The role of BimEL in the pathogenesis of Huntington’s disease

by

Rebecca Leon

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Abstract

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Huntington’s Disease (HD) is a devastating neurodegenerative disorder caused by an expanded polyglutamine repeat within the Huntingtin gene, IT15. In this study we demonstrated that Bcl-2 interacting mediator of cell death Extra Long (BimEL) protein expression was significantly increased in cells expressing mutant Huntingtin (mHtt). Moreover, striatal BimEL expression remained high in an R6/2 HD mouse model throughout the disease progression. Utilizing novel BimEL phospho-mutants we demonstrated the phosphorylation of Ser65 to be important for the stabilization of BimEL. We provided evidence that impaired proteasome function, increased JNK activity and reduced striatal BDNF lead to changes in the phosphorylation of BimEL, thereby promoting its stabilization specifically within the striatum of R6/2 mice. Furthermore, knocking down BimEL expression prevented mHtt-induced cell death in a HD cell culture. Taken together, these findings suggest that BimEL may contribute to the selective neurodegeneration and pathogenesis of HD.
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Chapter 1 Background

Genetics of Huntington’s disease

Huntington’s Disease (HD) is an autosomal dominant neurodegenerative disease affecting 1 in 10,000 people within the United States. The disease was named after the American physician, George Huntington, who in 1872 was the first to provide a detailed description of the disease and its dominant inheritance across several generations. It wasn’t until 1993 that the Huntingtin (Htt) gene, IT15, located on chromosome 4 was discovered. It was demonstrated that HD is a consequence of a mutation in exon 1 of IT15 (Duyao et al., 1993). Normally, the 5’ end of IT15 contains 14-39 CAG codon repeats, but individuals with HD may have 40-100 CAG repeats. The mechanism for this expansion is suspected to be a result of the susceptibility of CAG repeats to breakage and hairpin formation which may lead to expansions during DNA repair or synthesis. The unstable CAG expansion can increase, resulting in anticipation, an earlier onset of the disease with each generation. In addition, a CAG expansion greater than 100 produces juvenile HD, a severe and accelerated form that occurs in children. A more severe phenotype is also seen in homozygous patients compared to heterozygotes (Squitieri et al., 2003). Other trinucleotide repeat disorders including spinocerebellar ataxias (SCA types1, 2, 3, 6, 7), Dentatorubral-pallidoluysian atrophy (DRPLA), and Spinal bulbar muscular atrophy (S
BMA) each produce a unique pattern of neurodegeneration, and share in common single mutations encoding CAG expansions within the protein coding region, anticipation, and the formation of protein aggregates (Table 1).

### Table 1 Polyglutamine diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Genes</th>
<th>Protein encoded</th>
<th>Atrophy</th>
<th>Normal CAG tract</th>
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<td>Androgen receptor</td>
<td>motor neurons (Face/arms/legs)</td>
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<td>Dentatorubral-pallidolysian atrophy (DRPLA)</td>
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<td>Ataxia-1</td>
<td>Cerebellum, brain stem</td>
<td>4-39</td>
<td>40-80</td>
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<td>Ataxia-2</td>
<td>Ataxia-2</td>
<td>Cerebellum, brain stem</td>
<td>14-31</td>
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<td>spinocerebellar ataxia-3 (SCA-3)</td>
<td>Ataxia-3</td>
<td>Ataxia-3</td>
<td>Cerebellum, brain stem</td>
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<td>spinocerebellar ataxia-6 (SCA-6)</td>
<td>Ataxia-6</td>
<td>P/Q Calcium channel</td>
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<td>Cortex, striatum, cerebellum</td>
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**Brain areas and circuitry affected in HD**

The area of the brain mostly affected in HD is the basal ganglia (Fig.1). The basal ganglia is responsible for modulating voluntary movements, emotions, and reward systems. Throughout the majority of HD pathology, the striatum nucleus of the basal ganglia is drastically degenerated. The Medium Spiny Neurons (MSNs), which make up 90% of the striatal neurons, are predominately susceptible to degeneration in HD. Loss of gamma-aminobutyric acid (GABAergic) MSNs culminates in the loss of inhibitory transmission and thereby over-stimulation of the thalamus, pre-motor cortices and consequently motor neurons. Although striatal neurons are lost in HD, the substantia
nigra, the major source of dopamine in the basal ganglia, remains intact. In the striatum, dopaminergic transmission activates neurons expressing D1 receptors, facilitating movements but inhibits neurons expressing D2 receptors, thereby inhibiting movements (Fig. 2). The majority of MSNs affected in HD are the D2 expressing neurons. This neurodegeneration tips the balance of the basal ganglia output; over stimulation of D1 receptors by dopaminergic input from the substantia nigra causes an increase in thalamic output further contributing to the hyper-activation of motor neurons in HD. Interestingly, studies utilizing yeast artificial chromosome HD mouse model encoding 128 CAG repeats (YAC128) demonstrated that dopamine contributes to glutamate induced Ca\(^{2+}\) overload and apoptosis in YAC128 MSNs suggesting that imbalanced dopaminergic signaling may not only contribute to HD symptoms but also to the degeneration of MSNs (Tang et al., 2007). Notably, glutamate is the neurotransmitter used by cortical neurons projecting to and initiating the basal ganglia circuitry (Fig. 2). Moreover, glutamate receptors on the dendrites of MSNs have been implicated in the pathogenesis of HD due to measurable changes in pro-survival synaptic N-methyl-D-aspartate glutamate receptor (NMDAR) activation and expression compared to that of pro-apoptotic extra-synaptic NMDARs (Milnerwood and Raymond, 2010). In addition to the striatum, cortical tissue is also depleted, which is believed to be responsible for the cognitive defects observed in HD patients.
Figure 1  The neuroanatomy of the basal ganglia.

A. Anatomy of the human basal ganglia. B. The human brain slice of a patient who had HD (left) compared to a normal brain (right). There is marked degeneration of the basal ganglia and cortex in the HD brain slice.
Figure 2 The normal circuitry of the basal ganglia.

A schematic representation of the activating and inhibitory connectivity comprising the indirect and direct pathways of the basal ganglia. Modulating the activation of MSNs within the basal ganglia regulates thalamic activity and thereby motor movements.

As a result of these cellular, morphological and biochemical changes within the basal ganglia, HD is classified as a hyperkinetic disorder which manifests into the characteristic involuntary movement dysfunctions such as, chorea, uncontrollable tick-like movements; dystonia, repetitive twisting motions due to muscle spasms; dysphagia, difficulties controlling mouth and tongue. When these motor disturbances advance, it becomes difficult to stay seated, walk or talk. A common cause of death in HD is due to complications related to dysphagia. Cognitive decline and psychiatric symptoms are also
observed in HD including memory loss, dementia, depression, and anxiety. HD Symptoms typically begin at mid-life, but disease onset can vary depending upon the degree of expansion. Due to the onset of HD typically starting in adulthood; many people with HD have children with a 50/50 chance of acquiring the disease that is deteriorating their parent. The decision to get tested, after a parent has been diagnosed, is a difficult one often requiring genetic counseling. Collectively the pathogenesis of HD is devastating to the individual, leaving them unable to care for themselves or even communicate, as well as to their entire family.

**HD treatments**

There is no cure for HD and current treatments are aimed at alleviating the symptoms. The medications approved by the FDA for the treatment of chorea include: neuroleptics, antipsychotics known to reduce movements; Benzodiazepine, a depressant for its muscle relaxant properties, and tetrabenazine (TBZ), a vesicular monoamine transporter inhibitor that promotes dopamine depletion by preventing its entry into synaptic vesicles thereby enhancing its degradation by monoamine oxidase (Iversen, 2009). Speech therapy is also prescribed in order to improve dysphagia and communication. Potential treatments currently under investigation include histone deacetylase inhibitors to reduce the mutant Htt protein (mHtt)-induced transcriptional dysregulation and MK-801, an NMDAR antagonist to prevent excitotoxicity in sensitive MSNs (Iversen, 2009). At best the current treatments function to reduce some of the symptoms but none of them prevent the progression of the disease. Advances in the treatment of HD are dependent upon understanding of the molecular pathology of HD.
Molecular therapeutics of HD

Function of wild type Htt

To begin, it is important to consider the physiological functions of Htt in the brain. The huntingtin protein (Htt) is ubiquitously expressed throughout the body and brain (Strong et al., 1993). The normal functions of Htt include embryogenesis, neurogenesis, transcriptional regulation, post-synaptic organization and facilitating molecular motor formation (Cattaneo et al., 2005). Htt null mice display embryonic lethality demonstrating its essential role in embryogenesis. The HD mutation does not lead to any developmental abnormalities suggesting that the developmental functions of Htt are independent of the CAG tract, implicating the role of Htt in post-mitotic neurons to be important to the pathogenesis of HD. Although Htt is predominantly a cytosolic protein, upon proteolytic cleavage it can enter the nucleus. The polyQ tract on the N-terminus of Htt is believed to promote interactions with proteins including transcription factors or co-factors which also contain polyQ segments.

mHtt functions

The mHtt protein displays both loss of functions and gain of functions. As a result of the expanded tri-nucleotide repeat, mHtt contains an abnormally long N-terminal poly-glutamine (polyQ) segment. The polyQ expansion in mHtt interferes with functional protein interactions that lead to loss of transcriptional regulation and restricted vesicular transport (Table 2) (Cattaneo et al., 2005). Its propensity to form aggregates provides a gain of function by promoting the sequestration of cytosolic proteins including the transcriptional co-activator CREB binding protein (CBP) and components of the ubiquitin proteasome system (UPS), thereby reducing their activities (Bence et al., 2001,
Notably, the polyQ expansion makes mHtt a misfolded protein that undergoes ubiquitination and proteasomal degradation in order to reduce cellular stress. mHtt aggregates have been shown to be ubiquitinated suggesting that the UPS is unable to clear the toxic protein. A deficient UPS would contribute to the accumulation of toxic mHtt as well as other mis-folded or non-functional proteins.

**Table 2 Aberrant mHtt binding leads to loss of function.**

<table>
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<tr>
<th>Htt Interacting proteins</th>
<th>Loss of functions</th>
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<tr>
<td>AP-2 (adaptor protein 2)</td>
<td>AMPAR &amp; NMDARs trafficking</td>
</tr>
<tr>
<td>HAP1 (Huntingtin associated protein 1)</td>
<td>GABAA-receptor trafficking</td>
</tr>
<tr>
<td>PSD (post synaptic density protein)</td>
<td>NMDARs clustering</td>
</tr>
<tr>
<td>HIP1 (Huntingtin interacting protein 1)</td>
<td>reduced binding to AP-2 and NMDAR trafficking</td>
</tr>
<tr>
<td>HIP14 (Huntingtin interacting protein 14)</td>
<td>palmitoyl transferase</td>
</tr>
<tr>
<td>REST (repressor element-1 silencing transcription factor)</td>
<td>prevention of BDNF silencing</td>
</tr>
<tr>
<td>CBP (CREB binding protein)</td>
<td>transcriptional activation</td>
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The polyQ expansion also makes mHtt an insoluble protein prone to aggregate formation, one of the hallmarks of HD. Cytosolic and perinuclear aggregates are observed throughout the cortex and striatum of HD brains (Gutekunst et al., 1999). The contribution of mHtt aggregates to the pathogenesis of HD is the source of much debate. Larger mHtt aggregates are observed in neurons that are spared in HD suggesting that aggregation is a protective mechanism that functions to reduce soluble mHtt fragments (Gutekunst et al., 1999) (Fig). The increase in soluble mHtt by sumoylation of mHtt was reported to induce cytotoxicity in neuronal cells (Subramaniam et al., 2009). Nevertheless, mHtt aggregates demonstrate pathological interactions with the UPS and
transcriptional regulators (Bence et al., 2001, Cattaneo et al., 2001, Sugars and Rubinsztein, 2003). A group utilizing poly-glutamine binding peptoids, N-Substituted Glycines, have demonstrated that reduced mHtt aggregates confers neuroprotection in a cellular HD model and improved motor performance in YAC128 mice (Chen et al., 2011). In our studies we developed a HD cell culture model consisting of over expressed full length or truncated mHtt fused to enhanced green fluorescent protein (EGFP) in neuronal cells. The mHtt aggregates observed by the concentrated GFP signal was used as a molecular marker for mHtt induced neuronal dysfunction.

