been found to be a transcriptional regulator of a number of genes of the autism spectrum disorders (Bowers & Konopka, 2012). Foxp1 deletion in humans leads to developmental delays, severe language and speech impairment, motor deficits and learning disabilities (Newbury & Monaco, 2010). Mutations in Foxp2 leads to verbal dyspraxia and impaired language development (Feuk et al., 2006). While Foxp3 plays major role in T-cell development and humans with mutations in Foxp3 gene have dysfunction of the immune system and polyendocrinopathy (Bennett et al., 2001), Foxp3 is also found to be expressed in the brain in gliomas (L. Wang et al., 2014). Foxp4 is highly expressed in the developing brain and inactivation of Foxp4 leads to neural tube deficits (Rousso et al., 2012).

Foxp1 Knockout Mouse

Foxp1 knockout mice die at embryonic day E14.5 due to defects in cardiac development.(B. Wang et al., 2004). Brain specific knock out of Foxp1 was performed using a Cre-Lox system and these Nes-Cre $^{Foxp1-/-}$ mice have enlarged lateral ventricles and reduction in striatal volume (Bacon et al., 2015). The shrinkage of striatum was prominent at about 3 weeks of age but was very similar to wild type at E18 and P1. Even though the hypothesis was that the reduced striatal number might be due to the role played by Foxp1 in striatal development, the fact that the striatum from these knockout mice remained normal well after birth suggest that the striatal

neurons were lost due to apoptosis rather than development because neurogenesis in the striatum begins on E12 and peaks around E14 and E15 (Fentress, Stanfield, & Cowan, 1981).

Drosophila Foxp1

The Drosophila orthologue of Foxp1, dFoxP, plays an important role in normal brain development. The mutant dFoxP flies have reduced optic glomeruli and impaired self-learning and habit formation (Mendoza et al., 2014). The dFoxP flies also exhibited slower and less accurate perceptual decision making when compared to wild-type flies (DasGupta, Ferreira, & Miesenböck, 2014). Reduced dFoxP expression leads to impaired motor coordination and altered song structure(Lawton, Wassmer, & Deitcher, 2014).

Domains of Foxp1

Besides having the characteristic forkhead domain, Foxp1 has a leucine zipper domain, zinc finger domain and a poly-glutamine domain.

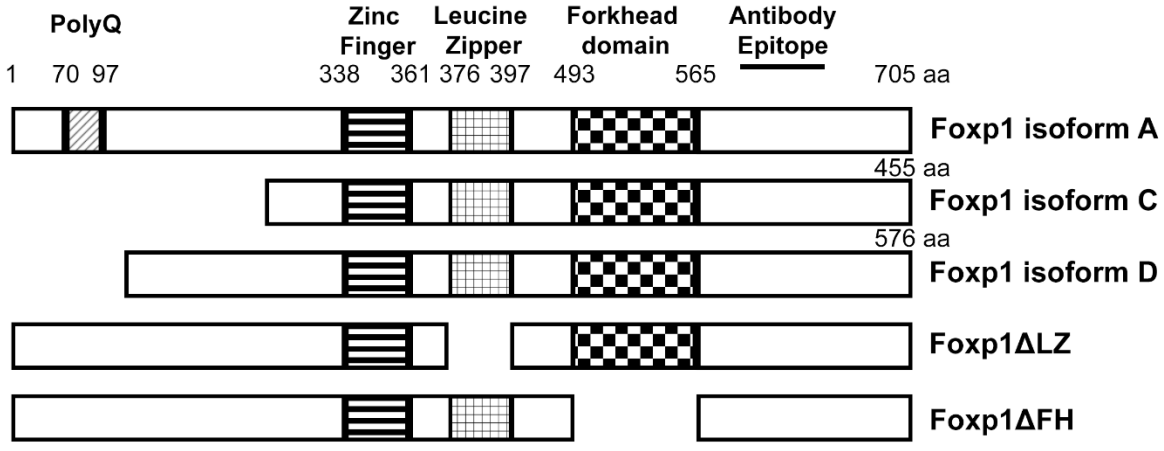

Figure 1.1. Various domains of Foxp1 and the differences between the isoforms.

Foxp1 has an N-terminal poly-glutamine stretch of 37-40 residues and this poly-Q domain has an effect on transcriptional repression strength of Foxp1. Foxp1 isoform D which lacks the poly-Q domain has a higher repressive effect than Foxp1 isoform A (B. Wang, Lin, Li, & Tucker, 2003). The leucine zipper domain is essential for the homo and hetro dimerization of Foxp1 with other proteins of the Fox family (S. Li, Weidenfeld, & Morrisey, 2004). Interaction between Foxp1 and Foxp3 is lost due to point mutation in the leucine zipper domain and these patients suffer from autoimmune disorders (B. Li et al., 2007). The zinc finger offers specificity to the dimerization of Foxp1. The forkhead domain of Foxp1 is a unique feature that is conserved among the proteins of the Fox family. Mutations in the forkhead domain, R525X has been associated with language disorders, autism and mental retardation (Bacon et al., 2015; Hamdan et al., 2010).

Isoforms of Foxp1

Foxp1 undergoes alternative splicing and has at least 9 isoforms in humans and 4 isoforms in mouse. These isoforms play various roles in cell development and differentiation and can also modulate the effect of one another. The smaller isoforms of Foxp1 are found to be increased in B-cell lymphomas (Brown et al., 2008). An embryonic stem cell (ESC) specific isoform of Foxp1 can stimulate the expression of OCT4, NANOG, GDF3 and NR5A2 (Gabut et al., 2011). The expression of these transcription factors helps in the maintenance of these cells in the pluripotent state. The isoforms also show differential expression in parts of the body and in brain and since these isoforms can bind with each other, they can regulate their transcriptional activity (B. Wang et al., 2003).

Regulation of Foxp1

Most of the studies on how Foxp1 is regulated, have utilized non-neuronal systems. In B-cells, the expression of Foxp1 is regulated by non-coding microRNA miR-150 (Mraz et al., 2014). The Foxp1 transcript also binds with microRNA, miR-1 and this regulates the expression of Foxp1 in bronchial epithelial cells (Nasser et al., 2008). Foxp1 is also a target of chromosomal translocations in MALT lymphomas (Streubel, Vinatzer, Lamprecht, Raderer, & Chott, 2005). Foxp1 is sumoylated at lysine 670 and this sumolylation is necessary for the interaction of Foxp1 and CtBP1, which modulates the transcription repression by Foxp1 (Rocca, Wilkinson, & Henley, 2017).

Proteins interacting with Foxp1

Besides forming homo and hetero dimers, Foxp1 also interacts with a number of transcription coactivators and corepressors and these proteins dock on the N-terminus of Foxp1 to regulate the differential gene expression (S. Li et al., 2004). In B-cells Foxp1 has been found to function with NF-kappa B to promote cell survival (van Keimpema et al., 2014). Foxp1 interacts with NCoR/SMRT co repressors and plays critical role in normal heart development and macrophage differentiation (Jepsen, Gleiberman, Shi, Simon, & Rosenfeld, 2008). CtBP1 is another co repressor which binds and increases the transcription repression activity of Foxp1 (S. Li et al., 2004). Foxp1 has been found to be interacting with the exon-1 of mutant huntingtin in R6/1 mice (Tang et al., 2012) and in our lab we found that Foxp1 only interacts with huntingtin aggregates and not with wild type or un-aggregated huntingtin. This interaction could also be a modulating mechanism in the progression of Huntington's disease.

The regulation of cell cycle

The somatic cell division occurs via mitosis and in eukaryotic cells the cell division has 4 distinct phases, G1, S, G2 and M. G1 is the growth phase in which the proteins needed for DNA replication are synthesized, S phase involves the synthesis of DNA, G2 phase marks the synthesis of proteins that help in arrangement of the mitotic spindles and M phase in which the two sets of chromosomes are organized and cytoplasm is separated into two daughter cells. The progression through these cell cycle stages is activated by the cyclin / cyclin dependent kinase (cdk) complexes (Graña & Reddy, 1995). Neurons and other highly differentiated cells which are post-mitotic reside in a specialized form of G1 called the G0 state and they do not enter the cell cycle unless they are stimulated (Frade & Ovejero-Benito, 2015a).

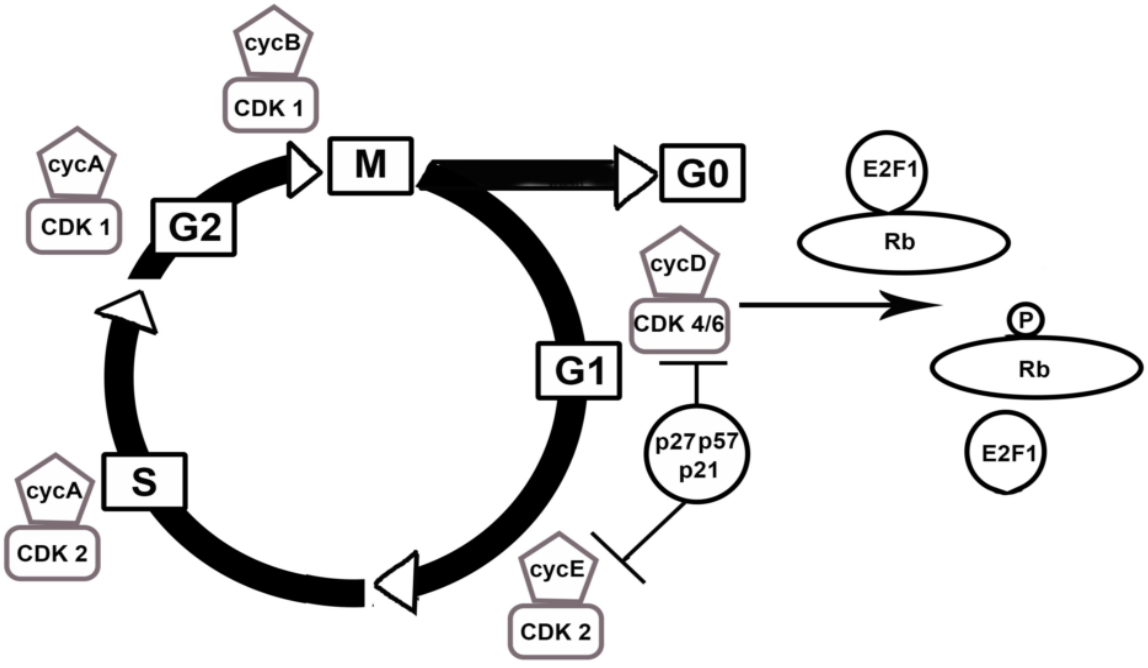

Figure 1.2. The regulation of cell cycle by cyclins, cyclin dependent kinases (cdk) and cdk-inhibitors.

There are three checkpoints in the cell cycle where a cell can abort from proliferation. The first is the G1/S checkpoint, which is regulated by E2F1. E2F1 is usually bound by Rb, when Rb is phosphorylated by cyclin D / CDK4/6, E2F1 is released and E2F1 activated cyclin E and cyclin A. If the favorable conditions for cell cycle don't exist the cells revert back to the G0 state (Kolupaeva & Janssens, 2013; Pfister & D'Mello, 2015). DNA damage can activate ATM kinases which activate p53 which in turn activates $p21^{Waf1/Cip1}$, thus inhibiting the cyclin/CDK complex and arresting the cell cycle (Chehab, Malikzay, Stavridi, & Halazonetis, 1999).
The second checkpoint is in the S phase where the integrity of DNA after the replication is checked. Any mistakes in DNA replication or DNA damage activates the tumor suppressor p53 and halts the cell cycle until it is rectified. If the damage is irrecoverable the cells are committed to apoptosis (Sancar, Lindsey-Boltz, Unsal-Kaçmaz, & Linn, 2004). The third checkpoint is the G2/M, mitotic checkpoint where the completion of DNA synthesis and distribution of chromosomes are checked. Even though this checkpoint is poorly studied, it involves activation of CDK1 complex by CDK activating kinase complex (Labbé et al., 1994)

Cell cycle regulation in neurons

Even though neurons are post-mitotic, several cell cycle proteins involved in the G1/S transition have been detected in neurons (Frade & Ovejero-Benito, 2015b). Several of the external and internal stress stimuli like trophic factor withdrawal, oxidative stress, DNA damage and neurotoxins have been shown to increase the expression level of these cell cycle genes in neurons (Becker & Bonni, 2004). For the progression of cell cycle, Cyclin D must translocate to the nucleus, but in neurons cyclin D is bound by p21 and p27 and sequestered in the cytoplasm (Sumrejkanchanakij, Tamamori-Adachi, Matsunaga, Eto, & Ikeda, 2003).

The first checkpoint in G1/S transition is the activation of Cyclin D / CDK4/6 complex which deregulates E2F1 and E2F1 mediates suppression of Rb is critical for survival of neurons. E2F1 may also cause dysregulation of other downstream targets which signals for apoptosis (D. X. Liu & Greene, 2001). This E2F1 mediated cell death involves the activation of caspase-3 in a p53 independent manner (Giovanni et al., 2000).

Another checkpoint in G2/M transition is the expression of Cdk1 which phosphorylates FOXO1 at Ser249, which dissociates it from 14-3-3 complex and results in translocation of FOXO1 to the nucleus where it begins the cascade of apoptosis. (Kim & Bonni, 2008; P. Liu, Kao, & Huang, 2008; Yuan et al., 2008). BDNF inhibits Cdk1 and blocks the G2/M transition resulting in tetraploid neurons (Ovejero-Benito & Frade, 2013).

Dysfunction of cell cycle in neurodegeneration

The activation of cell cycle in neurodegeneration was first identified in Alzheimer's disease (AD) patient brains (Vincent, Rosado, & Davies, 1996), where post-mitotic neurons undergo a partial to full DNA replication. In AD patients, a huge portion of the pyramidal neurons of the hippocampus and basal forebrain neurons exist in tetraploid state for months before dying by apoptosis, indicating a completion of S phase and blockage of mitosis (Yang, Geldmacher, & Herrup, 2001). Transgenic mouse models of Alzheimer's disease like the APP-Tg mice also shows cell cycle activation in neurons (Yang, Varvel, Lamb, & Herrup, 2006).

Cultured neurons which are subjected to apoptotic stimuli like beta amyloid require cell cycle activation (Copani et al., 2006) and the inhibition of cell cycle using chemical inhibitors can protect these neurons (Sala, Muñoz, Bartolomé, Bermejo, & Martín-Requero, 2008).

Hippocampal neurons express high levels of Cyclin D when subjected to ischemia and correlated

with the induction of apoptosis machinery (Timsit et al., 1999). Cerebellar granule neurons when subjected to BDNF withdrawal, up-regulated cyclin D1 before undergoing apoptosis. This induction of apoptosis can be inhibited by treating the neurons with cell cycle inhibitors (Sakai, Suzuki, Tanaka, & Koike, 1999). The expression of oncogenes like c-myc, ras and cell cycle activators like E2F1 in cultured neurons induce apoptosis (Konishi & Bonni, 2003; McShea et al., 2007).

