ECTOPIC EXPRESSION OF mnb AND Hip1 ALTERS DROSOPHILA DEVELOPMENT

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Ectopic expression of mnb and Hip1 alters Drosophila development

by

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Abstract

A powerful approach to studying the mechanisms of disease is through the use of transgenic models like *Drosophila melanogaster*. The Drosophila homologue of the human Down syndrome candidate gene, *Dyrk1A*, is *minibrain* (*mnb*). Studies have shown that *mnb* can participate in development of the Drosophila eye to control growth and survival through modification of insulin receptor signalling. Huntingtin-interacting protein 1 (*Hip1*), which differentially interacts with the mutant and normal forms of the Huntington disease protein, huntingtin, has been shown to play a role in Drosophila neurogenesis. There are two naturally occurring versions of the *Hip1* protein, a full length version and a truncated version missing the N-terminal ANTH domain, referred to as *Hip1ΔANTH*. As a biochemical interaction has been identified between *Hip1* and *Dyrk1A/mnb*, this study examines these two genes in Drosophila development. In this study the *UAS-Gal4* system was utilized to direct the expression of *Dyrk1A/mnb*, *Hip1* and components of the insulin signalling pathway, *akt* and *foxol*, in the developing dorsal notum and eye. While neither *mnb* nor *foxol* overexpression has any effect, we found that over-expression of *akt*, a component of the insulin signalling pathway, increases the microchaetae density on the dorsal notum. The over-expression of *mnb* and *Hip1* in the eye have no effect on eye development. Under the sensitized growth conditions where the *GMRGal4* also drives the *UASfoxol11* transgene, the full length versions of *Hip1* show decreased bristle number and decreased ommatidia number while the truncated versions of *Hip1* lacking the lipid-binding ANTH domain show decreased ommatidia number. Over-expression of *mnb* and *Hip1* together with *foxol* show changes in eye development, indicating a possible interaction between these two genes. As these are genes in two completely different diseases, finding how they interact could provide a key insight into finding therapies for neurodegenerative diseases.
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<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ANTH</td>
<td>AP-180 N-terminal homology</td>
</tr>
<tr>
<td>AP2</td>
<td>adapter protein 2</td>
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<tr>
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<td>androgen receptor-interacting protein 4</td>
</tr>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>DRPLA</td>
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</tr>
<tr>
<td>DS</td>
<td>Down syndrome</td>
</tr>
<tr>
<td>DCR-1</td>
<td>Down syndrome critical region</td>
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<tr>
<td>Dyrk1A</td>
<td>dual specificity tyrosine phosphorylation-regulated kinase</td>
</tr>
<tr>
<td></td>
<td>dual-specificity YAK1-related kinase A</td>
</tr>
<tr>
<td>eIF2Bε</td>
<td>eukaryotic initiation factor 2Bepsilon</td>
</tr>
<tr>
<td>ENTH</td>
<td>epsin N-terminal homology</td>
</tr>
<tr>
<td>ESEM</td>
<td>environmental scanning electron microscope</td>
</tr>
<tr>
<td>foxo1</td>
<td>forkhead homeobox type O</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
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<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>green fluorescent protein</td>
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<td>glass multiple reporter</td>
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<tr>
<td>KDa</td>
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</tr>
<tr>
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<td>polyglutamine</td>
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<tr>
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<td>glutamine</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SF3b1</td>
<td>splicing factor 3b, subunit 1</td>
</tr>
<tr>
<td>Sla2p</td>
<td>synthetic lethal with actin binding protein (ABP) 1</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
</tr>
<tr>
<td>YAC</td>
<td>yeast artificial chromosome</td>
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<td>X</td>
<td>times</td>
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Introduction

Drosophila as a model organism

A powerful approach to the study of mechanisms of disease is through the use of transgenic models. Notably, *Drosophila melanogaster* has many attributes that make it a valuable research tool, such as a rapid life cycle, large numbers of offspring and ease of maintenance, that make it ideal for genetic experimentation (Bier 2005). Approximately 75% of known human disease genes have homologues in Drosophila (Bier 2005), which makes it possible to characterize the biological properties associated with the Drosophila homologues. Furthermore, many developmental processes have been conserved throughout evolutionary history, so study in Drosophila can provide insights into the mechanisms of similar processes in humans (Bier 2005). Specifically, Drosophila has been used to study developmental disorders, neurological diseases such as Alzheimer, Parkinson and Huntington disease, trinucleotide repeat disorders (such as dentatorubral pallidoluysian atrophy), and cancer (reviewed in Bier 2005; Sang and Jackson 2005). Given the diversity of studies that have utilized Drosophila, it is an excellent choice as a model organism in which to study homologues of disease genes.

The UAS-Gal4 system in Drosophila

The UAS-Gal4 system, a directed or ectopic expression system used to express genes, is one of many molecular and genetic techniques available to manipulate Drosophila (Phelps and Brand 1998). This system utilizes the yeast transcription factor gene, *Gal4*, and its protein target sequence UAS (upstream activating sequence) to drive
the expression of genes in specific cells and tissues at precise times throughout the life of the organism. These two genes are maintained in separate transgenic fly lines. When crossed, the target gene is activated in the progeny due to the Gal4 protein binding to the UAS element in the enhancer region of the target gene to induce transcription (Phelps and Brand 1998). The UAS-Gal4 system will be utilized in this study to direct the expression of Dyrk1A/mnb, the Down syndrome candidate gene, Huntingtin-Interacting Protein 1 and genes of the insulin receptor-signaling pathway, akt and foxo1.

**Drosophila eye growth and differentiation**

The Drosophila eye normally contains a hexagonal pattern of 750-800 ommatidia (light-sensing units) that is the product of a series of precise events that take place during development (reviewed in Bonini and Fortini 1999; Thomas and Wassarman 1999; Baker 2001; Frankfort and Mardon 2002). Differentiation of the cells that comprise the adult eye begins in the mid-third instar in the eye imaginal disc. The morphogenetic furrow moves from posterior to anterior across the eye disc as progressively more anterior disk regions begin eye differentiation. Each ommatidia contain eight photosensitive neurons, or photoreceptors, for a total of over 6000 neurons in the eye. In addition, accessory cells, including four lens-secreting cone cells, two primary pigment cells, shared secondary and tertiary pigments cells, and a four-cell mechanosensory bristle organ comprise each ommatidia (reviewed in Bonini and Fortini 1999; Thomas and Wassarman 1999; Baker 2001; Frankfort and Mardon 2002). As the mechanism of Drosophila eye development is highly reproducible and consistent, the eye is a very useful tool for the study of subtle abnormalities such as subtle defects in a single
cell type or fraction of cells of a certain type. Moreover, in the laboratory, the phenotype of the eye does not influence viability and fertility.

Approximately two thirds of the 13,600 genes in Drosophila are required for eye development, with the vast majority of these being required for general cellular processes (Sang and Jackson 2005). The study of the Drosophila eye has proven to be a powerful means to investigate gene function and is used to study cell growth and differentiation, programmed cell death, and tissue patterning (Kramer and Staveley 2003; Kramer et al. 2003; reviewed in Thomas and Wassarman 1999; Frankfort and Mardon 2002; Sang and Jackson 2005). Given its ability to show subtle, yet significant results, the Drosophila eye is an important tool for studying the effects of gene expression.

The Drosophila notum as a model of neurogenesis

Microchaetae development on the dorsal notum of flies is a measure of neurogenesis, as bristles are mechanosensory organs formed from proneural cells (Jan and Jan 1994). For the most part, the dorsal notum is a two dimensional sheet of sensory bristles called microchaetae, or small bristles, and macrochaetae, or larger bristles. In the first stages of neural development, the proneural genes are turned on in the proneural clusters, giving those cells the potential to become neural precursors. Within those proneural clusters, the cells compete with each other through the actions of neurogenic genes, including Notch, Delta and the enhancer of split gene complex, so that only a subset of the cells give rise to neural precursors. These neural precursors will eventually give rise to the mechanosensory bristle organs. Therefore, the number of sensory bristles formed on the dorsal notum can be correlated to the number of sensory neurons (Jan and
Jan 1994). Although not as widely used as the eye, the dorsal notum has been used to study the Notch signaling pathway genes (Ramain et al. 2001) as well as the effects of Hipl gene expression on neurogenesis (Moores et al. 2008). With its regular pattern of macrochaetae and microchaetae, the Drosophila dorsal notum is a reliable means to study neurogenesis.

**Down syndrome: Characteristics of the disease**

Down syndrome (DS) is a neurogenerative disorder affecting one in 700 live births and is the most frequent cause of mental retardation in humans (Jacobs et al. 1959; Coyle et al. 1986). Individuals with DS typically have a reduction in brain size and brain weight, reduction of neurons in the cerebellum and the hippocampus, reduction of the cholinergic neurons of the basal forebrain and the granular layers of the cerebral cortex, and several abnormal neuronal differentiation processes (Guimerá et al. 1996). In addition to neuronal defects, DS patients have congenital heart abnormalities, seizure disorders, low muscle tone, gastrointestinal malformations, defects of the endocrine and immune system, increased rate of leukemia and early onset Alzheimer disease (Coyle et al. 1986; Korenberg et al. 1994). Given the prevalence and severity of this condition, the genetic and molecular causes of this condition must be fully understood.

**Down syndrome: Genetic causes**

DS is the result of a total or partial triplication of human chromosome 21. Studies of rare cases of partial trisomy of chromosome 21 have resulted in the identification of a small section of this chromosome, 21q22.2, known as the “Down syndrome critical
region” (DCR-1), which is responsible for the phenotypes commonly observed in DS patients (Delabar et al. 1993; Antonarakis 2001). One of the best studied candidate genes located in DCR-1 is the “dual specificity tyrosine phosphorylation-regulated kinase”, or “dual-specificity YAK1-related kinase A”, Dyrk1A, and this gene has been investigated in brain function and development (Guimera et al. 1996). Studies of the role of Dyrk1A is a promising avenue of research to understand the mechanisms underlying Down syndrome.

Identification and nomenclature of Dyrk1A/mnb

The acronym “Dyrk1A” refers to the ability of this family of kinases to phosphorylate serine/threonine and tyrosine residues and to sequence similarity with the protein kinase YAK1 from Saccharomyces cerevisiae (Becker and Joost 1999). In addition to the kinase domain, Dyrk1A contains a stretch of histidine repeats and a nuclear localization signal (Becker and Joost 1999). The first vertebrate Dyrk1A was identified in rodent models using a polymerase chain reaction (PCR) cloning method specific to the protein kinases (Kentrup et al. 1996). The human version of the gene was discovered by screening a human cDNA library with a putative exon isolated using an exon-trapping method, and verified to be a Dyrk1A gene based upon sequence similarities with the rat and Drosophila versions (Shindoh et al. 1996). The highly conserved nature of Dyrk1A suggests the importance of this gene in normal development.

The Drosophila homologue of the human Dyrk1A is minibrain (mnb), the first member of the Dyrk family discovered. The mnb gene was identified in Drosophila through analysis of the mnb mutations, named due to the phenotypic “smaller brain" in
adult \textit{mnb} mutants and was characterized by positional cloning (Tejedor \textit{et al.} 1995). The human and Drosophila versions of the \textit{mnb} protein exhibit a 75\% amino acid sequence similarity (Shindoh \textit{et al.} 1996). Although \textit{mnb} was first described over a decade ago, very little research has been described in Drosophila since then.