**The neurotoxicity of mHtt**

Although the mechanism by which mHtt induces the selective neurodegeneration of the striatum remains unclear, recent studies have shown that mHtt causes defects in the axonal transport of Brain-Derived Neurotrophic Factor (BDNF) in HD cortical and striatal cellular cultures (Gauthier et al., 2004,(Her and Goldstein, 2008)). In addition, studies investigating the striatal specific guanine nucleotide binding protein, Rhes, have implicated a role for posttranslational sumoylation of mHtt in the neuro-specific degeneration of the striatum (Subramaniam et al., 2009). mHtt has also been shown to enhance the activation of striatal NMDARs, containing the striatal specific subunit NRB2, making MSNs prone to Ca^{2+} overload and excitotoxicity (Zeron et al., 2002). Furthermore, altered interaction between mHtt and post-synaptic density protein 95 (PSD-95), adaptor protein 2 (AP-2), and huntingtin associated protein 1 (HAP1) are believed to underlie aberrant NMDA and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor trafficking which may further contribute to the sensitization of striatal neurons to excitotoxicity (Milnerwood and Raymond, 2010).
Studies using transgenic mice expressing expanded polyQ tracts linked to a gene unrelated to any tri-nucleotide repeat disorder was enough to produce a neurodegenerative phenotype demonstrating the neurotoxicity of polyQ tracts alone (Ordway et al., 1997). The proteolytic cleavage of mHtt C-terminal of the polyQ segment by pro-apoptotic proteases, caspases and calpains result in N-terminal mHtt fragments that are believed to be more toxic than full length mHtt. Cleaved products can aggregate with full length cytosolic mHtt or enter the nucleus and form intra-nuclear aggregates. We have previously demonstrated the cytotoxic effects of truncated mHtt expression in neurons to occur via apoptotic processes (Leon et al., 2010). Moreover, truncated mHtt fragments were present in HD brains where it may inhibit transcription due to altered interactions with co-repressors (Kim et al., 2001, Kegel et al., 2002). Studies utilizing transgenic mice expressing caspase-6 resistant mHtt lack the classic neurodegeneration and motor disturbances of HD transgenic mice (Graham et al., 2006).

Although the role for apoptosis in the neurodegeneration of HD remains to be elusive, clearly apoptotic processes are implicated by the cleavage of Htt by caspase-3 and caspase 6 (Kim et al., 2001, Warby et al., 2008). In addition, full length models have also linked the sensitivity of MSNs to apoptotic cell death (Tang et al., 2007). Despite insight into the toxic effects of mHtt, the mechanism for neurodegeneration and its selective neuropathology remains elusive.

**BimEL**

Bim (Bcl-2 mediator of cell death) is a prop-apoptotic BH3-only protein that undergoes alternative splicing resulting in three major isoforms: Bim short (BimS), Bim Long (BimL), and Bim Extra Long (BimEL). BH3-only proteins are unique members of
the Bcl-2 family; containing only one of the BCL-2 homology domains, BH3 (Fig.3). The BimEL isoform is predominately expressed in testes, B-lymphocytes, and neuronal cells. Although BimEL is the least potent of the three isoforms it is essential to neuronal apoptosis and is up-regulated in response to multiple cellular stresses including endoplasmic reticulum (ER) stress and trophic factor withdrawal (Whitfield et al., 2001, Putcha et al., 2003, Puthalakath et al., 2007).

BimEL plays an essential role in the apoptotic pathway; it promotes apoptosis by directly or indirectly inhibiting anti-apoptotic Bcl-2 members (Fig.4). The interaction of BimEL with Bcl-2 and Mcl-1 indirectly promotes apoptosis by inhibiting their interaction with Bax thereby promoting Bax homodimerization (Ewings et al., 2007). BimEL has also been reported to directly activate Bax and facilitate its translocation to the mitochondria (García-Martínez et al., 2007)(Kuwana et al., 2005). Although the evidence for both models seems to contradict each other, the pro-apoptotic mechanism for BimEL may occur in a signal or cell specific manner.
Figure 3 Schematic representation of the structure of Bcl-2 family members.

Bim is a member of the BH3-family. The BH3 domain allows it to interact with antiapoptotic proteins. (Cory and Adams, 2002)

Figure 4 The direct and indirect model proposed for the pro-apoptotic function of BimEL.

A. In the direct model BimEL directly binds Bax. In this model the interaction of BimEL and Bax promotes the activation of Bax and the downstream apoptotic mechanism including, the formation of the mitochondrial permeability pore, cytochrome c release and activation of caspases. B. In the indirect model BimEL binds to and inhibits anti-apoptotic proteins including Bcl-2 and Mcl-1. This prevents them from binding to
Bax thereby allowing the formation of Bax homodimerization and the downstream mitochondria dependent apoptotic pathway. (Strasser et al., 2011)

To ensure that apoptosis occurs only when necessary; there are multiple levels of regulating BimEL. Loss of BimEL regulation may induce a pathological state that could contribute to neuro-degeneration; accordingly BimEL is regulated both at the transcriptional and post-translational level (Huang and Strasser, 2000). The transcriptional regulation is evident in the unfolded protein response (UPR); a protective mechanism activated during ER stress. If ER stress is overwhelming the UPR initiates apoptotic mechanisms. During ER stress, the transcription factor, C/EBP homologous protein or growth arrest- and DNA damage-inducible gene 153 (CHOP/GADD153), mediates BimEL mRNA induction (Puthalakath et al., 2007). Notably, the expanded poly-glutamine segment of mHtt causes misfolding and aggregate formation both of which stimulate ER stress. Interestingly, CHOP has been shown to be increased within the striatum and cortex of HD R6/2 mice providing a link between HD and BimEL expression (Cho et al., 2009). In our previous studies we showed that mHtt expression caused the transcriptional up-regulation of BimEL as a result of ER stress (Leon et al., 2010).

The post-translational modifications of BimEL include phosphorylation and ubiquitinylation both of which regulate its pro-apoptotic activity. The mitogen-activated protein kinase (MAPK) signaling pathway regulates BimEL activity by way of phosphorylation (Fig. 5). The two major kinases that phosphorylate BimEL are the pro-survival Extracellular-signal-regulated kinase 1/2 (ERK1/2) and stress activated c-Jun N-terminal kinase (JNK) (Hübner et al., 2008). In the absence of cellular stress BimEL is
phosphorylated by ERK1/2 at ser65 thereby promoting its disassociation from anti-apoptotic Bcl-2 members and its degradation by the UPS (Luciano et al., 2003, Ewings et al., 2007, Wiggins et al., 2010). Although ERK mediated degradation of BimEL was not demonstrated in neurons, other studies report ERK signaling in neurons to negatively regulate BimEL transcriptionally (Hughes et al., 2011). Interestingly in neurons phosphorylation of BimEL by JNK, also at Ser65, promotes apoptosis (Putcha et al., 2003). Likewise, the Thr112 site has also been shown to be phosphorylated by JNK and to enhance it pro-apoptotic activity (Hubner et al., 2008). Phosphorylation by either kinase may occur as a result of kinase activity or cell specific mechanisms (Ley et al., 2005, Hubner et al., 2008).

Figure 5 The Bim isoforms created by alternative splicing.
BimEL, BimL, and BimS are produced by alternative slicing at corresponding exons. Exons 3 and 4 provide sites for post translational modifications. Exon 3 contains putative ERK1/2 phosphorylation sites while exon 4 contains the JNK phosphorylation site, Thr112. Phosphorylation of BimEL is believed to regulate its activity with ERK1/2.
phosphorylation reducing its pro-apoptotic activity and JNK phosphorylation enhancing it. Notably, BimS, the most potent Bim isoform, does not undergo this form of post translational regulation (Hubner et al., 2008).

The next major post-translational modification and regulation of BimEL is the poly-ubiquitination. Ubiquitinylation and degradation of BimEL by the UPS is a dynamic response to cell viability and apoptotic signaling. BimEL is ubiquitinylated and degraded frequently in healthy cells while cells undergoing stress have a reduced turnover of BimEL (Akiyama et al., 2003, Puthalakath et al., 2007, Ishihara et al., 2011). Components of the UPS have been found sequestered into mHtt aggregates causing decreased proteasome activity and implicating a role for protein degradation in the pathogenesis of HD (Bence et al., 2001, Jana et al., 2001). In this study we demonstrate that BimEL served as a molecular link between mHtt-induced proteasome dysfunction and cell death (Leon et al.).

**BDNF**

We also evaluated BimEL expression in other areas of the brain and provide a link for the selective up-regulation of striatal BimEL to striatal BDNF expression. BDNF plays an essential role in developmental and adult neurogenesis, maintenance of dendritic spine density and neuronal growth (Baquet et al., 2004, Bartkowska et al., 2007, Yuan, 2008, Rauskolb et al., 2010). It is expressed in the hippocampus, basal ganglia, retina, and cortex. The expression of this important neurotrophic factor has been linked to HD. HD patients and transgenic models display significantly reduced cortical BDNF (Zuccato et al., 2005, Zuccato et al., 2008). Although the substantia nigra pars compacta does
produce and transport BDNF to the striatum, the cortical striatal pathway is the major source of striatal BDNF (Baquet et al., 2004).

Studies on the normal functions of Htt have revealed its interesting relationship to BDNF. Htt regulates the transcription of neuronal genes, including BDNF, by preventing RE1-silencing transcription factor (REST) from entering the nucleus and binding to the neuron restrictive silencer element (NRSE) (Fig. 6) (Zuccato et al., 2003). Furthermore, the interaction of Htt with huntingtin-associated protein1 (HAP1) regulates the formation of the molecular motors necessary for anterograde and retrograde vesicular trafficking of BDNF (Fig. 6) (Cattaneo et al., 2005). The vesicular transport of BDNF along the axons of cortical neurons is essential for the delivery of BDNF to synaptic terminals and its release onto striatal neurons (Fig. 7) (Cattaneo et al., 2005). The polyQ expansion present in mHtt results in aberrant protein-protein interactions that abolish these neuro-protective Htt functions. Studies measuring the axonal transport of vesicular BDNF in a HD cell culture demonstrate the neurotoxic effects of mHtt on the disruption of cortical BDNF transport (Gauthier et al., 2004).
Figure 6 Loss of wild-type Htt function effects BDNF expression.

Top, Wild type Htt sequesters RE1-silencing transcription factor (REST) in the cytoplasm whereas mHtt allows it to enter the nucleus. The entrance of REST into the nucleus promotes the formation of a transcriptional silencing complex on the neuron restrictive silencer element (NRSE). Among the various neuronal genes regulated in this fashion is the BDNF gene. mHtt therefore negatively regulates BDNF transcription. Bottom, wild type Htt forms a complex with HAP1 and p150 to form the motor complex necessary for BDNF vesicular transport. The polyQ expansion present in mHtt causes a rigid interaction within the motor complex which hinders the vesicular transport of BDNF along microtubules (Cattaneo et al., 2005).
The expression of cortical BDNF is regulated by wild type Htt.

Htt drives the transcription of BDNF by inhibiting the repressor complex formation on the NRSE located on the BDNF promoter. Sufficient BDNF mRNA production is necessary for maintaining adequate trophic support in the striatum via the cortical-striatal pathway (Cattaneo et al., 2005).

Reduced striatal BDNF in HD has led to an interest in its contribution to the pathogenesis of HD. Exploring the role of BDNF in HD was confounded by early postnatal lethality of BDNF −/− mice. The generation of novel transgenic models has provided new insight into the functional requirements of BDNF in the striatum. The
Emx-BNDF\textsuperscript{KO} model, lacking cortical BDNF, not only display the hindlimb clasping phenotype characteristic of all HD transgenic models, but also reduced striatal volume and degeneration of MSNs late in life (Baquet et al., 2004). The \textit{cBDNF ko} model, depleting BDNF expression in all postmitotic neurons, displayed the clasping phenotype, reduced striatal volume and synaptic morphological dysfunctions localized to the striatum leaving other brain regions unaffected (Rauskolb et al., 2010). These studies provide evidence that the striatum is preferentially sensitive to BDNF expression and may account for the selective degeneration that occurs in HD despite the ubiquitous mHtt expression. Interestingly, striatal gene expression profiling of the Emx-BNDF\textsuperscript{KO} model revealed greatest similarities to human HD when compared to various HD transgenic models (Strand et al., 2007).

BDNF may also play a role in the cognitive impairments observed in HD. The early stage of Huntington’s disease is associated with cognitive deficits including memory impairment. This is believed to be a result of changes in neocortical synaptic plasticity including impaired long-term depression (LTD) and dendritic retraction (Cummings et al., 2006). These pre-symptomatic abnormalities are reminiscent of the aforementioned BDNF null studies that reported synaptic dysfunctions including reduced dendritic spines and complexity (Baquet et al., 2004, Rauskolb et al., 2010). Moreover, Choi et al. demonstrate that BDNF is required for learning and memory consolidation in the neocortex (Choi et al., 2010). Notably, reduced cortical volumes are observed in HD patients and transgenic models in the late stages of the disease (Mangiarini et al., 1996).

Although, it is easy to envisage BDNF as a key regulator of HD pathology it is unlikely that deficient striatal BDNF alone initiates all downstream pathological events.
The D9-N171-98Q transgenic mouse model, generated by directing mHtt expression to the DARPP-32 gene thereby confining mHtt expression to MSNs, does not abolish all HD phenotypes despite an unaffected cortical-striatal pathway (Brown et al., 2008). BDNF expression is therefore one contributing factor regulating neuronal dysfunction and degeneration in HD.