The dysfunction of cell cycle has also been observed in other neurodegenerative diseases, and positive Ki-67 staining (which labels cells in all stages of mitosis) has been found in Alzheimer's disease and Parkinson's disease (T. W. Smith & Lippa, 1995). Hyper-phosphorylated Rb has been detected in the brain and spinal cord tissue from AD patients as well as in amyotrophic lateral sclerosis (ALS) patients (Ranganathan, Scudiere, & Bowser, 2001). Parkin, a ubiquitin ligase, targets Cyclin E for degradation, and in the case of Parkinson's disease, the mutations in Parkin leads to accumulation of Cyclin E in the post-mitotic neurons leading to apoptosis.(Staropoli et al., 2003) In the post-mortem brains of HD patients, high levels on E2F1 and cyclin D were detected indicating an attempt by the neurons to re-enter the cell cycle (Pelegrí et al., 2008).

$p21^{Waf1/Cip1}$ a neuroprotective molecule

Cip/Kip family of proteins can inhibit a wide range of cyclin/cdk complexes and function as tumor suppressors. $p21^{Waf1/Cip1}$ is a regulator of the G1/S transition and is a transcriptional target of the well-known tumor suppressor p53. Upon DNA damage, p53 activates $p21^{Waf1/Cip1}$ which blocks the progression of cell cycle at G1/S checkpoint (el-Deiry et al., 1993). Post-mitotic neurons express high levels of $p21^{Waf1/Cip1}$ and exposure of neurons to neurotoxin AF64A

(ethylcholine aziridinium) leads to decrease in $p21^{Waf1/Cip1}$ levels followed by apoptosis (Harms et al., 2007). Overexpression of p21 can protect against NGF deprived toxicity in neurons. Apoptosis caused by this growth factor withdrawal can also be suppressed by overexpressing dominant negative Cdk4 or Cdk6 suggesting a definitive involvement of cell cycle activation (Park, Levine, Ferrari, & Greene, 1997). Neurotoxic molecules like DNAJB6 and NPM1 function by activation of cell cycle in neurons and their toxicity can be blocked by overexpression of $p21^{Waf1/Cip1}$ in these neurons (Pfister & D'Mello, 2016; C. Smith & D'Mello, 2016). Neuroprotective molecules like JAZ induce the transcription of $p21^{Waf1/Cip1}$ which can in turn protect against LK induced toxicity in cerebellar granule neurons, and mutant huntingtin induced toxicity in cortical neurons (Mallick & D'Mello, 2014). The neuroprotective effects of $p21^{Waf1/Cip1}$ are dependent on its phosphorylation by PI-3-kinase-Akt pathway and inhibitors of Akt blocks the neuroprotection by $p21^{Waf1/Cip1}$ (Harms et al., 2007). $p21^{Waf1/Cip1}$ has also been shown to protect against oxidative stress induced by honocysteic acid (HCA) in cortical neurons through a non-canonical pathway (Langley et al., 2008). $p21^{Waf1/Cip1}$ is involved in erythropoietin mediated neuronal preconditioning and lack of $p21^{Waf1/Cip1}$ abrogates the neuroprotection due to preconditioning (Mergenthaler et al., 2013). Several HDAC inhibitors (HDACi) have been found be neuroprotective in HD mouse models and recent studies have shown that these HDACi inhibitors activate the expression of $p21^{Waf1/Cip1}$ (Sleiman et al., 2011). Mithramycin, a potent cell cycle inhibitor which works via $p21^{Waf1/Cip1}$, is used in cancer treatment and has been shown to increase the survival rate in R6/2 HD mouse model (Osada, Kosuge, Ishige, & Ito, 2013).
Even though the causes of neurodegeneration and its manifestations are varied, there exists a few common themes and cell cycle re-entry being one of them offers an excellent therapeutic target

because of the availability of FDA approved chemotherapy drugs against cancer, which also targets the cell cycle.

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CHAPTER 2

REDUCED EXPRESSION OF FOXP1 AS A CONTRIBUTING FACTOR IN HUNTINGTON'S DISEASE

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II. ABSTRACT

Huntington's disease (HD) is an inherited neurodegenerative disease caused by a polyglutamine expansion in the huntington protein (*htt*). The neuropathological hallmark of HD is the loss of neurons in the striatum and, to a lesser extent, in the cortex. *Foxp1* is a member of the Forkhead family of transcription factors expressed selectively in the striatum and the cortex. In the brain, three major *Foxp1* isoforms are expressed – isoform-A (~90 kDa), isoform-D (~70 kDa) and isoform-C (~50 kDa). We find that expression of *Foxp1* isoforms A and D is selectively reduced in the striatum and cortex of R6/2 HD mice as well as in the striatum of HD patients. Furthermore, expression of mutant *htt* in neurons results in the downregulation of *Foxp1*. Elevating expression of isoform A or D protects cortical neurons from death caused by the expression of mutant *htt*. On the other hand, knockdown of *Foxp1* promotes death in otherwise healthy neurons. Neuroprotection by *Foxp*1 is likely to be mediated by the transcriptional stimulation of the cell cycle inhibitory protein, $p21^{Waf1/Cip1}$. Consistently, *Foxp1* activates transcription of the $p21^{Waf1/Cip1}$ gene promoter and overexpression of *Foxp1* in neurons results in the elevation of p21 expression. Moreover, knocking down of $p21^{Waf1/Cip1}$ blocks the ability of *Foxp1* to protect neurons from mut-Htt-induced neurotoxicity. We propose that the selective vulnerability of neurons of the striatum and cortex in HD is related to the loss of expression of *Foxp1*, a protein that is highly expressed in these neurons and required for their survival.

III. INTRODUCTION

Huntington disease (HD) is an inherited neurodegenerative disease caused by an abnormal expansion of a CAG repeat in the first exon of the huntingtin gene, resulting in a mutant protein with a poly-glutamine expansion (Zuccato, Valenza, & Cattaneo, 2010). Although mutant huntingtin (mut-*Htt*) is ubiquitously expressed in the brain, in both neurons and glial cells, neurodegeneration in HD is largely restricted to medium spiny neurons (MSNs) of the striatum and to a lesser degree some neurons in layers II, V, and VI of the cortex (Zuccato et al., 2010). We propose that a key factor in the selective vulnerability of the striatum and cortex is the altered expression of a survival-promoting gene called *Foxp1*, which is expressed most highly in neurons of the striatum and, to a lower level, in the cortex (Desplats et al., 2006; Desplats, Lambert, & Thomas, 2008). Foxp1 is one of four members of the *Foxp* subfamily of Forkhead (*FOX)* proteins (Bowers & Konopka, 2012). While *Foxp1*, *Foxp2* and *Foxp4* are expressed in the brain, *Foxp3* is not (Le Fevre et al., 2013; Tam, Leung, Tong, & Kwan, 2011). Mutations of *Foxp1* are linked to speech and language disorders, delays in motor development, autism and mental retardation (Bacon et al., 2015; Bowers & Konopka, 2012; Hamdan et al., 2010; Horn et al., 2010; Le Fevre et al., 2013; Sollis et al., 2016). MRI scans have revealed abnormally enlarged ventricles in humans with *Foxp1* mutations raising the possibility of neuronal loss (Pariani, Spencer, Graham, & Rimoin, 2009).

Conventional *Foxp1* knockout mice (*Foxp1-/-*) die at E14.5 with cardiac abnormalities (Wang et al., 2004). Analyses of *Foxp1+/-* mice have revealed a role in motor neuron diversification, connectivity, and differentiation of stem cells into dopaminergic neurons (Dasen, De Camilli, Wang, Tucker, & Jessell, 2008; Konstantoulas, Parmar, & Li, 2010). Knockdown of *Foxp1* in

the developing cortex has revealed a role in regulating neuronal migration and morphogenesis (Li et al., 2015). These observations demonstrate the importance of *Foxp1* in brain development. However, *Foxp1* is expressed throughout adulthood, but little is known about its function in the adult brain. Within the striatum *Foxp1* is expressed in MSNs with no expression in striatal interneurons (Tamura, Morikawa, Iwanishi, Hisaoka, & Senba, 2004). Brain-specific deletion of *Foxp1* causes pronounced reduction in striatal volume (Bacon et al., 2015) which was suggested to be due to reduced proliferation of neuronal progenitors. Interestingly, the striatal reduction was not observed until 3 weeks of age. Since neurogenesis in the striatum is complete before this time it is possible that degeneration is responsible for the smaller striatum.

The *Foxp1* gene can be alternatively spliced to produce multiple isoforms, the largest of which is approximately 90 kDa (also referred to as isoform-A). These isoforms are expressed tissue-specifically and may have distinct functions (Brown et al., 2008; Green, Gandhi, Courtney, Marlton, & Griffiths, 2009; Santos, Athanasiadis, Leitão, DuPasquier, & Sucena, 2011). For example, *ES-Foxp1*, an isoform expressed only in embryonic stem cells and referred to as isoform-B, contributes to the maintenance of stem cell pluripotency (Gabut et al., 2011). Almost all published work on *Foxp1* has focused on isoform-A. Although the brain expresses two other major isoforms of ~50 KDa and ~70 kDa molecular weight (isoforms C and D, respectively), the significance of these *Foxp1* isoforms for neurons or the brain is not known.

We have investigated the role of *Foxp1* in the regulation of neuronal survival. We describe that *Foxp1* expression is reduced in the R6/2 mouse model of HD and in the striatum of HD patients. Forced expression of mut-*Htt* results in a downregulation of *Foxp1* expression whereas elevating expression of either isoforms-A or D inhibits mut-*Htt* neurotoxicity. To our knowledge, this is

the first report demonstrating a protective role of *Foxp1* in HD. Furthermore, this is the first study that investigates the function of the smaller isoforms of *Foxp1* in the nervous system. We suggest that *Foxp1* protects neurons by stimulating the expression of $p21^{Waf1/Cip1}$, a cell cycle-regulatory protein with well documented neuroprotective effects.

IV. MATERIALS AND METHODS

Materials: All reagents and cell culture media were obtained from Invitrogen (Carlsbad, CA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Poly-L-Lysine for tissue culture was obtained from Trevigen (Gaithersburg, MD). The antibodies used in this paper were: Flag (catalog #F1804, Sigma-Aldrich), HA (Y-11 catalog# sc-805, F-7 catalog

Materials. Unless stated otherwise, all tissue culture media was purchased from Invitrogen (Carlsbad, CA) and chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Poly-L-Lysine for primary neuronal cultures was purchased from Trevigen (Gaithersburg, MD). Antibodies used in this study are as follows: Foxp1 from three sources (Cell Signaling Technologies (Danvers, MA), which recognizes Foxp1 aminoacids adjacent to Leu584, catalog #D35D10; Abcam (Cambridge, MA), catalog #ab134055; Bethyl Laboratories (Montgomery, TX), which recognizes Foxp1 aminoacids 627-677, catalog #A302-620A), FLAG (Sigma-Aldrich, catalog #F1804), α-Tubulin (Sigma-Aldrich, catalog #T9026), ERK1/2 (Cell Signaling Technology, catalog #9102), GFP antibodies were from Santa Cruz Biotechnology (Dallas, TX) (mouse-GFP catalog #sc-9996a and rabbit-GFP catalog #sc-8334), p21 (BD PharmingenTM (San Jose, CA), catalog#56430), HRP-conjugated secondary antibodies were obtained from Pierce (Waltham, MA) Immunocytochemistry secondary antibodies, Dylight 594 (catalog #115-585-146) and Alexa Fluor 488 (catalog #115545146), were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Human caudate tissue samples from both male and female HD patients along with unaffected controls were obtained from NIH Neurobiobank.

Expression plasmids. Flag-tagged *Foxp1* plasmid, pCMV10-mFoxp1 was a gift from Benjamin Blencowe (Addgene plasmid #35170). GFP tagged *Foxp1* plasmids and *Foxp1* isoform-D were

cloned in our lab. GFP and RFP tagged *Htt* plasmids were a kind gift from Dr. Troy Littleton (Massachusetts Institute of Technology, Cambridge, MA). The $p21^{Waf1/Cip1}$ promoter luciferase construct was a kind gift from Dr. Ming Zhang (Northwestern University Feinberg School of Medicine). FLAG tagged $p21^{Waf1/Cip1}$ was a gift from Mien-Chie Hung (Addgene plasmid #16240)

Culturing, treatment and transfection of cerebellar granule neurons (CGNs). CGNs were cultured from 7-9 day old Wistar rats and plated in Eagle's Basal Medium supplemented with 10% FBS, 25 mM KCl, 2 mM glutamine, and 0.2% gentamycin, as previously described (D'Mello, Galli, Ciotti, & Calissano, 1993). For 4 or 24 well dishes cells were plated at a density of 1 x 10^6/well and for 60 mm dishes at a density of 10 x 10^6. The anti-mitotic agent cytosine arabinofuranoside was added 16 – 20 hours later at a concentration of 10 µM. Treatment with high potassium (HK) or low potassium medium (LK) was performed 5 days after plating by switching the cultures to serum-free medium with 25 mM KCl (HK) or without KCl supplementation (LK). Cell death was quantified by DAPI (4'6'-diamidino-2-phenylindole hydrochloride) staining as previously described (Bardai et al., 2013; Dastidar, Landrieu, & D'Mello, 2011). Cells with condensed or fragmented nuclei were scored as dead. Transfection of CGN cultures was performed 4 - 5 days after plating using the calcium phosphate method as previously described (Bardai et al., 2013; Dastidar et al., 2011). Unless mentioned otherwise, neurons were switched to HK or LK treatment 24 h after transfection and viability quantified 24 h later using immunocytochemistry to detect transfected cells. The proportion of transfected cells undergoing apoptosis was quantified by DAPI staining. The viability is normalized to that of the

neurons transfected with GFP in HK condition. Transfections for each experiment were performed in duplicates and the experiments were repeated at least 3 times.

Culturing, treatment and transfection of embryonic cortical neurons. Cortical neurons were cultured from embryonic day 17 - 18 Wistar rat embryos as we have previously described (Bardai et al., 2013; Dastidar et al., 2011). The neurons were plated in Neurobasal media with B27 supplement. Transfection was performed on day 5 of culture using the calcium phosphate method and after 24 h of transfection the neurons were subjected to apoptotic stimuli by adding 1mM homocysteic acid (HCA) which induces oxidative stress (Ratan, Murphy, & Baraban, 1994). After 16-18 h the proportion of cells undergoing apoptosis was quantified by DAPI staining and viability was normalized to that of the neurons transfected with GFP in untreated condition. Transfections for each experiment were performed in duplicates and the experiments were repeated at least 3 times.

Culturing of cortical glial cells. The Cortex was isolated from postnatal day 2 –3 rat pups and placed in HBSS buffer. The tissue was then dissociated by the addition of trypsin followed by mechanical dissociation. The trypsin was then neutralized by adding serum and the cells were plated in DMEM with 10% FBS. The cell culture media was replaced every 2 days and the cells were sub cultured when they reach 80% confluence. After 2 passages the neurons are eliminated and the cortical glial cells are used for the experiments.