\textbf{Dyrk1A/mnb interacts with a large number of proteins}

Phosphorylation is an important mechanism of signal transduction in eukaryotic cells (Becker and Joost 1999). Dyrk1A/mnb’s ability to catalyze tyrosine-directed autophosphorylation and proline-directed phosphorylation of serine/threonine residues on exogenous substrates allows it to act with a high degree of versatility and substrate specificity. Dyrk1A/mnb has been shown to phosphorylate substrates in the nucleus such as STAT3 (signal transducer and activator of transcription 3) (Matsuo \textit{et al.} 2001), cyclin L2 proteins (de Graaf \textit{et al.} 2004), chromatin remodeling factors, SNR1 and TRX (Kinstrie \textit{et al.} 2006), and the splicing factor SF3b1/SAP155 (de Graaf \textit{et al.} 2006). In addition, the transcription factor Arip4 (androgen receptor-interacting protein 4) (Sitz \textit{et al.} 2004) and the eukaryotic protein-synthesis initiation factor eIF2Be (eukaryotic initiation factor 2Be) (Woods \textit{et al.} 2001a) are phosphorylated by Dyrk1A/mnb. Dyrk1A/mnb induces cAMP response element (CRE) mediated gene transcription by activating c-AMP response element binding protein (CREB) (Yang \textit{et al.} 2001), Gli1 (Mao \textit{et al.} 2002), the forkhead transcription factor, foxo1 (Woods \textit{et al.} 2001b), and the NFAT (nuclear factor of activated T cells) family of transcription factors (Gwack \textit{et al.} 2006), suggesting a role for \textit{Dyrk1A/mnb} in gene expression. Proteins in the cytoplasm including 14-3-3 (Kim \textit{et al.} 2004), glycogen synthase (Skurat and Dietrich 2004),
dynamin (Chen-Hwang et al. 2002), and the brain specific protein, PAHX-AP1 (phytanoyl-CoA α-hydroxylase associated protein 1) are also phosphorylated by Dyrk1A/mnb (Bescond and Rahmani 2005). Dyrk1A/mnb also interacts with tau, a microtubule-associated protein (Woods et al. 2001a). This broad range of interactions suggests that Dyrk1A/mnb may be involved in numerous signaling pathways in vivo.

**Dyrk1A/mnb studies in vertebrates**

An extra copy of Dyrk1A/mnb is hypothesized to play a major role in the developmental anomalies associated with DS (Wegiel et al. 2004). Dyrk1A/mnb is expressed in regions of the embryonic and adult rat brain that include the cerebellum, cerebral cortex, hippocampus and brain stem, as well as the heart (Okui et al. 1999; Marti et al. 2003). Northern blot analysis with mRNA from human and mouse fetal brain tissue revealed that Dyrk1A/mnb is over-expressed in the fetal DS human brain as well as in a mouse model of DS (Guimera et al. 1999). In the developing and adult human brain, Dyrk1A/mnb was found in the nucleus and cytoplasm of neurons (Wegiel et al. 2004). The presence of Dyrk1A/mnb in the nucleus suggests that it may play a role in the control of gene expression. In mouse brain development, the first wave of Dyrk1A/mnb expression participates in the transition of neural progenitor cells from proliferating to neurogenic divisions. This suggests that Dyrk1A/mnb plays a role in the neurogenesis of the vertebrate brain (Hämmerle et al. 2002). A second wave of Dyrk1A/mnb expression occurs in intermediate and late stage embryos during dendritic tree differentiation. In these stages, Dyrk1A/mnb expression is initiated by the translocation from the cytoplasm to the nucleus and then movement to the growing dendritic tree where the Dyrk1A/mnb
protein colocalizes with dynamin 1, a key protein in receptor-mediated endocytosis (Hämmerle et al. 2003). Murine models of DS that over-express DyrklA/mnb display motor, learning and memory deficits in behavioural studies that seem to recapitulate the cognitive defects observed in DS (Smith et al. 1997; Branchi et al. 2004; Martínez de Lagrán et al. 2004). Transgenic mice that contain only one copy of the DyrklA/mnb gene show significant impairment in hippocampal-dependent memory tasks (Ahn et al. 2006). These studies indicate a significant role for DyrklA/mnb in learning and memory. Mutant DyrklA/mnb mice have a decrease in brain size and changes in neuron density in selected brain regions (Fotaki et al. 2002). Alternatively, mice over-expressing DyrklA/mnb have an increase in brain weight (Branchi et al. 2004). Collectively, all of these studies suggest that DyrklA/mnb is involved in the neuropathological changes seen in patients with DS.

DyrklA/mnb studies in non-vertebrates

The DyrklA/mnb enzyme is highly conserved evolutionarily, showing structural, enzymatic and functional similarities in organisms as diverse as yeast and humans (Becker and Joost 1999). In Saccharomyces cerevisiae, the DyrklA/mnb homologue is Yaklp, which negatively regulates cell growth by acting as an antagonist to the Ras/cAMP-dependent protein kinase pathway (Smith et al. 1998). In Dictyostelium, YakA is needed for starvation-induced growth arrest and initiation of a developmental response, as well as to mediate developmental events and facilitate exit from the cell cycle (Souza, et al. 1998). Three homologues of DyrklA/mnb have been examined in Caenorhabditis elegans termed mbk-1, mbk-2 and hpk-1; the first is the most similar in
amino acid sequence to Dyrk1A/mnb. Although the loss of mbk-1 has no obvious defects, over-expression of mbk-1 causes behavioural defects in chemotaxis toward volatile chemoattractants, with the extent of the defects increasing with the gene dosage (Raich et al. 2003). The conservation of the Dyrk1A/mnb gene in organisms as unrelated as yeast and humans is a testament to the importance of its function.

**mnb studies in Drosophila**

Adult central nervous system development in *Drosophila melanogaster* involves a precise pattern of neuroblast proliferation. The Dyrk1A/mnb gene has been found to play a role in postembryonic neurogenesis by regulating the number and type of neurons that are formed. Flies with mutations in the Dyrk1A/mnb gene have significantly reduced optic lobes and central brain hemispheres due to a decrease in the number of cells generated during the proliferative process (Tejedor et al. 1995). This finding is consistent with the finding that mutant Dyrk1A/mnb mice have reduced brain size, and suggests a conserved mode of action determining brain size and normal growth in flies and mice (Lochhead et al. 2003). The Dyrk1A/mnb mutant flies also show learning and memory defects (Tejedor et al. 1995) similar to those found in humans (Coyle et al. 1986), showing the high conservation of the function of this gene in flies and humans.

Previous studies using Drosophila as a model organism have shown that Dyrk1A/mnb participates in development of the Drosophila eye and controls growth and survival through modification of insulin receptor signaling (Rotchford and Staveley, unpublished). Flies with mnb mutations were found to be phenotypically similar (ie. smaller than normal adult size) to flies with mutations in akt and over-expression of
foxl. This showed that mnb is required for normal growth and may act through the foxol protein to regulate cell size. Finally, over-expression of mnb was found to suppress loss of bristles and ommatidia in the eye caused by foxol over-expression, suggesting that mnb negatively regulates foxol (Ratchford 2006).

Huntington disease: Characteristics of the disease

Huntington disease (HD) is an autosomal dominantly inherited neurodegenerative disorder, affecting four to eight people per 100,000 (Harper 1992). Characteristic symptoms of the disease include cognitive, emotional and motor dysfunctions (Petersén et al. 1999). Cognitively, patients experience decreases in mental processes, reduced mental flexibility, and disturbances in memory and intellectual ability, which lead to dementia. Depression and manic-depression may accompany cognitive disturbances. Motor function defects are the most common and well known features of HD, and involve disturbances in both voluntary and involuntary movements. In addition, patients with juvenile HD have an increase rate of seizures, rigidity and tremors. Often patients display loss of body weight and muscle bulk. Symptoms often develop between the ages of 35 to 45, but age of onset can vary depending on the extent of genetic defect. In adult-onset HD, death usually occurs within 15 to 20 years of onset. Juvenile cases progress faster, with death occurring in 7 to 10 years. Pathologically, there is a loss of the GABAergic medium-sized spiny neurons in the striatum and cortex of the brain. These neurons innervate the substantia nigra and globus pallidus (Petersén et al. 1999). Aggregates of N-terminal fragments of mutant huntingtin form in the nucleus and cytoplasm of neurons in the brain (DiFiglia et al. 1997). The lack of treatment as well as
the great hardship for patients and their families highlights the importance of research on the mechanisms leading to HD.

**Huntington disease: Genetic causes**

HD is the result of an expanded CAG (cytosine, adenine, guanine) trinucleotide repeat in the gene *huntingtin* (*htt*), which encodes a protein of the same name whose routine function is relatively unknown (Ranen *et al.* 1995). The *htt* gene is located on chromosome 4 and was isolated using restriction-fragment length polymorphism mapping (Petersén *et al.* 1999). The *htt* protein has an amino terminal polyglutamine (polyQ) stretch and mutant forms have an expanse of Q’s that is greater than 36 to 39 residues in length. Below this pathogenic threshold, individuals are unaffected (Snell *et al.* 1993; Rubinsztein *et al.* 1997). However, there are some cases where individuals with 36 to 39 repeats survive into old age without developing symptoms, suggesting that the HD mutation is not fully penetrant in some cases (Rubinsztein *et al.* 1997). Nevertheless, there is an inverse relationship between the age of onset and the severity of the disease with the number of residues present, such that repeats above 100 cause juvenile-onset of HD, whereas 40 to 50 residues are seen in adult-onset HD (Snell *et al.* 1993). High-repeat-number alleles tend to undergo germline mutations that increase repeat number, leading to an increase in the length of the polyQ stretch from one generation to the next. This means that the disease can be present at earlier ages in successive generations (Rubinsztein *et al.* 1997). Although the proximate genetic cause is known, there are still many unanswered questions about the mechanism that leads to the trinucleotide repeat/polyQ stretch manifesting as HD.
Identification and studies of huntingtin

The finding that amyloid-like protein aggregates form in brains of the transgenic mouse model of HD led researchers to investigate this aspect of the pathology in humans (Scherzinger et al. 1997). Nuclear inclusions had previously been found in HD patients, but they had not been investigated (Petersén et al. 1999). This led researchers to perform immunohistochemical mapping of the striatal biopsies taken from HD individuals. The nuclear inclusions contained mutant forms of the huntingtin protein, which led to further research on this protein in the pathogenesis of HD (Petersén et al. 1999).

Several research groups have established mouse models of HD, such as a yeast artificial chromosome (YAC) transgenic mouse containing an htt gene with 46 CAG repeats (Hodgson et al. 1999). This mouse shows no behavioural defects or neuronal loss, but it does have a reduction in long-term potentiation in hippocampal sections. Another mouse model with 46 repeats shows striatal neuronal inclusions and hyperactivity (Laforet et al. 1998). Additionally, invertebrate models, such as C. elegans have been engineered to express polyQ expanded htt proteins, and show that htt fragments cause nuclear aggregation and neurodegeneration (Morley et al. 2002). Studies on mice, rats, flies, C. elegans and pufferfish suggest that the gene is highly conserved, indicating an important cellular function (Sharp and Ross 1996). Many studies have shown a role for huntingtin in vesicle trafficking in which both normal and mutant huntingtin associate with clathrin-coated vesicles (Velier et al. 1998). Mutant htt forms aggregates that impede the function of the endocytic pathway (Qin et al. 2004). Despite these studies, the role of htt is not well understood and proteins that interact with it are under investigation.
Studies of huntingtin in Drosophila

There are a number of Drosophila models of polyglutamine expansion disease which display cell death and protein aggregation much like that seen in human HD patients (reviewed in Rubinsztein 2002). These models show declined motor performance, decreased flying ability and a reduced survival rate (Romero et al. 2008). Drosophila models have shown that polyglutamine proteins can cause defects in axonal transport (Gunawardena et al. 2003) and induce neuronal degeneration of photoreceptors (Jackson et al. 1998) that occurs over a period of about 20 days (Romero et al. 2008), or about two-thirds of a fly’s life cycle. Attempts to find treatment for HD have been made using Drosophila as a model. Drosophila models of HD have been used to identify compounds that may be used as protein aggregation inhibitors (Desai et al. 2006). Lithium and rapamycin used in combination show greater protection against neurodegeneration in Drosophila than either treatment used on its own (Sarkar et al. 2008). Finally Drosophila models of HD can be used to test the effectiveness of drug combinations as testing is rapid and potentially therapeutic compounds can be administered in low doses, decreasing costs (Agrawai et al. 2005). Drosophila models of HD have proven to be effective models for testing the development of HD pathology as well as methods for treating the disease.