The purpose of this study was to identify the downstream molecular events associated mHtt expression. We provide evidence that BimEL is selectively up-regulated in mHtt expressing cells and in the striatum of R6/2 HD mice. Our investigation into the mechanism of BimEL up-regulation has led us to the effects of mHtt on the UPS, MAPK pathway, and BDNF expression.
Chapter 2 BimEL as a molecular link between proteasome dysfunction and cell death induced by mutant huntingtin

Full length mHtt is a misfolded protein that undergoes N-terminal proteolysis. Studies using transgenic mice expressing casapase-6 resistant mHtt, provide insight into the toxicity of truncated mHtt as these mice displayed reduced neurological disturbances (Graham et al., 2006). The ability of truncated mHtt to enter the nucleus was demonstrated to induce apoptosis in neurons; and is believed to interfere with transcriptional regulation by way of altered interactions with transcriptional regulators (Table 2). (Saudou et al., 1998). The transcriptional dysregulation associated with HD is thought to enhance the sensitivity of MSNs. In addition, N-terminal mHtt fragments form cytosolic and intra-nuclear protein aggregates in HD patients (DiFiglia et al., 1997, Lunkes et al., 2002).

The ability of mHtt to form aggregates and sequester cytosolic proteins has been linked to deficiencies in UPS activity implicating aberrant protein degradation as a contributing factor to the pathogenesis of HD (Bence et al., 2001, Jana et al., 2001). Interestingly, N-terminal mHtt aggregates were found to be ubiquitinated in HD brains further implicating a deficiency in the UPS (DiFiglia et al., 1997). Ubiquitination and
degradation by the UPS is a pivotal form of regulating BimEL. In viable cells BimEL is phosphorylated by ERK1/2 which signals its proteasomal degradation (Ley et al., 2003, O'Reilly et al., 2009). We also therefore, explored the possibility that mHtt may trigger molecular events that impede BimEL degradation. The molecule linking UPS dysfunction in HD to cell death had not been identified. In this study we reveal BimEL to be up-regulated in response to UPS dysfunction.

By the late stages of HD pathology the striatum and cortex of HD patients are severely degenerated. The neuronal and synaptic dysfunction including receptor trafficking, vesicular trafficking, dendritic retraction, and deficient BDNF production is believed to be responsible for reduced striatal volume in the early stages of the disease. However, the marked atrophy that results in the late stages is clearly due to neuronal death. Whether or not neurodegeneration occurs in an apoptotic manner has not been fully determined. Apoptotic markers such as caspase and calpain activity implicate the contribution of apoptotic mechanisms to the neuronal dysfunction in HD and perhaps apoptosis. In this study we provide evidence that the pro-apoptotic protein, BimEL, is essential for mHtt-induced cell death via an apoptotic pathway.

This study was conducted using two HD models. The first was a cellular HD model; mouse neuroblastoma cell line, Neuro-2a, expressing the N-terminus of the human Htt gene encoding 103 CAG repeats or full length mHtt. In these studies we investigated the effects of truncated and full length mHtt on the regulation of BimEL and determined that mHtt expression alters cell signaling pathways important for the expression of BimEL at the transcriptional and post-translational levels. This cellular HD model depicts the molecular changes associated with truncated and full length mHtt and
provided a means for exploring the MAPK pathway and the UPS by way of kinase and protease specific inhibitors.

The second model was the R6/2 transgenic HD mouse model. R6/2 mice express exon 1 of the human \textit{Htt} gene containing 144 CAG repeats, which was demonstrated to be enough to produce a neurological phenotype similar to HD. Although the \textit{Htt} gene is ubiquitously expressed in these mice, the striatum nucleus of the basal ganglia is selectively reduced. The R6/2 model displays an aggressive phenotype with an age of onset beginning at 7 weeks, and an average life span of 12-15 weeks. The observable motor disturbances include tick-like grooming, hunched posture, tremors, and limb clasping when held by the tail (Fig.8). The clasping phenotype is a characteristic motor disturbance of R6/2 mice. This motor dysfunction was graded and determined to occur in a progressive manner, consistent with human HD pathology (Table 3). R6/2 mice also have a noticeable weight loss, reduced brain weight, female sterility, disheveled coat, and commonly in advanced symptomology, die from seizures during handling. The R6/2 mouse model allowed us to analyze BimEL mRNA and protein expression in the parts of the brain mostly affected by HD.
Figure 8 The clasping of fore and hind limbs in 10 week old R6/2 HD mice compared to age matched wild-type mice.

Clasping is a characteristic uncontrollable motor disturbance displayed in HD transgenic models when held by the tail and is reminiscent of HD chorea in humans. This phenotype is observable in R6/2 mice as early as 5 weeks, beginning as a partial clasping of one fore limb and progressing to all limbs by 8 weeks of age.

Table 3 Clasping phenotype of R6/2 mice.

<table>
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<th>10</th>
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In this portion of the study we demonstrated that BimEL expression is up-regulated in mHtt expressing cells and R6/2 striatal tissue. We also investigate the molecular pathway for the up-regulation of BimEL including the UPS and the MAPK pathway.
Finally we demonstrate that mHtt-induced cell death occurs through an apoptotic pathway for which BimEL plays an essential role.
Materials and methods

Animals, genotyping and sample preparation

Breeding pairs of R6/2 transgenic mice were purchased from the Jackson Laboratories (Bar Harbor, ME), and the line was maintained by backcrossing to CBA3 C57BL/6 F1 in the animal facilities of Florida Atlantic University (FAU). All animals were maintained under temperature and light controlled conditions (20–23°C, 12-hour-light/12-hour-dark cycle) with continuous access to food and water. Primer sequences used for genotyping were: 5' - ATG AAG GCC TTC GAG TCC CTC AAG TCC TTC -3' and 5' - GGC GGC TGA GGA AGC TGA GGA -3'. PCR program was: 95°C for 5 min; 35 cycles of 95°C for 30 sec; 58°C for 30 sec and 72°C for 1.5 min; 72°C for 10 min as the final elongation step. The PCR product was run on a 2% agarose gel. A single band at ~524 bp indicated the R6/2 mice. Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of FAU.

For sample preparation, striatal tissue was dissected from 8-week-old R6/2 mice and their control littermates and then lysed in lysis buffer at 4°C for 30 min (50mM Tris. pH=7.5, 150mM NaCl, 5mM Ethylenediaminetetraacetic acid (EDTA), 1% triton X-100, 1X...
protease and phosphatase inhibitor cocktail, from Pierce). After lysis, the samples were centrifuged at 13,000 x g for 20 min at 4°C to remove any insoluble materials. Protein concentration was determined using Bradford method.

**Plasmid transfection**

Plasmid cDNA encoding the N-terminal region of human Htt (1-69) with polyQ repeat length of 25Q (Addgene plasmid 1187), 103Q (Addgene plasmid 1186) fused to enhanced green fluorescent protein (EGFP), full length Htt with a repeat of 23Q (fHtt23Q), and 103Q (fHtt103Q) were obtained from Addgene and CHDI (Cambridge, MA, USA). They were subcloned into a mammalian expression vector pcDNA3.1/HisB (Invitrogen, CA) and designated as pcDNA3.1/HisB-Htt25Q, pcDNA3.1/HisB-Htt103Q, pcDNA3.1/HisB-fHtt23Q, and pcDNA3.1/HisB-fHtt103Q respectively. The mouse neuroblastoma cell line, Neuro-2a, (ATCC, Rockville, MD, USA) was cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 0.1mM MEM non-essential amino acid (NEAA), 2mM L-glutamine, and 1 mM MEM sodium pyruvate. Neuro-2a cells were transiently transfected with the above plasmid constructs using the standard lipofectamine 2000 method according to the manufacturer’s instruction (Invitrogen, CA, USA). Unless stated elsewhere, the cells were harvested 48 hours after transfection and samples were lysed in lysis buffer (50mM Tris, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 1x complete protease inhibitor cocktail from Sigma, and 1x phosphatase inhibitor cocktail from Pierce).
**Mitochondria isolation**

Briefly, cells were harvested by centrifugation and washed twice in ice-cold PBS. The cell pellets were resuspended in five volumes of resuspension buffer (20 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH, pH 7.5, 10 mm KCl, 1.5 mm MgCl₂, 1 mm sodium EDTA, 1 mm sodium ethylene glycol tetraacetic acid (EGTA), 1 mm dithioerytrol, 250 mm sucrose, 1X complete protease inhibitor cocktail, 1X phosphatase inhibitor cocktail) and homogenized followed by centrifugation at 750 x g at 4°C for 10 minutes. The supernatant was collected and centrifuged again at 10,000 x g at 4°C for 15 minutes. The resulting mitochondria pellet was washed twice with cold phosphate buffered saline (PBS) and dissolved in 20 µl 2X sodium dodecyl sulfate (SDS) and the supernatant collected to get the cytosolic fraction. Mitochondrial and cytosolic fractions were used for western blot analysis.

**siRNA transfection**

Mouse BimEL ON-TARGET PLUS SMART pool small interfering RNA (siRNA) was obtained from Dharmacon (Chicago, IL, USA). Bim siRNA duplex and different Htt plasmid constructs were co-transfected into Neuro-2a cells using lipofectamine LTX method according to the manufacturer’s instruction. A FITC-conjugated fluorescent oligo (Invitrogen, CA, USA) was used to monitor the transfection efficiency. A transfection efficiency of ~90% was routinely obtained.

**MG-132 treatment**

MG-132, a proteasome inhibitor, was purchased from Calbiochem (Gibbstown, NJ, USA) and dissolved in dimethyl sulfoxide (DMSO). Neuro-2a cells were incubated with different concentrations of MG-132 (1 µM and 20 µM) for 4 hours before harvest. The
control group was treated with 1% DMSO to account for the 1% DMSO introduced by the MG-132 treatment.

**Quantitative RT-PCR**

Striatal tissue of 5 week old R6/2 and wild-type mice were dissected with ribonuclease (RNase) free equipment and placed in RNAlater solution (Ambion) overnight at 4°C. The striatal tissue was homogenized with 1ml TRIzol reagent (Invitrogen)/sample followed by chloroform ribonucleic acid (RNA) phase separation: mixed and incubated samples 30°C for 3 min then centrifuged 12,000 x g for 15 min at 4°C. The RNA was then precipitated using 0.5ml isopropyl alcohol followed by centrifugation at 12,000 x g for 10 min and then resuspension in nanopure water (1µl/mg tissue used). First-strand complementary deoxyribonucleic acid (cDNA) was synthesized using 1ng of RNA, dT23VN primer/dNTP mixture, and M-MuLV reverse transcriptase (New England Biolabs, MA, USA). The mixture was heated for 5 min at 70°C followed by a brief chill, then a 42°C 1 hour incubation, and a 90°C 5 min inactivation step. Amplification of cDNA was done using the forward and reverse primers for mouse BimEL (NM_007393): 5’-GACAGAACCAGCAAGGTAATCC-3’ and 5’-ACTTGTCACAACACTCATGGGTG-3’ respectively, and the following PCR program: 95°C for 1 min; 35 cycles of 94°C for 30 sec; 55°C for 30 sec and 68°C for 1min followed by a final extension of 68°C for 10min. The amounts of BimEL transcripts in striatal tissue from wild-type and R6/2 mice were analyzed by quantitative reverse transcription polymerase chain reaction (RT-PCR) using the SYBR green method (Stratagene, CA, USA). The samples were run in triplicates using the Mx3005PTM Real-Time PCR System and compared using β-actin as a normalizer gene. Fold increase was calculated using the relative Ct method. The
forward and reverse primers used for mouse β-actin (AF032459) were: 5’-GGCCTGTATTCCTCCATCG-3’ and 5’-CCAGTTGGTAACAATGCCATGT-3’ respectively. The PCR program was: 95°C for 10 min; 50 cycles of 95°C for 30 sec; 55°C for 1 min and 72°C for 1 min.

**Western blot**

Whole cell lysates of transfected neuro-2a cells, brain striatal lysate, or mitochondrial extracts (20-50 μg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and blocked with blocking buffer for 2 hours at room temperature. The membranes were incubated with different antibodies against BimEL (1:500), phospho-BimEL (Ser69; 1:500), JNK (1:500), phospho-JNK (Thr183/Tyr185; 1:500), ERK1/2 (1:500), phospho-ERK1/2 (Thr202/Tyr204; 1:500), Caspase-3 (1:500), PARP (1:500), PUMA (1:500), Cytochrome c (1:500, the above antibodies were purchased from Cell Signaling, MA, USA), Bid (1:500), β-actin (1:1000), Hsp70 (1:500, the above antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax (1:500, Chemicon, Billerica, MA, USA) phospho-BimEL (Thr112; 1:500, a generous gift from Dr. Davis, University of Massachusetts) followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence and analyzed with NIH image J. The immunoblotting for phospho-BimEL (Ser65) and (Thr112) were performed using LI-COR Odyssey Fc system with Odyssey blocking buffer.
ATP assay

Cells were seeded in a 6-well plate at 1 x 10^5 cells/well and transfected with different Htt plasmids in the presence or absence of BimEL siRNA using the lipofectamine LTX method. Forty-eight hours after transfection, cells were trypsinized and counted using a hemocytometer. Cells were further diluted into 1 x 10^4 cells/ml using culture medium and 100μl cells were transferred to a well in a 96-well plate. Adenosine triphosphate (ATP) content was measured in accordance with the protocol of the CellTiter-Glo™ luminescent cell viability assay kit (Promega, Madison, WI, USA). ATP content was measured using a microplate luminometer (Molecular Devices, CA, USA). The background luminescence of the culture medium was subtracted.