Culture and transfection of cell lines. Cell lines, HEK293T (catalog #CRL-11268) and N2A cells (catalog #CCL-131) were obtained from ATCC. Each were cultured in DMEM supplemented with 10% FBS and transfected with Lipofectamine 2000 (Life Technologies) in Opti-MEM

media according to manufacturer's instructions. Transfection efficiency was about 70-80% in N2A cells and about 80-90% in HEK293T cells.

Western blot analysis. Cell culture and tissue samples were lysed, protein concentration quantified, and Western blots performed as previously described (Bardai et al., 2013; Dastidar et al., 2011). Typically, 50 µg of protein was used/lane and transferred following PAGE to PVDF membrane. After blocking with milk, membranes were incubated overnight in primary antibodies that were used at a 1:500 to 1:1000 dilution in TBST with 5% BSA. Appropriate secondary antibodies were used at 1:20,000 dilution in 5% milk in TBST for 1 hour. Immunoreactivity was detected by enhanced chemiluminescence (GE Healthcare Bio-Sciences) according to manufacturer's directions.

cDNA synthesis and RT-PCR. RNA was extracted by lysing the cells in TRIzol (Life Technologies) and following the manufacturer's instructions. The RNA extracted was quantified and 3 µg of RNA was used to generate cDNA using Superscript First Strand Synthesis System (Life Technologies). GoTaq Green Master Mix (Promega) was used to perform PCR and initially 1 µl of cDNA was used to check the expression levels of Actin and then the quantity of cDNA used was adjusted according to Actin normalization. The following primers were used for amplification:

Foxp1 MR For: 5'-GGACGATAGAAGCACAGCTCAATGT-3' and *Foxp1* MR Rev: 5'-ATGGGCACGTTGTATTTGTCTGAGT-3'; *Foxp1* HUM For: 5'-GTTCCCGTGTCAGTGGCTAT-3' and *Foxp1* HUM Rev: 5'-TGTGATTGTTGCCTGTGGTT-3'; *Actin* For: 5'-GAGAGGGAAATCGTGCGTGAC-3' and *Actin* Rev: 5'-CATCTGCTGGAAGGTGGACA-3'; *c-Jun* For: 5'-GATGGAAACGAC CTTCTACG-3' and *c-*

Jun Rev: 5'-GTTGAAGTTGCTGAGGTTGG-3'; *p21* For: 5'-CCTGGTGATGTCCGACCTGTTCC-3' and *p21* Rev: 5'-GCGCTTGGAGTGATAGAAATCTG-3';

shRNA-mediated knockdown. Five shRNA constructs were obtained from Sigma-Aldrich to be used for knockdown analysis of *Foxp1* (TRCN0000072003 (shA), TRCN0000072004 (shB), TRCN0000072005 (shC), TRCN0000072006 (shD), andTRCN0000321549 (shE)). For knocking down the expression of $p21^{Waf1/Cip1}$ shRNA constructs TRCN0000042585(sh1), TRCN0000042587(sh2) from Sigma-Aldrich were used. The pLKO.1-TRC control vector which encodes a 18 bp non-hairpin forming insert purchased from Addgene (catalog #10879) was used as control. The shRNA's were transfected into cortical neurons on day 4 *in vitro* and allowed to express for 72 h before quantifying the viability of transfected cells. The transfected cells were visualized by co-transfecting the shRNA with EGFP in a ratio of 6.5:1. The viability of these transfected cells was quantified by DAPI staining.

Site directed mutagenesis. Foxp1 mutant plasmids *Foxp1ΔLZ* (lacking the leucine zipper region) and *Foxp1ΔFH* (lacking the Forkhead domain) were generated using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions. The GFP tagged *Foxp1* plasmid with the CMV promoter was used as the template. The plasmids were sequenced to confirm the mutations and then expressed in HEK293 cells to confirm protein expression. The following primers were used to generate the mutants. *Foxp1ΔLZ*-376-397 –For: 5′-GTAGAGTACAAATGCAGGTTCATGTGAAGTCTACAGAACC-3′; *Foxp1ΔLZ*-376-397 –Rev: 5′-GGTTCTGTAGACTTCACATGAACCTGCATTT -GTACTCTAC-3′; *Foxp1ΔFH*-493-

583-For: 5′-GAATTTTATAAGAACGCGGAAGTTATTAAAAACATGCAGAGCAGCCAC-3′; *Foxp1ΔFH*-493-583-Rev: 5′-GTGGCTGCTCTGCATGTTTTTAATAACTTCCGCGTTCTTATAAAATTC-3′.

Adenovirus Generation. Adenovirus was generated using the ViraPower Adenovirus Expression Kit from Invitrogen. The *Foxp1* gene was cloned into the pDONR 221 shuttle vector which was then transferred into the adenoviral vector pAd/CMV/V5-DEST™. The gene containing vector was then linearized with PacI and transfected into HEK293A cells for virus amplification. The crude lysate was then freeze thawed 4 times followed by CsCl gradient ultracentrifugation to purify the virus. The approximate titer of the virus ranged from 10^{10} - 10^{11} pfu/ml. CGNs and cortical neuron cultures were infected with adenovirus on day 5 *in vitro* and protein/ gene expression changes were analyzed 36-48 h later. The transduction efficiency of about 25-30% was observed using the adenovirus in neurons.

Bromodeoxyuridine (BrdU) assay. BrdU incorporation assays were performed as previously described (Majdzadeh et al., 2008; Mallick & D'Mello, 2014). Briefly, cell lines were transfected with the experimental plasmids by Lipofectamine 2000 and 22 h later BrdU (200µM) was added to the cell culture media and incubated for 2 h. The cells were fixed with 4% paraformaldehyde and immunocytochemistry was performed. The dividing cells (BrdU positive cells) were identified using a monoclonal mouse antibody against BrdU (Sigma, catalog #B8434) whereas transfected cells were identified with GFP or FLAG antibodies. The proportion of transfected cells that were BrdU-positive was quantified.

Luciferase assay for transcriptional activity. 3.75 µg *p21*$^{Waf1/Cip1}$ luciferase Reporter plasmid with firefly luciferase and 0.25 µg renilla luciferase were transfected into N2A cells along with 4 µg of experimental plasmids. After 24 h the cells were lysed with Passive Lysis Buffer and the transcriptional assay was performed using Promega Dual-Luciferase reporter assay system (catalog #E1910). First the substrate for firefly luciferase was added and the Relative Luminescence Units (RLU) was measured in a luminometer followed by the addition of Stop and Glow Reagent which has a substrate for renilla luciferase and then RLU measured for renilla. The RLU values of firefly luciferase, which were normalized with renilla, were used to calculate the fold induction.

CRISPR knockdown. CRISPR plasmids were constructed by cloning the target sequence of *Foxp1* in vector PX458 (Addgene plasmid #48138) which has the cas9 gene in the backbone. The oligos for target sequences were melted in a hotplate, allowed to anneal at room temperature and then cloned into the sgRNA scaffold of PX458 digested with BbsI. The oligo sequences are as follows

Foxp1-CPR-1 For: 5'-CACCGTGACACTCGGTCCAACGGAG-3'

Foxp1-CPR-1 Rev: 5'-AAACCTCCGTTGGACCGAGTGTCAC-3'

Foxp1-CPR-2 For: 5'-CACCGCGAGAGCTGTCCATTGGTAG-3'

Foxp1-CPR-2 Rev: 5'-AAACCTACCAATGGACAGCTCTCGC-3'

Foxp1-CPR-3-For: 5'-CACCGTCTTCAGGTTCCCGTGTCAG-3'

Foxp1-CPR-3-Rev: 5'-AAACCTGACACGGGAACCTGAAGAC-3'

The CRISPR plasmids were transfected into N2A cells by Lipofectamine 2000 to check for efficiency of knockdown by western blotting and into primary neurons by the calcium phosphate

method. 72 hours later immunocytochemistry was performed with the GFP antibody to detect the transfected cells and the viability of the neurons was determined using DAPI as described earlier.

Fly stocks and genetics. Mammalian GFP-tagged full-length *Foxp1* isoform-A and *Foxp1* isoform-D were excised from mammalian expression vectors and subcloned into the Not1-Xba1 site of the modified fly upstream activation sequence (UAS) expression vector (Exelixis, San Francisco, CA, USA). The expression vectors containing the *Foxp1* gene were then microinjected into flies by (BestGene, Chino Hills, CA, USA) as part of a fee-for service arrangement to obtain transgenic flies. The UAS-*Foxp1A* and UAS-*Foxp1D* flies were recombined with *Appl-GAL4* to drive their expression in all neurons. The GMR-*Htt* Q120 flies were obtained from the Bloomington stock center (Bloomington, Indiana University, IN, USA). All flies were maintained and crosses were set at room temperature (25ºC) in standard *D. melanogaster* medium.

Paraffin sections. Paraffin sections for determining retinal degeneration were prepared as described in (Bettencourt da Cruz, Schwarzel et al. 2005) Briefly, whole flies were fixed in Carnoy's solution and dehydrated in an ethanol series followed by incubation in methyl benzoate before embedding in paraffin. Sections were cut at 7μm and imaged using the auto-fluorescence caused by the dispersed eye pigment.

Fast phototaxis. Fast phototaxis assays were conducted in the dark using the countercurrent apparatus described by Benzer and a single light source (Benzer, 1967). A detailed description of the experimental conditions has been previously published (Strauss & Heisenberg, 1993). Flies were starved for 4h but had access to water before being tested. Five consecutive tests

were performed in each experiment with a time allowance of 6 seconds to make a transition towards the light and into the next vial.

The R6/2 transgenic mouse model of HD. Female C57BL/6J ovarian transplant mice hemizygous for exon-1 of the mutant *Htt* gene containing 120± 5 CAG repeats were bred with wild type C57 BL/6J males (Jackson Laboratory) and the genotype of the progeny was analyzed by PCR. Transgenic mice (R6/2) and their wild type gender matched litter mates were euthanized and the brains were dissected. Cortex (CTX), striatum (STR), cerebellum (CBM) and rest of the brain/other brain parts (OBP) were separated to be analyzed by western blotting. Mice of both genders were used.

The Hdh knock-in mouse model of HD. Dissected brain tissues from Hdh knock-in mice with different polQ lengths were a kind gift from Dr. Peter Detoff. The knock-in mice were as described in (Kumar et al., 2016) all congenic to C57BL/6. The tissue was homogenized in RIPA buffer and analyzed by western blotting.

Statistical analysis. GraphPad Prism 5 software was used to generate the graphs in this study. For RT-PCR Kodak 1D Software was used to quantify the intensity of the bands which were normalized to that of housekeeping genes. For Western blots Image J software was used to calculate the area and intensity of the bands. The intensity of the bands were then normalized with the loading control. Statistical analysis was performed by unpaired two-tailed Student's t test for sample sizes less than 5 (n=5). For larger sample sizes Mann-Whitney U test was used to calculate the significance level. For comparing more than two datasets one way analysis of variance (ANOVA) with posttest analysis was performed. The results are displayed as mean ± S.D. For viability experiments the transfections were performed in duplicate and the experiments

were repeated at least thrice and > 200 cells were counted for each condition. The p values <0.05 were considered as statistically significant and asterisks in legends indicate the p values as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

V. RESULTS

***Foxp1* is highly expressed in the cortex and striatum**. To examine the expression of *Foxp1* in the adult mouse brain we performed western blots. *Foxp1*-isoform-A (~90 kDa) is expressed widely, but with highest expression in the striatum and cortex (Fig. 2.1). This is consistent with the results of previous studies (Desplats et al., 2006; Desplats et al., 2008; Tang et al., 2012). In addition to isoform-A, expression of two other *Foxp1* isoforms is clearly detectable in the brain. These have previously been designated as isoform-C (~50 kDa) and isoform-D (~70 kDa) (Brown et al., 2008; Green et al., 2009; Santos et al., 2011). Isoform-D is also expressed selectively in the striatum and cortex and, in fact, more so than isoform-A (Fig. 2.1). Isoform-C is expressed at lower levels and in a pattern that is different from the two larger isoforms with expression in the hippocampus and other brain parts, but not cortex (Fig. 2.1).

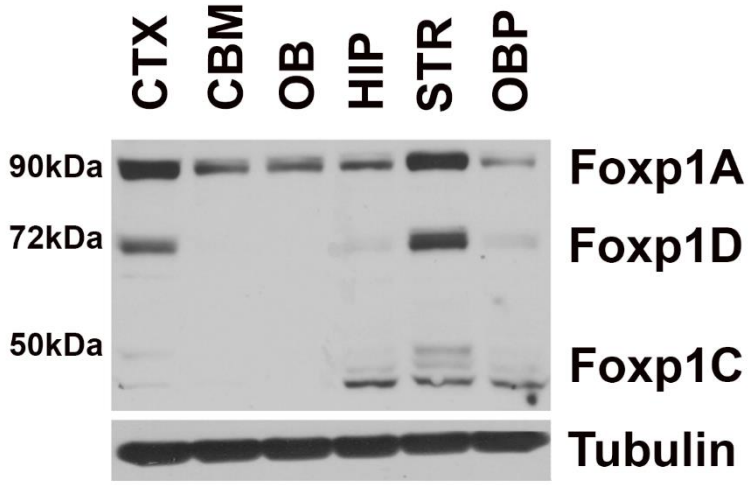
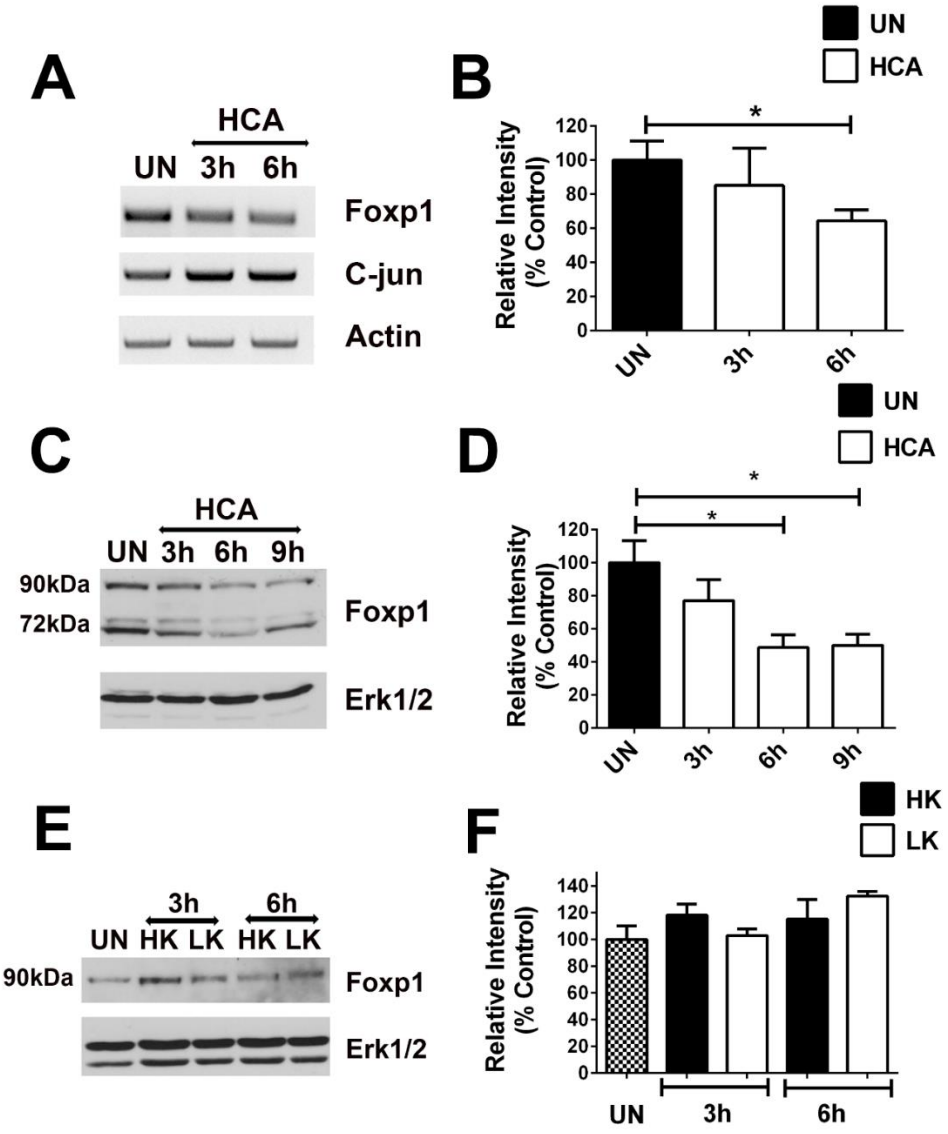


Figure 2.1. Foxp1 expression levels in different brain regions. A, Foxp1 protein expression levels in the mouse cortex (CTX), cerebellum (CBM), olfactory bulb (OB), hippocampus (HIP), striatum (STR) and other brain parts (OBP) at 12 months of age, analyzed using Foxp1 antibody (Bethyl Labs). α-Tubulin was used as a loading control.