Huntingtin-interacting proteins

Understanding the functions of proteins that interact with htt may help clarify the cellular mechanisms by which polyglutamine expansion of the htt protein manifests as HD. The first of the huntingtin-interacting proteins discovered was huntingtin-associated
protein (Hap1) (Li et al. 1995). Hap1 could play a role in HD as its expression is higher in the brain than in other tissues. However, it is found in areas of the brain that are not affected in HD and the interaction of Hap1 with htt is increased with increased polyglutamine expansion. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) interacts with both normal and mutant huntingtin (Burke et al. 1996). GAPDH is a multifunctional enzyme that plays a role in glycolysis, DNA repair, and replication and endocytosis. GAPDH directly interacts with the polyglutamine tail of huntingtin.

Huntingtin-interacting protein 2 (Hip2) was identified as a ubiquitin-conjugating enzyme, which may play a role in the ubiquitination of huntingtin (Kalchman et al. 1996). Although Hip2 is expressed in the brain regions affected in HD, there is no correlation between the length of polyglutamine residues and the extent of binding of Hip2.

Huntingtin has been show to form a complex with calmodulin (Bao et al. 1996). Normal huntingtin binds to calmodulin in a calcium-dependent manner, but mutant huntingtin has been demonstrated to not require calcium to interact with calmodulin.

Huntingtin-interacting protein 1 (Hip1) is perhaps the most interesting protein that binds to htt, due to its differential binding to normal and mutant htt (Kalchman et al. 1997; Wanker et al. 1997). Binding of Hip1 to huntingtin is reduced dramatically with polyglutamine expansion. The interaction between Hip1 and htt presumably occurs at the membrane, as both proteins are found bound there (Kalchman et al. 1997). Hip1 is predominantly expressed in the central nervous system, and colocalizes with htt in neuronal cells and clathrin-coated vesicles (Kalchman et al. 1997; Wanker et al. 1997). Hence, Hip1 is a strong candidate to reveal functional differences in normal and mutant htt.
**Hip1 characterization**

Hip1 is a 120kDa protein (Gervais et al. 2002) identified through a yeast-two hybrid screen because it binds to the amino terminus of huntingtin (Kalchman et al. 1997). Through comparative genomic analyses, Hip1 was shown to have 45% amino acid sequence similarity to Sla2p (synthetic lethal with actin binding protein [ABP] 1), the cytoskeletal assembly gene of *S. cerevisiae* (Holtzman et al. 1993). Sla2p encodes a protein involved in vesicle trafficking, endocytosis and cortical actin cytoskeleton formation. In addition, Hip1 is homologous to the *Caenorhabditis elegans* gene, ZK370.3, a gene of unknown function (Morley et al. 2002). The three proteins, Hip1, Sla2p and ZK370.3, have similar molecular masses and carboxy-terminal domains (Kalchman et al. 1997). Hip1 has four domains, an AP-180 N-terminal homology domain (ANTH), previously referred to as ENTH (epsin N-terminal homology domain), which allows the protein to interact with membranes that contain phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂), a pseudo-death effector domain (pDED) for protein-protein interactions, a central coiled-coil domain for clathrin binding and targeting to clathrin coated vesicles, and a C-terminal talin-like domain that binds to actin (Engqvist-Goldstein et al. 2001; Legendre-Guillemin et al. 2002; Hyun et al. 2004). This domain structure suggests that Hip1 plays a role mediating interactions between the cell membrane and/or membrane-bound vesicles and the cytoskeleton (Chen and Brodsky 2005). The complex structure of Hip1 suggests that the Hip1 protein has complex functions and knowing the structure of Hip1 and functions of its domains may provide clues to the mechanisms that give rise to HD.
**Hip1 functions in cell death, cell growth and endocytosis**

Hip1 has been identified as a pro-apoptotic protein as over-expression leads of 
*Hip1* to caspase-dependent cell death (Hackam et al. 2000). Furthermore, Hip1 contains 
a domain that has a sequence homology to the death effector domains found in other pro-
apoptotic proteins. When expressed alone in tissue culture, the pDED of Hip1 is 
sufficient to cause cell death, indicating the role of this domain in apoptosis. 
Interestingly, the severity of Hip1 toxicity is increased with a longer polyglutamine tail 
(Hackam et al. 2000). Polyglutamine expansion may cause the release of Hip1 to 
activate apoptosis in striatal neurons in a caspase-dependent manner. This theory is 
supported by the finding that the main pathological defect in HD patients is the loss of 
medium spiny neurons in the striatum (Gervais et al. 2002). Alterations in *Hip1* 
expression can lead to death of cells, much like the loss of neurons observed in HD 
brains.

*Hip1* has been implicated as a contributor of tumour formation and growth. 
Expression of *Hip1* has been found in many primary human epithelial cancers, including 
breast, colon and prostate cancer, and is correlated with increasing aggressiveness of 
prostate cancer (Rao et al. 2003). Clearly, *Hip1* plays a significant role in the balance 
between cell death and the uncontrolled growth seen in cancer.

Hip1 has been found to participate in receptor-mediated endocytosis by promoting 
clathrin-coated vesicle assembly (Chen and Brodsky 2005). Clathrin-coated vesicles are 
important for selectively transporting proteins for receptor mediated endocytosis, a 
process that ensures the fast and specific retrieval of synaptic vesicle membranes. The 
clathrin molecule has a triskelion shape and is composed of both heavy and light clathrin
chains (Chen and Brodsky 2005). Trimerized heavy chains, each with a covalently-bound light chain, form polyhedral lattice-coated triskelia. Typically, proteins affect receptor-mediated endocytosis by influencing coat assembly, membrane association, membrane fission and uncoating by binding to heavy chains (Chen and Brodsky 2005).

Hip1 has been identified to bind to the clathrin adapter protein (AP2) and the clathrin heavy chain (Metzler et al. 2001). The light chains function to prevent spontaneous assembly and promote controlled assembly by other regulatory proteins. Hip1 is one of the proteins that bind to light chains (Chen and Brodsky 2005). In addition, because Hip1 binds both clathrin and PtdIns (4, 5) P₂, it is thought to link the triskelia to the lipid membrane (Metzler et al. 2001). According to these studies, Hip1 certainly plays a role in receptor-mediated endocytosis, but the specific function of Hip1 has not yet been described in detail.

**Study of Hip1 in Drosophila**

To facilitate the biological characterization of Hip1, the Drosophila homologue of Hip1 was isolated in our laboratory (Moores et al. 2008). Compared to humans, the Drosophila genome has a highly conserved Hip1 gene that contains the AP180 N-terminal homology (ANTH) domain, pseudo death effector domain (pDED) and the talin-like domain. There are two versions of the putative protein, a full length version and an alternative transcript that yields a version missing the N-terminal ANTH domain, referred to as Hip1ΔANTH. Hip1 regulates neurogenesis in flies as revealed by microchaetae analysis in the adult dorsal notum (Moores et al. 2008). While over-expression of full length Hip1 decreases microchaetae bristle formation, the ΔANTH version, presumably
acts to interfere through an inhibitory activity act, produces increased bristle density on the notum (Moores et al. 2008). Since the number of bristles can be correlated to the number of sensory bristles formed, this indicates a dual-regulatory role for Hip1 in neurogenesis (Ramain et al. 2001; Moores et al. 2008). The mechanism by which Hip1 influences neurogenesis seems to be through the non-canonical Notch signaling pathway, where deltex appears to have the greatest influence (Moores et al. 2008). The study by Moores et al. was the first account of a role for Hip1 in neurogenesis.

A link between Hip1 and Dyrk1A/mnb

A biochemical interaction has been identified between Hip1 and Dyrk1A/mnb using the yeast two-hybrid assay (Kang et al., 2005). During neuronal differentiation of embryonic hippocampal neuroprogenitor H19-7 cells, Dyrk1A/mnb selectively bound to and phosphorylated Hip1, which was induced by the addition of bFGF, a fibroblast growth factor. Strong complexes formed between Hip1 and Dyrk1A/mnb. The binding of these two proteins resulted in blockage of Hip1-induced cell death and contributed to neurite outgrowth. Upon the addition of etoposide phosphate, the binding decreased and phosphorylation of Hip1 diminished. No longer bound to Dyrk1A, Hip1 was free to bind to caspase-3 to activate cell death. On the basis of a clear, functional interaction established in tissue culture cells, the current study aims to investigate the biological interaction between the Dyrk1A/mnb, and Hip1 in Drosophila melanogaster as a model organism.
Goals of this research

This project will utilize *Drosophila melanogaster* to study the biological effects of *mnb* and *Hip1* gene expression. Experiments that examined *Dyrk1A/mnb* ectopic expression in Drosophila have shown that *Dyrk1A/mnb* controls growth through modification of insulin receptor signaling (Rotchford 2006; Rotchford and Staveley, unpublished). The first goal of the current study is to investigate a potential role for components of the insulin receptor pathway and *Dyrk1A/mnb* in neurogenesis. Specifically, I will direct the expression of these genes using the *pannier-* and *apterous* *Gal4* lines to the dorsal notum and perform biometric analysis on the tissue. Second, previous studies in our laboratory have characterized the role of the two versions of the Hip1 protein, a full length version containing a lipid-binding domain, and a truncated version lacking the lipid-binding domain, Hip1ΔANTH, in Drosophila neurogenesis (Moores *et al.* 2008). My goal is to analyze the effect of *Hip1* on eye development by directing the expression of *Hip1* to the developing eye using the *GMR* transgene and performing biometric analysis. Finally, as a biochemical link has been made between *mnb* and *Hip1*, the biological consequences of aspects of this proposed interaction will be studied in the Drosophila eye by expressing the *mnb* and *Hip1* genes together in a *foxo1*-sensitized background and analyzing the eyes biometrically. Using the Drosophila model will allow the characterization of subtle alterations in the development and growth of the adult eye and notum.
Materials and Methods

Investigating the role of mnb, akt, and foxo1 in neurogenesis

Drosophila stocks

The y w; apterousGal4/CyO (apGal4) and y w; pannierGal4/TM3, UAS, y+ (pnrGal4) transgenics (Calleja et al. 1996) were obtained from Bloomington Drosophila Stock Center (University of Indiana, Bloomington). The y w^67c23 mnb^EY14320 (UASmnb) (Bellen et al. 2004) stocks were created by members of the Drosophila Gene Disruption Project and provided by Bloomington Drosophila Stock Center. The w; UASAkt^1.1/CyO line (UASakt) was described in Staveley et al. (1998), and the inducible murine foxo1 homologue, w; UASfoxo1/CyO (UASfoxo) was described in Kramer et al. (2003). The w; UASlacZ^4-1-2 (UASlacZ) (Brand and Perrimon 1994) and the w; UASGFP (UASGFP) (Yeh et al. 1995) control lines were obtained from the Bloomington Drosophila Stock Center. Table 1 summarizes these genotypes, their sources, and abbreviations used throughout the thesis.
Table 1. The genotypes of stocks used to study the potential role of *mnb*, *akt*, and *foxo1* in neurogenesis, their sources, and abbreviations used throughout the thesis.

