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 *foxo1,* in the developing dorsal notum and eye. While neither *mnb* nor *foxo1* over-expression 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 *UASfoxo11* 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 *foxo1* 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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List of Abbreviations

A	adenine
ANOVA	analysis of variance
ANTH	AP-180 N-terminal homology
AP2	adapter protein 2
Arip4	androgen receptor-interacting protein 4
bFGF	basic fibroblast growth factor
C	cytosine
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CRE	cAMP response element
CREB	cAMP response element binding
C-terminal	carboxy-terminal
DNA	deoxyribonucleic acid
DRPLA	dentatorubral pallidoluysian atrophy
DS	Down syndrome
DCR-1	Down syndrome critical region
Dyrk1A	dual specificity tyrosine phosphorylation-regulated kinase
	dual-specificity YAK1-related kinase A
eIF2Bε	eukaryotic initiation factor 2Bepsilon
ENTH	epsin N-terminal homology
ESEM	environmental scanning electron microscope
foxo1	forkhead homeobox type O
G	guanine
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GFP	green fluorescent protein
GMR	glass multiple reporter
HD	Huntington disease
Hap1	Huntingtin-associated protein
Hip1	Huntingtin-interacting protein 1
Hip2	Huntingtin-interacting protein 2
htt	huntingtin
KDa	kiloDalton
mnb	minibrain
mRNA	messenger ribonucleic acid
N-terminal	amino terminal
NFAT	nuclear factor of activated T cells
PAHX-AP1	phytanoyl-CoA α-hydroxylase associated protein 1
PCR	polymerase chain reaction
pDED	pseudo-death effector domain
polyQ	polyglutamine
PtdIns(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
Q	glutamine
SEM	standard error of the mean
SF3b1	splicing factor 3b, subunit 1
Sla2p	synthetic lethal with actin binding protein (ABP) 1
STAT3	signal transducer and activator of transcription 3
UAS	upstream activating sequence
YAC	yeast artificial chromosome
X	times

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 *Hip1* 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 (Guimerá *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 *mnb* mutants and was characterized by positional cloning (Tejedor *et al.* 1995). The human and Drosophila versions of the mnb protein exhibit a 75% amino acid sequence similarity (Shindoh *et al.* 1996). Although *mnb* was first described over a decade ago, very little research has been described in Drosophila since then.

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 *et al.* 2001), cyclin L2 proteins (de Graaf *et al.* 2004), chromatin remodeling factors, SNR1 and TRX (Kinstrie *et al.* 2006), and the splicing factor SF3b1/SAP155 (de Graaf *et al.* 2006). In addition, the transcription factor Arip4 (androgen receptor-interacting protein 4) (Sitz *et al.* 2004) and the eukaryotic protein-synthesis initiation factor eIF2Bε (eukaryotic initiation factor 2Bepsilon) (Woods *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 *et al.* 2001), Gli1 (Mao *et al.* 2002), the forkhead transcription factor, foxo1 (Woods *et al.* 2001b), and the NFAT (nuclear factor of activated T cells) family of transcription factors (Gwack *et al.* 2006), suggesting a role for *Dyrk1A/mnb* in gene expression. Proteins in the cytoplasm including 14-3-3 (Kim *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 *Dyrk1A/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 *Dyrk1A/mnb* gene show significant impairment in hippocampal-dependent memory tasks (Ahn *et al.* 2006). These studies indicate a significant role for *Dyrk1A/mnb* in learning and memory. Mutant *Dyrk1A/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 *Dyrk1A/mnb* have an increase in brain weight (Branchi *et al.* 2004). Collectively, all of these studies suggest that *Dyrk1A/mnb* is involved in the neuropathological changes seen in patients with DS.

Dyrk1A/mnb studies in non-vertebrates

The *Dyrk1A/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 *Dyrk1A/mnb* homologue is *Yak1p*, 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 *Dyrk1A/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

foxo1. This showed that *mnb* is required for normal growth and may act through the *foxo1* protein to regulate cell size. Finally, over-expression of *mnb* was found to suppress loss of bristles and ommatidia in the eye caused by *foxo1* over-expression, suggesting that *mnb* negatively regulates *foxo1* (*Rotchford 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 theurapeutic 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)P_2), 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) P_2, 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.

Full Genotype	Reference	Abbreviation
Transgenic transcription factors		
y w; apterousGal4/CyO	Calleja *et al.* 1996	*apGal4*
y w; pannierGal4/TM3 UAS, y^+	Calleja *et al.* 1996	*pnrGal4*
Control genes		
w; UASGFP	Yeh *et al.* 1995	*UASGFP*
w; UASlacZ^{4-1-2}	Brand and Perrimon 1994	*UASlacZ*
Experimental genes		
y w^{67c23} $mnb^{EY14320}$	Bellen *et al.* 2004	*UASmnb*
w; UASAkt $^{1.1}$/CyO	Staveley *et al.* 1998	*UASakt*
w; UASfoxo1/CyO	Kramer *et al.* 2003	*UASfoxo1*

Drosophila culture

To investigate the roles of *mnb* and components of the insulin signaling pathway, *akt* and *foxo1*, in neurogenesis, four to six males carrying the *UASmnb, UASakt*, and *UASfoxo1* 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.

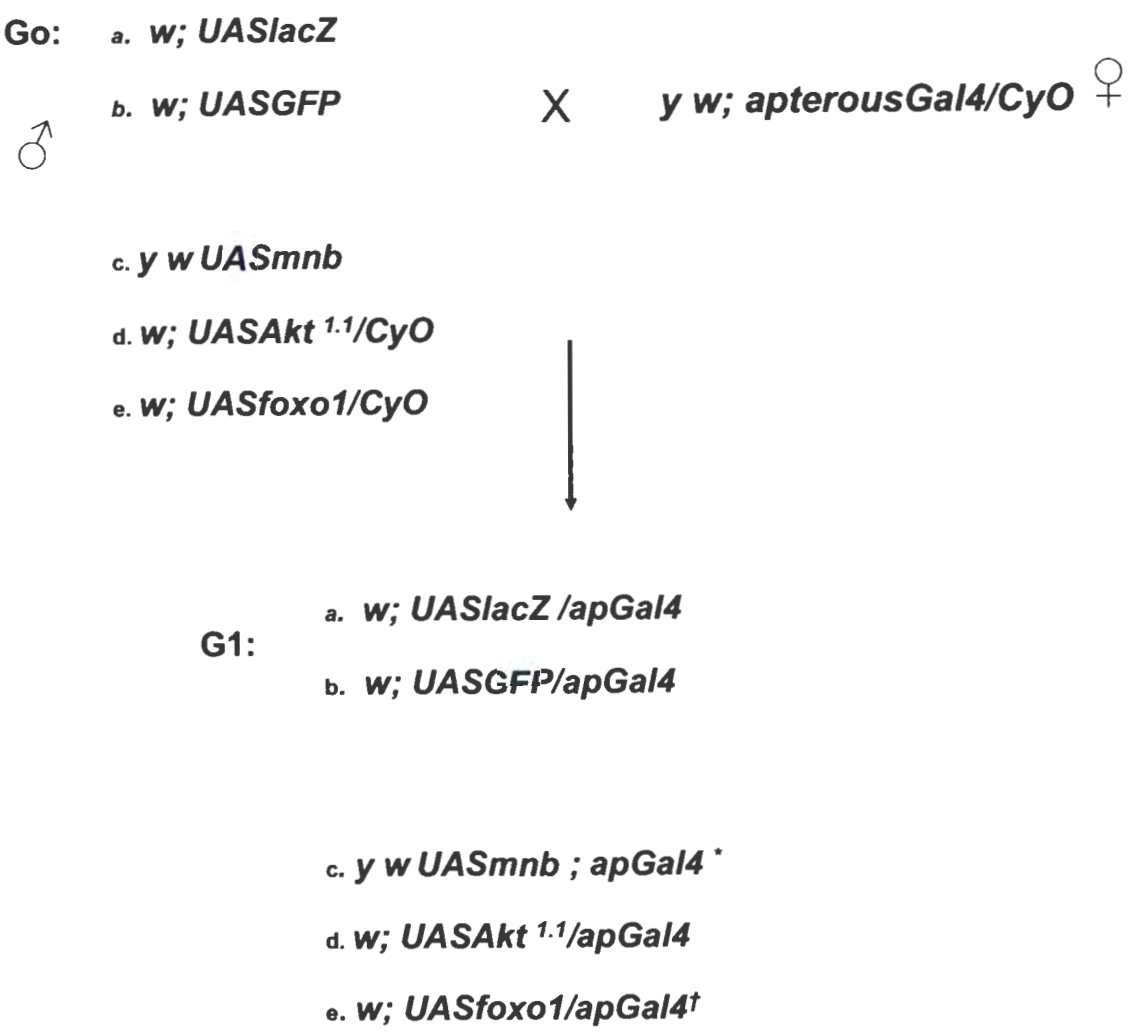


Figure 1. Schematic diagram of crosses made to examine the potential effects of *mnb, akt* and *foxo1* on neurogenesis using the *apterousGal4* transgene. Crosses to *UASlacZ* (a) and *UASGFP* (b) were conducted as controls. Experimental crosses were performed for *UASmnb* (c), *UASakt* (d) and *UASfoxo1*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

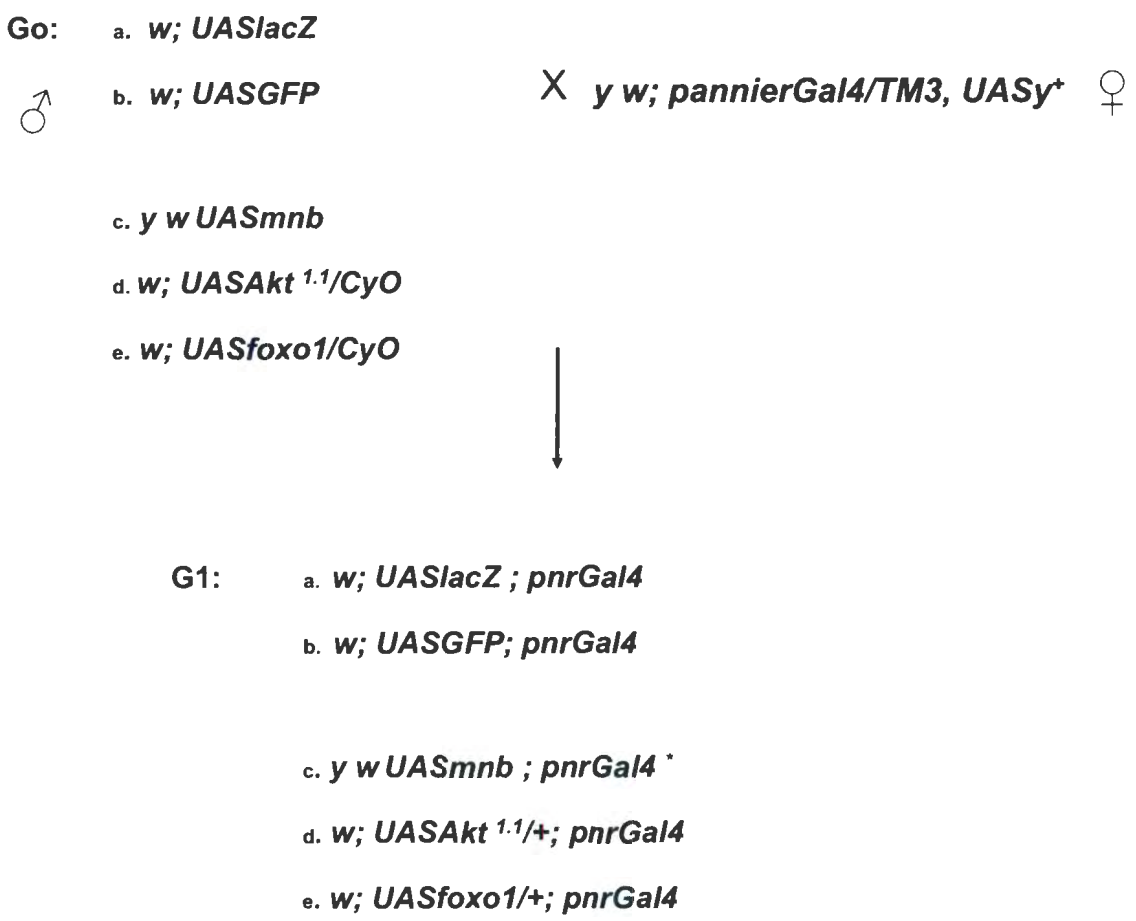


Figure 2. Schematic diagram of crosses made to examine the potential effects of *mnb*, *akt*, and *foxo1* on neurogenesis using the *pannierGal4* transgene. Crosses to *UASlacZ* (a) and *UASGFP* (b) were conducted as controls. Experimental crosses were performed for *UASmnb* (c), *UASakt* (d) and *UASfoxo1* (e). * Note: 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 250 X 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 (μm^2) was also calculated. The microchaetae counts and notum area were used to calculate the bristle density, expressed as number of microchaetae per 100 μm^2. 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; UASHip1^{L-2}/TM3,Sb* (*UASHip1^{L-2}*) and *w; L/CyO; UASHip1^{L-6}/TM3,Sb*, (*UASHip1^{L-6}*) and the two isolates of the amino terminal truncated form of the *Hip1* gene, *UASHip1$^{11.2}$* and *UASHip1$^{5.2}$* (referred to as *Hip-1ΔANTH*) 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; UASHip1^{L-2}/TM6B* and w; *UASHip1^{L-6}/TM6B* lines (Appendix 1). The *w; GMRGal4^{12}* 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.