***Foxp1* expression is reduced in cortical neurons exposed to oxidative stress.** Oxidative stress is an underlying feature in several neurodegenerative diseases, including HD (Johri & Beal, 2012; Schapira, Olanow, Greenamyre, & Bezard, 2014). Cortical neurons are particularly sensitive to oxidative stress and die when exposed to homocysteic acid (HCA) (Ratan et al., 1994). *Foxp1* mRNA and protein expression is reduced in HCA-treated neurons and this occurs well before neuronal death is observed (Fig. 2.2A - D). The reduction in mRNA suggests that the downregulation occurs at the transcriptional level. While *Foxp1* expression was reduced, the expression of c-jun, commonly used as a marker of neuronal death is increased by HCA (Fig. 2.2A). Although expressed in cultured CGNs at a low level, *Foxp1* expression is not reduced during low potassium (LK)-induced death (Fig. 2.2E and 2.2F). This suggests that expression of *Foxp1* during neuronal death is regulated cell-specifically.

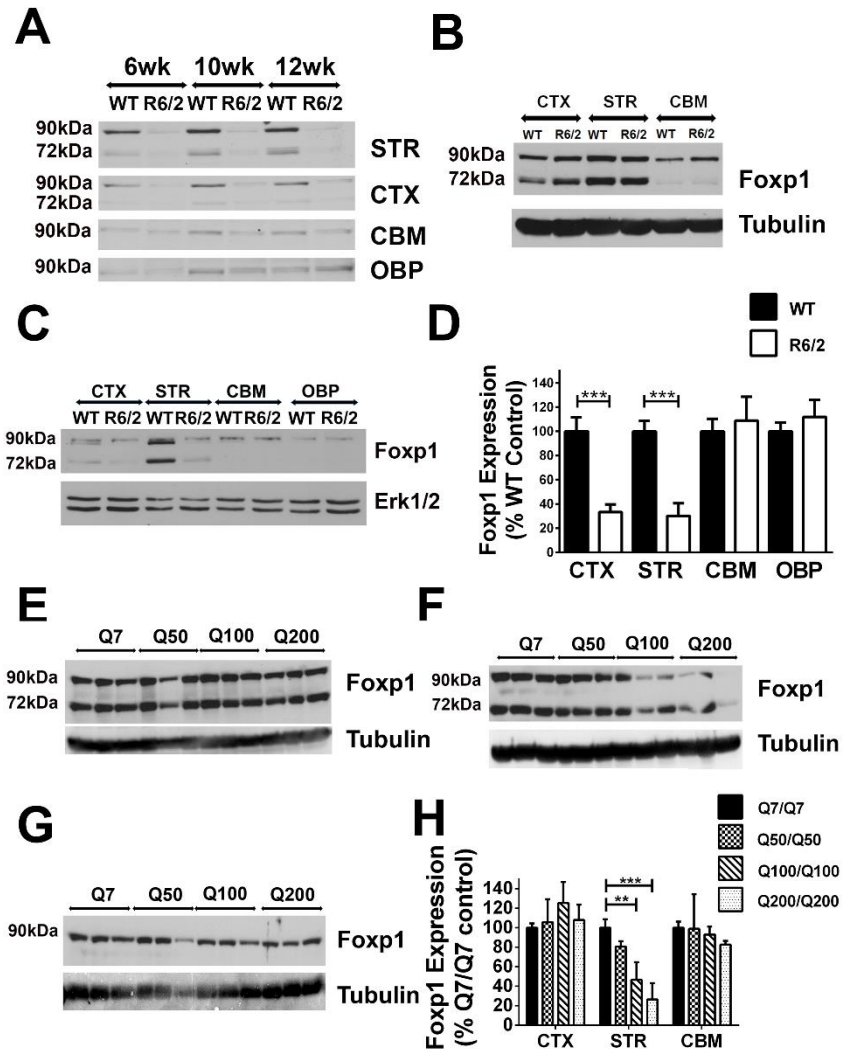
Next Page Figure 2.2. Foxp1 expression levels changes in neurons under apoptotic stress. **A,** *Foxp1* RNA expression analysis in cortical neurons subjected to apoptotic conditions. mRNA was prepared from cortical neurons treated with 1mM Homocysteic acid (HCA) for 3h and 6h. *C-jun* is used as a marker for apoptosis and actin is used as normalization control. **B,** Quantification of *Foxp1* RNA expression levels normalized to the expression levels of β-*actin*. *, $p<0.05$ (n=4). **C,** Foxp1 protein levels in cortical neurons under apoptotic stress from HCA treatment. Protein lysates were prepared at 3h, 6h and 9h of HCA treatment and subjected to western blotting using Foxp1 antibody (Cell Signaling Technology). Erk1/2 is used as a loading control. **D,** Quantification of Foxp1 protein levels in cortical neurons treated with HCA. Foxp1 protein levels were normalized to the expression levels of Erk1/2. *, $p<0.05$ (n=4). **E,** Foxp1 protein levels in CGNs subjected to apoptotic stress by low potassium (LK) treatment. Protein lysates were collected at 3h and 6h of treatment and subjected to western blotting using Foxp1 antibody (Cell Signaling Technology). Erk1/2 is used as loading control. **F,** Quantification of Foxp1 protein expression levels normalized with expression levels of ERK. n.s., not significant (n=3).



***Foxp1* expression is reduced in the striatum of HD mice and patients.** Since *Foxp1* is selectively expressed in the striatum and cortex, the same brain regions that are selectively affected in HD, we looked at the expression of *Foxp1* in the brains of R6/2 mice, a widely used transgenic mouse model of HD (Bates, Mangiarini, Mahal, & Davies, 1997). The expression of isoforms-A and D were dramatically reduced in the striatum and cortex (Fig. 2.3A). We and others have previously described that motor deficits in R6/2 mice can be observed at about 7

weeks and thus, *Foxp1* downregulation in the R6/2 brain occurs before motor deficits are obvious. The downregulation was selective for the striatum and cortex because no reduction was observed in the cerebellum or other brain parts (Fig. 2.3 C and 2.3D). *Foxp1* expression in the striatum of R6/2 was comparable to wild-type mice at 2 weeks of age (Fig. 2.3B), arguing against a developmental effect of expression and indicating that the reduction in *Foxp1* may trigger disease pathogenesis. In support of this conclusion, Foxp1 expression levels were also decreased in striatum of *Hdh* knock-in mice (Fig. 2.3F) but not to a significant extent in the cortex and cerebellum (Fig. 2.3E and 2.3G). The reduction in Foxp1 protein levels in the striatum of these mice correlated with the increase in length of the polyglutamine expansion, which previous studies have shown, correlates with disease severity (Fig. 2.3H)(Kumar et al., 2016).

Next Page Figure 2.3. Foxp1 expression levels in Huntington's disease models. A, Western blotting analysis of Foxp1 in various brain regions of wild-type (WT) and R6/2 transgenic littermates of mice at different ages using Foxp1 antibody from Bethyl Laboratories. Western blotting analysis of Foxp1 in various brain regions of WT and R6/2 mice at 2 weeks of age **(B)** and at 12 weeks of age **(C)** using Foxp1 antibody (Cell Signaling Technology). **D,** Quantification of protein expression levels of Foxp1 (isoform-A + isoform-D) in different brain regions of WT and R6/2 mice at 12 weeks of age. Erk1/2 was used as a normalization control. ***, $p<0.001$ compared to expression levels in WT mice (n=3). **E-G**, Western blotting analysis of Foxp1 expression levels in cortex **(E)**, striatum **(F)**, and cerebellum **(G)** of knock-in mice having various lengths of polyglutamine expansion in the Huntingtin gene using Foxp1 antibody (Abcam). Tubulin is used as a loading control. **H**, Quantification of Foxp1 protein levels in various brain regions of *Htt* knock-in mice normalized with expression levels of Tubulin. **,0.01; ***, $p<0.001$ (n=3)



In addition to our own studies, microarray analyses conducted by other groups also list *Foxp1* as one of the genes that is significantly downregulated in various mouse HD models.

Table 2.1. Decreased Foxp1 mRNA levels reported in published datasets from human HD caudate and HD mouse models

Model	Age/Grade	Region	Gene ID	Log2 FC	P-value	Reference
Human	Grades 0–4	Caudate	*FOXP1*	-0.578	2.57E-10	Hodges A et al., Hum Mol Genet. 2006 15:965-77.
R6/1	6 months	Striatum	*Foxp1*	-1.152	0.0017	Desplats P et al., J Neurochem. 2006 3:743-57.
R6/2-Q150	12 weeks	Striatum	*Foxp1*	-0.775	3.94E-06	Kuhn A et al. Hum Mol Genet. 2007 15:1845-61.
R6/2-Q150	12 weeks	Striatum	*Foxp1*	-0.304	0.0038	Tang B et al., Neurobiol Dis. 2011 42:459-67.
Hdh^{Q92}	18 months	Striatum	*Foxp1*	-1.737	0.0102	Kuhn A et al., Hum Mol Genet. 2007 15:1845-61.
Yac128	12 months	Striatum	*Foxp1*	-0.403	0.0431	Kuhn A et al., Hum Mol Genet. 2007 15:1845-61.
CHL2 (Hdh^{Q150})	22 months	Striatum	*Foxp1*	-0.979	7.55E-06	Kuhn A et al., Hum Mol Genet. 2007 15:1845-61.
Het-KI-Q111	10 months	Striatum	*Foxp1*	-0.223	0.00051	Langfelder P et al., Nat Neurosci. 2016 19(4):623-33.
Het-KI-Q140	10 months	Striatum	*Foxp1*	-0.341	1.06E-07	Langfelder P et al., Nat Neurosci. 2016 19(4):623-33.
Het-KI-Q175	10 months	Striatum	*Foxp1*	-0.325	4.09E-07	Langfelder P et al., Nat Neurosci. 2016 19(4):623-33.

We extended our analyses to lysates from the caudate of human HD patients and found a dramatic decrease in expression of *Foxp1* mRNA levels, both in males (Fig. 2.4A) and females (Fig. 2.4B) compared to age and gender matched normal individuals (see Table-2.2 for sample details).

Table 2.2. Summary of neuropathology and clinical diagnosis of human caudate tissue from NeuroBioBank

NeuroBioBank Sample ID	Age (years)	Gender	Post Mortem Interval	HD Vonsattel grade	Neuropathology	Other pathology
13082	41	Male	27h	n.a.	Normal	n.d.
4340	47	Male	12.5h	n.a.	Normal	Esophageal cancer with metastases to liver
4615	49	Male	15h	n.a.	Normal	Colon cancer with metastases to liver
13058	50	Male	8h	n.a.	Normal	n.d.
13069	53	Male	25h	n.a.	Normal	n.d.
4827	55	Male	11.4h	n.a.	Normal	Schizophrenia, Tourette's syndrome and ethanol abuse
13106	59	Male	5h	n.a.	Normal	n.d.
HCtZU_16_08	62	Male	29h	n.a.	Normal	n.d.
2983	30	Male	2h	NG	HD	Early Huntington's disease
2706	43	Male	17h	1-2	HD	Dementia
4518	49	Male	12.7h	3	HD	Dementia, Ataxia
2858	50	Male	12h	2	HD	n.d.
3573	51	Male	32h	3	HD	n.d.
4254	53	Male	9.3h	3	HD	Depression, Dementia, Pneumonia
3744	55	Male	19h	1-2	HD	n.d.
2488	59	Male	9.5h	4	HD	n.d.
5293	41	Female	11.5h	n.a.	Normal	Liver failure, Pneumonia,
4836	52	Female	15.1h	n.a.	Normal	Depression, suicide, ethanol abuse
3175	54	Female	21.5h	n.a.	Normal	Pancytopenia, Diabetes Type I, Hypothyroidism
4715	55	Female	4h	n.a.	Normal	n.d.
3558	59	Female	19.5h	n.a.	Normal	Lymphoma, Non-Hodgkins
HBJO_16_01	67	Female	21h	n.a.	Normal	Asperger's Disorder, Opioid abuse
HCtYD_16_01	68	Female	19h	n.a.	Normal	n.d.
HCtZZC_16_0	82	Female	14h	n.a.	Normal	n.d.
3383	40	Female	13h	1	HD	n.d.
2669	44	Female	11h	NG	HD	Advanced Huntington's disease
HBGY_16_01	48	Female	32h	NG	HD	Dementia, Depression, malnutrition
4072	50	Female	16.3h	2	HD	Breast Cancer
HBLF_16_01	62	Female	14h	NG	HD	n.d.
HBCS_16_01	68	Female	43h	NG	HD	n.d.
HBDY_16_01	73	Female	15h	NG	HD	n.d.
HBFO_16_01	80	Female	38h	NG	HD	n.d.