<table>
<thead>
<tr>
<th>Full Genotype</th>
<th>Reference</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td><strong>Transgenic transcription factors</strong></td>
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<td><em>y w; apterousGal4/CyO</em></td>
<td>Calleja <em>et al.</em> 1996</td>
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<td>Calleja <em>et al.</em> 1996</td>
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<td><strong>Control genes</strong></td>
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<td><em>w; UASGFP</em></td>
<td>Yeh <em>et al.</em> 1995</td>
<td><em>UASGFP</em></td>
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<tr>
<td><em>w; UASlacZ+/−</em></td>
<td>Brand and Perrimon 1994</td>
<td><em>UASlacZ</em></td>
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<td><strong>Experimental genes</strong></td>
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</tr>
<tr>
<td><em>y w⁶⁷c²³ mnb**EY14320</em></td>
<td>Bellen <em>et al.</em> 2004</td>
<td><em>UASmnb</em></td>
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<tr>
<td><em>w; UASAkt**1−1/CyO</em></td>
<td>Staveley <em>et al.</em> 1998</td>
<td><em>UASakt</em></td>
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<td><em>w; UASfoxo1/CyO</em></td>
<td>Kramer <em>et al.</em> 2003</td>
<td><em>UASfoxo1</em></td>
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</table>
Drosophila culture

To investigate the roles of mnb and components of the insulin signaling pathway, akt and foxol, in neurogenesis, four to six males carrying the UASmnb, UASakt, and UASfoxol responsive genes were each crossed to eight to ten virgin females of the apGal4 and pnrGal4 fly lines. Control crosses were set up using four to six males of UASlacZ and UASGFP, which were crossed to eight to ten virgin females from both the apGal4 and pnrGal4 fly lines. Crosses were maintained on standard cornmeal yeast molasses agar media at 25°C. Parental flies were transferred to fresh media after four days to increase production of progeny. Critical class males and females were collected based on an absence of CyO for apGal4 crosses and TM3; UAS, y⁺ for pnrGal4 crosses (Figure 1 and 2). Critical class individuals were aged in fresh vials for three to five days, then placed in 1.5 mL microcentrifuge tubes and frozen at -70°C.
Go:  

a. *w; UASlacZ*  
b. *w; UASGFP*  

\[ \times \]  
c. *yw UASmnb*  
d. *w; UASAkt^{1.1}/CyO*  
e. *w; UASFoxo1/CyO*  

G1:  

a. *w; UASlacZ/apGal4*  
b. *w; UASGFP/apGal4*  

\[ \downarrow \]  
c. *yw UASmnb; apGal4^{*}  
d. *w; UASAkt^{1.1}/apGal4*  
e. *w; UASFoxo1/apGal4^t*  

Figure 1. Schematic diagram of crosses made to examine the potential effects of *mnb, akt* and *foxo1* on neurogenesis using the *apterousGal4* transgene. Crosses to *UASlacZ* (a) and *UASGFP* (b) were conducted as controls. Experimental crosses were performed for *UASmnb* (c), *UASAkt* (d) and *UASFoxo1* (e). *Note: no critical class offspring were recovered from this cross.*  
*Note: only female progeny were collected from this cross.*
Figure 2. Schematic diagram of crosses made to examine the potential effects of mnb, akt, and foxo1 on neurogenesis using the pnierrGal4 transgene. Crosses to UASlacZ (a) and UASGFP (b) were conducted as controls. Experimental crosses were performed for UASmnb (c), UASAkt1 (d), and UASfoxo1 (e). *Note: only female progeny were collected from this cross.
Biometric analysis of dorsal notum microchaetae

The density of microchaetae on the dorsal notum has been characterized in Notch mutants as a measure of neurogenesis (Ramain et al. 2001). Flies were mounted on aluminum scanning electron microscope studs with the dorsal notum facing upward. Studs were desiccated overnight and then gold coated using either a S150 Gold Sputter Coater or an EMSK550 Gold Sputterer. Samples were photographed using a Hitachi 570 scanning electron microscope or a FEI Quanta 400 environmental scanning electron microscope. Micrographs taken on the Hitachi 570 scanning electron microscope were photographed at 80X magnification, while those taken on the FEI Quanta 400 environmental scanning electron microscope were photographed at 250X magnification due to the differences in the calibrations of the microscopes. Micrographs were analyzed using Image J digital image analysis software (http://rsbweb.nih.gov/ij/) (Abramoff et al. 2004). The number of microchaetae on the dorsal notum were counted for each image. Total dorsal notum area (μm²) was also calculated. The microchaetae counts and notum area were used to calculate the bristle density, expressed as number of microchaetae per 100 μm². Bristle density values were exported into the GraphPad Prism 4 program (by GraphPad Software, Inc.) and mean ± standard error of the mean (SEM) are plotted for each individual genotype. Males and females were analyzed separately due to expected phenotypic differences in size. Groups were subjected to a one-way ANOVA analysis with Neuman-Keuls post-tests, using GraphPad Prism 4, to determine significance between pairs.
The potential role of Huntingtin interacting protein-1 and minibrain in eye development using GMRGal4

Drosophila stocks

The UASmnb stock was described above. The two independent isolates of the full length version of the Hip1 gene, w; L/CyO; UASHip1L-2/TM3,Sb (UASHip1L-2) and w; L/CyO; UASHip1L-6/TM3,Sb, (UASHip1L-6) and the two isolates of the amino terminal truncated form of the Hip1 gene, UASHip111.2 and UASHip15.2 (referred to as Hip12ANTH) were described in Moores (2006). Both full length versions of Hip1 were crossed to w; Ly/TM6B (obtained from Staveley laboratory stock) to eliminate the second chromosome balancer. Male and female progeny with CyO and TM6B were backcrossed to each other to obtain w; UASHip1L-2/TM6B and w; UASHip1L-6/TM6B lines (Appendix 1). The w; GMRGal412 transgenic line was described in Freeman (1996). Table 2 summarizes these genotypes and their sources.
Table 2. The genotypes of stocks used to study the potential role of *Hip1*, *Hip1ΔANTH* and *mnb* in eye development, their sources, and abbreviations used throughout the thesis.

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<td>UASmnb</td>
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</table>
Drosophila culture

To examine the role of mnb in eye development, two to four males of UASmnb were crossed to six to eight virgin females of GMRGal4. To investigate the role of both the full length and truncated versions of the Hip1 gene on eye development, two to four males of UASHip1L-2, UASHip1L-6, UASHip111.2 and UASHip15.2 lines were each crossed to four to six virgin female GMRGal4. Control crosses were conducted using two to four males of UASlacZ and UASGFP and four to six virgin females from the GMRGal4 line (Figure 3). Crosses were maintained at 25°C on standard cornmeal yeast molasses agar media. Parental flies were transferred to fresh media after four days to increase production of progeny. All progeny were collected for GMRGal4 crosses as there was no balancer chromosome to select against. Collected flies were aged in fresh vials for three to five days, placed in microcentrifuge tubes and frozen at -70°C.
Figure 3. Schematic diagram of crosses made to examine the potential effects of *Hipl*, *HiplANTH* and *mnb* in eye development using the *GMRGal4* transgene.

Crosses to *UASlacZ* (a) and *UASGFP* (b) were conducted as controls. Experimental crosses were performed for *UASmnb* (c), *UASHiplL-2* (d), *UASHiplL-6* (e), *UASHipl15.2* (f) and *UASHipl111.2* (g).
Biometric analysis of the adult eye

Analysis of adult Drosophila eye structures has been used to reveal subtle aspects of growth and cell survival (for example Kramer et al. 2003). Flies were mounted on aluminum scanning electron microscope studs using double sided sticky tape with the left eye facing upward. Studs were then desiccated overnight and gold coated using either a S150 Gold Sputter Coater or an EMSK550 Gold Sputterer. Eyes were photographed using a Hitachi 570 scanning electron microscope or a FEI Quanta 400 environmental scanning electron microscope. Micrographs taken on the Hitachi 570 scanning electron microscope were photographed at 170X magnification, while those taken on the FEI Quanta 400 environmental scanning electron microscope were photographed at 450X magnification due to differences in the calibration of the microscopes. Micrographs were analyzed using Image J digital image analysis software (http://rsbweb.nih.gov/ij/) (Abramoff et al. 2004). The number of ommatidia and number of bristles were counted for each image. The area (in \( \mu m^2 \)) of seven ommatidia in a “honey comb” pattern was determined for three sets and the average area per ommatidia was calculated. Ommatidia number, bristle number and area of ommatidia values were exported into the GraphPad Prism 4 program (by GraphPad Software, Inc.) and means ± standard error of the means (SEM) were plotted for each of the three parameters for each individual genotype. Male and female flies were analyzed separately due to expected phenotypic differences in size. Using GraphPad Prism 4, groups were subjected to a one-way ANOVA analysis with Neuman-Keuls post-tests to determine significance between pairs and mean ± standard error of the mean (SEM) were plotted for each individual genotype.
**Interaction of foxo1 with mnb and Hip1**

*Drosophila stocks, culture and analysis*

The w; GMRGal4 UASfoxo1/CyO (GMRGal4 UASfoxo1) recombinant line was described in Kramer et al. (2003). Additional stocks used were as above in “The potential role of Huntingtin interacting protein-1 and minibrain in eye development using GMRGal4” section. To investigate the interaction of foxo1 with mnb and Hip1, two to four males of GMRGal4 UASfoxo1/CyO were crossed to six to eight virgin females of UASGFP, both versions of UASHipl, both versions of UASHip1ΔANTH, and mnb. Crosses were maintained at 25°C on standard cornmeal yeast molasses agar media. Parental flies were transferred to fresh media every four days to increase production of progeny. Both male and female progeny were collected based on a lack of CyO (Figure 4). Collected flies were aged in fresh vials for three to five days, placed in 1.5 ml microcentrifuge tubes and frozen at -70°C. Analysis was conducted as per above in the “Biometric analysis of dorsal notum microchaetae” and “Biometric analysis of the adult eye” sections.
Go:

- w; UASlacZ
- w; UASGFP
- y w UASmnb
- w; UASHip1L-2/TM6B
- w; UASHip1L-6/TM6B
- w; UASHip15.2
- w; UASHip111.2

X w; GMRGal4UASfoxo1/CyO

G1:

- w; UASlacZ/GMRGal4UASfoxo1
- w; UASGFP/GMRGal4UASfoxo1
- y w UASmnb; GMRGal4UASfoxo1
- w; GMRGal4UASfoxo1; UASHip1L-2
- w; GMRGal4UASfoxo1; UASHip1L-6
- w; UASHip15.2/GMRGal4UASfoxo1
- w; GMRGal4UASfoxo1; UASHip111.2

Figure 4. Schematic diagram of crosses made to examine the potential effects of Hip1, Hip1ΔANTH and mnb in eye development with the GMRGal4 and UASfoxo1 transgenes. Crosses to UASGFP (a) were conducted as controls. Experimental crosses were performed for UASmnb (b), UASHip1L-2 (c), UASHip1L-6 (d), UASHip15.2 (e) and UASHip111.2 (f).
Interaction of mnb and Hip1 with each other

Drosophila stocks

The w; L/CyO; Ki/TM3,Sh line was created and obtained from B. E. Staveley (Memorial University of Newfoundland). Additional stocks used were as above.