Trypan blue exclusion

Cell death was determined using the trypan blue exclusion assay. Briefly, 48 h after transfection with Htt plasmids, 10μl cell suspension was diluted (1:2) using 4% trypan blue solution (Sigma, St Louis, MO, USA). Trypan blue stains only non-viable cells leaving viable cells clear. 10μl of the diluted suspension was loaded onto a hemocytometer and observed under a light microscope. The number of stained (dead) cells and unstained (live) cells were counted. The percentage of dead cells in each group was calculated by dividing the number of dead cells by the total number of cells present.

Proteasome assay

Cell pastes were homogenized in homogenization buffer (15mM Tris-HCl, pH=8.0, 60 mM KCl, 15mM NaCl, 5mM EDTA, 1mM EGTA and 2mM ATP) at 4°C. The homogenate was centrifuged at 1,300 x g for 10 min to remove unbroken tissues and nuclei fractions. Protein concentration was measured and adjusted to 0.5 mg/ml total
protein by dilution with homogenization buffer. All assays were done in triplicate. Chymotrypsin-like activity of 20S proteasome was determined using substrate Suc-LLVY-aminomethycoumarin (AMC) (50μM, Calbiochem, La Jolla, CA). Caspase-like activity was measured using Z-LLE-AMC (50μM, Calbiochem, La Jolla, CA). Trypsin-like activity was measured using Z-ARR-AMC (50μM, Calbiochem, La Jolla, CA). 10 μg of cell lysate was incubated with each of the substrate in 100μl proteasome activity assay buffer (50mM Tris-HCl, pH=8.0, 0.5mM EDTA, 1mM dithiothreitol (DTT), 1mM ATP) for 30 min at 37°C. The reactions were stopped by the addition of 100μl of stop solution (2% SDS). Released fluorogenic AMC was measured at 360nm excitation and 460nm emission in a microplate luminometer (Molecular Devices, CA, USA). Fluorescence units were converted to AMC concentration using standard curves generated from free AMC.

**Statistical Analysis**

All data were expressed as means ± S.E.M. The data were analyzed and P value was calculated using unpaired student’s t-test.
I. Results

A cell culture model of HD was established in Neuro-2a cells

Our HD cellular model was established by expressing mHtt in a mouse neuroblastoma cell line, Neuro-2a. We expressed two forms of mHtt; N-terminal mHtt containing 103 CAG repeats fused with EGFP (Htt103Q), and full length mHtt containing 145 CAG repeats (fHtt 145Q). As a control we expressed wild-type N-terminus (Htt25Q) or full length Htt (fHtt23Q) encoding non-pathological repeats: 25 CAGs and 23 CAGs respectively. Cells were transfected with plasmids using standard lipofectamine transfection method resulting in 80% efficiency and analyzed 48 hours after transfection. Neuro-2a cells over-expressing wild-type Htt25Q displayed a GFP signal that is diffuse throughout the cell body and neuronal processes whereas cells expressing Htt103Q display a concentrated GFP signal (Fig. 9). The condensed signal observed in mHtt expressing cells indicated the formation of mHtt aggregates. Hoechst staining, to reveal nuclear localization, demonstrated the characteristic perinuclear and cytosolic protein aggregates found in HD. The expression of mHtt and formation of aggregates represented a HD pathological state at the cellular level that allowed us to explore the cell signaling mechanisms altered in HD.
Figure 9 Detection and localization of over-expressed Htt and mHtt in Neuro-2a cells by fluorescence microscopy.

A. plasmid map of the Htt25Q-GFP and Htt103Q-GFP construct. B-E. Neuro-2a cells were transfected with the Htt25Q plasmid (B, D), or Htt103Q plasmids (C, E). Images were taken 48 hours after transfection. The background in D & E is the differential interference contrast (DIC) image of the cells.

**mHtt induced an up-regulation of BimEL in Neuro-2a cells**

The pathology of HD has been demonstrated to be associated with the activation of key regulators of the apoptotic pathway including caspase and calpain activation (Kim et al., 2001, Graham et al., 2006). We set to determine the role BH3-only pro-apoptotic proteins play in the pathogenesis of HD. Htt and mHtt expressing Neuro-2a cells were collected forty-eight hours after transfection for western blot analysis. Interestingly cells transfected with Htt103Q expressed greater amounts of BimEL than cells expressing Htt25Q (Fig. 10A). Quantitative analysis revealed that BimEL protein levels were increased by 2 fold in cells expressing Htt103Q compared to those expressing Htt25Q.
There were no significant increases in the expression of the other BH3-only protein, PUMA or the BH3-only interacting protein Bid. We detected full length Bid to be decreased in cells expressing mHtt compared to those expressing wild-type Htt indicating that Bid cleavage had occurred. Bid is cleaved by caspase-8 during apoptosis suggesting that apoptotic pathways had been activated by mHtt expression. The heat shock protein 70 (Hsp70) has a role in protein folding, we therefore determined if it is up-regulated in response to mHtt expression. Surprisingly there were no detectable changes in Hsp70 (Fig. 10A). Taken together we identify a link between the expression of truncated mHtt and BimEL. Among the proteins analyzed, only BimEL expression was significantly elevated. Together with the reduction of full length Bid, we established that apoptotic mechanisms are activated in response to truncated mHtt.

Figure 10 Analysis of BimEL expression in Neuro-2a cells expressing Htt25Q or Htt103Q.

A. Western blot analysis of cell lysates from Neuro-2a cells transfected with Htt25Q or Htt103Q using BimEL, PhosphoSer65-Bim, PUMA, HSP70, Bid, and β-actin antibodies.
B. Densitometric analysis of BimEL increase from three independent western blots. ***
p<0.001 compared with cells expressing Htt25Q n=3.

To determine if a similar effect would be observed with the expression of full length mHtt, we transfected Neuro-2a cells with full length Htt plasmids. Similar to what we observed with truncated mHtt, full length mHtt resulted in an increase in BimEL protein levels. We next asked whether phosphorylation, a major form of regulating BimEL, played a role in the increase of BimEL. Utilizing phospho-specific antibodies we analyzed the two major regulatory phosphorylation sites for BimEL, serine 65 and threonine 112 (from now on referred to as phosphoSer65-BimEL and phosphoThr112-BimEL). The expression of truncated mHtt or full length mHtt led to an increase in the phosphorylation of BimEL at both sites implicating phosphorylation as a contributing factor in the up-regulation of BimEL as a result of mHtt expression (Fig.11). Furthermore, these results indicate that truncated mHtt is enough to elicit the effects of full length mHtt on BimEL expression.
Figure 11 The phosphorylation of BimEL at Ser65 and Thr112 in mHtt expressing cells.

Neuro-2a cells expressing full length mHtt displayed an upregulation of BimEL, phosphoSer65-BimEL and phosphoThr112-BimEL. Neuro-2a cells expressing the N-terminus of mHtt displayed significantly increased phospho$^{\text{Ser65}}$BimEL and phosphoThr112-BimEL. Densitometric analysis was calculated from three independent blots.

**BimEL was up-regulated in an R6/2 mouse model of HD.**

We next sought to determine if BimEL is also up-regulated in an in vivo model that more closely resembles HD; the R6/2 HD mouse model. The striatum, the area of the brain mostly affected in HD, of R6/2 mice was dissected and prepared for western blot and qRT-PCR analysis. Consistent with our observation from the cell culture models, 12 week old R6/2 mice displayed increased BimEL protein expression compared to age matched wild-type mice (Fig. 12A). Quantitative analysis of BimEL mRNA in striatal
tissue from 5 week old R6/2 and wild-type mice did not reveal any significant differences (Fig. 12B). The increase of BimEL within the striatum of R6/2 mice confirmed our hypothesis that BimEL expression is altered in HD and suggests that BimEL may play an essential role in the molecular pathology of HD. The increase in BimEL protein level is not likely to result from transcriptional up-regulation alone as we were unable to detect a significant difference in BimEL mRNA in 5 week old R6/2 mice compared to wild-type mice.

Figure 12 BimEL protein and mRNA expression in R6/2 HD mice.
A. Striatum dissected from 12-week-old R6/2 and wild type mice were lysed and subjected to western blot analysis using Bim, and β-actin antibodies. B. qRT-PCR was used to measure the amount of BimEL mRNA in the striatum of 5 week old R6/2 and wild-type mice.

Impaired UPS in mHtt expressing cells
mHtt aggregates have been implicated in the dysfunction of the UPS in HD. To determine if there was any connection between UPS dysfunction and BimEL expression
we measured the protease activity of the UPS in our HD cellular model. A proteasome activity assay was performed on Neuro-2a cells expressing Htt plasmids utilizing three luminogenic proteasome substrates: Suc-LLVY-aminomethycoumarin (AMC), Z-LLE-AMC, and Z-ARR-AMC to measure the Chymotrypsin-like activity of 20S proteasome, Caspase-like activity, and Trypsin-like activity respectively. Neuro-2a cells expressing Htt103Q exhibited significant reductions in all protease activity measured (Fig. 13).

Moreover, treatment with proteasome inhibitor, MG-132, produced an up-regulation of BimEL in a dose dependent manner. The phosphorylation of BimEL at Ser65 has been demonstrated to promote the proteasomal degradation of BimEL (Luciano et al., 2003, Wiggins et al., 2010). We therefore determined the phosphorylation state of Ser65 during MG-132 treatment. Likewise the phosphorylation of BimEL at Ser65 was also increased in a dose dependent manner. The treatment of Neuro-2a cells with MG-132 produced similar effects on BimEL to that measured in cells expressing mHtt (Fig. 13). These results suggest that BimEL may be partially up-regulated due to reduced protein turnover.
Figure 13 The proteasome activity of mHtt expressing cells.

A, B, and C. Neuro-2a cells transfected with Htt25Q or Htt103Q were subjected to a proteasome activity assay which measured the Chymotrypsin-like, Caspase-like, and Trypsin-like activity. *p<0.05, ***p<0.001 as compared with cells expressing Htt25Q. D. Neuro-2a cells were either untreated or treated with 1 or 20µM MG-132, a proteasome inhibitor. Cells were then lysed and subjected to western blot analysis using BimEL, phosphoSer65BimEL, and β-actin antibodies.

**ERK1/2 and JNK were activated in cells expressing mHtt**

An increase in BimEL phosphorylation may reflect an increase in kinase activity. To determine if elevated phospho-BimEL occurs due to altered kinase activity or simply because of increased availability to kinases as a result of increased BimEL, we evaluated the activity of the two major kinases that phosphorylate BimEL, ERK1/2 and JNK. Cells expressing mHtt were analyzed by western blot to determine if mHtt induced any changes in the activation of ERK1/2 or JNK through the use of phospho-ERK1/2 and phospho-JNK antibodies. Cells expressing mHtt had greater phosphorylated ERK1/2 and JNK with no changes in the total amounts of ERK1/2 and JNK, indicating that the increase in phosphorylated ERK1/2 and JNK is a result of increased kinase activation and not an up-regulation in total kinase protein levels (Fig. 14).
Figure 14 ERK1/2 and JNK are activated in mHtt expressing cells.
Western blot analysis of ERK1/2 and JNK activation in Neuro-2a cells transfected with Htt25Q or Htt103Q using JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, and β-actin antibodies.

mHtt induced Bax translocation from cytosol to the mitochondria membrane

A major event during apoptosis is the translocation and activation of Bax to the mitochondrial membrane. BimEL has been shown to promote Bax dependent apoptosis (Putcha et al., 2003). To determine whether BimEL induced Bax translocation in mHtt expressing cells, Neuro-2a cells expressing Htt plasmids were harvested followed by fraction centrifugation to obtain cytosolic and mitochondrial fractions. BimEL was present in the mitochondria fractions of cells expressing wild-type Htt and mHtt. Notably, cells expressing mHtt contained much higher amounts of BimEL in the mitochondria fractions (Fig. 15). In addition, cells expressing mHtt contained more Bax in the mitochondrial fraction; whereas the localization of Bax in cells expressing wild-type Htt was predominantly in the cytosol (Fig. 15; lanes 2 and 4). These results demonstrate that mHtt expression induced the translocation of Bax from the cytosol to the mitochondria as shown by the reduction of Bax in the cytoplasmic fraction (Fig. 15; lanes 3 and 4). In addition, we also assayed cytochrome c release from mitochondria.
Cells expressing Htt103Q displayed more cytochrome c in the cytosolic fraction compared to the control indicating the release of cytochrome c. The purity of the mitochondria isolation was shown by the enrichment of mitochondrial marker, COXIV and the absence of cytosolic marker GADPH. These results implicate a role for BimEL in initiating apoptotic mechanisms as a result of mHtt expression; including BimEL expression, Bax translocation, and cytochrome c release. This apoptotic process may be essential to the neuronal dysfunction that occurs in HD.

![Figure 15 The translocation of Bax to the mitochondria due to mHtt expression.](image)

Neuro-2a cells expressing either Htt25Q or Htt103Q were subjected to mitochondrial isolation and western blot analysis. The presence of mHtt resulted in the up-regulation of BimEL which had greater mitochondrial localization when compared to the control. Bax was preferentially translocated from the cytosol to the mitochondria in cells expressing mHtt. The release of cytochrome c was used as a marker for apoptosis. Cytochrome c was increased in the cytosolic fractions of mHtt expressing cells compared to the control. Cytochrome c oxidase IV (Cox IV) is a mitochondrial enzyme that served as the mitochondrial marker, glyceraldehyde-3-phosphate dehydrogenase (GADPH) is a
cytosolic enzyme that was used as a cytosolic marker. Western blot was performed using BimEL, Bax, Cox IV, GADPH antibodies.