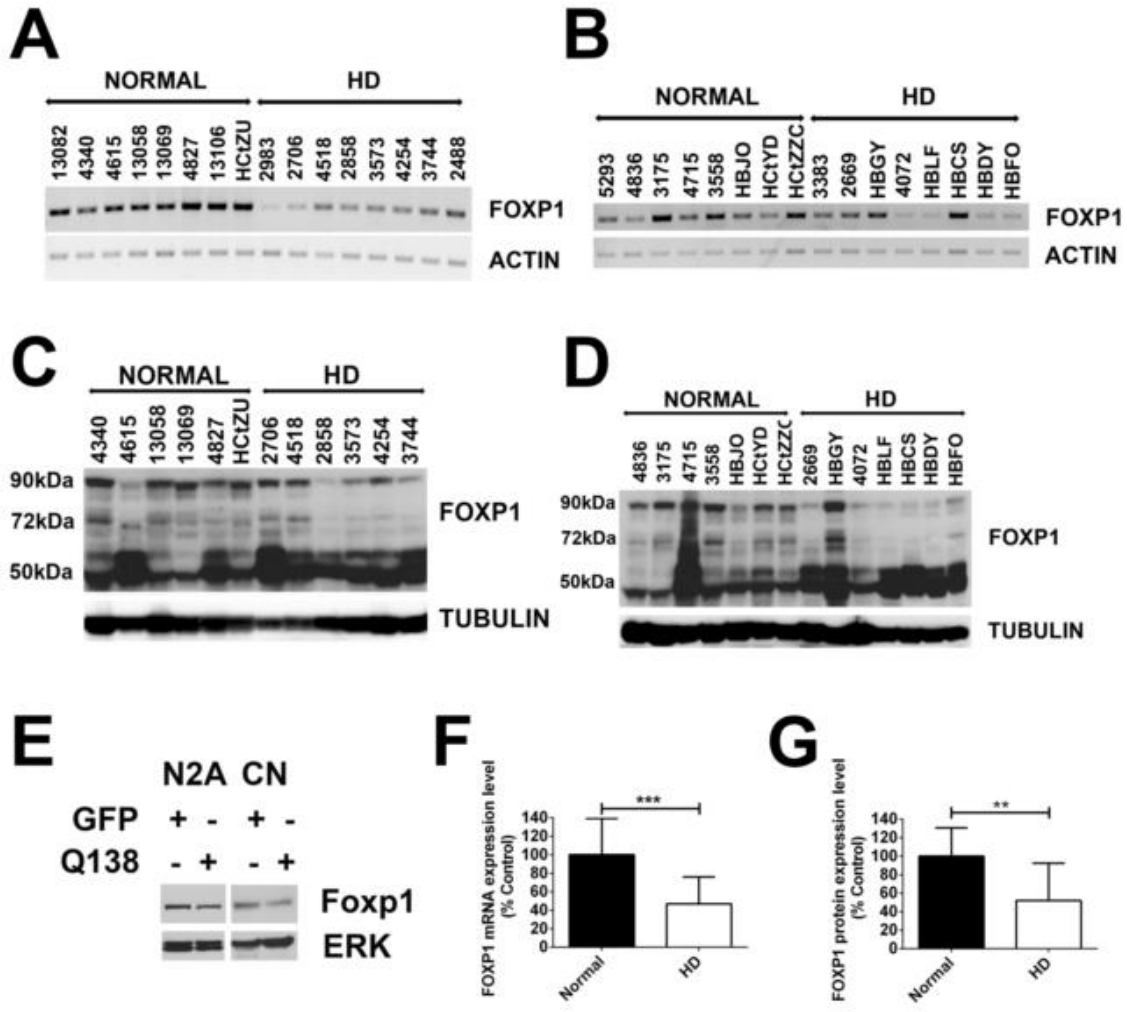
n.d. :- not determined; n.a. :- not applicable; NG :- No grade

In western blots Foxp1 isoform-A and isoform-D were decreased in lysates of HD patients compared to normal controls, whereas expression of isoform-C appeared to be increased (Fig. 2.4C and Fig. 2.4D). Reduced *Foxp1* expression has also been described by other labs in R6/1 mice, a related HD model (Desplats et al., 2006; Tang et al., 2012). And while expression of *Foxp1* protein has not been studied previously in HD patients, reduction of *Foxp1* mRNA levels

in the HD caudate has been described by another lab (Desplats et al., 2006; Tang et al., 2012). It is noteworthy that *Foxp1* expression is not altered in the striatum or substantia nigra of 6-hydroxy dopamine-administered rats, a commonly used model of Parkinson's disease (Tang et al., 2012). Together with the absence of a downregulation in LK-treated CGNs, this observation suggests that *Foxp1* downregulation is both cell type and disease-selective.

To examine if we could recapitulate the downregulation of *Foxp1* expression seen in mouse models (Fig. 2.3D and Fig 2.3H) and human HD patients (Fig. 2.4F and Fig. 2.4G) in a cell culture model of HD, we expressed mut-*Htt* in primary cortical neurons and Neuro2A cells, a mouse striatal cell line. As observed in HD mice and patients, substantial downregulation of *Foxp1* was observed in mut-*Htt* expressing neurons as well as N2A cells (Fig. 2.4E)

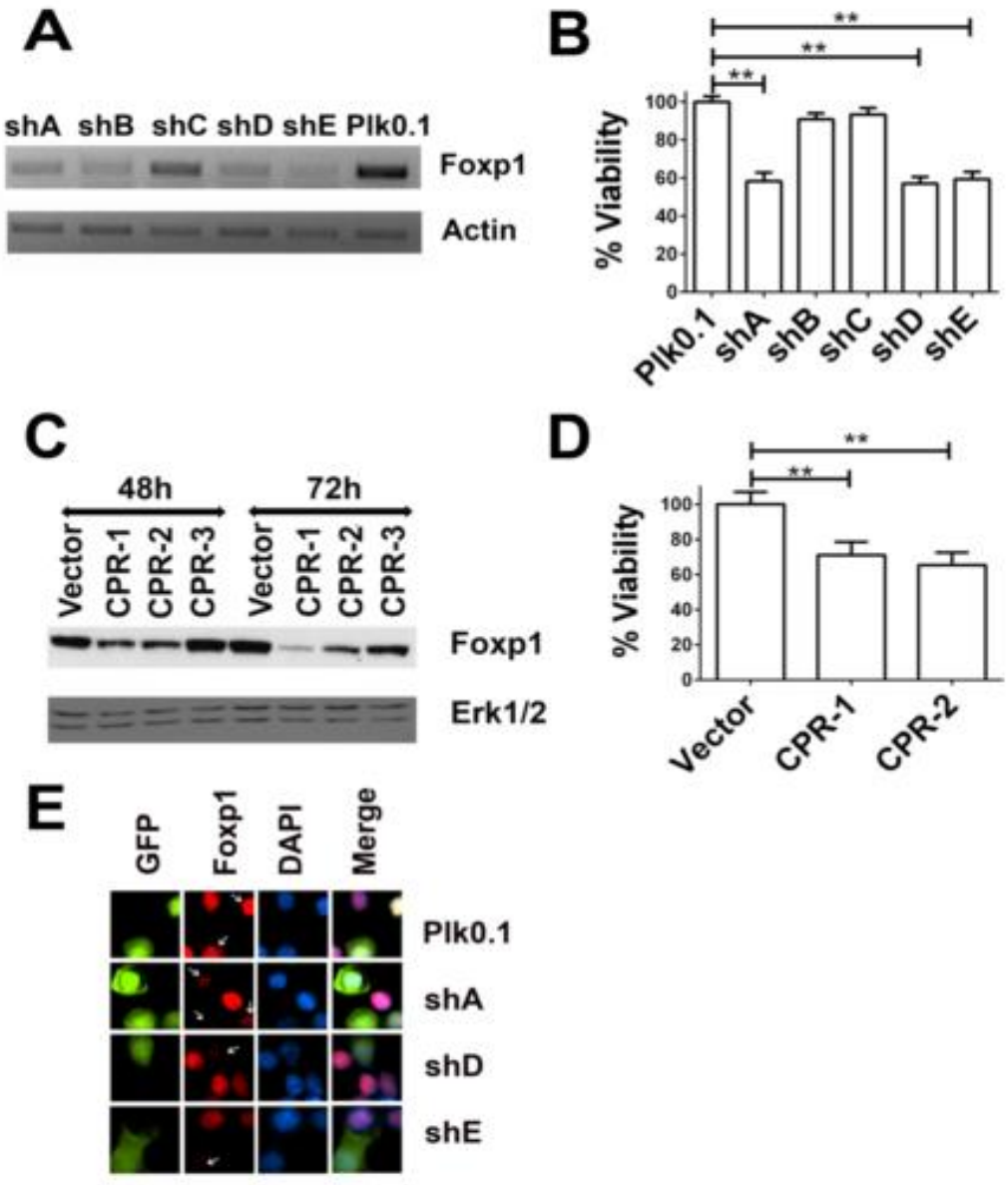
Next Page Figure 2.4. *FOXP1* mRNA and protein expression in caudate of HD patients. **A&B,** RNA extracted from caudate of age matched normal male individuals and HD patients **(A)** or normal females individuals and HD patients **(B)** was subjected to RT-PCR analysis using primers amplifying regions common to all the isoforms of *FOXP1*. β-*ACTIN* was used as normalization control. **C&D**, Foxp1 protein expression levels in normal male individuals and HD patients **(C)** or normal females individuals and HD patients **(D). E**, Western blotting analysis of Foxp1 protein levels in N2A neuroblastoma cells and in cortical neurons (CN), upon infection with GFP or Q138 adenovirus. **F&G**, Quantification of *Foxp1* RNA expression levels **(F)** and protein expression levels **(G)** in caudate of normal individuals and HD patients.



***Foxp1* is needed for neuronal survival.** The downregulation of *Foxp1* isoform-A expression in both oxidatively-stressed cortical neurons and degenerating regions of the R6/2 and human HD brain suggests that elevated expression of *Foxp1* isoform-A is necessary for the full survival capacity of neurons. To investigate this possibility, we tested five commercially available shRNAs against *Foxp1* and identified three that reduced *Foxp1* protein levels significantly, designated as shA, shD, and shE (Fig. 2.5A). Expression of these three shRNAs induced cell death in otherwise healthy cortical neurons (Fig. 2.5B). In contrast, another shRNA, shC, which was incapable of knocking down *Foxp1* expression and shB which only binds to *Foxp1* isoform-

A, but not to the other isoforms, had no effect on neuronal survival (Fig. 2.5B). Similarly, when *Foxp1* expression was knocked down using CRISPR/Cas9 technology using two separate guide RNA constructs, CPR-1 and CPR-2, the survival of cortical neurons was reduced (Fig. 2.5C and 2.5D). The knockdown of *Foxp1* was confirmed in cortical neurons by immunocytochemistry using endogenous Foxp1 antibodies (Fig. 2.5E).

Next Page-Figure 2.5. Suppression of *Foxp1* expression induces apoptosis in healthy neurons. A, RNA was collected from cortical neurons infected with lentivirus generated from shRNA constructs (shA-shE) to knockdown *Foxp1* expression or by lentivirus Plk0.1 as control. β-*Actin* is used as normalization control. **B,** Cortical neurons were co-transfected with Plk0.1 (Control) or shRNA plasmids and GFP in a ratio of 6.5:1 for 72 hrs. Immunocytochemistry was performed using a GFP antibody to identify the transfected cells and viability was determined by DAPI staining. **, $p<0.01$ (n=3), compared to the viability of cells transfected with Plk0.1. Data is expressed as mean ± S.D. **C,** CRISPR plasmids CPR-1, CPR-2, CPR-3 and a vector control (PX-458) were transfected into N2A cells for 48hrs and 72 hrs. Protein lysates were collected and analyzed by western blotting using Foxp1 antibody (Cell Signaling Technology). Erk1/2 is used as loading control. **D,** Cortical neurons were transfected with PX-458 (vector) or CRISPR plasmids CPR-1 and CPR-2 for 72hrs and immunocytochemistry was performed using a GFP antibody and viability was quantified by DAPI staining. Viability was normalized to cortical neurons transfected with PX-458. The data is represented as mean ±S.D. **, $p<0.01$ compared with neurons transfected with PX-458 (n=3). **E,** Cortical neurons were co-transfected with Plk0.1 or shRNA plasmids along with GFP in a ratio of 6.5:1 and 72 hours later immunocytochemistry was performed to detect the endogenous levels of Foxp1 protein and determine the efficiency of the shRNA in knocking down Foxp1.



Elevating Foxp1 levels protects against mut-*Htt* neurotoxicity. While reduced *Foxp1* expression results in loss of neuronal viability, restoring elevated levels of *Foxp1* isoform-A by ectopic expression protected cortical neurons from HCA-induced death (Fig. 2.6A) and against mut-*Htt* neurotoxicity (Fig. 2.6B). The two domains essential for *Foxp1* transcriptional activity

are the leucine zipper (LZ) and the Forkhead (FH) domains. We therefore generated mutant forms of *Foxp1* lacking these two domains, *Foxp1ΔLZ* and *Foxp1ΔFH*, and tested their ability to protect against mut-*Htt* neurotoxicity. When ectopically expressed in neurons, *Foxp1ΔLZ* maintains a nuclear localization like wild type *Foxp1* whereas *Foxp1ΔFH*, which lacks the nuclear localization signal, shows a cytoplasmic localization (Fig. 2.6C). Neither of these mutant constructs could protect against mut-*Htt* neurotoxicity (Fig. 2.6B).

As shown in Fig. 2.2E and 2.2F, *Foxp1* is expressed relatively weakly in CGNs and its expression is not reduced in these neurons when cell death is induced by LK treatment. Yet, the overexpression of *Foxp1* completely prevents LK-induced death of CGNs (Fig. 2.6D). This indicates that *Foxp1* is a *bona fide* neuroprotective protein.

Virtually nothing is known about the functional significance of the smaller isoforms of *Foxp1* in the nervous system. Because *Foxp1* isoform-D is also downregulated in the striatum of patients and R/2 mice, we investigated whether it was also capable of protecting neurons. As shown in Fig. 2.6B, *Foxp1* isoform-D also protected cortical neurons against mut-*Htt* toxicity.