Generation of mnb; Hip1 lines

To investigate the possible interactions between mnb and Hip1, combinations of mnb transgenics with both versions of Hip1 were generated as follows:

For Hip1 on the third chromosome

Eight to ten virgin females of UASmnb were crossed to four to six males of w; Ly/TM6B. Males were selected with either Ly or TM6B and crossed to UASmnb virgin females. The UASmnb; Ly/+ progeny were crossed to UASmnb; +/TM6B progeny to obtain UASmnb; Ly/TM6B flies. The UASmnb; Ly/TM6B flies were then crossed together to establish a stock line that can be used in future crosses (Figure 5). Six to eight virgin females of UASmnb; Ly/TM6B were crossed to four males of UAS Hip1L-2, UAS Hip1L-6, and UAS Hip111.2. Male progeny was collected on the basis of TM6B presence, and crossed back to virgins with mother-like genotype (UASmnb; Ly/TM6B). Virgin females and males were collected on the basis of TM6B presence and crossed together to establish UASmnb; UASHip1L-2, UASmnb; UASHip1L-6 and UASmnb; UASHip111.2 combination lines (Figure 6).
Figure 5. Schematic diagram of crosses made to establish UASmnb; Ly/TM6B stocks to examine the potential interaction of Hip1 and Hip1ΔANTH with mnb in eye development with the GMRGal4 and UASfoxo1 transgenes.
Figure 6. Schematic diagram of crosses made to establish $UASmnb; UASHip1$ combination stock lines for $Hip1$ on the third chromosome that will be used to examine the potential interaction of $Hip1$ and $Hip1\Delta ANTH$ with $mnb$ in eye development with the $GMRGal4$ and $UASfoxo1$ transgenes.
For *Hip1* on the second chromosome

Eight to ten virgin females of *UASmnb* were crossed to four to six males of *w; L/CyO; Ki/TM3, Sb*. Males were selected with either *L/+; +/TM3, Sb* or *+/CyO; +/TM3, Sb* and crossed to *UASmnb* virgin females. The *UASmnb; L/+; +/TM3, Sb* progeny were crossed to *UASmnb; +/CyO; +/TM3, Sb* progeny to obtain *UASmnb; L/CyO; +/TM3, Sb* flies. The *UASmnb; L/CyO; +/TM3, Sb* flies were then crossed together to establish a stock line that can be used in future crosses (Figure 7). The *UASmnb; L/CyO; +/TM3, Sb* flies were crossed to *UASHip1*\(^{5.2}\). Male progeny was selected for *CyO* and TM3,Sb or *CyO* alone, and crossed back to virgins with mother-like genotype (*UASmnb; L/CyO; +/TM3,Sb*). Virgin females and males were collected on the basis of *CyO* presence and crossed together to establish *UASmnb; UASHip1*\(^{5.2}\) combination lines (Figure 8).
Figure 7. Schematic diagram of crosses made to establish UASmnb; L/CyO; +/TM3, Sb stocks to examine the potential interaction of Hip1 and Hip1ΔANTH with mnb in eye development with the GMRGal4 and UASfoxol transgenes.
Figure 8. Schematic diagram of crosses made to establish UASmnb; UASHipl combination stock lines for Hipl on the second chromosome that will be used to examine the potential interaction of Hipl and HiplΔANTH with mnb in eye development with the GMRGαM and UASfoxo1 transgenes.
Drosophila culture and analysis

To investigate the interaction of \textit{mnb} and \textit{Hip1} together with \textit{foxo1}, two to four males of \textit{GMRGal4 UASfoxo1/CyO} were crossed to six to eight virgin females of \textit{UASmnb; UASHip1^{L-2}, UASmnb; UASHip1^{L-6}, UASmnb; UASHip1^{5-2} and UASmnb; UASHip1^{11-2}}. Crosses were maintained at 25°C on standard cornmeal yeast molasses agar media. Parental flies were transferred to fresh media at four days to increase production of progeny. Both male and female progeny were collected based on a lack of \textit{CyO} (Figure 9). Collected flies were aged in fresh vials for three to five days, placed in 1.5 ml microcentrifuge tubes and frozen at -70°C.

Analysis was conducted as above.
Go:

a. UASmnb; UASHip1L-2/TM6B
b. UASmnb; UASHip1L-6/TM6B
c. y w UASmnb; Hip15.2/CyO
d. UASmnb; UASHip111.2/TM6B

X w; GMRIGal4UASfoxo1/CyO

G1:

a. UASmnb; GMRIGal4UASfoxo1; UASHip1L-2
b. UASmnb; GMRIGal4UASfoxo1; UASHip1L-6
c. UASmnb; UASHip15.2/GMRIGal4UASfoxo1
d. UASmnb; GMRIGal4UASfoxo1; UASHip111.2

Figure 9. Schematic diagram of crosses made to examine the potential effects of Hip1 and Hip1ΔANTH with mnb in eye development with the GMRIGal4 and UASfoxo1 transgenes. Experimental crosses were performed for UASmnb; UASHip1L-2 (a), UASmnb; UASHip1L-6 (b), UASmnb; UASHip15.2 (c) and UASmnb; UASHip111.2 (d).
Results

Investigation of neurogenesis in the dorsal notum

*there is no phenotypic effect of mnb on neurogenesis in the dorsal notum*

With the demonstrated role for Hip1 in neurogenesis and the intriguing interaction between mnb and Hip1 in cell culture, this study investigates the role of mnb on neurogenesis, using pnrGal4 and apGal4 to direct the over-expression of genes throughout the dorsal notum. The expression of pannier is greatest at the midline and diminishes laterally while the expression of apterous is highest laterally and is lowest at the midline (Calleja et al. 1996). Microchaetae density per 100 μm² was determined for electron micrographs of males and females of each genotype. As well, macrochaetae gross morphology was observed as well as differences in position and number on the dorsal notum. Results are summarized in Table 3. There was no statistically significant difference in microchaetae density in response to mnb by apterousGal4 compared to controls in females based on a one-way ANOVA (Figure 10). Similarly, expression of mnb by pannierGal4 in females showed no differences in microchaetae density compared to controls based on a one-way ANOVA (Figure 11). It should be noted that no statistically significant difference between the lacZ and GFP controls using either apterousGal4 or pannierGal4 based on a one-way ANOVA (Figures 10 and 11). Results are summarized in Table 3. There was no difference in the gross morphology, position or number of macrochaetae in response to mnb as shown in the electron micrographs in Figures 10 and 11.
A

GFP
lacZ

mnb
Figure 10. Biometric analysis of a potential role of mnb in neurogenesis with the apterousGal4 transgene in females. Directed expression of mnb in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA (n=30). Micrographs of dorsal notums are shown in A. Graphic representations of microchaetae density is shown in B (values represent mean ± SEM). The genotypes are as follows:

**GFP** UASGFP/apGal4, **lacZ** UASlacZ/apGal4, **mnb** UASmnb\textsuperscript{EY14320}/+; apGal4/+. Note: GFP and lacZ controls are the same as those used for apterousGal4 driving the expression of akt.
A

GFP

lacZ

mnb
Figure 11. Biometric analysis of a potential role of *mnb* in neurogenesis with the *pannierGal4* transgene in females. Directed expression of *mnb* in the dorsal notum had no phenotypic effect on microchaetae density based on a one-way ANOVA (n=30). Micrographs of dorsal notums are shown in A. Graphic representations of microchaetae density are shown in B (values represent mean ± SEM). The genotypes are as follows: GFP UASGFP/++; *pnrGal4*/*+, lacZ UASlacZ/++; *pnrGal4*/*+, *mnb* UASmnb^{EY14320}/++; *pnrGal4*/*+. Note: GFP and lacZ controls are the same as those used for *pannierGal4* driving the expression of *akt* and *foxo1*. 
akt over-expression affects neurogenesis in the dorsal notum but foxo1 over-expression does not show a phenotypic effect

The potential roles of akt and foxo1, two well-studied insulin receptor signaling components, were investigated in neurogenesis. The pannierGal4 and apterousGal4 transgenes were used to direct the over-expression of akt and foxo1 throughout the dorsal notum. Microchaetae density was determined for electron micrographs of males and females except that in flies with apterousGal4 driving foxo1 did not survive and could not be analyzed. As well, differences in gross morphology, position and number of macrochaetae on the dorsal notum were noted. There was no statistically significant difference, based on a one-way ANOVA, in microchaetae density in response to akt expression by apterousGal4 compared to controls for either males or females (Figures 12 and 13). Expression of foxo1 by pannierGal4 showed no differences in microchaetae density compared to controls for males and females based on a one-way ANOVA (Figures 14 and 15). However, there was an increase in the microchaetae density with akt expression by pannierGal4 compared to both the lacZ and GFP controls in females but not males. Although not different from the controls, the microchaetae density of female flies expressing foxo1 with pannierGal4 is significantly lower than the microchaetae density of flies expressing akt (Figure 14). Comparisons are summarized in Table 3. There was no difference in the gross morphology, position or number of macrochaetae in response to akt or foxo1 expression under either transgene as seen in the electron micrographs shown in Figures 12 to 15.
Figure 12. Biometric analysis of a potential role of akt in neurogenesis with the apterousGal4 transgene in females. Directed expression of akt in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA (n=30). Micrographs of dorsal notums are shown in A. Graphic representations of microchaetae density are shown in B (values represent mean ± SEM). The genotypes are as follows: GFP UASGFP/apGal4, lacZ UASlacZ/apGal4, akt UASakt/apGal4. Note: GFP and lacZ controls are the same as those used for apterousGal4 driving mnb.
Figure 13. Biometric analysis of a potential role of \( \text{akt} \) in neurogenesis with the \textit{apterousGal4} transgene in males. Directed expression of \( \text{akt} \) in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA (\( n = 30 \)).

Micrographs of dorsal notums are shown in A. Graphic representations of microchaetae density are shown in B (values represent mean ± SEM). The genotypes are as follows:

Figure 14. Biometric analysis of a potential role of akt and foxo1 on neurogenesis with the pannierGal4 transgene in females. Directed expression of foxo1 in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA (n=20), while expression of akt increases microchaetae density compared to both GFP and lacZ controls (n=30) (p < 0.01 by Neuman-Keuls post-test). Micrographs of dorsal notums are shown in A. Graphic representations of microchaetae density are shown in B (values represent mean ± SEM). The genotypes are as follows: GFP UASGFP/++; pnrGal4/+, lacZ UASlacZ/++; pnrGal4/+, akt UASakt/++; pnrGal4/+, foxo1 UASfoxo1/++; pnrGal4/+. Note: GFP and lacZ controls are the same as those used for pannierGal4 driving mnb. Bars with dissimilar superscripts indicate groups that differ significantly.
Figure 15. Biometric analysis of a potential role of *akt* and *foxo1* in neurogenesis with the *pannierGal4* transgene in males. Directed expression of *akt* (n=30) and *foxo1* (n=7) in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA. Micrographs of dorsal notums are shown in A. Graphic representations of microchaetae density are shown in B (values represent mean ± SEM). The genotypes are as follows: **GFP UASGFP/+; pnrGal4/+, lacZ UASlacZ/+; pnrGal4/+, akt UASakt/+; pnrGal4/+, foxo1 UASfoxo1/+; pnrGal4/+**.
Table 3. Comparison of microchaetae density changes in the dorsal notum in response to *mnb*, *akt*, and *foxo1* over-expression.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Transcription factor</th>
<th>Sex</th>
<th>Significance</th>
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<tbody>
<tr>
<td><em>akt</em> vs. <em>GFP</em></td>
<td>apterous</td>
<td>F</td>
<td>NS</td>
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<td><em>akt</em> vs. <em>lacZ</em></td>
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<td><em>foxo1</em> vs. <em>GFP</em></td>
<td>apterous</td>
<td>F</td>
<td>flies died</td>
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<td><em>foxo1</em> vs. <em>lacZ</em></td>
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<td>flies died</td>
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<tr>
<td><em>akt</em> vs. <em>GFP</em></td>
<td>pannier</td>
<td>F</td>
<td>↑ p &lt; 0.001</td>
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<td><em>akt</em> vs. <em>lacZ</em></td>
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<td><em>foxo1</em> vs. <em>lacZ</em></td>
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<td><em>akt</em> vs. <em>foxo1</em></td>
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<td><em>akt</em> vs. <em>foxo1</em></td>
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Arrows indicate increase (↑) or decrease (↓) in treatment.