**Silencing BimEL attenuated mHtt-induced cell death**

Thus far we have identified apoptotic processes to be activated in the presence of mHtt expression in neurons. About 48 hours after transfecting neuronal cells with mHtt, we observed and measured a significant amount of cell death. The increase in cytochrome c led us to believe that cell death occurred by apoptosis. We next tested whether BimEL is essential to apoptosis in HD. Neuro-2a cells were co-transfected with Htt plasmids and BimEL on-target plus SMARTpool siRNA. Cell viability was measured 48 hours after transfection using a luminescent cell viability assay and measured using a microplate luminometer; values were expressed as Relative Luminescence Units (RLU). Cells expressing Htt103Q displayed a significant reduction in cell viability when compared to cells expressing Htt25Q. Treatment of mHtt expressing cells with BimEL siRNA prevented mHtt-induced cell death and returned cell viability to levels comparable to the control (Fig. 16A). As expected, BimEL siRNA caused little difference in the cell viability of cells expressing Htt25Q (Fig. 16A). In addition, cell death was measured using the trypan blue exclusion method which demonstrated a significant increase in the cell death of mHtt expressing cells that was prevented by BimEL SiRNA (Fig. 16B).

To further examine the role of apoptosis in the cell death measured in mHtt expressing cells we examined the presence of other apoptotic markers. Pro-caspase-3 is an effector caspase that undergoes proteolytic cleavage during early apoptosis resulting in active caspase-3. Poly (ADP-ribose) polymerase (PARP) is a DNA polymerase that is cleaved
by caspase-3 during apoptosis. A western blot analysis using caspase-3 and PARP antibodies revealed that cells expressing Htt103Q are undergoing apoptosis as marked by the increase in cleaved caspase-3 and PARP. The cleavage of caspase-3 and PARP is inhibited in the presence of BimEL siRNA (Fig. 16C; lanes 3 and 4). Cells expressing Htt25Q are undergoing minimal apoptosis as determined by the lack of cleaved caspase-3 and PARP. There were no observable apoptotic affects by BimEL siRNA in the control cells indicating that siRNA treatment itself did not produce any changes in cell viability (Lane 1 vs. Lane 2). The cleavage of caspase3 and PARP demonstrated that mHtt-induced cell death occurs via apoptosis. Moreover, the up-regulation of BimEL is essential to the apoptotic pathways activated in the presence of mHtt. Taken together results indicate that BimEL may play a vital role in the neurodegeneration that occurs in HD.
Figure 16 Effect of silencing BimEL expression on mHtt-induced cell death.

A. Assessment of cell viability by ATP measurements, cellular ATP is an indicator of cell viability, after co-transfection of Neuro-2a cells with BimEL siRNA and Htt
plasmids: Htt25Q or Htt103Q. B. Measurement of cell death after co-transfection of Htt25Q or Htt 103Q and BimEL SiRNA using the trypan blue exclusion assay, which stains only dead cells. C. Western blot analysis of Neuro-2a cells 48 hours after co-transfection with BimEL siRNA and Htt plasmids using Bim, Poly (ADP-ribose) polymerase (PARP), caspase-3, and β-actin antibodies.
I. Discussion

How neurodegeneration occurs in HD remains elusive. There is evidence implicating apoptotic processes in various HD models, including the cleavage of mHtt by caspases and calpains (Saudou et al., 1998, Gafni and Ellerby, 2002). The increased sensitivity of MSNs could be a result of apoptotic processes activated in the presence of mHtt. It is likely that misfolded mHtt or mHtt aggregates may serve as the initial signal for stress activated apoptotic responses. In this aspect of the study we provide a novel link between mHtt expression and apoptosis.

BimEL is a pro-apoptotic protein that is up-regulated in response to cellular stress, such as ER stress, trophic factor withdrawal, and excitotoxicity. The mechanism for the up-regulation of BimEL includes mRNA induction and reduced protein turnover. We demonstrate that mHtt does cause an up-regulation of BimEL in Neuro-2a cells expressing both full length and truncated mHtt. Likewise, our R6/2 HD mouse model, also expressing human truncated mHtt, displayed elevated levels of BimEL. Interestingly, the increase in BimEL protein occurred in the striatum nucleus of the basal ganglia, the area of the brain predominately affected in HD. This was observed in 12 week old mice that displayed advanced motor disturbances characteristic of HD mice. Together these results suggest that mHtt somehow alters the regulation of BimEL to the extent that it is either up-regulated or stabilized; implicating a role for BimEL in the pathogenesis of HD.
To investigate the mechanism for mHtt-induced up-regulation of BimEL, we first examined the post-translational and transcriptional regulation of BimEL in the presence of mHtt. In addition to total BimEL levels being increased we also detected an increase in phosphorylation of BimEL at Serine 65 and threonine 112; the two best characterized BimEL phosphorylation sites, in mHtt expressing Neuro-2a cells. It is well established that phosphorylation of BimEL at Ser65 promotes its poly-ubiquitination and proteasomal degradation. Interestingly, we detected a significant reduction of UPS activity in Neuro-2a cells expressing mHtt; which is consistent with reports of UPS inhibition as a result of mHtt aggregation and sequestration (Bence et al., 2001). These results suggested that although the signal for BimEL turnover had been given, phosphorylation at Ser65, the deficiency measured in the UPS resulted in the inability to properly degrade BimEL and the accumulation of BimEL. In support of this, we demonstrate that treating Neuro-2a cells with MG-132, a UPS inhibitor, in the absence of Htt plasmids led to a similar increase in both BimEL and phosphoSer65 BimEL to that observed in mHtt expressing cells (Fig. 13). Viable cells maintain low levels of BimEL by way of proteasomal degradation providing evidence for the importance of this pro-survival mechanism to BimEL levels and cell survival. Importantly, the induction of BimEL degradation initiated by phosphorylation of BimEL at serine 65 has been repeatedly demonstrated in non-neuronal cells (Luciano et al., 2003, Hubner et al., 2008). In neurons phosphoSer65-BimEL has actually been shown to enhance cell death due to the phospho-65-dependent interaction of BimEL with Pin1, a neuron specific prolyl-isomerase, which prevented BimEL degradation (Fig. 17) (Becker and Bonni, 2006). We explore the physiological significance of phosphoSer65-BimEL in neurons in further
detail in the next study. Nevertheless, the inhibitory effects of mHtt on the UPS provide one mechanism for the up-regulation of BimEL in mHtt expressing cells. In addition, we also detected an increase in phosphoThr112-BimEL, an important site shown to enhance the pro-apoptotic activity of BimEL by promoting its binding to Bcl-2 thereby inhibiting its pro-survival functions (Hubner et al., 2008). The increase in BimEL but not PUMA, another BH3-only protein that is regulated by proteasomal degradation in viable cells, suggest that there is also a selective up-regulation of BimEL that cannot be explained simply by reduced protein turnover (Arbour, 2009). Together our findings suggest that BimEL is up-regulated in the presence of mHtt partially due to UPS dysfunction.

![Schematic representation of JNK mediated phosphorylation of BimEL in neurons.](image)

The phosphorylation of BimEL by JNK at Ser65 was shown to be necessary for its interaction with the neuron specific peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (Pin1). This interaction prevented the proteasomal degradation of BimEL and thereby promoted its pro-apoptotic activity and facilitated apoptosis in neurons undergoing serum deprivation (Becker and Bonni, 2006).
To further investigate the mechanism for the up-regulation of BimEL in our HD models, we explored the transcriptional regulation of BimEL. The transcription factor for BimEL, growth arrest- and DNA damage-inducible gene 153 (GADD153), is activated during ER stress and leads to BimEL mRNA induction. We detected an increase in the expression of GADD153 in HEK293 cells expressing mHtt compared to control indicating that mHtt, as a misfolded protein, does cause ER stress (Leon et al., 2010). However, qRT-PCR did not reveal a significant difference in BimEL mRNA expression between 5 week old R6/2 and wild-type striatal tissue, indicating that post-translational regulation plays the major role in the increase of BimEL during mHtt expression.

To further investigate a possible mechanism for the selective increase of BimEL, we examined ERK1/2 and JNK activity during mHtt expression in Neuro-2a cells. The kinase activities were evaluated by immunoblotting 48 hours after Htt plasmid transfection; revealing both kinases to be increased. Although the changes observed in the phosphorylation of BimEL could be a result of increased availability of BimEL to kinases as a result of reduced BimEL degradation; this would not explain the increase in kinase activity. It is our conclusion that mHtt has multiple effects in neurons that, thus far include UPS dysfunction and altered MAPK signaling resulting in reduced BimEL turnover and changes in the phosphorylation of BimEL.

BimEL is a pro-apoptotic protein that functions to initiate apoptosis suggesting that the cellular toxicity due to mHtt expression, occurred via an apoptotic pathway, which we provide a variety of evidence to support. Neurons predominantly contain BimEL, when present, on the mitochondria where it is able to facilitate apoptosis by interacting via its BH3 domain with Bcl-2 or directly with Bax. In our studies we demonstrate that an up-
regulation of BimEL was associated with increased translocation of Bax to the mitochondria and the release of cytochrome c into the cytosol, both pivotal events for the induction of apoptosis. The silencing of BimEL by BimEL siRNA was enough to prevent both apoptotic events. We also demonstrate the proteolytic cleavage of PARP and caspase-3 to occur in mHtt expressing neurons, which was also prevented by BimEL siRNA. Moreover, the silencing of BimEL provided neuro-protection from mHtt induced cell death, establishing BimEL as an essential molecule for mHtt-induced cell death in our HD cellular model.

This study provides evidence that mHtt perturbs normal cellular processes including protein degradation that lead to cellular toxicity (Fig. 18). We identify various apoptotic markers to be present in neurons expressing mHtt compared to those expressing wild-type Htt. In this study we present the novel finding of altered BimEL expression in a HD model. Still these findings reveal many topics left unclear: what accounts for the altered MAPK pathway; why is BimEL up-regulated specifically in the striatum of R6/2 mice, and what is the significance of phosphorylation of BimEL in neuronal systems? We investigate these topics in the following chapters.
Figure 18 Proposed model for the role of BimEL in mHtt induced cell death.

The inhibitory effects of mHtt on the UPS prevents proper degradation of BimEL. Furthermore, mHtt causes ER stress and the activation of GADD153, a BimEL transcription factor. This further contributes to the up-regulation of BimEL in mHtt expressing cells and consequently, downstream apoptotic events, including the translocation of Bax to the mitochondria, cytochrome c release, and caspase activation.

The role for protein phosphatase 2A (PP2A) in this mechanism remains to be explored. The activity of this enzyme in the presence of mHtt may provide important information; this enzyme may also alter the phosphorylation of BimEL. (Leon et al., 2010)
Chapter 3 Multifaceted up-regulation of striatal BimEL in an R6/2 mouse model of Huntington’s disease

In HD there is a selective and dramatic loss of MSNs within the striatum. Apoptosis has been proposed as a key mechanism underlying (mHtt)-induced cell death. However, how mhtt leads to cell death is not well understood. In the previous studies, we showed that BimEL is the molecule that links UPS dysfunction to apoptotic cell death in a HD cell culture. We further demonstrated that phosphorylation of BimEL was altered in HD. Nevertheless, how phosphorylation of BimEL is regulated in HD and its functional significance remains unclear.

The phosphorylation of BimEL is dictated by pro-survival or pro-apoptotic signaling. Viable cells maintain low levels of BimEL by the induction of proteasomal degradation, which requires BimEL to be primed by phosphorylation. This is initiated by ERK1/2 mediated phosphorylation of BimEL at Ser 55/65/73, for which Ser65 is believed to be the most important for mediating downstream effects, including Rsk1/2 mediated phosphorylation of Ser 93/94/98 (Akiyama et al., 2003) (Dehan et al., 2009). The molecular events following the coordinated phosphorylation of BimEL by ERK1/2 and Rsk1/2 include poly-ubiquitination and proteasomal degradation of BimEL providing a means for cellular survival. On the other hand, pro-apoptotic signaling leads to JNK
activation and phosphorylation of BimEL at Thr112 resulting in its enhanced pro-apoptotic activity (Putcha et al., 2003). Interestingly, in neurons JNK also phosphorylates BimEL at Ser65 but with the opposite effect to that mediated by ERK1/2; by facilitating apoptosis (Putcha et al., 2003, Becker and Bonni, 2006). The discrepancies between models suggest that phosphorylation of BimEL occurs in a cell specific manner, which we address further in Neuro-2a cells. Nevertheless, from these studies it is apparent that the two major kinases that determine the fate of BimEL are ERK1/2 and JNK. The focus of this study is on the regulation of BimEL, mediated by phosphorylation of Ser65 and Thr112, by ERK1/2 and JNK during HD pathogenesis.