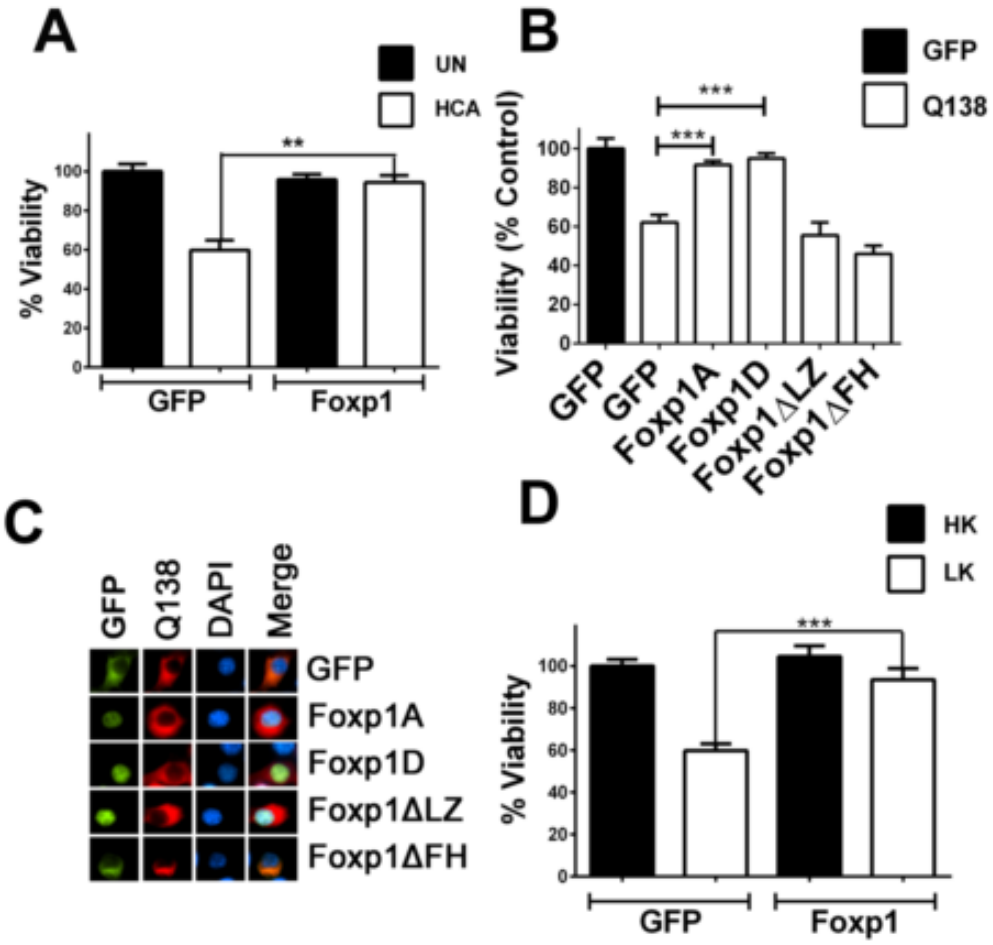


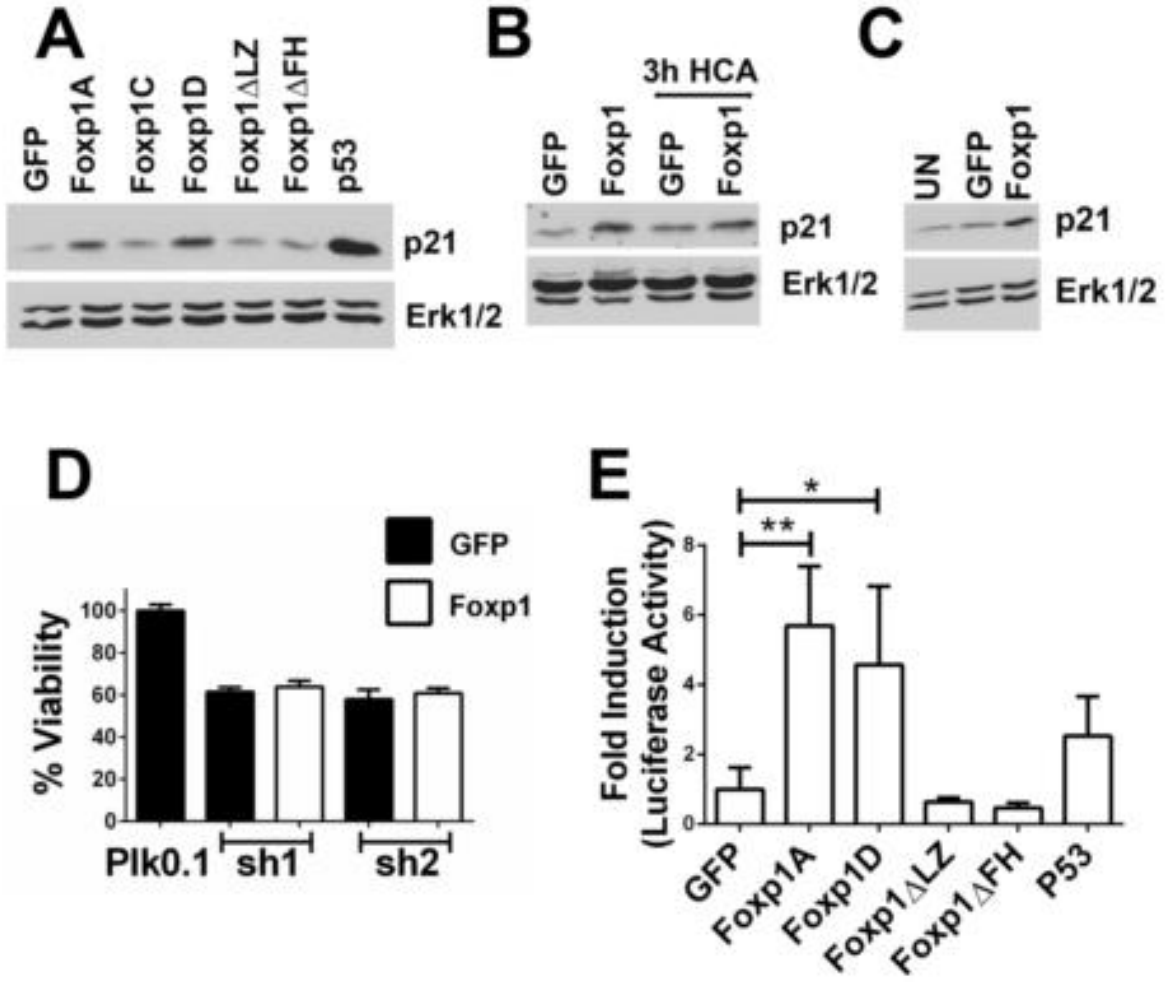
Figure 2.6. Overexpression of *Foxp1* protects neurons from apoptosis. A, Cortical neurons were transfected with *GFP* or *Foxp1* for 24h and then treated with medium with or without 1 mM HCA for another 24h. Viability of transfected neurons was quantified by DAPI staining and the viability was normalized to *GFP* transfected cortical neurons in media without HCA. The data is represented as mean ± S.D. **, $p<0.01$ compared with neurons transfected with *GFP* and treated with HCA (n=4). **B,** Cortical neurons were transfected with either *GFP* alone or *GFP*, *Foxp1A*, *Foxp1D*, *Foxp1ΔLZ,* and *Foxp1ΔFH* along with Q138-RFP. After 24h of transfection the cells were fixed and immunocytochemistry was performed with GFP and RFP antibodies and the viability of transfected cells was quantified by DAPI staining. Viability was normalized to neurons transfected with *GFP* alone. The data is represented as mean± S.D. ***, $p<0.001$ compared to *GFP* and Q138 co-transfected cells. **C,** N2A cells were co-transfected with *GFP* or *Foxp1* plasmids along with Q138-RFP and immunocytochemistry was performed to determine the intracellular localization of the proteins. **D,** CGNs were transfected with *GFP* or *Foxp1* plasmids for 24hrs and then the media was changed to HK or LK medium for another 24h. Immunocytochemistry was performed with GFP/FLAG antibody to identify the transfected cells and viability was determined by DAPI staining. Viability was normalized to cells transfected

with *GFP* in HK media. The data is represented as mean ±S.D. ***, p<0.001 compared with neurons transfected with *GFP* and treated with HK media (n=6).

***Foxp1* stimulates *p21*$^{Waf1/Cip1}$ expression and inhibits cell cycle progression.** Tang et al., (2012) conducted an RNA-Seq analysis to identify genes regulated by *Foxp1* isoform-A overexpression in cultured striatal neurons (Tang et al., 2012). One of the genes identified in that screen was *CDKN1A*, which encodes the cell cycle inhibitory protein p21$^{Waf1/Cip1}$ (Tang et al., 2012). Interestingly, a separate RNA-Seq study of the striatum of *Foxp1*$^{+/-}$ mice also identified *p21*$^{Waf1/Cip1}$ as a gene that was positively regulated by *Foxp1* (Araujo et al., 2015). We and several other labs have previously described neuroprotective effects of *p21*$^{Waf1/Cip1}$, including protection against mut-*Htt* toxicity (Harms et al., 2007a; Langley et al., 2008; Ma et al., 2013; Mallick & D'Mello, 2014; Poluha et al., 1996; Tomita et al., 2006). We therefore investigated whether *Foxp1* exerts its neuroprotective effect through stimulation of *p21*$^{Waf1/Cip1}$. Overexpression of *Foxp1* isoform-A in striatally-derived N2A cells led to an increase in *p21*$^{Waf1/Cip1}$ protein expression (Fig 2.7A). In contrast, *Foxp1* mutants *Foxp1ΔLZ* and *Foxp1ΔFH* failed to stimulate *p21*$^{Waf1/Cip1}$. Like *Foxp1* isoform-A, expression of *Foxp1* isoform-D also increased *p21*$^{Waf1/Cip1}$ expression, while *Foxp1* isoform-C had no effect (Fig. 2.7A). *Foxp1* stimulated expression of *p21*$^{Waf1/Cip1}$ in cortical neurons both under normal and apoptotic conditions (Fig. 2.7B). Similar results were obtained in CGNs upon overexpression (Fig. 2.7C). If stimulation of *p21*$^{Waf1/Cip1}$ is necessary for neuroprotection by *Foxp1*, then knockdown of *p21*$^{Waf1/Cip1}$ would be expected to abolish the ability of *Foxp1* to protect. Indeed, knocking down *p21*$^{Waf1/Cip1}$ using two separate shRNA constructs blocked the ability of *Foxp1* to protect neurons from cell death (Fig. 2.7D). To examine whether *p21*$^{Waf1/Cip1}$ was a direct target of *Foxp1*, we

performed transcriptional assays in N2A cells by co-expressing *Foxp1* plasmids with a *p21*$^{Waf1/Cip1}$ promoter-Luciferase reporter plasmid. Both *Foxp1* isoform-A and *Foxp1* isoform-D stimulated the activity of the *p21*$^{Waf1/Cip1}$ promoter (Fig.2.7E).

Next Page-Figure 2.7. Foxp1 induces the expression of p21$^{Waf1/Cip1}$. A, N2A cells were transfected with *GFP* or *Foxp1* plasmids for 24hrs. The protein lysates were subjected to western blotting with p21 antibody. Erk1/2 was used as loading control. **B,** Adenovirus for *GFP* or *Foxp1* was used to infect cortical neurons and 48h later the neurons were maintained in either normal survival condition or HCA induced apoptotic condition for 3h and protein lysates were collected and subjected to western blotting with a p21 antibody. Erk1/2 was used as a loading control. **(C)** CGNs were infected with *GFP* or *Foxp1* adenovirus and 48h later the protein lysates were collected and subjected to western blotting with p21 antibody. Erk1/2 was used as a loading control. **D,** *p21* shRNA plasmids were co-transfected with *GFP* or *Foxp1*-GFP plasmids in cortical neurons for 72h and the viability was accessed by immunocytochemistry and DAPI staining. Viability is normalized to cortical neurons transfected with Plk0.1 along with *GFP*. The data is represented as mean ± SD (n=3). **E,** N2A cells were transfected with *GFP* or *Foxp1* plasmids along with a p21 promoter luciferase construct for 24h. Renilla was used as a transfection control. Promoter activity is expressed as a ratio of firefly luciferase to renilla luciferase. Fold change represents the ratio of p21 promoter activity in experimental transfected cells to p21 promoter activity in GFP transfected cells. **, $p<0.01$; *, $p<0.05$ Foxp1 fold induction compared with the fold induction in GFP transfected cells.



A large body of evidence suggests that aberrant re-entry into the cell cycle underlies cell loss in neurodegenerative diseases (Becker & Bonni, 2004; Greene, Liu, Troy, & Biswas, 2007). Since *Foxp1* stimulates $p21^{Waf1/Cip1}$ expression and given that $p21^{Waf1/Cip1}$ is a strong cell cycle inhibitor, it was likely that *Foxp1* has an inhibitory effect on the cell cycle machinery. Indeed BrdU-incorporation assays indicate that *Foxp1* does inhibit cell cycle progression in N2A cells (Fig. 2.8A, 2.8B) and in rat primary cortical astrocytes. (Fig. 2.8C).

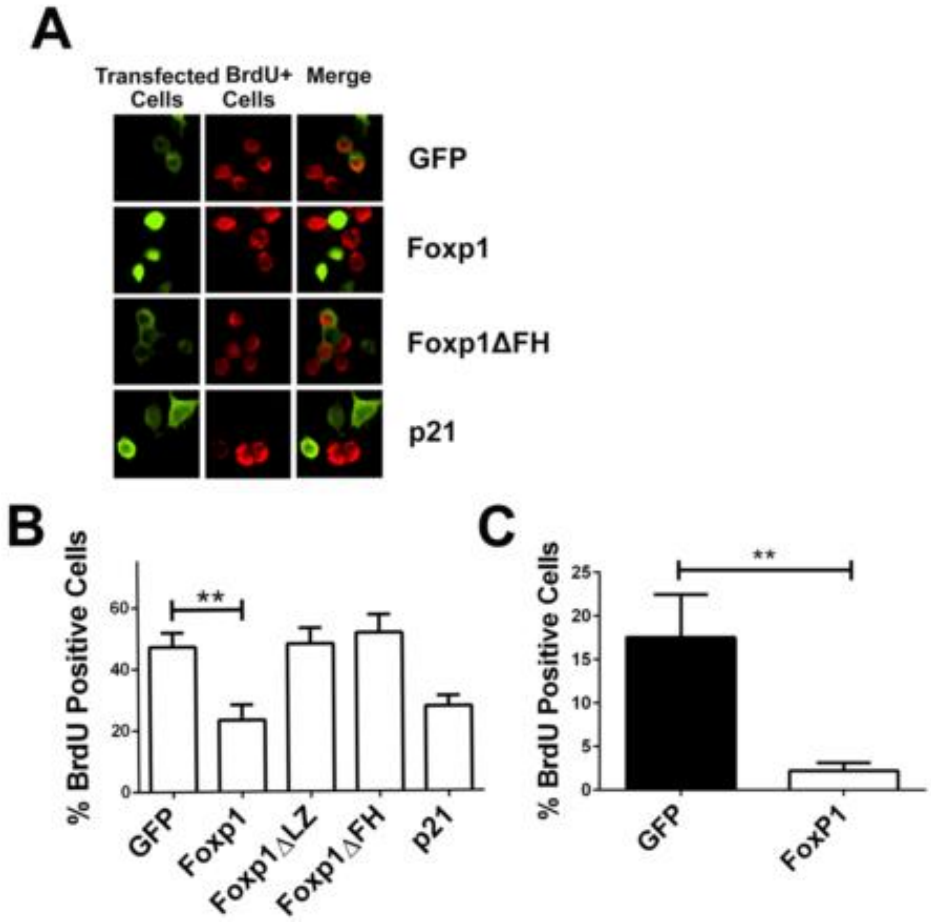


Figure 2.8. Foxp1 inhibits cell cycle progression. A, N2A cells were transfected with plasmids expressing *GFP*, *Foxp1* or *p21*$^{Cip1/Waf1}$ which serves as a positive control for cell cycle inhibition. BrdU was added to the culture medium 22h later for 2h. Immunocytochemistry was performed with GFP or FLAG antibodies to label the transfected cells and BrdU antibodies to label the dividing cells. BrdU assay in N2A cells **(B)** and in cortical glial cells **(C)** %BrdU positive cells was calculated as BrdU positive cells/ Transfected Cells. **, p<0.01 (n=3), *Foxp1* transfected cells compared with *GFP* transfected N2A cells.