Full Genotype	Reference	Abbreviation
Transgenic transcription factors		
w; GMRGal4^{12}	Freeman 1996	*GMRGal4*
Control genes		
w; UASGFP	Yeh *et al.* 1995	*UASGFP*
w; UASlacZ^{4-1-2}	Brand and Perrimon 1994	*UASlacZ*
Experimental genes		
w; UASHip1^{L-2}/TM6B	Moores 2006	*UASHip1^{L-2}*
w; UASHip1^{L-6}/TM6B	Moores 2006	*UASHip1^{L-6}*
w; UASHip1$^{5.2}$	Moores 2006	*UASHip1$^{5.2}$*
w; +; UASHip1$^{11.2}$	Moores 2006	*UASHip1$^{11.2}$*
y w^{67c23} mnb^{EY14320}	Bellen *et al.* 2004	*UASmnb*

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 $UASHip1^{L-2}$, $UASHip1^{L-6}$, $UASHip1^{11.2}$ and $UASHip1^{5.2}$ 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.

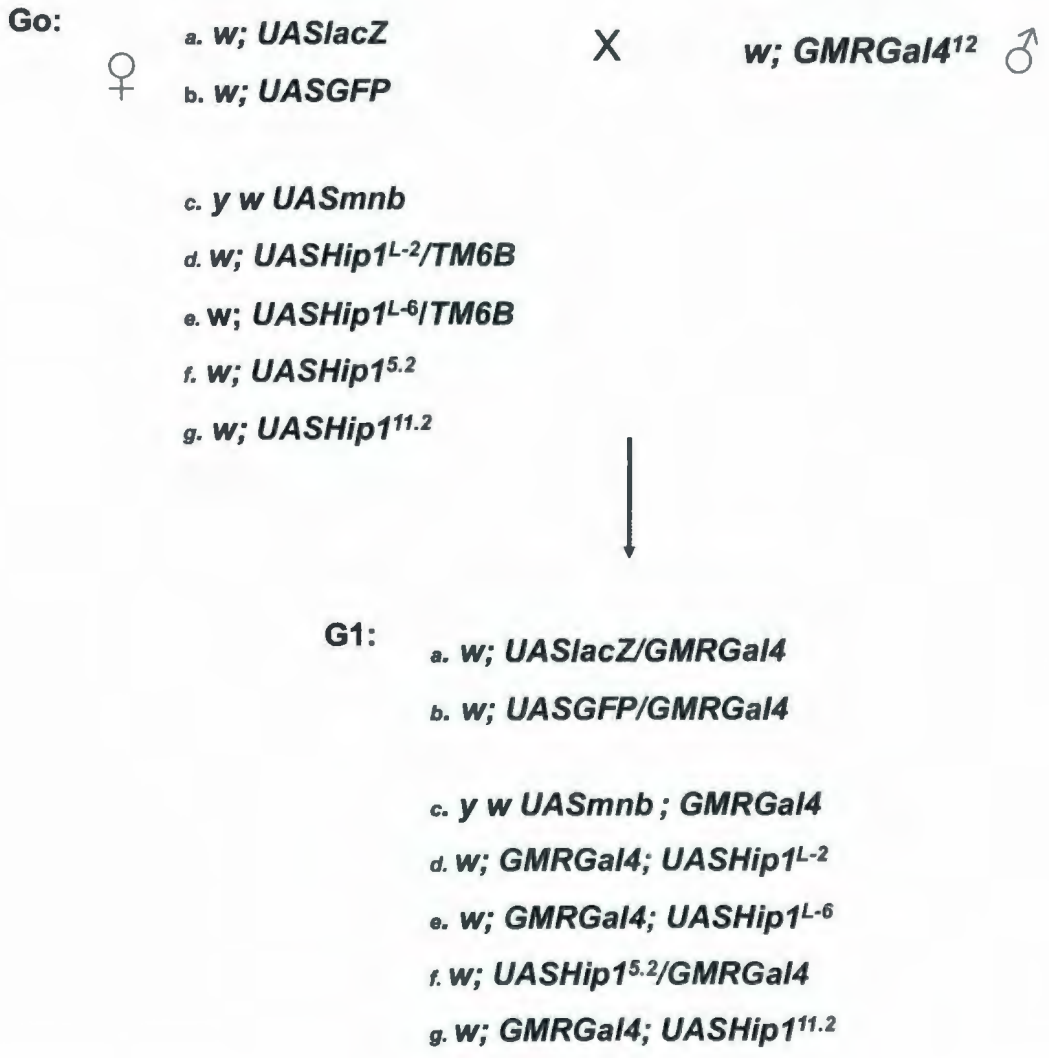


Figure 3. Schematic diagram of crosses made to examine the potential effects of *Hip1, Hip1ΔANTH* and *mnb* in eye development using the *GMRGal4* transgene. Crosses to *UASlacZ* (a) and *UASGFP* (b) were conducted as controls. Experimental crosses were performed for *UASmnb* (c), *UASHip1*$^{L-2}$ (d), *UASHip1*$^{L-6}$ (e), *UASHip1*$^{5.2}$ (f) and *UASHip1*$^{11.2}$ (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 μm^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 *UASHip1,* 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.

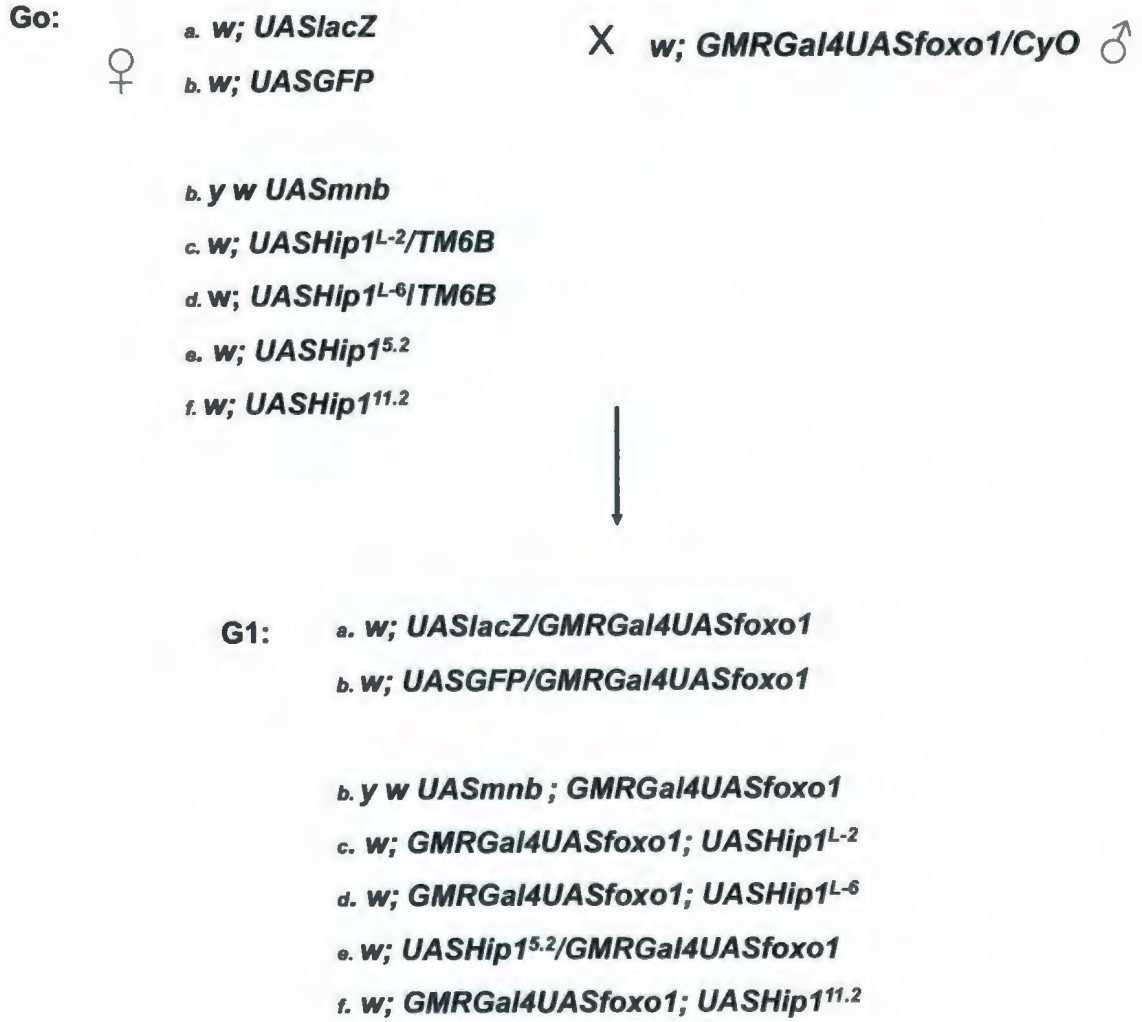


Figure 4. Schematic diagram of crosses made to examine the potential effects of *Hip1, Hip1ΔANTH* and *mnb* in eye development with the *GMRGal4* and *UASfoxo1* transgenes. Crosses to *UASGFP* (a) were conducted as controls. Experimental crosses were performed for *UASmnb* (b), *UASHip1^{L-2}* (c), *UASHip1^{L-6}* (d), *UASHip1$^{5.2}$* (e) and *UASHip1$^{11.2}$* (f).

Interaction of *mnb* and *Hip1* with each other

Drosophila stocks

The *w; L/CyO; Ki/TM3,Sb* 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* $Hip1^{L-2}$, *UAS* $Hip1^{L-6}$, and *UAS* $Hip1^{11.2}$. 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; UASHip1*$^{L-2}$, *UASmnb; UASHip1*$^{L-6}$ and *UASmnb; UASHip1*$^{11.2}$ combination lines (Figure 6).