p values based on Neuman-Keuls post tests

NS= not significant      F=female      M=male
The potential role of *Huntingtin interacting protein-1* and *mnb* in eye development using *GMRGal4*

*Hip1 has no phenotypic effect on eye development under standard growth conditions*

As *mnb* has been found to play a role in eye development, the role of *Hip1* on eye development was investigated. The *GMRGal4* transgene was used to direct the over-expression of genes throughout the developing eye. *GMRGal4* directs the expression of genes posterior to the morphogenetic furrow, a dorsoventral indentation, late in development (Freeman 1996). Ommatidia number, bristle number and ommatidia area were determined for electron micrographs of males and females. There was no difference within males or females in ommatidia number, bristle number or ommatidia area for either of the full length versions of *Hip1, Hip1L-2* and *Hip1L-6* or the truncated versions of *Hip1, Hip1ΔANTH5.2* or *Hip1ΔANTH11.2*, based on a one-way ANOVA (Figures 16 and 17). Based on a one-way ANOVA, there was no difference between the two full length versions of *Hip1* or between the two truncated versions of *Hip1* (Figures 16 and 17). The controls, *GFP* and *lacZ* were not different in males or females based on one-way ANOVA, to suggest that either of these are good controls. Electron micrographs of eyes demonstrate the similarities among experimental conditions and controls (Figures 16 and 17).
A

GFP

lacZ

Hip1^{L-2}

Hip1^{L-6}

Hip1^{ΔANTH^{5.2}}

Hip1^{ΔANTH^{11.2}}
Figure 16. Biometric analysis of a potential role of *Hip1* and *Hip1ΔANTH* on eye development using the *GMRGal4* transgene in females. Directed expression of two full length versions of *Hip1*, *Hip1L*-2 and *Hip1L*-6 and two truncated versions of *Hip1*, *Hip1ΔANTH*5.2 and *Hip1ΔANTH*11.2. *Hip1* in the eye has no phenotypic effect on bristle number (n=10), ommatidia number (n=10) or ommatidia area (n=10) compared to controls based on a one-way ANOVA. Micrographs of eyes are shown in A. Graphic representations of ommatidia area, bristle number and ommatidia size are shown in B (values represent mean ± SEM). The genotypes are as follows: GFP

UASGFP/GMRGal4, lacZ UASlacZ/GMRGal4, *Hip1L*-2 UASHip1L*-2/*; GMRGal4/*,

*Hip1L*-6 UASHip1L*-6/*; GMRGal4/*, *Hip1ΔANTH*5.2 UASHip15.2/GMRGal4,

*Hip1ΔANTH*11.2 UASHip111.2/*; GMRGal4/*.
Figure 17. Biometric analysis of a potential role of Hip1 and Hip1ΔANTH on eye development using the GMRGal4 transgene in males. Directed expression of two full length versions of Hip1, Hip1L-2 and Hip1L-6, two truncated versions of Hip1, Hip1ΔANTH5.2 and Hip1ΔANTH11.2 have no phenotypic effect on bristle number (n=10), ommatidia number (n=10) and ommatidia area (n=10) compared to controls based on a one-way ANOVA. Micrographs of eyes are shown in A. Graphic representations of ommatidia area, bristle number and ommatidia number are shown in B (values represent mean ± SEM). The genotypes are as follows: GFP UASGFP/GMRGal4, lacZ UASlacZ/GMRGal4, Hip1L-2 UASHip1L-2/+; GMRGal4/+, Hip1L-6 UASHip1L-6/+; GMRGal4/+, Hip1ΔANTH5.2 UASHip15.2/GMRGal4, Hip1ΔANTH11.2 UASHip111.2/+; GMRGal4/+.
*mnb has no phenotypic affect on eye development*

Studies in our laboratory have found that the over-expression of *mnb* along with *foxol* over-expression disrupts wild-type eye development (Rotchford 2006). However, the directed expression of *mnb* has not been carefully studied in eye development. This study utilized the GMRGal4 transgene to direct the over-expression of *mnb* throughout the developing eye. Ommatidia number, bristle number and ommatidia area were determined for electron micrographs of females only. There was no difference in ommatidia number, bristle number for ommatidia area formed on the eye between experimental samples and controls based on one-way ANOVA (Figure 18). Electron micrographs show the similarity between *mnb* and control eyes (Figure 18).
A

GFP

lacZ

mnb
Figure 18. Biometric analysis of a potential role of mnb in eye development using the GMRGal4 transgene in females. The directed expression of mnb has no phenotypic effect on ommatidia area (n=10), bristle number (n=10), or ommatidia number (n=10). Micrographs of eyes are shown in A. Graphic representations of ommatidia area, bristle number and ommatidia number are shown in B (values represent mean ± SEM). The genotypes are as follows: GFP UASGFP/GMRGal4, lacZ UASlacZ/GMRGal4, mnb UASmnbEY14320/+; GMRGal4/+.
Interaction of foxo1 with mnb and Hip1

Hip1 and Hip1ΔANTH differentially affect eye development under conditions of foxo1-sensitized eye development

Expression of mnb has been shown to rescue the phenotype caused by over-expression of foxo1 by GMR-Gal4 (Rotchford 2006), which results in a decrease in ommatidia number, bristle number and ommatidia area (Kramer et al. 2003). To determine if Hip1 interacts with foxo1, GMR-Gal4 was used to direct the over-expression of murine foxo1 plus other genes throughout the developing eye and ommatidia area, bristle number and ommatidia number were measured. Results are summarized in Table 4. Notably, directed expression of the truncated versions of Hip1, Hip1ΔANTH5.2 and Hip1ΔANTH11.2, and mnb in the eye along with foxo1, have no effect upon the ommatidia area in males or females (Figures 19 and 20). In males only, flies in which Hip1L-2 and Hip1L-6 were over-expressed had significantly lower ommatidia area than controls (Figures 19 and 20). There is no difference with respect to ommatidia area between the two truncated versions of Hip1, but there is a difference in the full length versions of Hip1 in males only (Figure 20).

For flies in which Hip1L-2, Hip1L-6, and mnb were over-expressed, there was a statistically significant decrease in bristle number compared to both lacZ and GFP controls (Figures 19 and 20). There is no difference in bristle number for the two truncated versions of Hip1, Hip1ΔANTH5.2 and Hip1ΔANTH11.2 compared to controls, with the exception of Hip1ΔANTH5.2, which has increased bristle number compared to lacZ in females (Figure 19). There was no statistical difference between the two full length versions of Hip1, Hip1L-2 and Hip1L-6, or between the two truncated versions of
*Hip1, Hip1ΔANTHδ.2 and Hip1ΔANTHt1.2* in males and females in bristle number based on a one-way ANOVA.

Flies over-expressing all of the *Hip1* genes produced fewer ommatidia than the controls with the exception of *Hip1ΔANTHδ.2* females which show no difference compared to *lacZ* controls (Figures 19 and 20). As well, *mnb* females had significantly lower ommatidia number than the *GFP* control only (Figure 19). There was no statistical difference when examining ommatidia number between the two full length versions of *Hip1, Hip1t2* and *Hip1t6*, but there was a difference between the two truncated versions of *Hip1, Hip1ΔANTHδ.2* and *Hip1ΔANTHt1.2* (Figures 19 and 20).

There was no difference in the controls, *GFP* and *lacZ* for any condition observed (Figures 19 and 20). Electron micrographs of eyes demonstrate the differences in experimental genes compared to controls (Figures 19 and 20).
Figure 19. Biometric analysis of a potential role of Hip1, Hip1ΔANTH and mnb in eye development along with the GMRGal4 and UASfoxo1 transgenes in females.

Directed expression of both full length versions of Hip1, Hip1L-2 and Hip1L-6, the truncated versions of Hip1, Hip1ΔANTH6.2 and Hip1ΔANTH11.2, and mnb effect eye development. Micrographs of eyes are shown in A. Graphic representations of ommatidia area, bristle number and ommatidia number are shown in B (values represent mean ± SEM). The genotypes are as follows: GFP UASGFP/GMRGal4UASfoxo1, lacZ UASlacZ/GMRGal4UASfoxo1, Hip1L-2 UASHip1L-2/+; GMRGal4UASfoxo1/+, Hip1L-6 UASHip1L-6/+; GMRGal4UASfoxo1/+, Hip1ΔANTH5.2 UASHip1ΔANTH5.2/GMRGal4UASfoxo1, Hip1ΔANTH11.2 UASHip1ΔANTH11.2/+; GMRGal4UASfoxo1/+, mnb UASmnbEY14320/++; GMRGal4UASfoxo1/+. Bars with dissimilar superscripts indicate groups that differ significantly.
Figure 20. Biometric analysis of a potential role of Hip1, Hip1ΔANTH and mnb on eye development along with the GMRGal4 and UASfoxol transgenes in males.

Directed expression of both full length versions of Hip1, Hip1<sup>1-2</sup> and Hip1<sup>1-6</sup>, the truncated versions of Hip1, Hip1ΔANTH<sup>5·2</sup> and Hip1ΔANTH<sup>11·2</sup>, and mnb effect eye development. Micrographs of eyes are shown in A. Graphic representations of ommatidia area (n=10), bristle number (n=10) and ommatidia number (n=10) are shown in B (values represent mean ± SEM). The genotypes are as follows: **GFP**