Foxp1 is protective in a *Drosophila* model of HD. To extend our results on *Foxp1*-dependent neuroprotection to an *in vivo* model, we used a *Drosophila* HD model. In these flies the coding region for an N-terminal *Htt* fragment (aa 1-170) containing 120 CAG repeats is fused to the GMR promoter, resulting in expression in the eye (Jackson et al., 1998). While the eye develops normal in these flies, they develop severe photoreceptor degeneration as they age (Jackson et al.,

1998). We therefore analyzed the degenerative phenotype in 6d and 10d old GMR-*Htt* Q120 flies with and without *Foxp1* expression. For this analysis, we generated transgenic flies that express UAS constructs of either *Foxp1* isoform-D or *Foxp*1 isoform-A, the two isoforms we found to be reduced in R6/2 mice and HD patients. These UAS constructs can be readily induced by crossing the flies with flies carrying a promoter-GAL4 construct (Brand and Perrimon, 1993). However, inducing their expression in the retina with the strong GMR-GAL4 promoter construct resulted in defects in the development of the eye, rendering them unsuitable for our analysis of age-related degeneration. As an alternative we used *Appl*-GAL4, which induces expression in all neurons (including photoreceptors) but at much lower levels. Indeed, expressing *Foxp1* isoform-A or *Foxp*1 isofrom-D with this driver had no detectable effect on the development of the eye (data not shown). Intriguingly, expression of either isoform with *Appl*-GAL4 suppressed the degenerative eye phenotype in GMR-*Htt* Q120 flies. Wild type flies show a regular pattern of ommatidia in the eye that contain eight photoreceptors (each with its own light-sensitive rhabdomere) but because photoreceptors R7 and R8 are positioned above each other, only seven of the photoreceptors/rhabdomeres are detectable in cross sections (arrows, Fig. 2.9A, fly was 6d old). In contrast, 6d old GMR-*Htt* Q120 control flies (carrying the *Appl*-GAL4 promoter construct) showed a highly disorganized retina, with reduced numbers of rhabdomeres (Fig. 2.9B). When we induced UAS-*Foxp1* isoform-A (via *Appl*-GAL4) in these flies, their retinae appeared less disrupted and more rhabdomeres were present (Fig. 2.9C) and a similar result was obtained with UAS-*Foxp1* isoform-D (not shown). To quantify this phenotype, we counted the number of rhabdomeres per ommatidia in each line. Wild type flies showed an average of 6.6 rhabdomeres (Fig. 2.9D), close to the expected 7 (the difference is most likely due to a

rhabdomere occasionally not being discernable). GMR-*Htt* Q120 flies with only the *Appl*-GAL4 driver showed a significantly reduced number of rhabdomeres, with an average of 2.5 per ommatidia. Expression of *Foxp1* isoform-A or *Foxp1* isoform-D increased this number to 4.2 and 4.4, confirming a protective function for both isoforms. When we examined flies that only expressed the Foxp1 constructs, we found no effect on rhabdomere numbers with *Foxp1*-isoform-D but *Foxp*1-isoform-A did show a slight reduction to 6.3 rhabdomeres per ommatidia, which was significantly different compared to wild type (Fig. 2.9D). Although this construct induced a weak degenerative phenotype, it nevertheless reduced the retinal degeneration caused by mutant *Htt*, showing that both isoforms are protective in combination with *Htt* Q120. A similar protective effect was detected in 10d old flies (data not shown) but was more difficult to quantify due to the eye becoming progressively more disrupted with age. Lastly, to determine whether the increased survival of photoreceptor cells also improved visual function, we performed phototaxis assays, which measure behavioral responses to a light source. As expected from the severe retinal degeneration seen in 10d old GMR-*Htt* Q120 flies (with *Appl*-GAL4), they showed a significantly reduced performance in this assay (Fig. 2.9E). In contrast, expressing *Foxp1* isoform-A or *Foxp*1 isoform-D with *Appl*-GAL4 in GMR-*Htt* Q120 flies dramaticaly increased their performance in this assay. Remarkably, the *Foxp*1-isoform-D expressing flies actually performed as well as wild type flies, revealing a dramatic functional improvement. Inducing *Foxp1*-isoform-A or *Foxp1*-isoform-D in the wild type background did not cause detectable changes in phototaxis behavior.

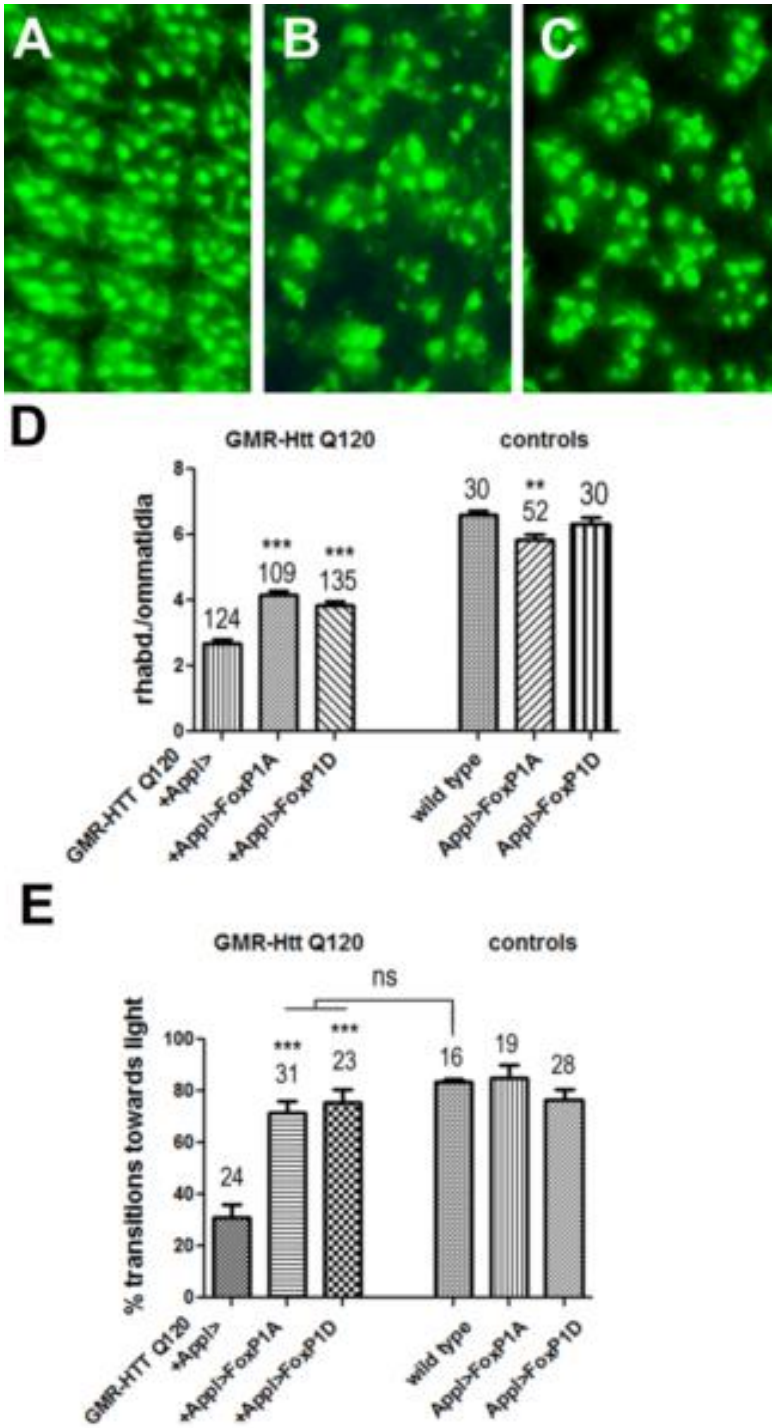


Figure-2.9. *Foxp1* reduces photoreceptor loss in 6d old Htt Q120 expressing flies. Section through the retina of a 6d old wild type fly (A), 6d old GMR-*Htt* Q120 fly (B), and 6d GMR-*Htt* Q120, Appl-GAL4>UAS-*Foxp1A* fly (C). Some rhabdomeres are indicated by arrows. (D) Counting the number of rhabdomeres per ommatidia revealed a significant increase from 2.5 rhabdomeres in GMR-*Htt* Q120 to 4.2 when *Foxp1A* was induced and 4.4 when *Foxp1D* was

induced. The number of ommatidia analyzed is indicated (4-6 ommatidia were counted per retina and at least 5 flies were used).**p<0.01, ***p<0.001. (D) The performance in the fast phototaxis assay is reduced in 10d GMR-*Htt* Q120 flies compared to age-matched wild type and this is also significantly improved by the expression of *Foxp1A* or *Foxp1D*. The number of flies tested is indicated. *p<0.05, ***p<0.001. All flies were females.

As a first step to determine whether the protective effects of *Foxp1* were specific for *Htt*, we also determined the effects of *Foxp1* isoform-A and *Foxp1* isoform-D on amyloidβ (Aβ) induced degeneration and behavioral deficits but could not detect significant effects (data not shown). These studies confirm that both of these *Foxp1* isoforms have a neuroprotective effect in vivo and they indicate that Foxp1 specifically interacts with Htt, supporting the hypothesis that downregulation of *Foxp1* is a key event in the pathology of HD.

VI. DISCUSSION

A defining feature of neurodegenerative diseases is the abnormal loss of neurons for which there is no cure or treatment strategy that can slow down the relentless death of neurons. A number of genes have been identified whose mutations cause familial forms of many of these diseases. An intriguing but yet unexplained feature about neurodegenerative diseases is the selective nature of neuronal loss. Specifically, although the mutant genes responsible for the various diseases are generally expressed widely in the brain, only specific brain regions and neuronal populations are affected in each disease. One possibility that may explain this selective vulnerability is that the mutant gene may affect, either directly or through other biochemical or metabolic changes, the expression of genes that are selectively expressed in the vulnerable brain regions and that regulate the survival of neurons in these regions. Reduced expression of such region or cell-specific neuroprotective genes, or increased expression of region or cell-specific neurotoxic genes, would thus explain the selective vulnerability of specific brain regions. Focusing on HD, we suggest that *Foxp1* is one such gene. In support of this possibility, and as previously described (Desplats et al., 2006; Desplats et al., 2008; Tang et al., 2012), we find that *Foxp1* is expressed most highly in the striatum and to a lower level, the cortex of HD mice. Within the striatum *Foxp1* is expressed only in MSNs, which are the neuronal population that degenerates in HD (Desplats et al., 2006; Desplats et al., 2008; Tang et al., 2012). Expression of *Foxp1* is selectively reduced in the striatum and cortex of R6/2 HD mice and even more dramatically in the caudate of human HD patients. Forcibly knocking down *Foxp1* induces death in otherwise healthy cortical neurons supporting the idea that *Foxp1* is necessary for their survival. In support of our hypothesis that the loss of specific neuronal populations in neurodegenerative diseases is

due to an effect of the disease gene on molecules selectively expressed in those cell populations we found that overexpression of mut-*Htt* is sufficient to reduce *Foxp1* expression. Indeed, re-establishment of elevated levels of *Foxp1* protects cultured cortical neurons from mut-*Htt* neurotoxicity. Furthermore, elevated *Foxp1* protects against neuronal loss and improves behavioral performance in a *Drosophil*a model of HD. It deserves mention that while neurons of the striatum and cortex selectively degenerate, other brain regions and neuronal types are also known to be affected in HD. Further investigation is needed to determine if *Foxp1* is expressed in these other neuronal types and whether its expression is reduced these cells in HD. It is quite possible that while *Foxp1* protect cortical and striatal neurons, the survival of other neuronal populations that are affected in HD may be safe-guarded by molecules other than members of the *Foxp* family or by other proteins all together.

The importance of *Foxp* for neuronal function is also supported by the finding that a neuronal knockdown of endogenous *Foxp* in flies causes defects in motor coordination (Lawton, Wassmer, & Deitcher, 2014). However, whether this may be due to neuronal death was not investigated. Together with the protective function of *Foxp1* in flies, this suggests that the neuroprotective function and the molecular pathways that mediate this function are conserved between *Drosophila* and mammals. An intriguing aspect of *Foxp1*-mediated neuroprotection is that while it can protect cultured cortical neurons and CGNs against HCA and LK-induced death, and can protect retinal neurons in *Drosophila* against mut-*Htt* toxicity, it cannot protect retinal neurons against Aβ toxicity. This suggests that *Foxp1* cannot protect neurons in all cirumstances . Certain neurotoxic stimuli may activate molecular mechanisms or a form of cell death that

Foxp1 is unable to protect against. In the case of Aβ, it is possible that Aβ induces molecular alterations that *Foxp1* cannot prevent or block the effects of.

Although it is known that the *Foxp1* gene encodes multiple isoforms as a result of alternative splicing, the functional significance of the smaller isoforms of *Foxp1* are poorly understood. Interestingly, isoform-D is even more selectively expressed in the striatum and cortex than *Foxp1* isoform-A and is more robustly downregulated in R6/2 mice than the full length form. That this isoform is also neuroprotective is shown by our findings that ectopic expression of isoform-D also protects neurons and flies from mut-*Htt* toxicicity. Although further investigation is necessary, it is possible that downregulation of isoform-D is even more relevant to HD pathogenesis than downregulation of isoform-A. In contrast to these two other isoforms, isoform-C has a different pattern of expression and its levels are increased in the human HD striatum.

Our results point to $p21^{Waf1/Cip1}$ as one target of *Foxp1*'s neuroprotective action because elevating *Foxp1* expression results in a stimulation of $p21^{Waf1/Cip1}$ expression. Several other labs have described neuroprotective effects for $p21^{Waf1/Cip1}$, including in the context of HD (Harms et al., 2007b; Langley et al., 2008; Ma et al., 2013; Mallick & D'Mello, 2014; Poluha et al., 1996; Tomita et al., 2006). It is possible that once stimulated by *Foxp1*, $p21^{Waf1/Cip1}$ acts by inhibiting cell cycle progression. Aberrant cell cycle progression has been described as a contributing factor in a variety of neurodegenerative diseases, including HD (Becker & Bonni, 2004; Greene et al., 2007; Liu et al., 2015). While our results point to $p21^{Waf1/Cip1}$ as a contributing factor, it is likely that there are other *Foxp1* targets that contribute to its neuroprotective activity in the striatum and cortex. And it is also likely that there are other alterations besides reduced *Foxp1*

levels that combined result in HD. Indeed, while mutations in *Foxp1* are associated with autism, speech/language deficiency, motor deficiencies, and mental retardation, they do not result in striatal degeneration or other major pathological features of HD. It may be noted, however, that mutations in other genes whose reduced function are widely believed to contribute to HD pathogenesis and considered as therapeutic targets, don't produce HD characteristics either. For example, mutations of BDNF, a molecule whose reduced activity and function is widely believed to contribute to HD, are associated with bulimia, anorexia, and congenital hypoventilation (Ribasés et al., 2004; Weese-Mayer, Bolk, Silvestri, & Chakravarti, 2002).