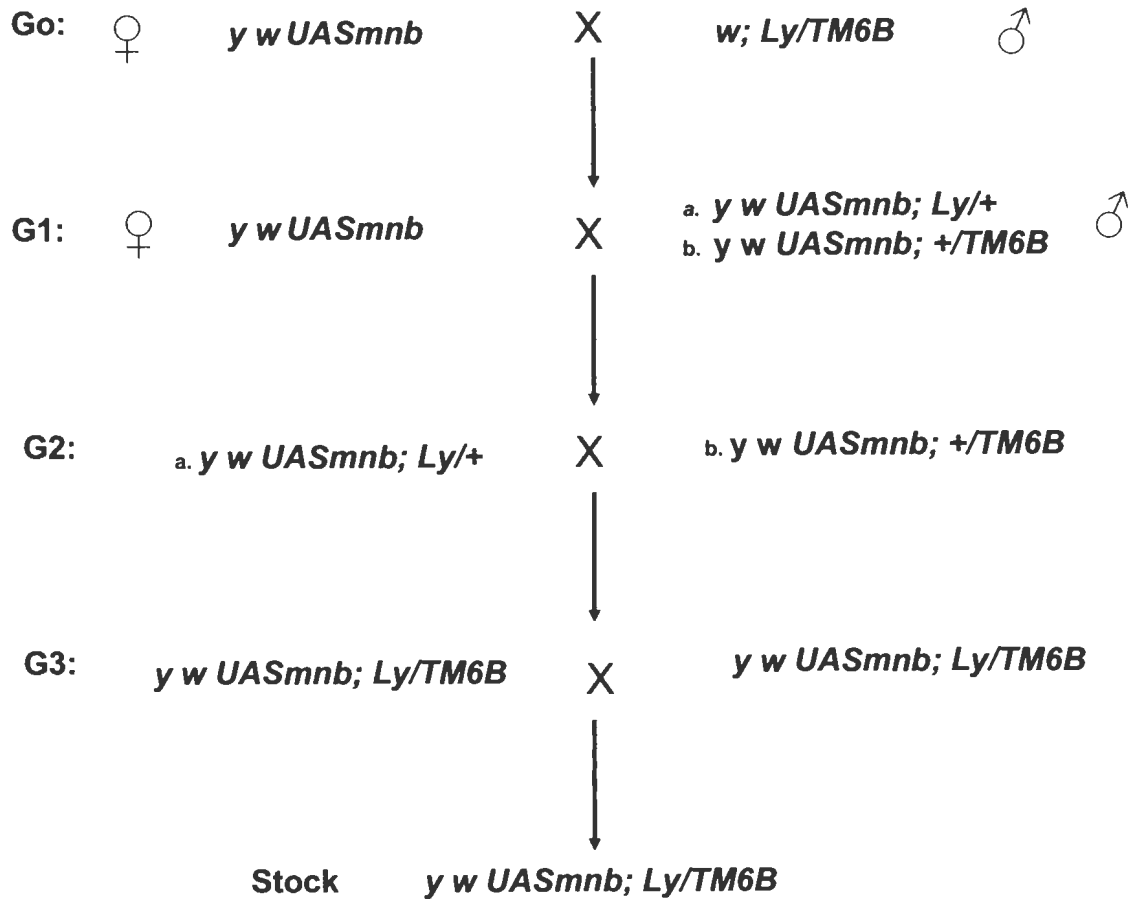


Figure 5. Schematic diagram of crosses made to establish *UASmnb; Ly/TM6B* stocks to examine the potential interaction of *Hip1* and *Hip1ΔANTH* with *mnb* in eye development with the *GMRGal4* and *UASfoxo1* transgenes.

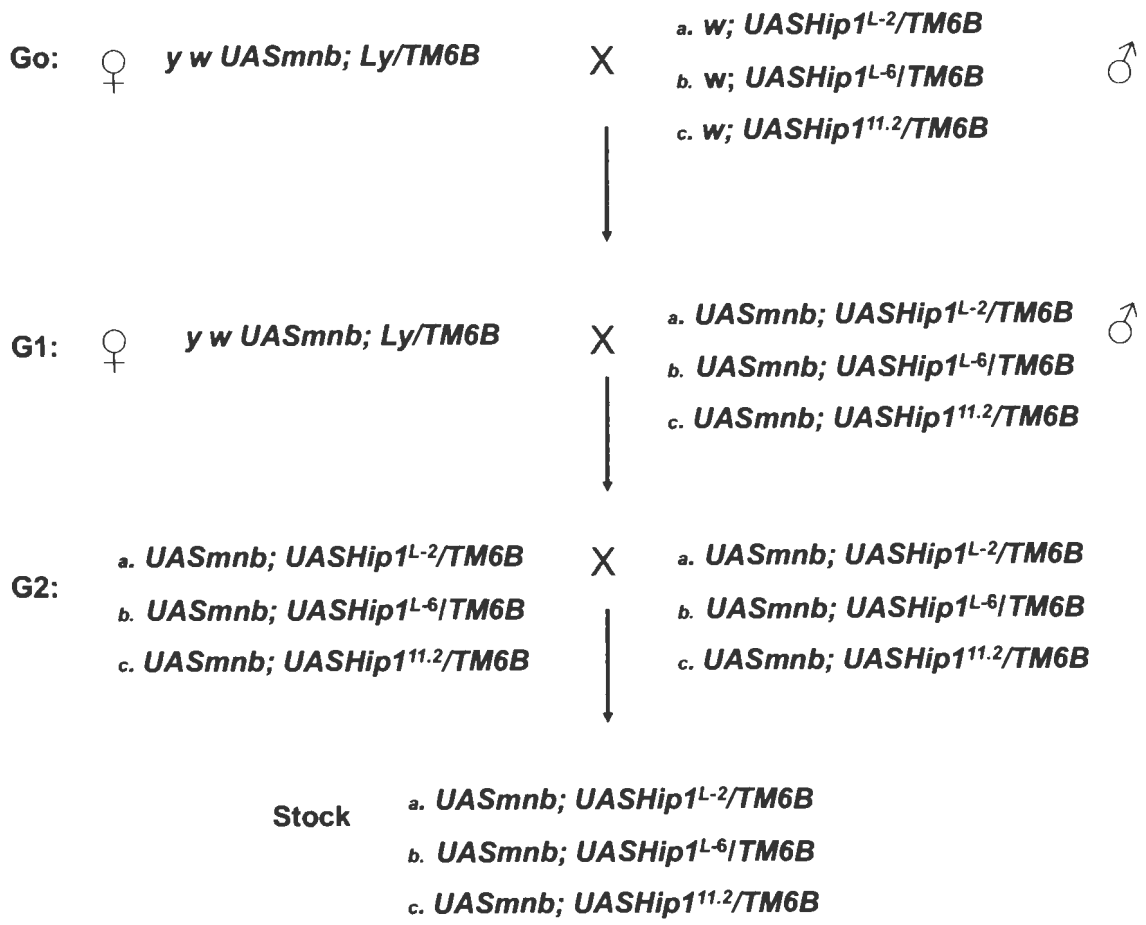


Figure 6. Schematic diagram of crosses made to establish *UASmnb; UASHip1* combination stock lines for *Hip1* on the third chromosome that will be used to examine the potential interaction of *Hip1* and *Hip1ΔANTH* with *mnb* in eye development with the *GMRGal4* and *UASfoxo1* transgenes.

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).

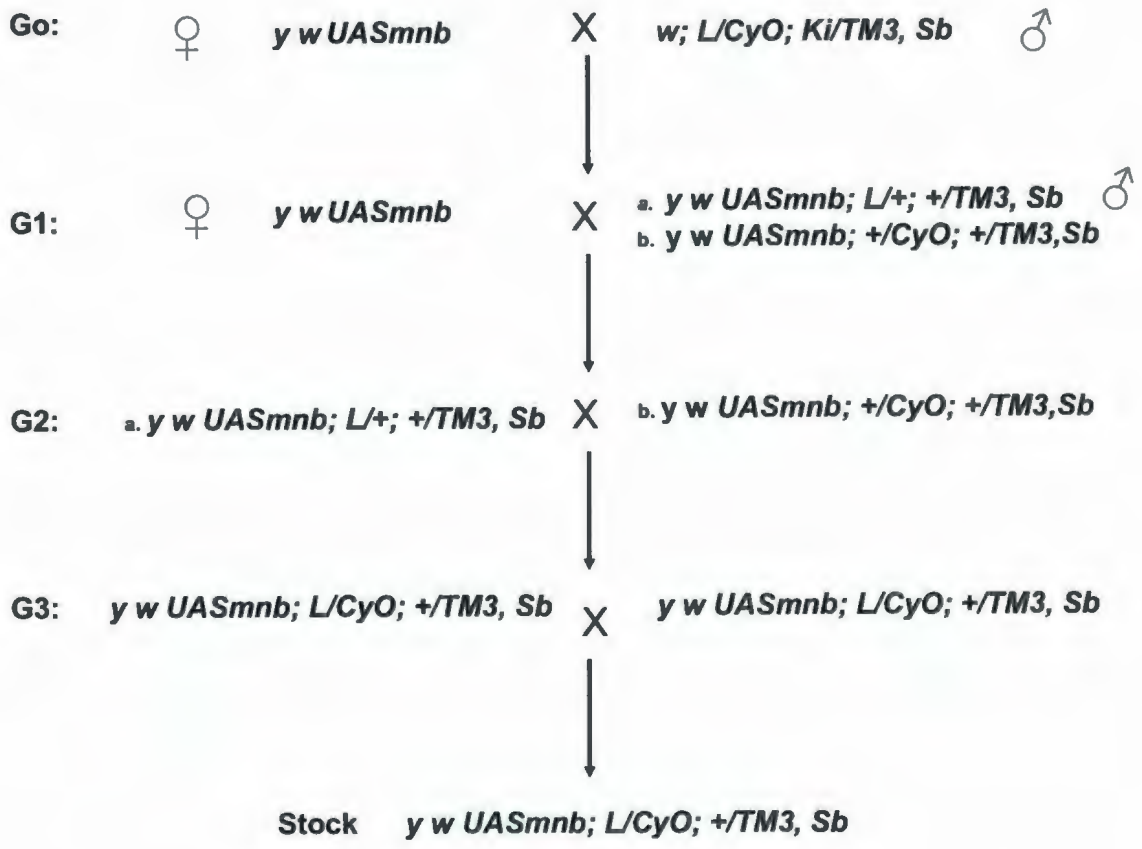


Figure 7. Schematic diagram of crosses made to establish *UASmnb; L/CyO; +/TM3, Sb* stocks to examine the potential interaction of *Hip1* and *Hip1ΔANTH* with *mnb* in eye development with the *GMRGal4* and *UASfoxo1* transgenes.

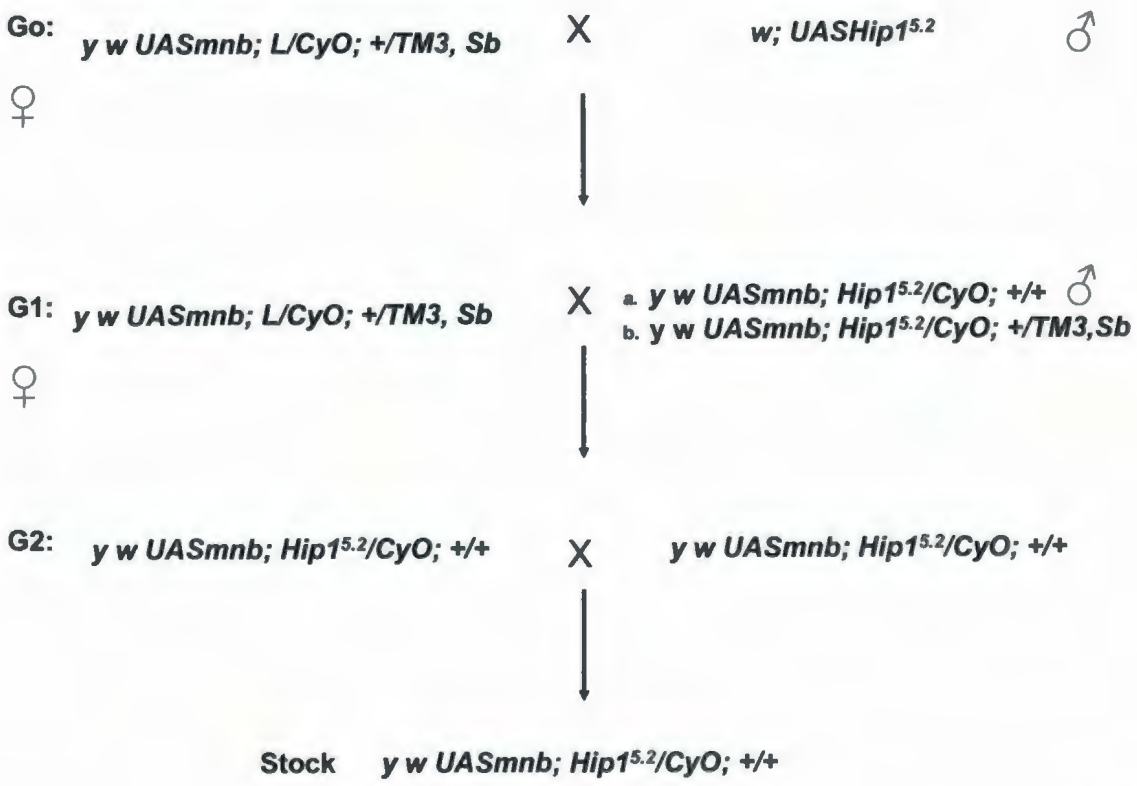


Figure 8. Schematic diagram of crosses made to establish *UASmnb; UASHip1* combination stock lines for *Hip1* on the second chromosome that will be used to examine the potential interaction of *Hip1* and *Hip1ΔANTH* with *mnb* in eye development with the *GMRGal4* and *UASfoxo1* transgenes.