**UASGFP/GMRGal4UASfoxol, lacZ UASlacZ/GMRGal4UASfoxol, Hip1**<sup>1-2</sup> UASHip1<sup>1-2</sup>;<br>**GMRGal4UASfoxol/+**, **Hip1**<sup>1-6</sup> UASHip1<sup>1-6</sup>/+; **GMRGal4UASfoxol/+**, **Hip1ΔANTH**<sup>5·2</sup> UASHip1<sup>5·2</sup>/GMpGal4UASfoxol, **Hip1ΔANTH**<sup>11·2</sup> UASHip1<sup>11·2</sup>/+;<br>**GMRGal4UASfoxol/+**, **mnb UASmnb**<sup>EY14320</sup>/--; **GMRGal4UASfoxol/+**. Bars with dissimilar superscripts indicate groups that differ significantly.
Table 4. Comparison of differences in ommatidia area, bristle number and ommatidia number when *mnb* or *Hip1/Hip1ΔANTH* is expressed along with *foxol* via the *GMRGa4* transgene.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Sex</th>
<th>Ommatidia Area</th>
<th>Bristle Number</th>
<th>Ommatidia Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hip1</em>&lt;sup&gt;L-2&lt;/sup&gt; vs. <em>GFP</em></td>
<td>F</td>
<td>NS</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td><em>Hip1</em>&lt;sup&gt;L-6&lt;/sup&gt; vs. <em>GFP</em></td>
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<td>NS</td>
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<td>M</td>
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<td>p &lt; 0.0001</td>
<td>NS</td>
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<td><em>Hip1ΔANTH</em>&lt;sup&gt;5,2&lt;/sup&gt; vs. <em>GFP</em></td>
<td>F</td>
<td>NS</td>
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<td>M</td>
<td>NS</td>
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<td>NS</td>
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<td><em>Hip1ΔANTH</em>&lt;sup&gt;11,2&lt;/sup&gt; vs. <em>GFP</em></td>
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<td>NS</td>
<td></td>
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<td></td>
<td>M</td>
<td>NS</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
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<tr>
<td><em>mnb</em> vs. <em>GFP</em></td>
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<td>NS</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
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<td></td>
<td>M</td>
<td>NS</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
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<td><em>Hip1</em>&lt;sup&gt;L-2&lt;/sup&gt; vs. <em>lacZ</em></td>
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<td>NS</td>
<td></td>
<td>p &lt; 0.0001</td>
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<td>M</td>
<td>NS</td>
<td>p &lt; 0.0001</td>
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<td><em>Hip1</em>&lt;sup&gt;L-6&lt;/sup&gt; vs. <em>lacZ</em></td>
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<td>M</td>
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<td><em>Hip1ΔANTH</em>&lt;sup&gt;5,2&lt;/sup&gt; vs. <em>lacZ</em></td>
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<td>NS</td>
<td>p &lt; 0.0001</td>
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<td><em>Hip1ΔANTH</em>&lt;sup&gt;11,2&lt;/sup&gt; vs. <em>lacZ</em></td>
<td>F</td>
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<td>NS</td>
<td>p &lt; 0.0001</td>
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<td><em>mnb</em> vs. <em>lacZ</em></td>
<td>F</td>
<td>NS</td>
<td>p &lt; 0.0001</td>
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<td>NS</td>
<td>p &lt; 0.0001</td>
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<td>NS</td>
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<td>NS</td>
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<td>M</td>
<td>p &lt; 0.0001</td>
<td>NS</td>
<td>NS</td>
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<td><em>Hip1ΔANTH</em>&lt;sup&gt;5,2&lt;/sup&gt; vs. <em>Hip1ΔANTH</em>&lt;sup&gt;11,2&lt;/sup&gt;</td>
<td>F</td>
<td>NS</td>
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<td>NS</td>
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<td>M</td>
<td>NS</td>
<td>p &lt; 0.01</td>
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<td><em>Hip1</em>&lt;sup&gt;L-2&lt;/sup&gt; vs. <em>Hip1ΔANTH</em>&lt;sup&gt;5,2&lt;/sup&gt;</td>
<td>F</td>
<td>NS</td>
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<td>F</td>
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<td><em>mnb</em> vs. <em>mnb</em></td>
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<td>NS</td>
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<td><em>Hip1</em>&lt;sup&gt;L-2&lt;/sup&gt; vs. <em>mnb</em></td>
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<td><em>Hip1</em>&lt;sup&gt;L-6&lt;/sup&gt; vs. <em>mnb</em></td>
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<td>vs. mnb</td>
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<td>GFP</td>
<td>F</td>
<td>NS</td>
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<tr>
<td>vs. lacZ</td>
<td>M</td>
<td>NS</td>
<td>NS</td>
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</table>

Arrows indicate increase (↑) or decrease (↓) in treatment.

p values based on Neuman-Keuls post-tests

F= female  M= male

NS= not significant
Interaction of mnb and Hip1 with each other

The interaction between mnb and Hip1 alters foxol sensitized eye development when compared to mnb with foxol and Hip1 with foxol

As mnb and Hip1 have been found to interact with foxol in the process of eye development and have been shown to interact biochemically, the interaction of mnb and Hip1 with each other was investigated under the conditions of foxol-sensitized eye development. To determine if Hip1 and mnb interact, GMR-Gal4 was used to direct the over-expression of murine foxol plus other genes throughout the developing eye and ommatidial area, bristle number and ommatidial number were measured. A summary of these results can be seen in Table 5. Notably, when compared to Hip1 with foxol controls, Hip1 and mnb together with foxol increase ommatidial area for both full length versions, Hip1L-2 and Hip1L-6 and one truncated version of Hip1, Hip1ΔANTH11,2 (Figures 21 and 22). Compared to mnb and foxol alone, Hip1 and mnb together with foxol increase ommatidial area for one full length version, Hip1L-2, and one truncated version of Hip1, Hip1ΔANTH11,2 (Figures 21 and 22). Differences in ommatidial area were found when comparing the two full length versions of Hip1 with mnb and the two truncated versions of Hip1 with mnb (Figures 21 and 22).

Directed expression of Hip1 with mnb and foxol decreased bristle number for all versions of Hip1 compared to the Hip1 with foxol controls (Figures 21 and 22). When compared to the mnb with foxol control, both full length versions of Hip1 were found to decrease the bristle number, whereas the truncated versions on Hip1 increased the bristle number (Figures 21 and 22). In both males and females, there was no statistical difference when examining bristle number between the two full length versions of Hip1,
HipL^{1-2} and HipL^{6-6}, or in females expressing the truncated versions of Hip1, but there was a difference between the two truncated versions of Hip1, Hip1ΔANTH^{5-2} and Hip1ΔANTH^{11-2} in males (Figures 21 and 22).

There was no significant difference in ommatidia number between the full length versions of Hip1 with mnb and foxol compared to Hip1 with foxol controls. (Figures 21 and 22) Ommatidia number was increased in flies over-expressing the two truncated versions of Hip1, Hip1ΔANTH^{5-2} and Hip1ΔANTH^{11-2} compared to Hip1 with foxol controls (Figures 21 and 22). When compared to mnb with foxol controls, both the full length versions and one truncated version of Hip1 increased ommatidia number, while Hip1ΔANTH^{11-2} decreased ommatidia area (Figures 21 and 22). There was no difference between the two full length versions of Hip1 with mnb and foxol, but there was a difference between the two truncated versions in ommatidia area (Figures 21 and 22).

Electron micrographs show the effects of expressing the two genes, mnb and Hip1, together (Figures 21 and 22). These results suggest that mnb and Hip1 interact during foxol-sensitized eye development to alter the ommatidia area, bristle number and ommatidia number compared to controls.
A

\textit{GMRGal4UAS foxo1} plus ...

\begin{align*}
\text{mnb} & \quad \text{Hip1}^{L-2} & \quad \text{mnb; Hip1}^{L-2} \\
\text{Hip1}^{L-6} & \quad \text{mnb; Hip1}^{L-6}
\end{align*}
A

\textit{GMRGal4UAS foxo1 plus ...}

\begin{itemize}
\item Hip1\textsuperscript{\Delta ANTH\textsuperscript{5.2}}
\item mnb; Hip1\textsuperscript{\Delta ANTH\textsuperscript{5.2}}
\item Hip1\textsuperscript{\Delta ANTH\textsuperscript{11.2}}
\item mnb; Hip1\textsuperscript{\Delta ANTH\textsuperscript{11.2}}
\end{itemize}

80
Figure 21. Biometric analysis of the interaction between *mnb* and *Hip1* during eye development along with the *GMRGal4* and *UASfoxo1* transgenes in females. Directed expression of both full length versions of *Hip1, Hip1L-2* and *Hip1L-6*, the truncated versions of *Hip1, Hip1ΔANTH5,2* and *Hip1ΔANTH11,2*, with *mnb* in the eye effect ommatidia area, bristle and ommatidia number. Micrographs of eyes are shown in A. Graphic representations of ommatidia area (n=10), bristle number (n=10) and ommatidia number (n=10) are shown in B (values represent mean ± SEM). The genotypes are as follows: *mnb UASmnb<sup>EY14320</sup>/+, GMRGal4UASfoxo1/+, Hip1<sup>L-2</sup> UASHip1<sup>L-2</sup>/+; GMRGal4UASfoxo1/+, *mnb; Hip1<sup>L-2</sup> UASmnb<sup>EY14320</sup>/+, UASHip1<sup>L-2</sup>/+; GMRGal4UASfoxo1/+, *Hip1<sup>L-6</sup> UASHip1<sup>L-6</sup>/+, GMRGal4UASfoxo1/+, *mnb; Hip1<sup>L-6</sup> UASmnb<sup>EY14320</sup>/+, UASHip1<sup>L-6</sup>/+; GMRGal4UASfoxo1/+, *mnb; Hip1ΔANTH<sup>5,2</sup> UASHip1<sup>5,2</sup>/GMRRGal4UASfoxo1, *mnb; Hip1ΔANTH<sup>5,2</sup> UASmnb<sup>EY14320</sup>/+; UASHip1<sup>5,2</sup>/GMRRGal4UASfoxo1, *mnb; Hip1ΔANTH<sup>11,2</sup> UASHip1<sup>11,2</sup>/+; GMRGal4UASfoxo1/+, *mnb; Hip1ΔANTH<sup>11,2</sup> UASmnb<sup>EY14320</sup>/+, UASHip1<sup>11,2</sup>/+; GMRGal4UASfoxo1/+.* Bars with dissimilar superscripts indicate groups that differ significantly.
A

**GMRRGal4UAS foxo1 plus ...**

- mnb
- **Hip1**
- mnb; **Hip1**
- **Hip1**
- mnb; **Hip1**
A

GMRGal4UAS foxo1 plus ...

Hip1ΔANTH^{5.2}  
mnb; Hip1ΔANTH^{5.2}

Hip1ΔANTH^{11.2}  
mnb; Hip1ΔANTH^{11.2}
Figure 22. Biometric analysis of the interaction between *mnb* and *Hip1* during eye development along with the *GMRGal4* and *UASfoxo1* transgenes in males. Directed expression of both full length versions of *Hip1, Hip1<sup>L-2</sup>*, and *Hip1<sup>L-6</sup>*, the truncated versions of *Hip1, Hip1ΔANTH<sup>5.2</sup>* and *Hip1ΔANTH<sup>11.2</sup>* with *mnb* in the eye affect ommatidia area, bristle and ommatidia number. Micrographs of eyes are shown in A. Graphic representations of ommatidia area (n=10), bristle number (n=10) and ommatidia number (n=10) are shown in B (values represent mean ± SEM). The genotypes are as follows: *mnb* UAS<sup>EY14320</sup>*mnb/+; GMRGal4 UAS<sup>foxo1</sup>/+, *Hip1<sup>L-2</sup> UASHip<sup>L-2</sup>/+; GMRGal4 UAS<sup>foxo1</sup>/+, *mnb; Hip1<sup>L-2</sup> UAS<sup>EY14320</sup>*mnb/+; UASHip<sup>L-2</sup>/+; GMRGal4 UAS<sup>foxo1</sup>/+, *Hip1<sup>L-6</sup> UASHip<sup>L-6</sup>/+; GMRGal4 UAS<sup>foxo1</sup>/+, *mnb; Hip1<sup>L-6</sup> UASM<sup>EY14320</sup>*mnb/+; UASHip<sup>L-6</sup>/+; GMRGal4 UAS<sup>foxo1</sup>/+, *Hip1ΔANTH<sup>5.2</sup> UASHip<sup>5.2</sup>/GM<sup>RGal4 UAS<sup>foxo1</sup>/+, mnb; Hip1ΔANTH<sup>5.2</sup> UAS<sup>EY14320</sup>*mnb/+; UASHip<sup>5.2</sup>/GM<sup>RGal4 UAS<sup>foxo1</sup>/+, mnb; Hip1ΔANTH<sup>11.2</sup> UASHip<sup>11.2</sup>/+; GMRGal4 UAS<sup>foxo1</sup>/+, mnb; Hip1ΔANTH<sup>11.2</sup> UAS<sup>EY14320</sup>*mnb/+; UASHip<sup>11.2</sup>/+; GMRGal4 UAS<sup>foxo1</sup>/+. Bars with dissimilar superscripts indicate groups that differ significantly.
Table 5. Comparison of differences in ommatidia area, bristle number and ommatidia number when *mnb* and/or *Hip1/Hip1ΔANTH* is expressed along with *foxol* via the *GMRGal4* transgene.