How does the presence of mut-*Htt* lead to a reduction in *Foxp1* expression? It is possible that this reduction is mediated by *HDAC3*. We have previously described that *HDAC3* is required for mut-*Htt* neurotoxicity (Bardai & D'Mello, 2011). Other labs have described beneficial effects of *HDAC3* inhibitors in mouse models of HD (Jia et al., 2012; Thomas et al., 2008). While expressed in the healthy brain and required for proper brain development, *HDAC3* promotes neuronal death when phosphorylated by *GSK3*β, whose increased activity has been noted in HD and other neurodegenerative diseases (Carmichael, Sugars, Bao, & Rubinsztein, 2002; Rangone et al., 2005; Wei et al., 2001). Other events that contribute to the transformation of *HDAC3* to a neurotoxic protein include disassociation from wild-type *Htt* and association with *HDAC1* (Bardai et al., 2013).

In summary, we describe for the first time that *Foxp1*, a gene expressed selectively in the striatum and cortex, is neuroprotective. Using both cultured neurons and an *in vivo* model, we present evidence indicating that the downregulation of *Foxp1* in HD contributes to neurodegeneration. We show that a smaller isoform of *Foxp1* is also neuroprotective and likely

to be involved in HD pathogenesis. To our knowledge this is the first attempt to investigate the functions of the smaller isoforms of *Foxp1* in the nervous system. Finally, we suggest that *Foxp1* preserves neuronal viability by stimulating the expression of $p21^{Waf1/Cip1}$. We propose that elevating *Foxp1* expression in vulnerable populations either pharmacologically or delivering it using viruses or other biological means could represent at attractive therapeutic approach for HD.

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CHAPTER 3

DISCUSSION

I. ACKNOWLEDGEMENT

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II. SUMMARY

Neurodegenerative diseases affect millions of individuals in the U.S. alone and cost the economy billions of dollars annually. Although symptomatic treatments are available for some neurodegenerative diseases, there is no treatment that slows down the pathological loss of neurons for any disease. A better understanding of the molecular and cellular mechanisms underlying neurodegenerative diseases will molecules and mechanisms that can be targeted in the development of therapies. While there are a variety of common features that contribute to neuronal loss in these diseases including aggregation of proteins and their accumulation because of dysfunction of protein clearance mechanisms, mitochondrial dysfunction and oxidative stress, abortive activation of the cell cycle apparatus, and excitotoxicity, a feature of these diseases that is not understood and that that vexed scientists for decades is why only certain neuronal populations are vulnerable to neurodegeneration in each disease.

My research addresses this issue in the context of Huntington's disease, an inherited polyglutamine disorder in which neurons of the striatum and to a lesser extent, the cortex are selectively lost. Even within these brain regions there is selectivity in the neurons that degenerate. In the striatum only medium spiny neurons (MSNs) die whereas in the cortex, projection neurons in layers V and VI are most vulnerable. My research has focused on Foxp1, a

gene that is normally expressed selectively in the MSN neurons of the striatum and in layers V and VI of the cortex. We have found that Foxp1 expression is downregulated selectively in these brain areas. We tested the hypothesis that Foxp1 serves as a guardian for neurons of the striatum and cortex ensuring their survival. However, Foxp1 expression is reduced by the presence of mutant Huntington. We observed this in mouse models, in the striatum of HD patients and in cultured cortical neurons in which mutant Huntington is overexpressed. A reduction in the expression of Foxp1 selectively in the striatum and cortex leads to the selective vulnerability of these regions but not in other brain areas where Foxp1 is not highly expressed and its expression is not reduced in HD. Our results suggest that up-regulating Foxp1 expression either pharmacologically or through other molecular genetic approaches could represent an attractive therapy for HD.

While we have shown that the neuroprotection by Foxp1 requires the presence of $p21^{Waf1/Cip1}$ and works via the upregulation of $p21^{Waf1/Cip1}$ and suppressing the cell cycle re-entry, there may be other mechanisms behind the neuroprotection by Foxp1. One such mechanism is that Foxp1 can oppose the transcriptional effects of Foxo, a member of the forkhead family of proteins that has been shown to promote neuronal apoptosis (Maiese, 2015). Both Foxp1 and Foxo can bind to the same transcriptional targets but have opposing effects on the expression of the target gene. Thus Foxo can induce the expression of pro-apoptotic genes like Bik and Foxp1 can suppress the expression of these genes (van Boxtel et al., 2013).

Another mechanism of neuroprotection might be due to protein-protein interactions of Foxp1. Foxp1 has been shown to interact with the Huntington aggregates in R6/1 mouse (Tang et al., 2012). While we in our lab, we found no interaction between Foxp1 and un-aggregated

Huntington. Thus Huntington aggregates might suppress the activity of Foxp1 or Foxp1 might alleviate the toxicity of Huntington aggregates. The N-terminus of Foxp1 has a poly-glutamine domain which have interactions with other poly-Q binding proteins (Wang, Lin, Li, & Tucker, 2003). Foxp1 also has a C-terminal binding domain which binds with CtBP-1 and the SUMOylation of Foxp1 is essential for this interaction which is crucial for the repression of target genes during neurogenesis (Rocca, Wilkinson, & Henley, 2017).

The brain specific deletion of Foxp1 in mice using a Nestin-Cre driver results in a decrease in area of the striatum (Bacon et al., 2015). While the authors had implicated neurogenesis as the cause for the loss of striatal neurons, the loss of striatal neurons was only prominent after 3 weeks of age and so we currently generating the $Foxp1^{flox/flox}$ $Nes\text{-}Cre^{+/-}$ mice to see if we can identify cell death by tunnel staining. Another approach would be to use an inducible-Cre system such as a tamoxifen induced Cre and knockdown the expression of Cre after the mice reach adulthood. This would completely eliminate the effects of neurogenesis and neurodevelopment in the reduction of striatal neurons.

While it is known that there are several Foxp1 isoforms produced as a result of alternative splicing and differential promoter usage, most studies have focused exclusively on the full-form, commonly referred to as isoform-A. Besides isoform-A, isoforms C and D are expressed in the brain. To our knowledge the significance of these isoforms in the brain or to neurons has never been investigated prior to our study. In Chapter-2 we describe how the isoforms A and D of Foxp1 are neuroprotective and there are smaller isoforms of Foxp1 which increase in HD patients. We find that FoxP1-isoform-D is downregulated in HD mice and patients. As in the case of isoform-A, the overexpression of this isoform is also neuroprotective. Interestingly,

isoform-C is robustly upregulated in the striatum of HD patients. It is possible that this upregulation contributes to neurodegeneration. Indeed, other studies conducted in non-neuronal systems has shown that isoform-C promotes cell cycle progression and cancer (van Keimpema et al., 2017). Further studies are needed to establish the contributions of isoform C and D in neurodegenerative diseases.

Another interesting aspect of Foxp1 we discovered in our lab was that Foxp1 is a very stable protein under normal circumstances when treated with cycloheximide but in the presence of toxic stimulus, such as homocysteic acid or mutant-huntington, the levels of Foxp1 rapidly decrease indicating a sensitivity of Foxp1 towards apoptotic stimuli. Uncovering the mechanism behind the downregulation of Foxp1 might also shed some information about the apoptotic pathways in neurodegeneration.

HDAC3 is a neurotoxic molecule when phosphorylated by GSK3β (Bardai & D'Mello, 2011) and in ChIP analysis Foxp1 has been found to be a target of HDAC3. Preliminary data from our lab has also shown the reduction in Foxp1 levels upon over-expression of HDAC3. We have also identified reduction of Foxp1 expression in MeCP2 transgenic mice. Further studies are needed to see if HDAC3 and MeCP2 work synergistically to down regulate Foxp1 and other neuroprotective genes.

While Foxp1 can protect neurons from various apoptotic stimuli such as oxidative stress by homocysteic acid in cortical neurons, low potassium induced cell death in cerebellar granule neurons, huntington mediated toxicity in cortical neurons and mutant huntington toxicity in Drosophila, it cannot protect against amyloid beta toxicity. Thus there are limitations to the

neuroprotection by Foxp1 and might not be the silver bullet against all forms of neurodegeneration.

Studies in the field of neurodegeneration have progressed far from the idea of considering neurons to be post-mitotic(Frade & Ovejero-Benito, 2015), to identifying the increase of cell cycle proteins in neurons under stress conditions (Pelegrí et al., 2008), to identify the DNA synthesis in neurons by BrdU assimilation as well as Ki-67 staining (T. W. Smith & Lippa, 1995), to identification of neurons progressing beyond DNA synthesis, to the discovery of tetraploid neurons in the brains of AD patients (Yang, Geldmacher, & Herrup, 2001).

There have been several studies which show that inhibition of cell cycle re-entry by pharmacological anti-proliferation drugs or by other molecular biology approaches is a possible therapeutic option in the treatment of neurodegenerative disorders in various mouse models. (Becker & Bonni, 2004; Giovanni et al., 2000; Liu & Greene, 2001; Park, Morris, Greene, & Geller, 1997; Pelegrí et al., 2008; Yang, Varvel, Lamb, & Herrup, 2006). In our lab also we have previously identified a neuroprotective molecule JAZ which works up-regulating $p21^{Waf1/Cip1}$ (Mallick & D'Mello, 2014) and also found cell cycle being the cause behind the neurotoxicity of two other molecules NPM1 and DNAJB6 (Pfister & D'Mello, 2016; C. Smith & D'Mello, 2016).

In summary, we show that Foxp1 a forkhead protein, can prevent cell death by mutant Huntington and this neuroprotection occurs by up-regulation of $p21^{Waf1/Cip1}$. Although there are plenty of evidences of cell cycle re-entry in neurodegenerative diseases, the purpose of this attempt by neurons in not very clear. Further studies are needed to uncover the mechanisms behind the cell cycle dysfunction in neurons. This might pave way for repurposing FDA approved drugs for the treatment of cancer to be used for treatment of neurodegenerative

diseases. There are further challenges in targeting these drugs and delivering them across the Blood-Brain-Barrier but nevertheless this will be a step-forward in the treatment of neurodegenerative diseases.

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BIOGRAPHICAL SKETCH

Anto Sam Crosslee Louis Sam Titus was born on July 27, 1987 in Tamil Nadu, India to Louis Sam Titus and C.M. Sarojam. After completing his schooling in Rose Mary Matriculation School in Tirunelveli, he joined the Bachelor of Technology in Biotechnology program at Anna University, Chennai in 2005. After finishing his bachelor's degree in 2009, he joined the Master of Technology in Biopharmaceutical Technology program in the same university. After graduating in 2011, he joined the Department of Molecular and Cell Biology at The University of Texas at Dallas. He married Angela Steffi in December 2014.

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EDUCATION

PhD in Molecular and Cell Biology, Univ of Texas at Dallas (2011-2016)

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M.Tech in Biopharmaceutical Technology, Anna Univ, India (2009-2011) GPA : 9.2/10

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TECHNICAL SKILLS

Molecular Biology: DNA and RNA Isolation, Plasmid Isolation (Mini Prep and Medi Prep), Stable and transient transfection, Co-Immunoprecipitation, ChIP, Agarose Gel Electrophoresis, RT-PCR,SDS-PAGE, Western Blotting, ELISA, Affinity column chromatography, UV-spectrophotometry.

Cloning: Restriction digestion & ligation, transformation, Site directed Mutagenesis (SDM), CRISPR, Adenovirus and Lentivirus cloning, production and amplification , Transgenic mice vector cloning.

Cell Culture: Mammalian and Microbial Cell culture, Primary neuronal cultures(Cortical neurons and Cerebellar Granule Neurons), Glial cell culture, Calcium Phosphate transection, Lipofectamine transfection and viral infection.

Viability Techniques: Immunocytochemistry and fluorescence microscopy, Cell viability assays (DAPI, Cresyl Violet and MTT).

Animal handling: Handling mice and rats, genotyping by PCR, colony management, brain region dissections, behavioral analysis by TruScan locomotor analysis.

Computational skills: Bioinformatics Tools (BLAST, CLUSTALW, Primer designing, DAVID), Microsoft office, Programming in C.

RESEARCH EXPERIENCE

Doctoral thesis **(Dec 2011- Aug 2017)**

Reporting to principal investigator Dr.Santosh D'Mello, working on **"Reduction of Foxp1 as a contributor to Huntington's disease"** and mechanism of neuroprotection by Foxp1.

Essential functions

- Culturing primary neurons from cortex and cerebellum of rats.
- Gene expression analysis by PCR and western blot.
- Protein interactions by Co-Immunoprecipitation, ChIP
- Cell viability analysis
- Handling mice and rats, genotyping and locomotor analysis

Masters Thesis **(Dec 2010 – May 2011)**

Reporting to Dr.B.S.Lakshmi, Tissue Culture and Drug Discovery Lab, Taramani, working on "Isolation and Characterization of a Novel Anti-Adipogenic molecule"

Essential functions

- Extraction of metabolites from plant sources by solvents
- 3T3L1 pre-adipocytes differentiation to mature adipocytes.
- Gene expression analysis by PCR.
- Glucose and fat content measurement by Radio-glucose and Oil Red-O assays.

Research Project **(Dec 2008 - Apr 2011)**

Reporting to Dr. P. Gautam, working on **"Novel Drug Delivery System using modified Starch Bases"**, to investigate the possibility of using biopolymer nanoparticles as drug delivery vehicle.

Essential Functions

- Extraction of Curcumin
- Production on starch nanoparticles

- Sulphonation, crosslinking and incorporation of drug in nanoparticles.
- Drug Release kinetics.

Research Project **(Feb 2008 – June 2008)**

Reporting to Dr. P. Gautam, working on **"Generation of electricity in Microbial Fuel Cells using Halophilic microorganisms".**

Essential Functions

- Isolation of halophilic strains of bacteria from crystallization pond.
- Design and construct a microbial fuel able to generate was 31.2 mW/m2

MEMBERSHIPS

- Member of Society of Neuroscience (SFN) since May 2015

PUBLICATIONS

- Reduced expression of Foxp1 as a contributing factor in Huntington's disease.J Neurosci. 2017 May 26. pii: 3612-16. doi: 10.1523/JNEUROSCI.3612-16.2017.
- Inhibition of expression of FoxG1 and its association with HDAC3 in Huntington's disease (In review)
- Elevated MeCP2 in Mice Causes Neurodegeneration Involving Tau Dysregulation and Excitotoxicity (In review)