Drosophila culture and *analysis*

To investigate the interaction of *mnb* and *Hip1* together with *foxo1*, two to four males of *GMRGal4 UASfoxo1/CyO* were crossed to six to eight virgin females of *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 *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.

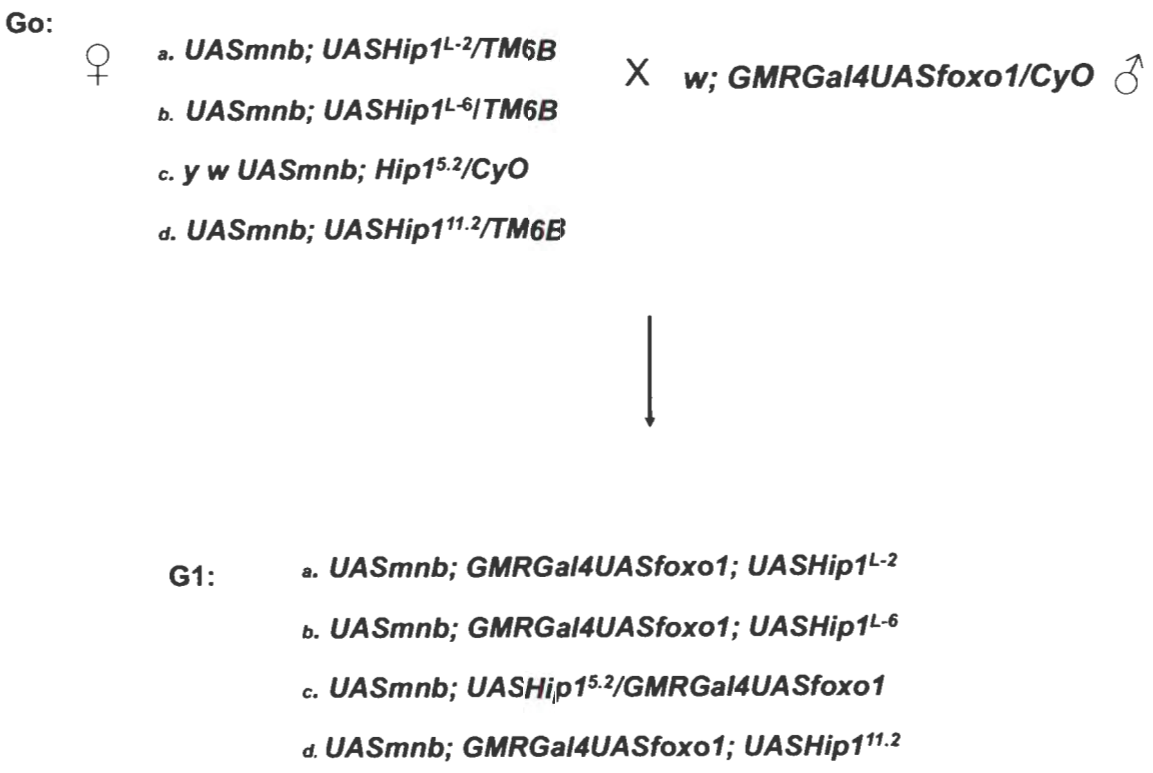


Figure 9. Schematic diagram of crosses made to examine the potential effects of *Hip1* and *Hip1ΔANTH* with *mnb* in eye development with the *GMRGal4* and *UASfoxo1* transgenes. Experimental crosses were performed for *UASmnb; UASHip1*$^{L-2}$ (a), *UASmnb*; *UASHip1*$^{L-6}$ (b), *UASmnb; UASHip1*$^{5.2}$ (c) and *UASmnb; UASHip1*$^{11.2}$ (d).

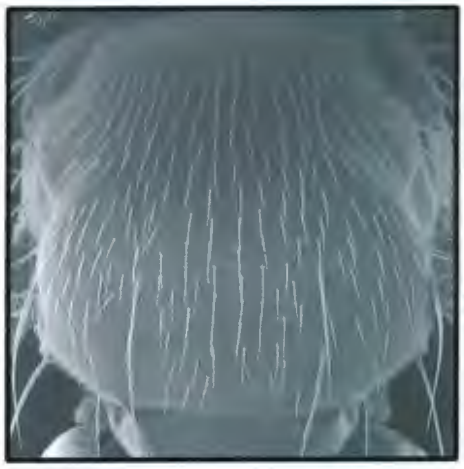
Results

Investigation of neurogenesis in the dorsal notum

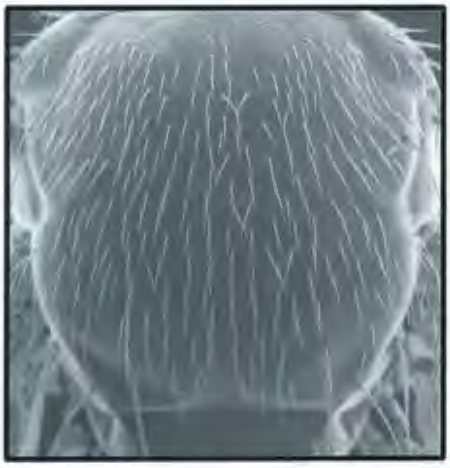
there is no phenotypic effect of mnb on neurogenesis in the dorsal notum

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 μm^2 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.

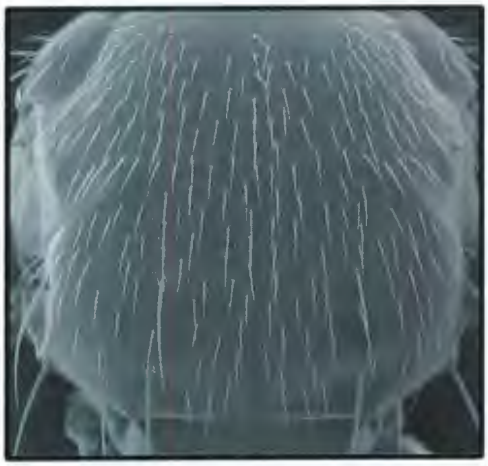
A



GFP



lacZ



mnb

B

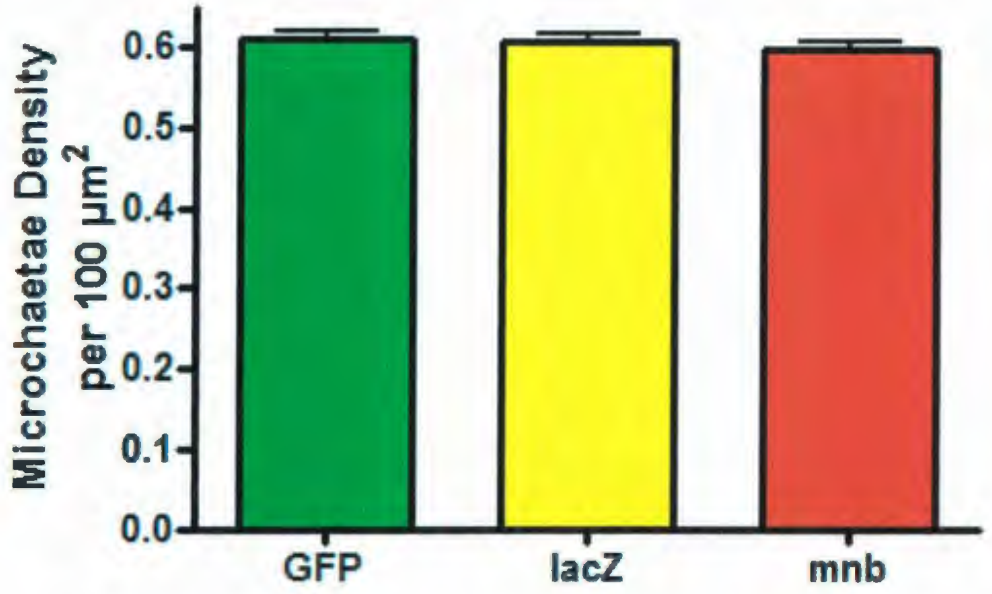
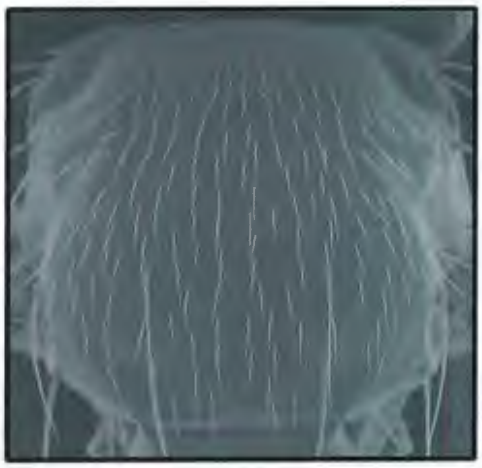
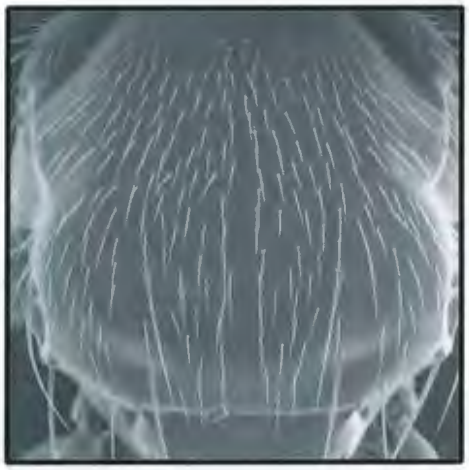


Figure 10. Biometric analysis of a potential role of *mnb* in neurogenesis with the *apterousGal4* transgene in females. Directed expression of *mnb* in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA (n=30). Micrographs of dorsal notums are shown in A. Graphic 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*^{EY14320}*/+; apGal4/+*. Note: *GFP* and *lacZ* controls are the same as those used for *apterousGal4* driving the expression of *akt*.

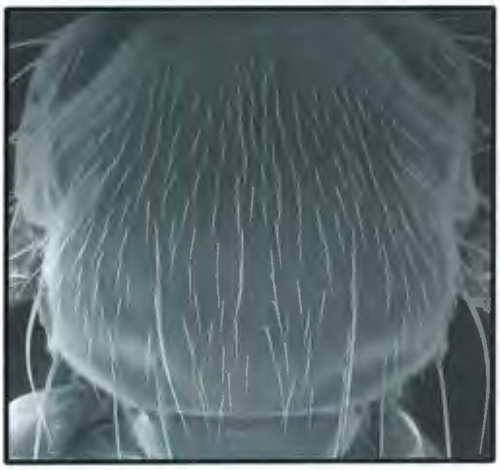
A



GFP



lacZ



mnb

B

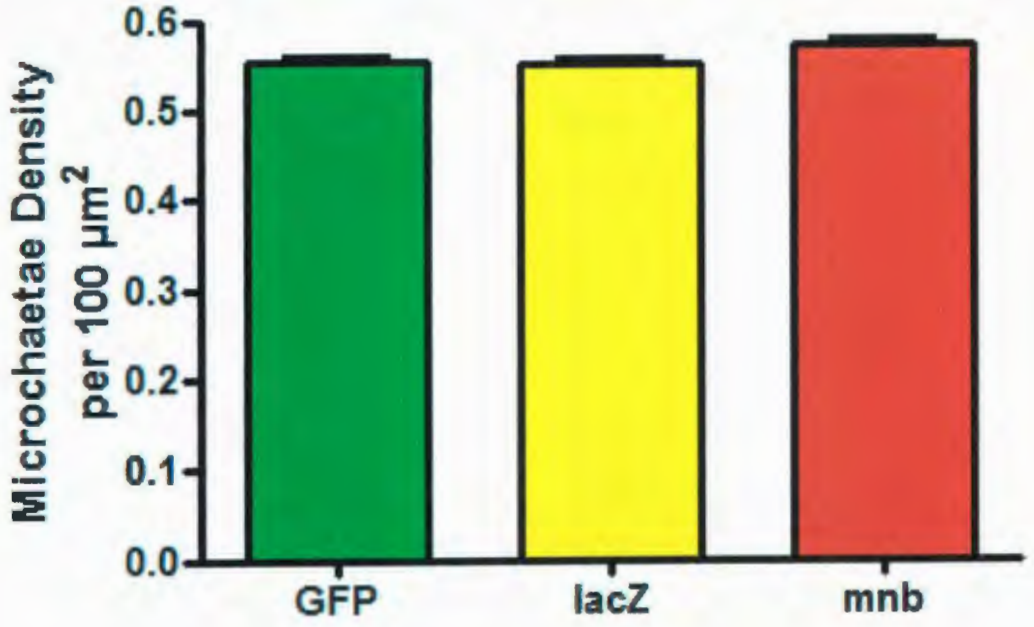
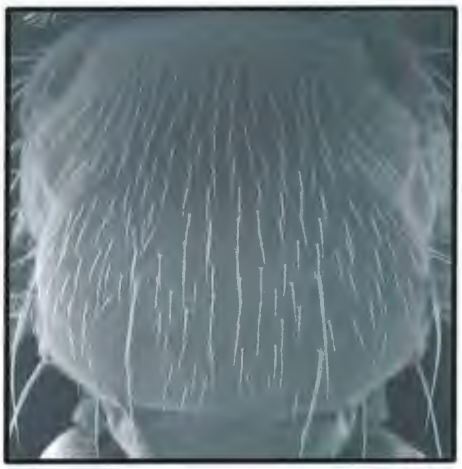


Figure 11. Biometric analysis of a potential role of *mnb* in neurogenesis with the *pannierGal4* transgene in females. Directed expression of *mnb* in the dorsal notum had no phenotypic effect on microchaetae density based on a one-way ANOVA (n=30). Micrographs of dorsal notums are shown in A. Graphic 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*.