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<tr>
<th>Comparison</th>
<th>Sex</th>
<th>Ommatidia Area</th>
<th>Bristle Number</th>
<th>Ommatidia Number</th>
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<td>vs. *Hip1&lt;sup&gt;l-2&lt;/sup&gt;</td>
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<td>p &lt; 0.0001</td>
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<td>vs. *Hip1&lt;sup&gt;l-6&lt;/sup&gt;</td>
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<td>mnb; *Hip1ΔANTH&lt;sup&gt;l-2&lt;/sup&gt;</td>
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<td>NS</td>
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<tr>
<td>vs. *Hip1ΔANTH&lt;sup&gt;l-2&lt;/sup&gt;</td>
<td>M</td>
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<td>p &lt; 0.0001</td>
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Summary:

- All comparisons show a significant decrease in ommatidia area and bristle number when *mnb* and/or *Hip1/Hip1ΔANTH* is expressed along with *foxol* via the *GMRGal4* transgene.
- The effects are more pronounced in females than in males.
- The number of ommatidia does not change significantly.

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Arrows indicate increase (↑) or decrease (↓) in treatment.

p values based on Neuman-Keuls post-tests
F= female  M= male
NS= not significant
Discussion

One of the goals of this research was to investigate the role of mnb in Drosophila neurogenesis by analysis of the microchaetae bristle formation on the dorsal notum. The original studies of mnb mutants have demonstrated a role for mnb during post-embryonic neurogenesis in Drosophila (Tejedor et al. 1995). However, since its discovery over a decade ago, few studies have been done on mnb in Drosophila. Mutant mnb flies with a reduction in gene expression show decreased volume of the optic lobes and central brain regions in adults and thus it was hypothesized that mnb may have a role in neurogenesis during the development of the dorsal notum. Recent study has indicated a novel role for Hip1 during Drosophila neurogenesis, where the over-expression of Hip1 affects the formation of microchaetae bristles (Moores et al. 2008). As a biochemical interaction was found between mnb and Hip1 (Kang et al. 2005), it was imperative to determine if this interaction could be involved in the process of mechanosensory bristle formation on the dorsal notum. If mnb was found to play a role in bristle formation, this could be a source for the biological interaction between mnb and Hip1. No difference in the bristle density when mnb is expressed via either apterousGal4 or pannierGal4 was determined. This suggests that mnb is not the limiting factor in this process.

The insulin-receptor signalling pathway describes a well-studied mechanism whereby cell size, cell number and cell death are regulated (reviewed in Burgering 2008). Two components of this pathway, akt and foxo1, play opposite roles in this process, with akt promoting cell growth, and foxo1 promoting cell death or cell cycle arrest. The dorsal notum has been used as a model to study the role of Notch (Ramain et al. 2001) and has been exploited to show that Hip1 plays a role in neurogenesis (Moores et al. 2008).
Although insulin signalling and neurogenesis have both been well-studied, research has not investigated the role of these two genes, \( \text{akt} \) and \( \text{foxo1} \), in Drosophila neurogenesis. To study this potential novel role for insulin-receptor signalling components on mechanosensory bristle formation, \( \text{akt} \) and \( \text{foxo1} \) were ectopically expressed in the dorsal notum. Considering the important role that \( \text{akt} \) and \( \text{foxo1} \) play in the growth of cells, it is reasonable to think they would play a role in the development of mechanosensory bristle cells in the dorsal notum.

These experiments demonstrate that \( \text{akt} \) causes an increase in the bristle density under the direction of the \textit{pannierGal4} transgene, to indicate a role for \( \text{akt} \) in bristle formation on the back in females. As \( \text{akt} \) plays a role in many developmental processes (Bhaskar and Hay 2007; Song and Wolfe 2007; Jiang and Lui 2008; Juntilla and Koretzky 2008; Tokunaga \textit{et al.} 2008), it is reasonable to believe that \( \text{akt} \) may play a role in mechanosensory bristle formation. To the best of my knowledge this is the first time that a role for \( \text{akt} \) in neurogenesis on the Drosophila dorsal notum has been described. An increase in the level of \( \text{akt} \) is sufficient to shift the balance towards neurogenesis, which suggests that indeed the insulin signaling pathway is active in the formation of mechanosensory bristles on the dorsal notum.

When \( \text{foxo1} \) is expressed in the dorsal notum under the control of the \textit{pannierGal4} transgene, there is no difference in bristle density compared to the controls, suggesting that \( \text{foxo1} \) does not interfere with neurogenesis. In contrast, over-expression of \( \text{foxo1} \) with \textit{apterousGal4} leads to lethality. Further investigation of the ectopic expression of \( \text{foxo1} \) will be required to fully understand this finding.
During this study it was found that there is no statistical difference in any of the phenotypes studied for GFP and lacZ. The fact that there was no difference in these controls indicates that either of these genes can be used as controls in future experiments. As there is always a question on what controls to use when designing an experiment, this study brings useful knowledge to the scientific community as either of these controls will yield virtually the same results.

With minor exceptions, the two full length insertions of the UASHip1 and two truncated versions of UASHip1 transgenes do not give statistically different results from each other when over-expressed in the eye with GMR and with GMR and foxo1. This suggests that the two independently inserted transgenes do not have different activities. Examination of Hip1’s ability to influence Drosophila neurogenesis (Moores et al. 2008), and Drosophila eye development can certainly add to our understanding of this protein in the progression of HD.

Studies have suggested a role for Hip1 as a pro-apoptotic protein, where over-expression of Hip1 leads to caspase-dependent cell death (Hackam et al. 2000). Hip1 over-expression with GMR, however, did not show any indication of cell death as bristle and ommatidia number were similar to controls. This suggests that although Hip1 may act to increase apoptosis in cell culture, it does not play this role in live organisms.

The over-expression of foxo1 in the developing eye leads to decreased ommatidia number, bristle number and ommatidia size (Kramer et al. 2003) presumably by altering aspects of the growth and cell survival signaling mechanisms. Ectopic mnb expression has been shown to interact with foxo1 in the process of eye development through the suppression of the effects of foxo1, to result in ommatidia number, bristle number and
ommatidia size more similar to controls (Rotchford 2006). Under the sensitized growth conditions where the GMRGal4 drives the foxo1 transgene, both the full length versions and truncated versions of Hip1 alter the growth of the eye compared to the controls. The full length versions of Hip1 show decreased bristle number and decreased ommatidia number with no change in ommatidia size compared to the controls. The truncated versions of Hip1 lacking the lipid-binding ANTH domain show normal ommatidia area, normal bristle number but decreased ommatidia number. These transgenes lead to significantly higher ommatidia number and bristle number than full length Hip1 in the presence of foxo1. These findings show that the alternative versions of Hip1 can play a slightly different role in eye development that is reminiscent of their dual role in Drosophila neurogenesis (Moores et al. 2008). Studies of Hip1 in neurogenesis indicate that full length Hip1 decreases microchaetae density and Hip1ΔANTH increases microchaetae density (Moores et al. 2008). The presence or absence of the ANTH domain causes two very distinct phenotypes and suggests that Hip1 may regulate eye development through a receptor-mediated endocytotic or vesicle trafficking mechanism, a mode similar to that hypothesized for Hip1 in neurogenesis (Moores et al. 2008).

Removal of the ANTH domain of S. cerevisiae’s Sla2P protein causes the protein to lose its endocytic ability (Sun et al. 2005), showing the importance of this domain in endocytosis. Like Drosophila, humans have multiple protein isoforms of Hip1 (Curtis et al. 2005), so it is possible that this dual role of Hip1 may occur in humans. This mechanism that employs two isoforms of the same protein to have opposing activities may be important in understanding the underlying mechanisms that contribute to HD.
This is the first account of a role for *Hip1* being described in Drosophila eye development.

The overexpression of *mnb* with *foxo1* shows decreased bristle number in males and females, and decreased ommatidia number in females. As it interacts with *foxo1*, it was hypothesized that *mnb* would increase cell growth by preventing *foxo1* activity. These results are inconsistent with the hypothesis and differ from previous results obtained for *mnb* (Rotchford 2006). It is unclear why results vary, but could be due to differences in experimental condition or sampling. There may very well be a delicate balance between the levels of *mnb* and *foxo1* and further research is needed to fully understand the role of *mnb* in insulin-receptor signaling and development.

Since both *mnb* and *Hip1* have been found to influence eye development under sensitized conditions, it is possible that these two genes may interact during this process. To investigate this, *Hip1* and *mnb* were ectopically expressed along with *foxo1* and GMR in the developing Drosophila eye. Compared to both *mnb* with *foxo1* and *Hip1* with *foxo1* as controls, *Hip1* with *mnb* and *foxo1* was found to increase ommatidia area, indicative of an increase in cell size. A previous biochemical interaction between *Hip1* and *mnb* resulted in the phosphorylation of *Hip1* by *mnb* and an increase in the growth of neurons (Kang *et al.* 2005). This study suggests that a similar role for *Hip1* and *mnb* may be happening in eye development to increase the growth of ommatidia. When compared to *Hip1* with *foxo1* controls, all versions of *Hip1* with *mnb* and *foxo1* were found to decrease the number of bristles formed. This suggests that *mnb* increases the ability of *Hip1* to decrease neurogenesis. Alternatively, when compared to *mnb* with *foxo1*, both full length versions of *Hip1* were found to decrease the bristle number, whereas the
truncated versions on *Hip1* increased the bristle number. This indicates that the two different versions of *Hip1* act differently with *mnb*, with the full length version decreasing neurogenesis and the truncated version increasing neurogenesis. This is reminiscent of the role that *Hip1* plays in Drosophila neurogenesis in the dorsal notum (Moores *et al.* 2008). Compared to the truncated versions of *Hip1* with *foxo1*, the truncated versions of *Hip1* with *mnb* and *foxo1* show a decrease in ommatidia number, with the full length *Hip1* having no effect. It is possible that *mnb* is influencing the truncated version only in the process of cell growth, but does not influence full length *Hip1* in this process. When compared to *mnb* with *foxo1* controls, both the full length versions and *Hip1ΔANTH* increased ommatidia number, while *Hip1ΔANTH* decreased ommatidia area. The two versions of *Hip1* may be influencing the activity of *mnb*, but appear to be doing so differentially.

It is clear that *mnb* and *Hip1* are interacting during the process of *foxo1*-sensitized eye development. As *mnb* has been shown to interact with *foxo1* and participate in the insulin signaling pathway, it could be that *Hip1* participates in this pathway as well. As little research has been done on *Hip1*, it is possible that participation in the insulin signaling pathway could be a novel role for *Hip1*. A second source for their interaction could be during receptor mediated endocytosis. It has been thought for the last two decades that *mnb* over-expression may alter receptor-mediated endocytosis (Wegiel *et al.* 2004). More specifically, *mnb* is thought to be involved in synaptic vesicle recycling and synaptic signal transmission by phosphorylating dynamin (Wegiel *et al.* 2004). *Hip1* has also been found to play a role in endocytosis, by binding to components of the endocytic apparatus, such as the adapter protein AP2, the heavy and light chains of the triskelia and
the lipid membrane via the ANTH domain (Metzler et al. 2001; Chen and Brodsky 2005). It is likely that Hip1 and mnb are interacting during receptor mediated endocytosis during the process of eye development.

Overall, this study has shown that akt and foxo1 may play a role in bristle development in the dorsal notum, adding to the understanding of the insulin signaling pathway and its components. Hip1 has a role in the process of Drosophila eye development, similar to the role found in Drosophila neurogenesis. During foxo1-sensitized eye development, mnb and Hip1 interact to influence cell size and cell number. Drosophila has proven to be a useful model to study these genes and how they interact in a living organism. In the future, Drosophila melanogaster will continue to be useful for studying the genes of diseases like Down syndrome and Huntington disease, a neurogenerative disease and neurodegenerative disease in humans. As these are genes in two completely different diseases, finding how they interact could provide a key insight into finding therapies for neurodegenerative diseases.
References


Appendix 1

Schematic diagram for crosses made to eliminate the second chromosome balancer. 

\( UASHip1^{L-2}/TM6B \) was generated in a and b shows w; \( UASHip1^{L-6}/TM6B \).