The pathogenesis of HD is progressive, spanning decades before the age of onset leading to severe symptomology and death within 15 years after disease onset. Age dependent neurological deficits are observed in R6/2 mice; we were therefore interested in examining the molecular changes previously noted in our cell culture model across several ages of R6/2 mice that are representative of the disease progression in this model. The expression of BimEL, ERK and JNK activation, and UPS activity were determined from striatal tissue obtained at different ages that collectively span the life of R6/2 mice. Furthermore, utilizing novel BimEL mutants we characterize the post-translational regulation of BimEL at ERK1/2 and JNK phosphorylation sites in neuronal cells and demonstrate its significance to the pathogenesis of HD in R6/2 mice.

**BDNF**

BDNF is an important neurotrophic factor necessary for the long term survival and growth of striatal neurons (Baquet et al., 2004, Rauskolb et al., 2010). BDNF is synthesized in the cortex and transported to the striatum via the corticostriatal pathway.
(Altar et al., 1997, Fusco et al., 1999). Interestingly R6/2 HD mice and HD patients display reduced striatal BDNF (Ferrer et al., 2000, Zhang et al., 2003). In this study we explore the contribution of reduced striatal BDNF to the regulation of BimEL expression in the striatum of R6/2 HD mice.

In this study, we investigated the molecular mechanism altered in HD. We utilized a HD cellular model, R6/2 HD mice, and R6/2 mice cross bred with BDNF overexpressing mice. We demonstrated that the post-translational regulation of BimEL is pivotal to its potency in neurons and altered in the presence of mHtt. Importantly, mHtt initiates multifaceted dysregulation of BimEL in the striatum of R6/2 mice.
II. Materials and Methods

DNA constructs

Plasmid cDNA encoding the N-terminal region of human Htt (1-69) with a polyQ repeat length of 25Q (Addgene plasmid 1187) or 103Q (Addgene plasmid 1186) fused to EGFP, full length Htt with a repeat of 23Q (fhtt23Q), or 103Q (fhtt103Q) were obtained from Addgene (Cambridge, MA, USA) and CHDI (Los Angeles, Ca, USA) respectively and subcloned as previously described in Chapter 3. BimEL point mutations of plasmid cDNA encoding Bim (pCMV sport6, Open Biosystems) were created using the polymerase incomplete primer extension (PIPE) method as described previously with the following modifications: used with Phusion High-Fidelity PCR master mix (NEB, Ipswich, MA, USA). (Klock et al., 2008). The single mutations were completed using specific primers (Table 4). The PCR program was: 98°C for 1min; 25 cycles of 98°C for 30sec; 55°C for 45sec; followed by 72°C for 7min as the extension step.
### Table 4 Primers used for BimEL phospho-mutants

| Ser65 to A65 | Forward: 5’- CCA CCG GCC GCA CCT GGC CCT TTT GCT ACC AGA TCC CCA C -3’  
| Reverse: 5’- AGG TGC GGC CGG TGG GGC CAG CGG GCC CTG AGG GCT GCC G -3’ |
| Ser65 to E65 | Forward: 5’- CCA CCG GCC GAA CCT GGC CCT TTT GCT ACC AGA TCC CCA C -3’  
| Reverse: 5’- AGG TTC GGC CGG TGG GCC CAG CGG GCC CTG AGG GCT GCC G -3’ |
| Thr112 to A112 | Forward: 5’- CAA CAC AAG CAC AAT CTC GTC CTT GCC AGG CCT TCA ACC AC -3’  
| Reverse: 5’- GGA CTG GGT GCT TGT GTC ACC TTG CAA CTC ATG GGT G -3’ |
| Thr112 to E112 | Forward: 5’- CAA CAC AAG AAC CAC AAT CTC GTC CTT GCC AGG CCT TCA ACC AC -3’  
| Reverse: 5’- GGA CTG GGT TCT TGT GTC ACC TTG CAA CTC ATG GGT G -3’ |

### Cell Culture and transfection

Human embryonic kidney 293 (HEK293) cells and Neuro-2a cells (ATCC, Rockville, MD, USA) were cultured in EMEM medium containing 10% FBS. Neuro-2a cells were transiently transfected with Htt or BimEL plasmid constructs using 0.5µg, and 0.02µg of DNA respectively; HEK293 cells with BimEL plasmids using 0.02µg of DNA; per well for a 24 well plate using the standard lipofectamine LTX method according to the manufacturer’s instruction (Invitrogen, CA, USA). Cells were harvested 48 hours after Htt transfection or 24 hours after Bim transfection.

### U0126 and SP00125 treatment

U0126 and SP00125 (Cell signaling, MA, USA), MEK1/2 and JNK inhibitors respectively, were dissolved in DMSO. After an overnight transfection Neuro-2cells were treated with 20µM of U0126, SP00125 or both and collected 48 hours after the
initial transfection. The control group was treated with .1% DMSO to account for the .1% DMSO introduced by the U0126, and SP00125 treatments.

CIP treatment

The brain samples were homogenized and lysed in lysis buffer without EDTA (50mM Tris, pH=7.5, 150mM NaCl, 1% triton X-100, and 1% protease inhibitor cocktail). About 200µg of tissue lysate was incubated with 50 units (0.25U/µg protein) of calf intestinal phosphatase (CIP, New England Biolabs) at 37°C for 1 hour. After incubation, the lysates were subjected to immunoblotting analysis.

Animals

The R6/2 transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed in the animal care facility at Florida Atlantic University. Genotyping was performed as previously described in Chapter 3. Breeding pairs of R6/2 transgenic mice was conducted as described in Chapter 3. R6/2 mice were crossbred with BDNF overexpressing mice (BDNF:BTg), a transgenic mouse line expressing the rat BNDF cDNA sequence under the control of the neuronal specific mouse calcium/calmodulin-dependent protein kinase II alpha (Camk2a) promoter. Primer sequences used for genotyping were: 5'-GTC CTT GGG GTC TTC TAC CTT TCT C-3' and 5'-GTG AAG GAA CCT TAC TTC TGT GGT G-3'. PCR program was: 94°C for 1.5 min; 40 cycles of 94°C for 30 sec; 54°C for 30 sec and 72°C for 1.5 min; 72°C for 10 min as the final elongation step. Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of FAU.

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For sample preparation, the striatum, cortex, cerebellum, and hippocampus from 6, 8, 10, and 12 week old R6/2 mice; striatum of 4, and 10 week old R6/2:BTg and their control littermates were dissected and then lysed in lysis buffer (50mM Tris, pH=7.5, 5mM EDTA, 150mM NaCl, 1% Triton X-100, protease and phosphatase inhibitor cocktail) at 4°C for 30 min while shaking. After lysis, the samples were centrifuged at 13,000 x g for 20 min at 4°C to remove any insoluble materials. Protein concentration was determined using the Bradford method.

**Cell culture sample preparations and western blot**

Following treatment, cells were lysed in lysis buffer (50mM Tris, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 1x complete protease inhibitor cocktail from Sigma, and 1x phosphatase inhibitor cocktail from Pierce). Mitochondrial and cytosolic fractions were prepared as previously described Chapter 3. After lysis, whole cell lysates (50μg), brain lysates (50μg) or mitochondrial extracts (~20μg) were resolved by SDS-PAGE and then transferred to a PVDF membrane. The membranes were blocked using Odyssey blocking buffer (Li-COR) and then incubated over night with the following primary antibodies: BimEL (1:500), phospho-BimEL(Ser65; 1:500), JNK (1:500), phospho-JNK (Thr183/Tyr185; 1:500), ERK (1:500), phospho-ERK (Thr202/Tyr204; 1:500), cleaved caspase 3 (1:500), PARP (1:500, the above antibodies were purchased from cell Signaling, Danvers, MA, USA), actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax (1:500, Chemicon, Billerica, MA, USA) and p-BimEL (Thr112; 1:1000, a generous gift from Dr. Roger Davis, University of Massachusetts). The membranes were incubated with Odyssey secondary antibodies for one hour at room temperature;
Goat anti rabbit IRDye 800 (1:5000) and Goat anti mouse IRDye 680 (1:20,000). Blots were imaged using an Odyssey Infrared Imaging System (Li-COR Biosciences).

**Assessment of cell survival**

The effect of different BimEL mutants on cell survival was assessed as reported with minor modifications (Putcha et al., 2003, Becker et al., 2004). Cells were seeded in a 24-well plate at $3 \times 10^5$ cells/well and co-transfected with different BimEL mutants and 0.2 μg of pcDNA3.1-RFP plasmid using standard lipofectamine 2000 method. Twenty-four hours later, the numbers of intact, RFP-positive cells were counted in a defined field under a fluorescent microscope. A total of 6-8 individual fields were randomly chosen in each well for the counting and the average cell number per field was determined. The total number of transfected cells counted in the control group was at least 400. The percentage of surviving cells was calculated relative to the numbers present in control wells. All experiments were performed at least in triplicate. And the results were reported as means ± S.E.M.

**Mitochondria Isolation**

The mitochondria fractions were prepared as described in chapter 3.

**Proteasome activity assay**

Striatal tissue of 6, 8, 10, and 12 week old wild-type and R6/2 mice were homogenized in homogenization buffer (15 mM Tris-HCl, pH=8.0, 60mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA and 2 mM ATP) at 4°C. The homogenates were centrifuged at 1,300 x g for 10 min to remove unbroken tissues and nuclei fractions. Protein concentration was measured and adjusted to 0.5mg/ml total protein by dilution.
with homogenization buffer. All assays were done in triplicate. Chymotrypsin-like activity of 20S proteasome was determined using substrate Suc-LLVY-AMC (50μM, Calbiochem, La Jolla, CA). Caspase-like activity was measured using Z-LLE-AMC (50μM, Calbiochem, La Jolla, CA). Trypsin-like activity was measured using Z-ARR-AMC (50μM, Calbiochem, La Jolla, CA). About 10μg of cell lysate was incubated with each of the substrate in 100μl proteasome activity assay buffer (50mM Tris-HCl, pH=8.0, 0.5mM EDTA, 1mM ATP, 1mM DTT) for 30 min at 37°C. The reactions were stopped by addition of 100μl of stop solution (2% SDS). Released fluorogenic AMC was measured at 360nm excitation and 460nm emission in a microplate luminometer (Molecular Devices, CA, USA). Fluorescence units were converted to AMC concentration using standard curves generated from free AMC.

**Quantitative RT-PCR**

RNA of 8 and 12 week old wild-type and R6/2 mice was isolated from striatal tissue, first-strand cDNA was synthesized, and the amounts of BimEL transcripts were analyzed as described in Chapter 3.

**BDNF assay**

Striatal BDNF of 4 and 10 week old wild-type, R6/2, and R6/2:BTg mice was measured in accordance with the protocol of Promega’s BDNF Emax immunoassay system (Promega, Madison, WI, USA). 10 week old wild type, R6/2, and R6/2:BTg mice were euthanized and the striatum dissected. The samples were lysed in BDNF Emax lysis buffer (100mM PIPES (pH= 7), 500mM NaCl, 0.2% Triton X-100, 0.1% NaN3, 2% bovine serum albumin (BSA), 2Mm EDTA, Na2.2H2O) followed by sonication at power level 4 for 15 seconds pulsed at 1sec intervals. Samples were centrifuged at 16,000 x g
for 30 min at 4 °C and the supernatant collected, diluted (1:4) and incubated for 2 hours at room temperature with shaking (400-100rpm) in a 96 well plate pre-coated with a carbonate coating buffer (0.025M sodium carbonate, ph.9.7) containing monoclonal anti-BDNF. The plate was washed and incubated with polyclonal anti-BNDF in sample buffer for 2 hours at room temperature with shaking (400-100rpm). The plate was then washed again and incubated for 1 hour at room temperature with shaking (400-100rpm) with 50µl Anti-IgY HRP in sample buffer. After a final wash 100µl TMB one solution (Promega, Madison, WI, USA) was added to each well and incubated for 10 minutes at room temperature with shaking followed by the addition of 100µl 1N HCL to stop the reaction. BDNF content was measured at 450nm using a microplate luminometer (Molecular Devices, CA, USA). Absorbance units were converted to BDNF concentration using standard curves generated from BDNF standard.

**BimEL degradation assay**

Neuro-2a cells were transfected with different BimEL mutants using lipofectamine 2000 method. After 24 hours, cells were incubated with 10µg/ml cycloheximide (CHX) to block new protein synthesis. Cells were harvested at indicated time points for western blot analysis.

**Statistical analysis**

All data were expressed as means + S.E.M. To establish significance, data were subjected to unpaired student’s t-tests or one way ANOVA followed by the Turkey's multiple comparison test using the GraphPad Prism software statistical package (GraphPad Software, San Diego, CA, USA). The criterion for significance was set at $P \leq 0.05$. 
Acknowledgement

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II. Results

Expression of BimEL throughout the pathogenesis of HD.

To investigate the role of BimEL in HD pathology, we evaluated BimEL expression in R6/2 mice at several ages spanning the disease progression. R6/2 mice display a severe phenotype with a life span of 12-15 weeks. For this reason our analysis consisted of 4, 6, 8, 10, and 12 week old mice. Densitometric analysis of 3 independent blots revealed no difference in BimEL levels between wild-type and R6/2 mice at 4 weeks of age (Fig. 19A). Further analysis demonstrated that in wild-type mice, the striatal protein expression of BimEL steadily decreased in an age dependent manner (Fig. 19B and C). On the contrary, the BimEL expression in the striatum of R6/2 mice remained high throughout all ages measured, with significantly more BimEL present in 12 week old R6/2 mice compared to 12 week old wild-type mice (Fig. 19B and C).
Figure 19 BimEL expression throughout the pathogenesis of HD in R6/2 mice.