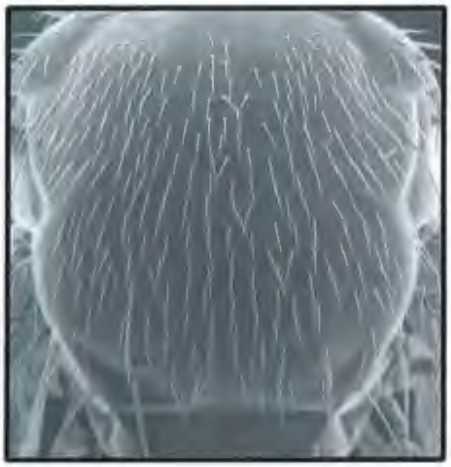
akt over-expression affects neurogenesis in the dorsal notum but foxo1 over-expression does not show a phenotypic effect

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.

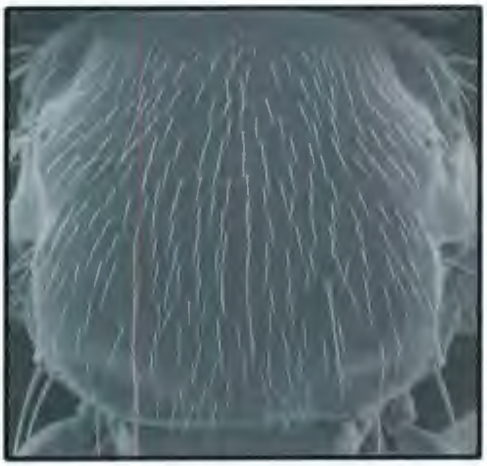
A



GFP



lacZ



akt

B

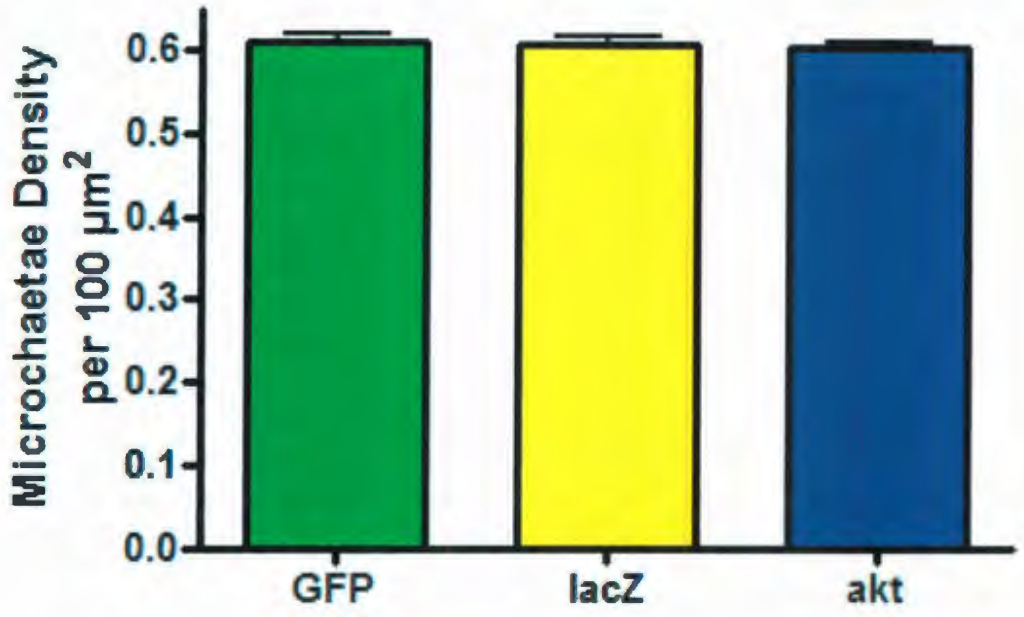
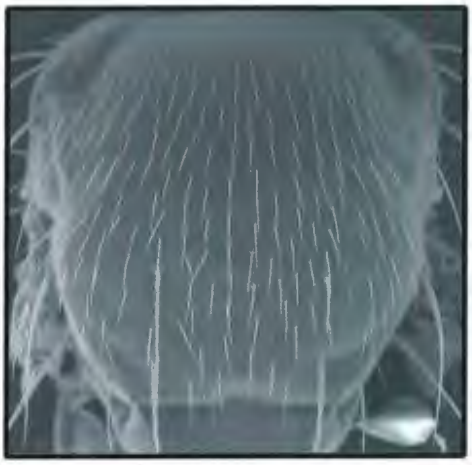
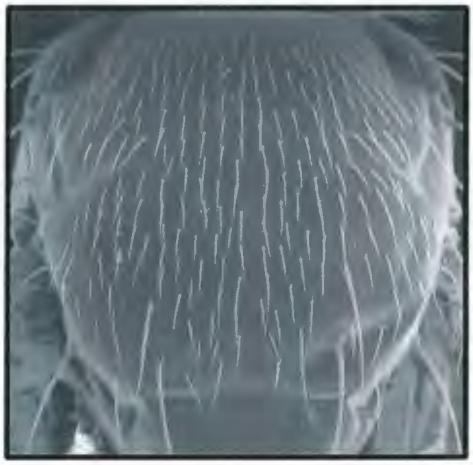


Figure 12. Biometric analysis of a potential role of *akt* in neurogenesis with the *apterousGal4* transgene in females. Directed expression of *akt* in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA (n=30). Micrographs of dorsal notums are shown in A. Graphic 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.*

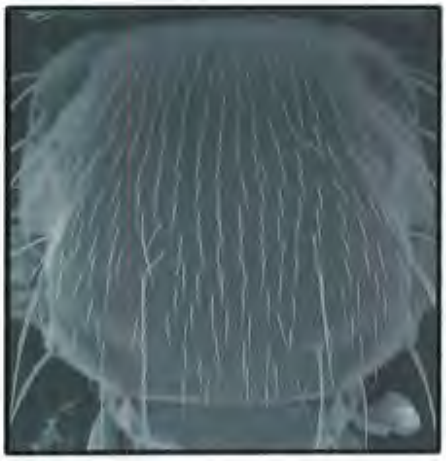
A



GFP



lacZ



akt

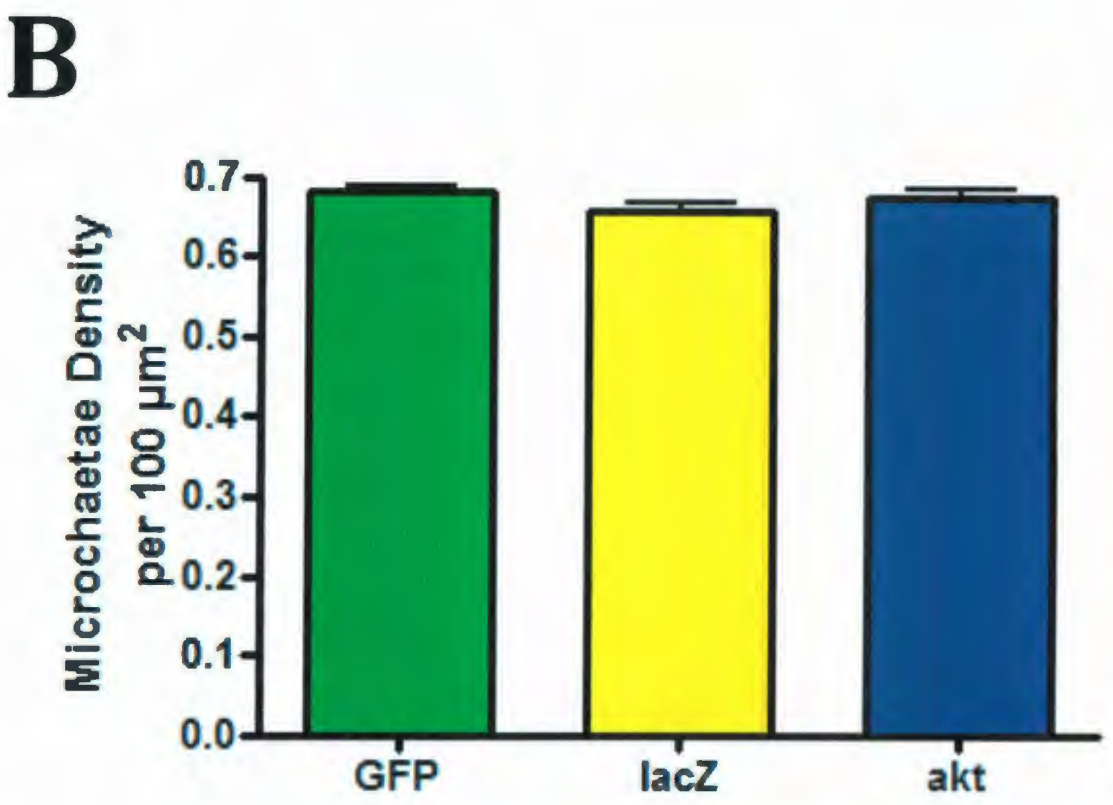
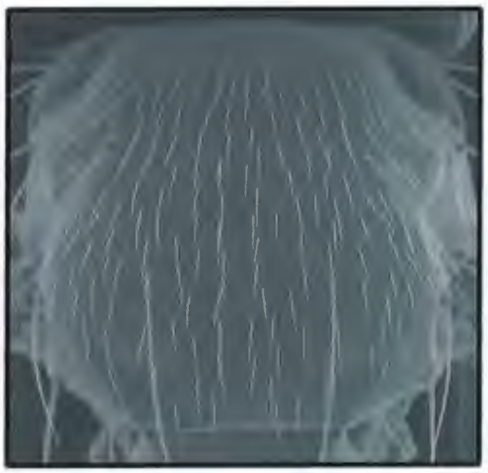
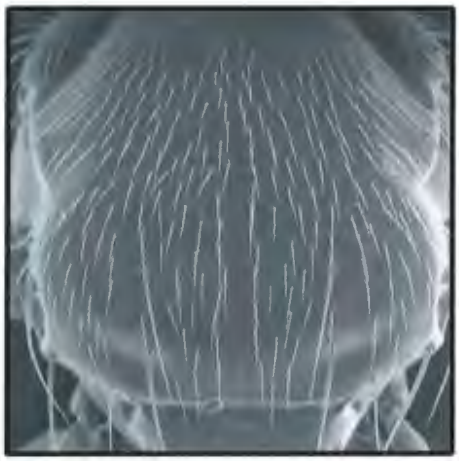


Figure 13. Biometric analysis of a potential role of *akt* in neurogenesis with the *apterousGal4* transgene in males. Directed expression of *akt* in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA (n=30). Micrographs of dorsal notums are shown in A. Graphic representations of microchaetae density are shown in B (values represent mean ± SEM). The genotypes are as follows: ***GFP*** *UASGFP/+; pnrGal4/+*, ***lacZ*** *UASlacZ/+; pnrGal4/+*, **akt** *UASakt/+; pnrGal4/+*.

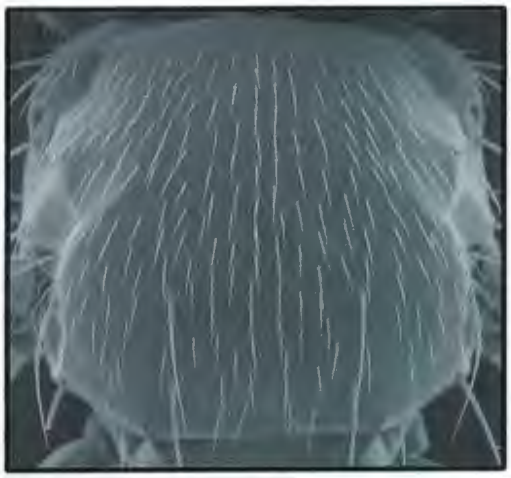
A



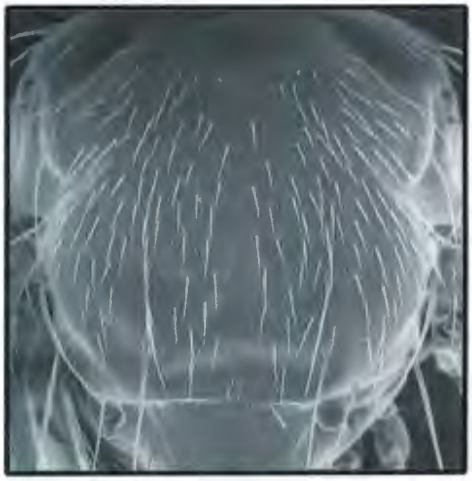
GFP



lacZ



akt



foxo

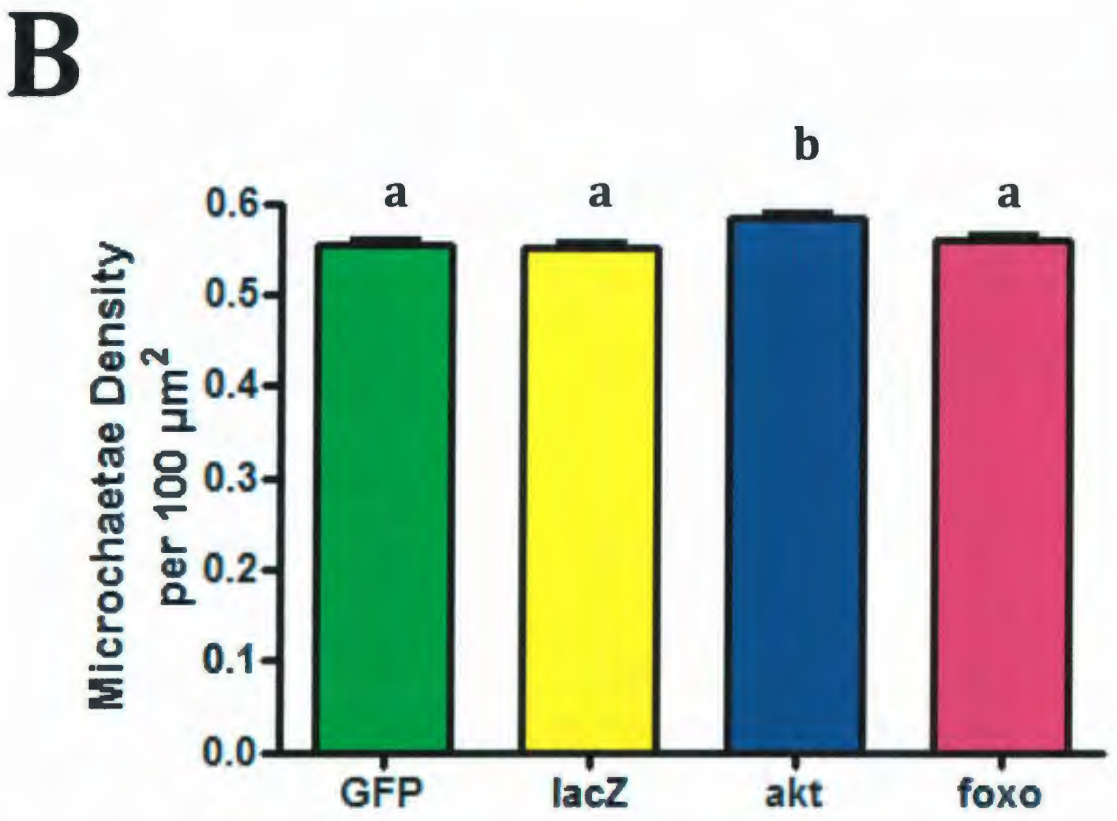
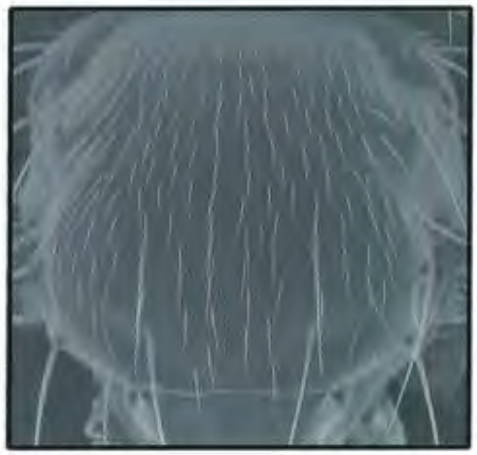
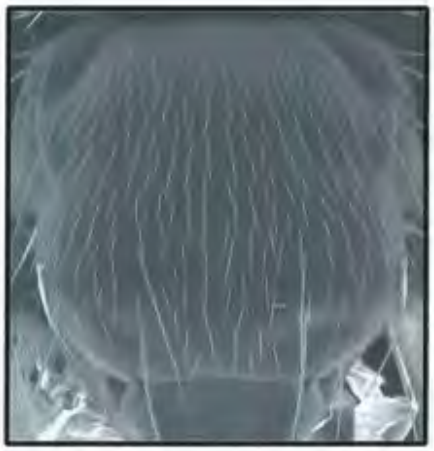


Figure 14. Biometric analysis of a potential role of *akt* and *foxo1* on neurogenesis with the *pannierGal4* transgene in females. Directed expression of *foxo1* in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA (n=20), while expression of *akt* increases 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.

A

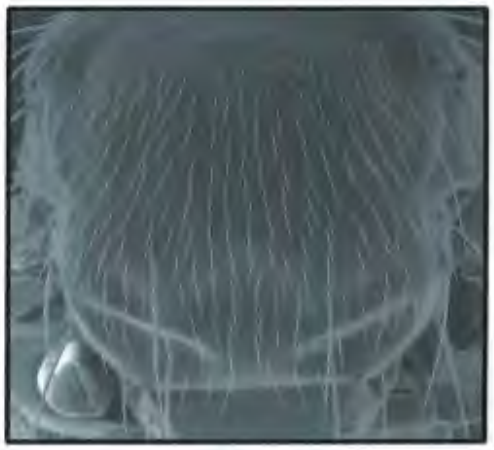


GFP



lacZ

akt



foxo

B

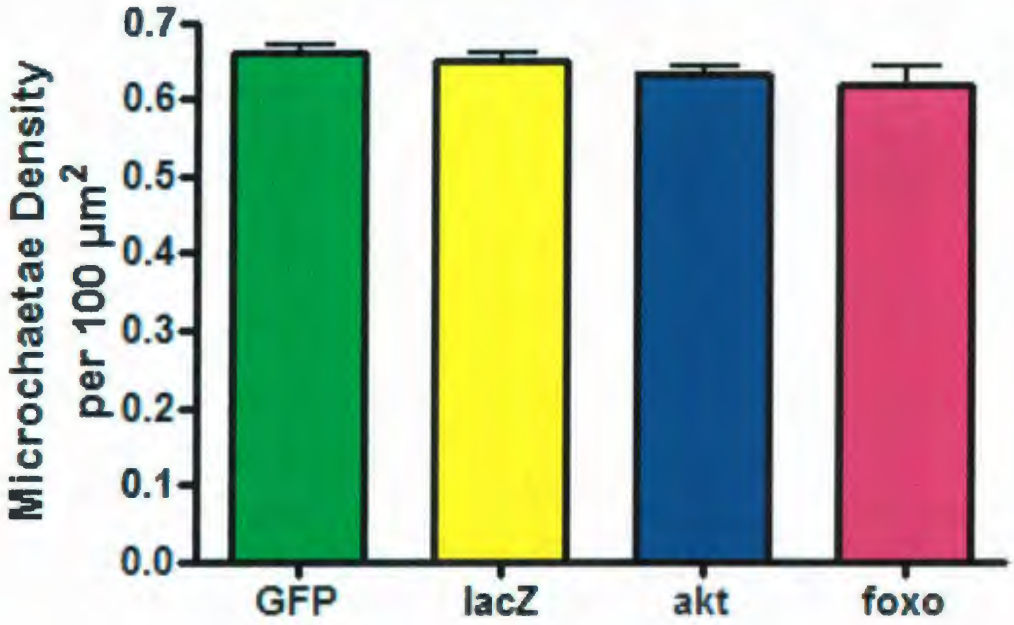


Figure 15. Biometric analysis of a potential role of *akt* and *foxo1* in neurogenesis with the *pannierGal4* transgene in males. Directed expression of *akt* (n=30) and *foxo1* (n=7) in the dorsal notum has no phenotypic effect on microchaetae density based on a one-way ANOVA. Micrographs of dorsal 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.

Comparison	Transcription factor	Sex	Significance
akt vs. *GFP*	*apterous*	F M	NS NS
akt vs. *lacZ*	*apterous*	F M	NS NS
foxo1 vs. *GFP*	*apterous*	F M	flies died flies died
foxo1 vs. *lacZ*	*apterous*	F M	flies died flies died
akt vs. *GFP*	*pannier*	F M	↑ $p < 0.001$ NS
akt vs. *lacZ*	*pannier*	F M	↑ $p < 0.001$ NS
foxo1 vs. *GFP*	*pannier*	F M	NS NS
foxo1 vs. *lacZ*	*pannier*	F M	NS NS
akt vs. *foxo1*	*apterous*	F M	NA NA
akt vs. *foxo1*	*pannier*	F M	↑ $p < 0.01$ NS
mnb vs. *GFP*	*apterous*	F	NS
mnb vs. *lacZ*	*apterous*	F	NS
mnb vs. *GFP*	*pannier*	F	NS
mnb vs. *lacZ*	*pannier*	F	NS
GFP vs. *lacZ*	*apterous*	F M	NS NS
GFP vs. *lacZ*	*pannier*	F M	NS NS

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

p values based on Neuman-Keuls post tests

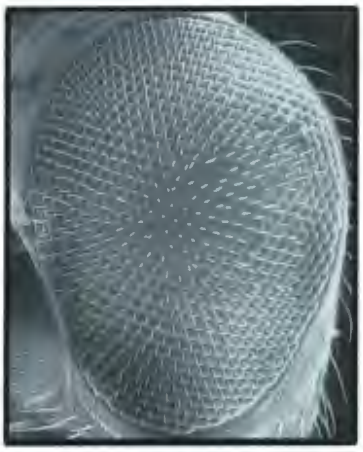
NS= not significant F=female M=male

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*, $Hip1^{L-2}$ and $Hip1^{L-6}$ or the truncated versions of *Hip1*, $Hip1\Delta ANTH^{5.2}$ or $Hip1\Delta ANTH^{11.2}$, 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