A. No difference in striatal BimEL expression was detected at 4 weeks of age. B. & C. BimEL is reduced in an age dependent manner in wild type mice. B. & C. R6/2 mice maintain unchanged BimEL protein levels at all ages measured. Significant reductions in BimEL protein was measured in the striatum of 12 week old R6/2 mice compared to wild
type mice B & C. Western blot is a representative image of 3 independent blots. Densitometric analysis was calculated from 3 independent blots A & C.

To determine if the changes in striatal BimEL is due transcriptional up-regulation, we analyzed striatal tissue of R6/2 mice by qRT-PCR. We previously analyzed striatal BimEL mRNA of R6/2 mice at 5 weeks and detected no significant difference when compared to wild type mice. Consequently in this analysis we chose 8 and 12 week age groups, ages groups in which a neurological phenotype is observable in R6/2 mice. In wild type mice we measured a decrease in BimEL mRNA in 12 weeks of age when compared to 8 weeks, consistent with the age dependent decrease in BimEL protein levels (Fig. 20). Although we also detected a decrease in BimEL mRNA in 12 week old R6/2 mice compared to 8 week olds, the reduction was not as impressive as observed in the 12 week wild type group (Fig. 20). These results indicate that BimEL elevations may be partially due to a deficiency in the normal developmental down-regulation of BimEL. However, transcriptional dysregulation cannot completely account for the elevation of BimEL protein levels, as a reduction in mRNA was measured in 12 week old R6/2 mice (Fig. 20).
**Figure 20 Transcriptional regulation of BimEL in R6/2 HD mice.**

qRT-PCR demonstrated a reduction of BimEL mRNA in 12 week old wild type mice. Although there was a measureable decrease in BimEL mRNA in 12 week R6/2 mice the difference was not as great as that observed in wild type mice.

**Phosphorylation of BimEL in R6/2 mice**

The previous results indicated that post-translational regulation may be an important mechanism for the elevations of striatal BimEL in R6/2 mice. As expected, western blot analysis of striatal wild-type tissues also displayed an age dependent reduction in phosphoSer65-BimEL and phosphoThr112-BimEL; corresponding to the reduction in total BimEL (Fig. 21). On the contrary, R6/2 mice maintained unvarying levels of phosphoSer65-BimEL and of phosphoThr112-BimEL suggesting that phosphorylation may contribute to the stability of BimEL in the striatum of R6/2 mice (Fig. 21).
Figure 21 The phosphorylation of BimEL at Thr112 and Ser65 in R6/2 HD mice.

Western blot analysis of striatal tissue throughout the disease pathogenesis of R6/2 mice demonstrated a reduction in phosphoThr112-BimEL and phosphoSer65-BimEL in wild type mice that occurred in an age dependent manner. R6/2 mice maintained high levels of phosphoThr112-BimEL and phosphoSer65-BimEL in all groups measured. Calf intestinal alkaline phosphatase (CIP) was used as a negative control and demonstrates the specificity of the antibodies. Antibodies used: phosphoThr112-BimEL, phosphoSer65-BimEL, and β-actin.

ERK and JNK activity is altered in the striatum of R6/2 HD mice

The changes in phosphorylation of BimEL detected in R6/2 mice could reflect increased availability to corresponding kinases or a change in kinase activity. We therefore determined the kinase activity of the two major kinases known to phosphorylate BimEL; the pro-survival ERK1/2 and the stress activated JNK. ERK1/2 activity, was evaluated by immunoblotting using a phospho-ERK antibody, and revealed to be notably reduced in the striatum of R6/2 mice mostly during the early ages analyzed (Fig. 22). The JNK activity from striatal R6/2 mice was measured with a phospho-JNK antibody, and was found to be increased when compared to wild-type mice throughout all ages measured (Fig. 22). There were no observable changes in total ERK1/2 and JNK indicating that the changes observed were due to increased kinase activity. Together these results suggest that altered ERK1/2 and JNK activity in striatal R6/2 tissue may
cooperate with other post-translational mechanisms of regulating BimEL expression to prevent the age dependent down regulation of BimEL seen in wild type mice.

**Figure 22 The activity of ERK and JNK in R6/2 HD mice.**

Western blot analysis of striatal tissue demonstrated increased JNK activity at all ages measured when compared to wild type mice. No increase was observed in ERK1/2 activity. No changes in total JNK or ERK1/2 was detected. Antibodies used: phospho-JNK, JNK, phospho-ERK1/2, ERK1/2, β-actin

**ERK and JNK inhibitors prevent BimEL phosphorylation in mHtt expressing Neuro-2a cells**

Thus far we have demonstrated that mHtt expression leads to alterations of the MAPK pathway, ERK1/2 and JNK activity. We also linked mHtt expression to the stabilization of BimEL, phosphoSer65-BimEL, and phosphoThr112-BimEL within the striatum of R6/2 mice. To determine if ERK1/2 and JNK are the kinases responsible for the phosphorylation of BimEL in our HD models we treated mHtt expressing Neuro-2a cells with specific kinase inhibitors; U0126 or SP600125, which inhibit ERK and JNK respectively. Treatment of mHtt expressing cells with either U0126 or SP600125 prevented the phosphorylation of BimEL at Ser65, while only SP600125 treatment
prevented the phosphorylation of Thr112 (Fig. 23). The presence of phosphoSer65-BimEL during U0126 treatment suggests that this site is not essential for phosphorylation at Thr112. These results reveal that changes in the phosphorylation state of BimEL in HD are mediated by ERK1/2 and JNK, as inhibiting their activities was enough to abolish phosphorylation even in the presence of mHtt. Furthermore, we provide evidence that in neurons, JNK can phosphorylate BimEL at both Thr112 and Ser65. Taken together this suggests a series of events that first involve kinase activation followed by BimEL phosphorylation to be important to the pathogenesis of HD. The association of ERK1/2 to cell survival and JNK to cell death; imply the differential phosphorylation of BimEL during mHtt expression to be a results of an imbalance between these regulatory pathways; which may be important to determine the fate of BimEL and cellular survival.
Neuro-2a cells expressing mHtt and treated with specific kinase inhibitors. 20uM SP600125 or U0126, specific JNK and ERK inhibitors respectively, prevented the phosphorylation of BimEL at Ser65. SP600125 treatment also prevent the phosphorylation of BimEL at Thr112.

**UPS activity is deficient in R6/2 striatal tissue**

We next investigated another major form of post-translational regulation of BimEL, UPS mediated degradation. The UPS activity was measured in striatal R6/2 and wild-type tissue. Three separate proteasome substrates were used: Suc-LLVY-aminomethycoumarin (AMC) to measure the Chymotrypsin-like activity of 20S, Z-LLE-AMC to measure Caspase-like activity, and Z-ARR-AMC to measure the Trypsin-like activity. Released fluorogenic AMC was measured by a microplate luminometer. Significant reductions in enzyme activity were observed in R6/2 mice compared to wild-type mice as early as 8 weeks of age (Fig. 24 A-C). In healthy cells BimEL is targeted for UPS mediated degradation. We have previously shown that the proteasome inhibitor, MG-132, elicits an up regulation of BimEL. Moreover, mHtt expression inhibits the UPS activity in a neuronal cell culture (Leon et al., 2010). These results indicate that reduced protein degradation via mHtt-induced inhibition of the UPS could contribute to the accumulation of BimEL observed in the striatum of R6/2 mice.
A. **Trypsin-like**

![Bar chart showing Trypsin-like activity across weeks 6 to 12 for WT and R6/2 mice.]

B. **Chymotrypsin-like**

![Bar chart showing Chymotrypsin-like activity across weeks 6 to 12 for WT and R6/2 mice.]

C. **Peptidyl-glutamyl-like**

![Bar chart showing Peptidyl-glutamyl-like activity across weeks 6 to 12 for WT and R6/2 mice.]

**Legend:**
- WT (white bars)
- R6/2 (gray bars)
- **P** (p-value significance symbol)
- *P* (p-value significance symbol)
The UPS is impaired in R6/2 HD mice.

The proteasome activity was measured using trypsin like, chymotrypsin like, and peptidyl glutamyl like luminogenic substrates. A significant reduction in enzymatic activity was observed in R6/2 striatal tissue as early as 8 weeks of age.

The significance of BimEL phosphorylation at Ser65 and Thr112 on neuronal survival

To determine the physiological significance of BimEL phosphorylation at Ser65 and Thr112 in HD, we created BimEL constructs containing mutations at Ser65 and Thr112: BimEL-S65A, BimEL-S65E, BimEL-T112A, and BimEL-T112E. Site directed mutagenesis of these sites to either alanine (A) or glutamate (E) allowed us to mimic the effects of non-phosphorylated and constitutively phosphorylated forms of BimEL respectively. In order to determine the effects of these phosphorylation sites we transfected Neuro-2a cells with BimEL constructs and quantified cell viability. The over expression of wild type BimEL proved to be very toxic to cells; we therefore measured survival 24 hours after transfection, a time point in which 50% of the cells underwent cell death. The BimEL-E112 mutation was the most potent inducer of cell death (Fig. 25A). These results are consistent with the hypothesis that phosphorylation of Thr112 enhances its pro-apoptotic activity (Hubner et al., 2008). In addition, the BimEL-E65 also displayed a significant reduction in cell viability when compared to wild-type BimEL (Fig. 25A). Although the phosphorylation of BimEL at this site has been shown to promote its degradation by the UPS in non-neuronal cells, in neuronal systems it has been determined to promote apoptosis (Becker and Bonni, 2006). As a control the alanine mutations were not as toxic as either glutamate mutations. To confirm the cell death
measured was due to the role of BimEL in initiating apoptosis, we analyzed the levels of cleaved caspase-3 in BimEL overexpressing cells. Consistently the E65 and E112 did demonstrate greater amounts of active caspase-3 compared to the wild type BimEL and alanine mutations (Fig. 25B). All BimEL mutants resided within the mitochondria fraction; indicating that the effects of these mutations were not due to a change in the sub-cellular localization from that of the wild-type BimEL (Fig. 25C).
Figure 25 Phosphorylation of BimEL regulates its apoptotic activity.
Site directed mutagenesis of BimEL substituting serine and threonine for alanine or glutamine. The cell viability of Neuro2a cells over expressing BimEL mutants was measured. BimELS65E and BimELT112E significantly reduced cell viability when compared to wild type BimEL. Mitochondrial fractionation of N2a cells expressing BimEL demonstrated mitochondrial localization of all Bim mutants.

The neuron specific effects of BimEL phosphorylation at Ser65 and Thr112 on the stability of BimEL

How does the phosphorylation of BimEL at Ser65 and Thr112 induce neuronal cell death? To address this question we examined the effects of phosphorylation on BimEL turnover, a regulatory mechanism previously characterized in non-neuronal cells. Neuro-2a cells were transfected with BimEL constructs; treated with cycloheximide 24 hours after transfection to inhibit protein synthesis; and collected at different time points for western blot analysis. This allowed for the detection of BimEL degradation in the absence of new BimEL synthesis. Both WT-BimEL and BimEL-E112 displayed a steady time dependent degradation beginning at 2 hours (Fig. 26A). Interestingly, the BimEL-E65 was resistant to degradation throughout all time point and remained present after 8 hours (Fig. 26A). These results indicate a cell specific mechanism that is unique to neurons; phosphorylation of BimEL at Ser65 did not initiate the proteasomal degradation of BimEL in neuronal cells. Consistent with previous reports of BimEL degradation in non-neuronal cells, the opposite effect was observed in HEK293 cells; BimEL-E65 underwent a steady turnover (Fig. 26B). Taken together these results indicate that in
neurons BimEL is stabilized by the phosphorylation of Ser65 and this occurs in a cell type specific manner.

Figure 26 Phosphorylation of BimEL at Ser65 provides stability in neurons.
Site directed mutagenesis of BimEL. Degradation of BimEL was measured by treating cells with cyclohexamide to prevent further protein synthesis. Neuro-2a cells expressing BimEL mutants were collected at 0, 2, 4, and 8 hours after cycloheximide treatment.
Both wild type BimEL and BimELT112E were significantly reduced by 8 hours after treatment. BimELS65E remained high even at 8 hours after treatment.