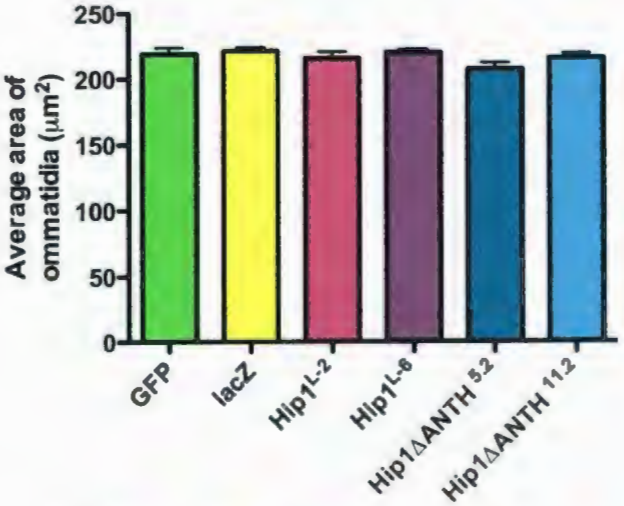
Hip1^{L-2}

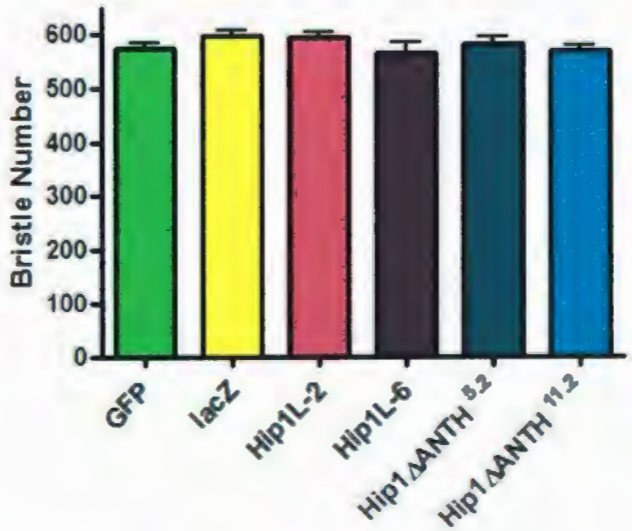
Hip1^{L-6}

Hip1ΔANTH$^{5.2}$

Hip1ΔANTH$^{11.2}$

B


Average area of ommatidia (μm²)
250
200
150
100
50
0
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2


Bristle Number
600
500
400
300
200
100
0
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2

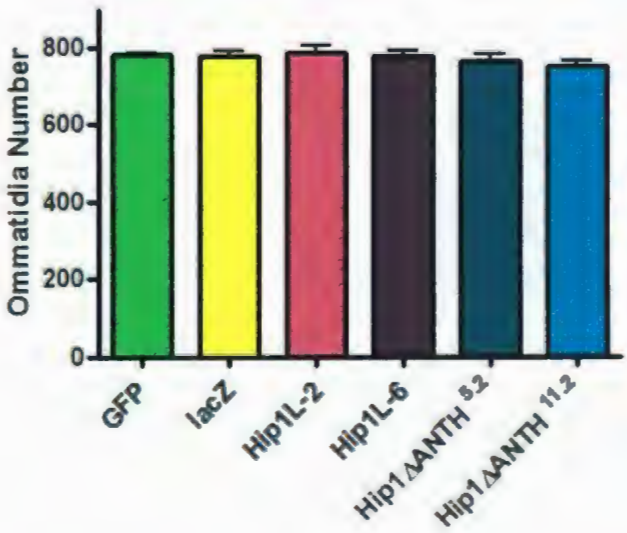

Ommatidia Number
800
600
400
200
0
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2

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*, $Hip1^{L-2}$ and $Hip1^{L-6}$ and two truncated versions of *Hip1*, $Hip1\Delta ANTH^{5.2}$ and $Hip1\Delta ANTH^{11.2}$. *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*, $\boldsymbol{Hip1^{L-2}}$ $UASHip1^{L-2}/+$; *GMRGal4/+*, $\boldsymbol{Hip1^{L-6}}$ $UASHip1^{L-6}/+$; *GMRGal4/+*, $\boldsymbol{Hip1\Delta ANTH^{5.2}}$ $UASHip1^{5.2}$*/GMRGal4*, $\boldsymbol{Hip1\Delta ANTH^{11.2}}$ $UASHip1^{11.2}/+$; *GMRGal4/+*.

A

GFP

lacZ

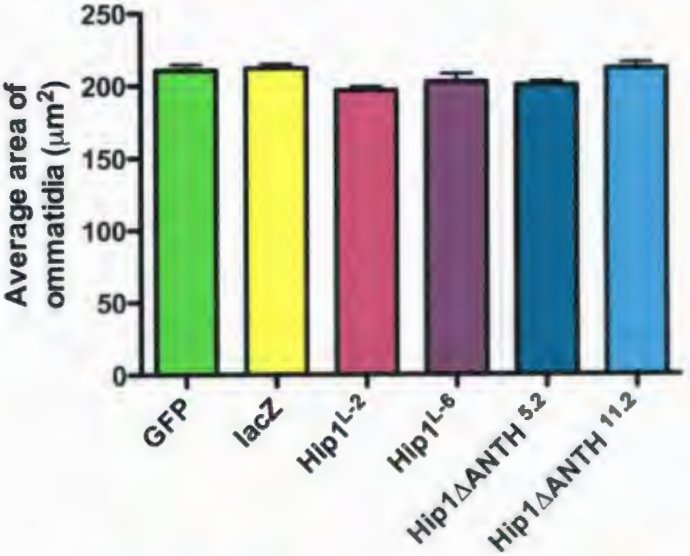
Hip1^{L-2}

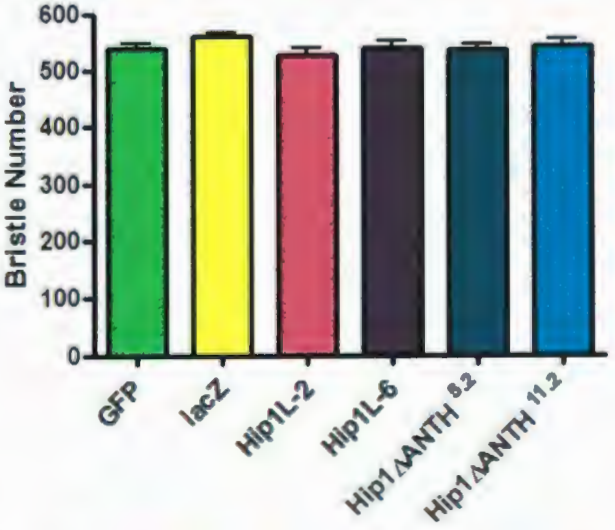
Hip1^{L-6}

Hip1ΔANTH$^{5.2}$

Hip1ΔANTH$^{11.2}$

B


Average area of ommatidia (μm²)
250
200
150
100
50
0
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2


Bristle Number
600
500
400
300
200
100
0
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2

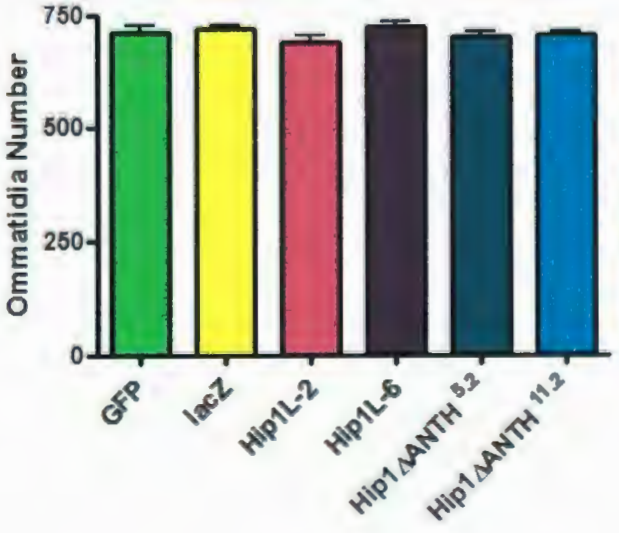
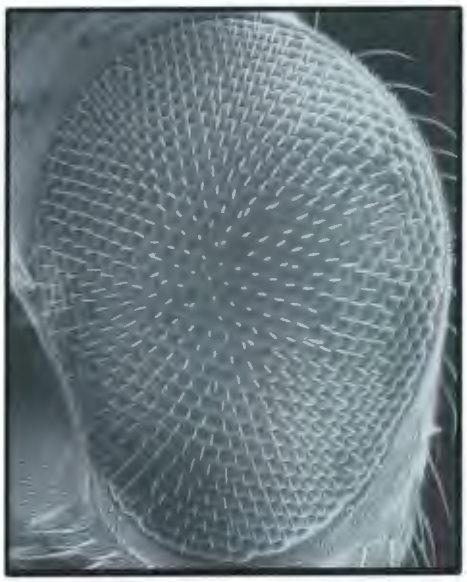

Ommatidia Number
750
500
250
0
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2

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*, $Hip1^{L-2}$ and $Hip1^{L-6}$, two truncated versions of *Hip1*, $Hip1\Delta ANTH^{5.2}$ and $Hip1\Delta ANTH^{11.2}$ 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*, $\boldsymbol{Hip1^{L-2}}$ $UASHip1^{L-2}/+$; *GMRGal4/+*, $\boldsymbol{Hip1^{L-6}}$ $UASHip1^{L-6}/+$; *GMRGal4/+*, $\boldsymbol{Hip1\Delta ANTH^{5.2}}$ $UASHip1^{5.2}$/*GMRGal4*, $\boldsymbol{Hip1\Delta ANTH^{11.2}}$ $UASHip1^{11.2}/+$; *GMRGal4/+*.

mnb has no phenotypic affect on eye development

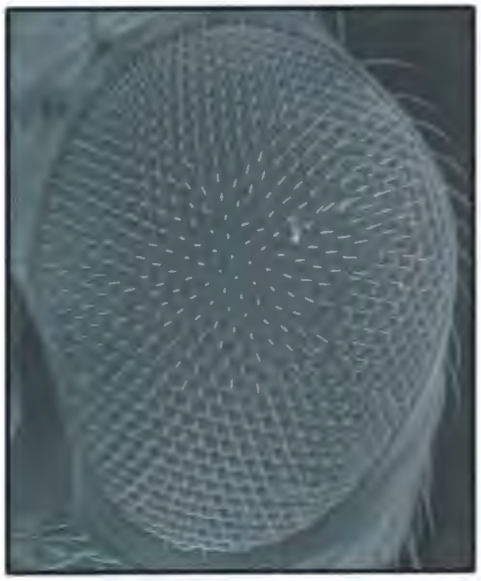
Studies in our laboratory have found that the over-expression of *mnb* along with *foxo1* over-expression disrupts wild-type eye development (Rotchford 2006). However, the directed expression of *mnb* has not been carefully studied in eye development. This study utilized the *GMRGal4* transgene to direct 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



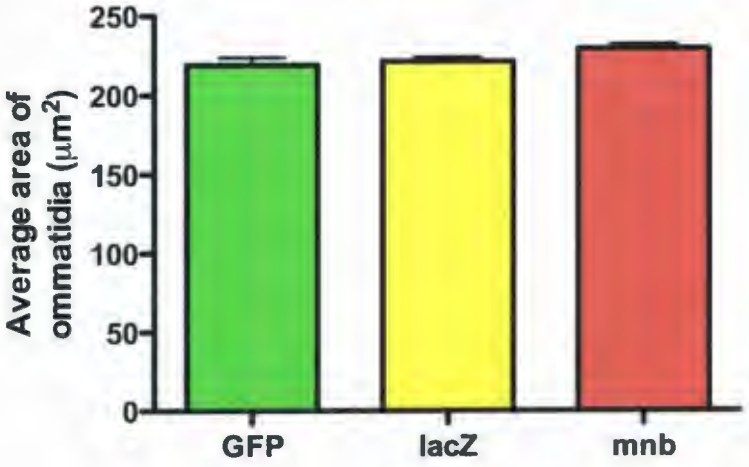
GFP

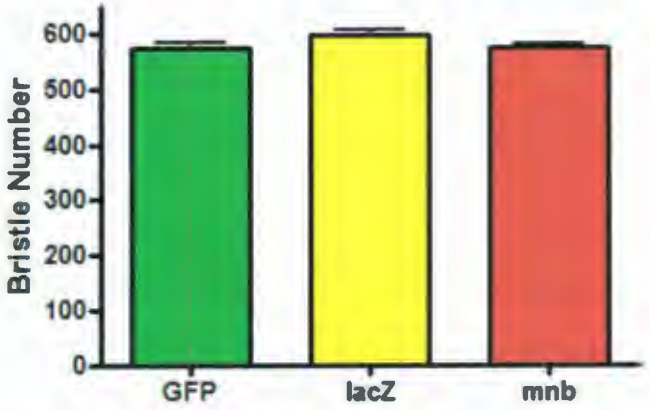
lacZ



mnb

B


Average area of ommatidia (μm²)
250
200
150
100
50
0
GFP
lacZ
mnb


Bristle Number
600
500
400
300
200
100
0
GFP
lacZ
mnb

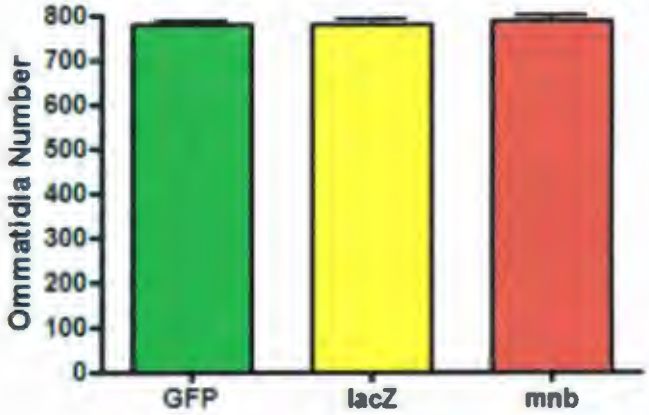

Ommatidia Number
800
700
600
500
400
300
200
100
0
GFP
lacZ
mnb

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*** *UASmnb*^{EY14320}*/+; 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 *GMRGal4* (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*, $Hip1\Delta ANTH^{5.2}$ and $Hip1\Delta ANTH^{11.2}$, 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 $Hip1^{L-2}$ and $Hip1^{L-6}$ 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 $Hip1^{L-2}$, $Hip1^{L-6}$, 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*, $Hip1\Delta ANTH^{5.2}$ and $Hip1\Delta ANTH^{11.2}$ compared to controls, with the exception of $Hip1\Delta ANTH^{5.2}$, 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*, $Hip1^{L-2}$ and $Hip1^{L-6}$, or between the two truncated versions of