**BimEL regional specificity in R6/2 HD mice**

We have thus far established an up-regulation of BimEL with in the striatum of R6/2 mice throughout the progression of the disease. To further demonstrate the selective increase of BimEL in HD we analyzed BimEL protein expression in other areas of the brain. The striatum, hippocampus, cerebellum, and cortex were dissected in wild type and R6/2 mice at 10 weeks of age. An increase in BimEL expression was observed in the cortex and striatum of R6/2 mice when compared to the wild type mice (Fig. 27A). No differences were detected in the hippocampus or cerebellum between wild type and R6/2 mice. Densitometry also demonstrated a difference in the ratio of BimEL expression in the cortex and striatum of R6/2 mice as measured by three independent blots (Fig. 27B). Likewise phosphoSer65-BimEL was also elevated in the cortex and striatum of R6/2 mice, implicating an increase in the kinase activity in both areas of the brain of R6/2 mice (Fig. 27A). A western blot was performed to assay the kinase activity of ERK1/2 and JNK in the brain regions specified. The activity of ERK1/2 was unaltered in all brain regions examined in wild type and R6/2 mice, as determined by the use of a phospho-ERK1/2 antibody (Fig. 27C). Interestingly, the activity of JNK was selectively increased in the striatum of R6/2 mice compared to the other brains regions (Fig. 27C). No observable difference in JNK activity was seen in the cortex, hippocampus, or cerebellum of R6/2 mice. Wild type mice also did not demonstrate any changes in the activity of JNK. Taken together these results implicate the activity of JNK in the stabilization of BimEL specifically with in the region of the brain predominantly affected in HD.
Figure 27 Regional specificity of BimEL expression in R6/2 HD mice.

A. Western blot analysis of striatal, cortex, hippocampus, and cerebellum demonstrated an increase in BimEL protein expression specifically in the striatum of 10 week old R6/2 mice. B. phosphoSer65-BimEL was also elevated in the striatum when compared to other regions. C. There was an increase in the activity of JNK within the striatum of R6/2 mice when compared to other regions. No observable changes were measured in ERK1/2 activity of R6/2 mice in the brain regions.
BDNF as the link to the regional specific expression of BimEL in HD

BimEL is a pro-apoptotic protein that responds to multiple stress factors including trophic factor withdrawal (Putcha et al., 2001, García-Martínez et al., 2007). To determine the mechanism for the regional specificity of BimEL expression we investigated the effects of BDNF levels in HD on BimEL expression. Reduced striatal BDNF in HD is well established but the molecular consequence of this impaired trophic support is not fully understood. To address this question we crossbred R6/2 mice with mice over expressing BDNF (Btg:BDNF) to produce R6/2:Btg mice. The levels of striatal BDNF was measured by ELISA analysis in 10 week old wild-type, R6/2, and R6/2:Btg mice. Consistent with previous studies, we observed a reduction in striatal BDNF in R6/2 mice compared to wild-type mice (Fig.28A). The levels of striatal BDNF was rescued in the R6/2:Btg mice (Fig. 28A). Importantly, western blot analysis revealed striatal BimEL to be greatly reduced in 10 week old R6/2:Btg mice compared to 4 weeks old R6/2:Btg mice (Fig. 28B). In addition, the levels of BimEL in 10 week old R6/2:Btg was less than the 10 week old R6/2 mice (Fig. 28C). These results indicate that reduced striatal BDNF in R6/2 mice also contributes to the up-regulation of BimEL in this region of the brain. Although the activity of JNK was unaltered in the R6/2:Btg mice there was an increase in the activity of ERK1/2 suggesting that a balance between these two kinases is pivotal to BimEL regulation (Fig. 28B). These findings highlight another level of regulation upon BimEL, and the potential of BDNF to elicit its pro-survival effects precisely in the area of the brain affected in HD pathology.
Figure 28 BimEL expression in R6/2:BTg mice.

A. ELISA analysis of striatal tissue demonstrated a reduction of BDNF within the striatum of R6/2 mice when compared to wild type mice. B. Western blot analysis of striatal tissue of transgenic mice. Increased ERK activity was measured in R6/2:BDNF at 10 weeks when compared to 4 week old R6/2:BTg mice. BimEL protein levels were reduced in the striatum of R6/2:BTg mice. C. R6/2:Btg mice expressed less BimEL within the striatum than did R6/2 mice at 10 weeks.
II. Discussion

In this study we characterized the role of BimEL in the pathogenesis of HD. We found that BimEL was stabilized in striatal R6/2 tissue whereas non transgenic mice displayed reduced levels of BimEL in an age dependent manner. These findings are consistent with reports that postnatal expression of BH3-only proteins decline with age (Donovan et al., 2006). Although we demonstrate a loss of transcriptional repression in R6/2 mice this dysregulation cannot account for the elevations observed in the striatum of R6/2 mice; hence we focused our attention to the post-translational regulation of BimEL.

The two major kinases that regulate BimEL are the pro-survival ERK1/2 and pro-apoptotic JNK. The phosphorylation of BimEL at Ser65 can signal either its degradation or stabilization depending upon the cell type; by way of ERK in non-neuronal cells or JNK in neuronal cells (Luciano et al., 2003, Becker and Bonni, 2006). Other studies have shown phosphorylation at Thr112 by JNK to enhance its apoptotic activity (Hubner et al., 2008). We therefore sought to explore the phosphorylation of BimEL amid mHtt-induced cellular dysfunction. To our knowledge, this is the first time that phosphoThr112-BimEL has been explored in neuronal systems. We found elevated phosphoSer65-BimEL and phosphoThr112-BimEL in mHtt-expressing Neuro-2a cells and striatal R6/2 tissue. Further analysis revealed JNK activity to be increased in striatal
R6/2 tissue. Although the activation of ERK1/2 was previously observed in a HD inducible cell line and our HD cell line we report ERK1/2 activity to be unaltered in R6/2 mice (Apostol et al., 2006). This disparity may relate to the different models used. Taken together these results suggest that in HD striatal BimEL is stabilized via a JNK-BimEL pathway.

To determine the significance of BimEL phosphorylation in HD we sought to understand its regulation in neurons utilizing BimEL phospho-mutants. Constitutively phosphorylated BimEL-S65E and BimEL-T112E caused significant cell death in Neuro-2a cells with BimEL-T112E being the most potent inducer of cell death. Notably, the BimEL-T112A restored cell viability to that of the wild-type BimEL. Other studies utilizing BimEL-T112A demonstrated reduced co-immunoprecipitation with Bcl-2 (Hubner et al., 2008). Interestingly, Bcl-2 has been shown to be expressed throughout the striatum while the large interneurons spared in HD express Bcl-xL (Perez-Navarro et al., 2005). This differential expression may render MSNs dependent upon Bcl-2 and specifically susceptible to elevations of phosphoThr112-BimEL.

Furthermore, a protein degradation assay of the BimEL mutants was performed. We demonstrated that in neurons phosphorylation of Ser65 prevented BimEL from degradation while the phospho-Thr112 had no effect on BimEL turnover. In neurons JNK dependent phosphorylation of BimEL at Ser65 has been implicated in stabilizing BimEL by promoting its interaction with a neuron specific prolyl isomerase located on the mitochondria (Fig. 17) (Becker and Bonni, 2006). Interestingly, all BimEL mutants were located in the mitochondria fractions. The increased phosphoSer65-BimEL and JNK
activity in the striatum of R6/2 mice suggest this neural specific regulation of BimEL may underly mHtt-induced cellular dysfunction.

In addition to phosphorylation, BimEL is also regulated by the UPS. In this study we demonstrate the UPS activity to be reduced in the striatum of R6/2 mice. We have previously shown that mHtt expression is enough to inhibit the UPS and that MG-132, a UPS inhibitor, induces the up-regulation of BimEL in a neuronal culture (Leon et al., 2010). Studies inhibiting the proteasome in vivo by means of stereotactic injection into the hippocampus also demonstrate an up-regulation of BimEL (Tsuchiya et al., 2011).

Moreover, mHtt aggregates have been shown to inhibit the UPS via sequestration of enzymatic components into mHtt aggregates (Bence et al., 2001). These findings implicate the UPS dysfunction in HD to the accumulation of BimEL, which may increase its availability to JNK.

To identify a mechanism for the striatal specific molecular changes we described in the R6/2 mice, we examined the BNDF pathway. The cortex delivers BDNF to striatal post-synaptic densities thereby providing essential trophic support. HD is associated with reduced striatal BDNF which is believed to contribute to the selective degeneration of the striatum (Ferrer et al., 2000). Interestingly cortical BDNF KO mice exhibit the hindlimb clasping phenotype characteristic of motor dysfunction in R6/2 mice (Baquet et al., 2004). Wild-type Htt but not mHtt functions to promote BDNF gene transcription and the vesicular transport of BDNF along microtubules (Zuccato et al., 2001, Gauthier et al., 2004). To identify the molecular dysfunction associated with the specificity of HD neurodegeneration, we examined if there was a correlation between BDNF expression and BimEL in the striatum of R6/2 mice. R6/2:Btg mice exhibited restored BDNF levels
within the striatum and reduced expression of striatal BimEL. This is consistent with
other studies that demonstrated that BDNF functions to down-regulate BimEL by way of
TrkB activation, suggesting that reduced BDNF affects pathways upstream of ERK1/2
and JNK activation that lead to the stabilization of BimEL (Li et al., 2006, Almeida et al.,
2009). These results suggest that reduced striatal BDNF due to loss of wild type Htt
function may further contribute to the up-regulation of BimEL and the selective
neurodegeneration of the striatum in HD.

In this study we further explored the significance of BimEL phosphorylation and
provide a mechanism for the selective up-regulation of BimEL within the striatum of
R6/2 mice. The degradation of BimEL was demonstrated to not be dependent upon
phosphorylation of Ser65 as this actually led to its stabilization. We conclude that
increased JNK activity by way of a reduced BNDF pathway provides an environment
within the striatum that leads to phosphoSer65-BimEL and consequently the stabilization
of BimEL. This is further confounded by reduced UPS as a result of mHtt aggregates.
This study highlights a novel pathway linking mHtt-induced molecular dysfunctions to
apoptotic events that we believe could cause MSNs to be more vulnerable to insult.
Chapter 4 Final discussion

Together our findings identify a novel pathway in HD pathogenesis, which involves reduced BDNF, altered MAPK pathway, and UPS dysfunction acting synergistically to promote BimEL expression (Fig. 29). It is well established that mHtt interferes with the proper production and transportation of BDNF resulting in deficient BDNF within the striatum. We believe this deficiency to be important to striatal BimEL levels; BDNF activates pro-survival pathways that can antagonize the JNK pathway. BDNF leads to the activation of Akt which inhibits ASK1, an important up stream kinase necessary for the activation of JNK (Fig 30). We believe that reduced striatal BDNF due to loss of wild-type Htt function may promote the activation of JNK specifically within the striatum of R6/2 mice. We demonstrate the significance of BimEL phosphorylation in neurons; Thr112 enhances apoptotic activity independent of the stability inferred by the phosphorylation of Ser65. The treatment of mHtt expressing cells with kinase specific inhibitors provide evidence that JNK activation is a necessary step for the phosphorylation of BimEL at Ser65 and Thr112, and corresponds to the next step in a series of events that collectively contribute to an increase of BimEL. The inhibitory effects of mHtt on the UPS allows for the further accumulation of BimEL within the striatum. This accumulation allows BimEL to become available for JNK mediated
phosphorylation at Ser65 and Thr112 enhancing its activity and stability, completing a pathway that favors BimEL expression (Fig. 29).

Our analysis on the R6/2:Btg mice thus far have revealed only a slightly prolonged life, 15-16 week, demonstrating that neuronal dysfunction cannot be rescued by BDNF alone. This is also supported by studies confining mHtt expression to the striatum thereby maintaining normal striatal BDNF levels; which did not completely abolish HD symptoms (Brown et al., 2008). These findings support our hypothesis that deficient BDNF contributed to increased BimEL within the striatum. We demonstrate that BimEL, and phosphorylated BimEL resided in the mitochondrial fraction suggesting that mitochondrial dysfunction may contribute to neuronal dysfunction and vulnerability of MSNs in HD. Interestingly, the striatal specific NRB2 subunit of NMDA receptors has been suggested to play an essential role in sensitizing the striatum to glutamatergic and dopaminergic input resulting in Ca$_{2+}$ overload and excitotoxicity (Tang et al., 2007). Thus it is likely that HD pathogenesis involves a highly complex interplay of mechanism that together result in neuronal dysfunction including the ability of the mitochondria to buffer Ca$_{2+}$. Notably, the activation of JNK can occur during trophic factor withdrawal and excitotoxicity.

This study provides important insight into the complex regulation of BimEL in neurons and its dysregulation amid HD pathogenesis. In addition, we demonstrate changes in the activation of ERK1/2 and JNK which may represent an imbalance between pro-survival and pro-apoptotic signaling during mHtt expression. These findings suggest various points for which intervention may be beneficial. Previous studies utilizing JNK inhibitors in HD and Parkinson’ disease models have demonstrated
neuroprotective properties (Perrin et al., 2009, Chambers et al., 2011). Although other studies have linked increased BimEL levels to the pathogenesis HD, none have described its expression throughout the disease progression, provided a detailed mechanism for it nor examined the post-translational regulation of BimEL in neurons (Zhang et al., 2003, García Martínez et al., 2007, Kong et al., 2009).
Figure 29 mHtt elicits multiple deleterious effects in neurons.

We propose that deficient UPS activity, increased GADD153 expression and reduced BDNF promotes the accumulation of BimEL within the striatum. Together with enhanced JNK activation, due to reduced BDNF signaling, these events facilitate the pro-apoptotic activity of BimEL. The role of BimEL on the mitochondria may contribute to the sensitivity of MSNs in HD.

Figure 30 Pathway leading to the activation of JNK.

The activation of ASK1 is a necessary step for the activation of JNK. Notably ASK1 can be inhibited by Akt during BDNF signaling. (Perrin et al., 2009)
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