Hip1, *Hip1ΔANTH*$^{5.2}$ and *Hip1ΔANTH*$^{11.2}$ 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 *Hip1ΔANTH*$^{5.2}$ females which show no difference compared to *lacZ* controls (Figures 19 and 20). As well, *mnb* females had significantly lower ommatidida 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*, *Hip1*$^{L-2}$ and *Hip1*$^{L-6}$, but there was a difference between the two truncated versions of Hip1, *Hip1ΔANTH*$^{5.2}$ and *Hip1ΔANTH*$^{11.2}$ (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).

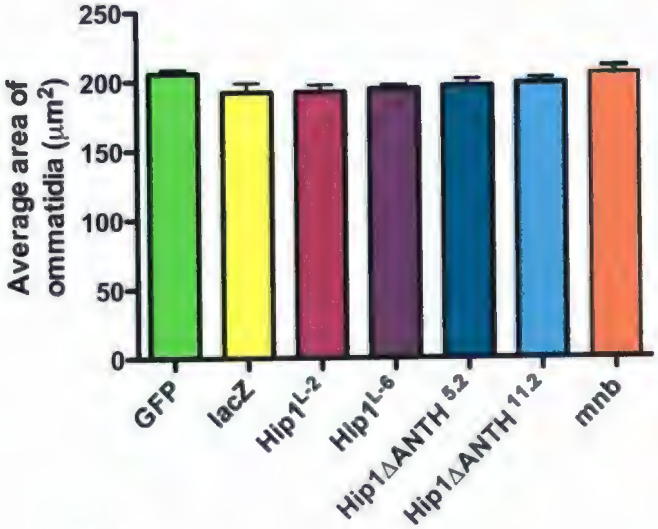
A

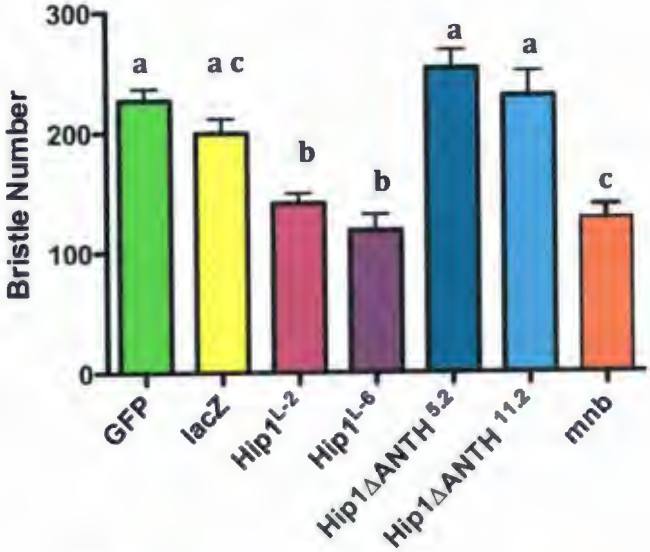
GFP lacZ mnb

$Hip1^{L-2}$ $Hip1^{L-6}$

$Hip1\Delta ANTH^{5.2}$ $Hip1\Delta ANTH^{11.2}$

B


Average area of ommatidia (μm²)
250
200
150
100
50
0
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2
mnb


Bristle Number
300
200
100
0
a
a c
b
b
a
a
c
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2
mnb

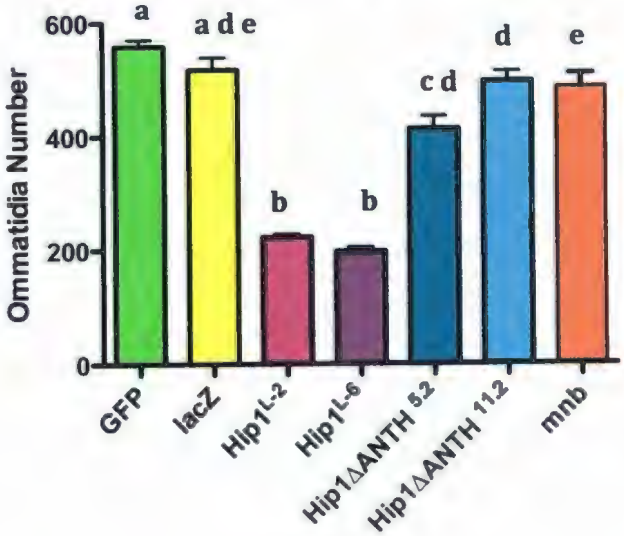
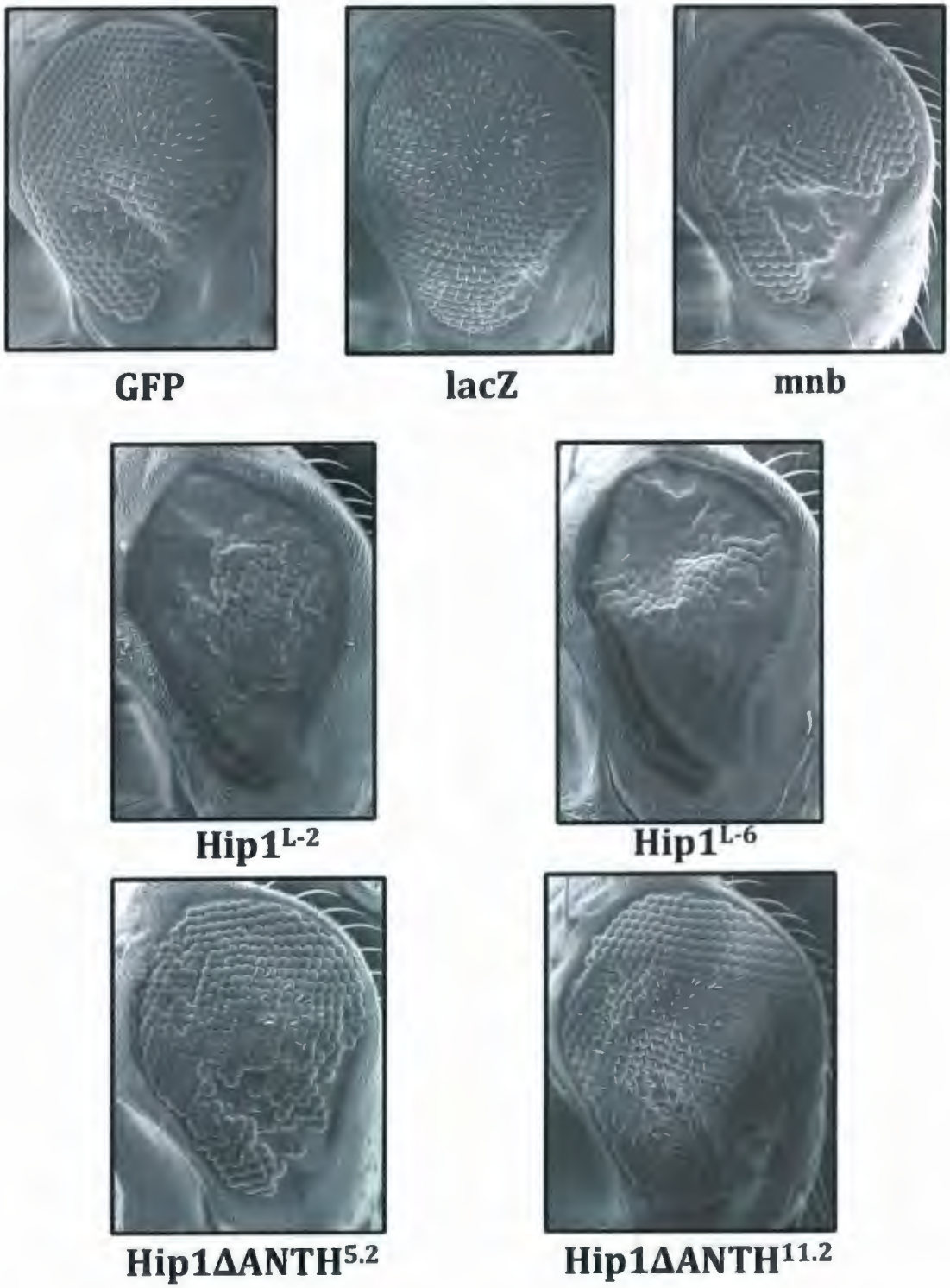
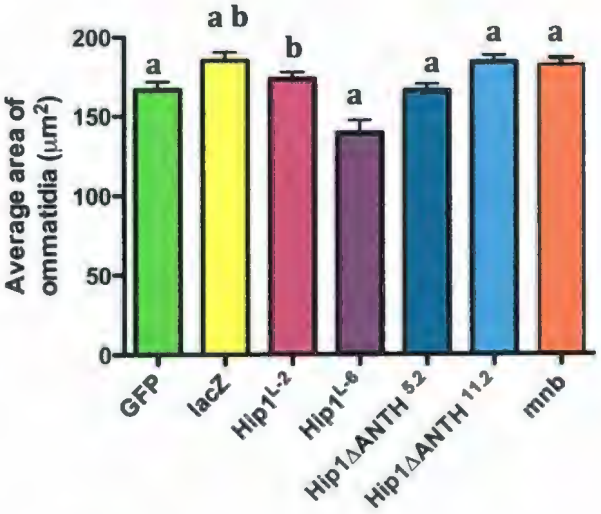

Ommatidia Number
600
400
200
0
a
a d e
b
b
c d
d
e
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2
mnb

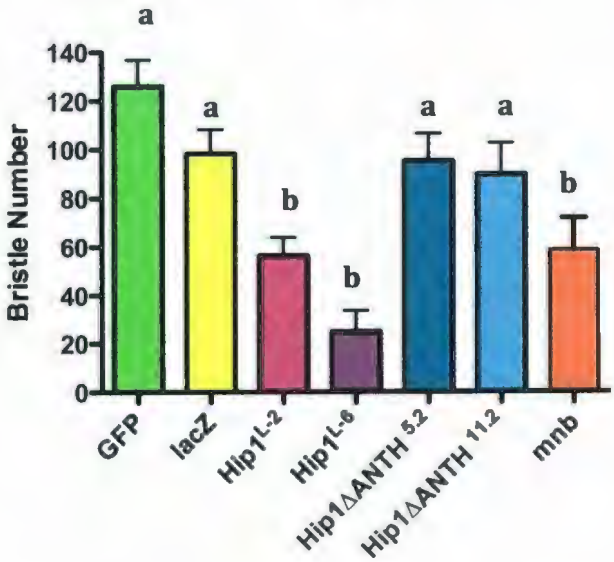
Figure 19. Biometric analysis of a potential role of *Hip1*, *Hip1ΔANTH* and *mnb* in eye development along with the *GMRGal4* and *UASfoxo1* transgenes in females. Directed expression of both full length versions of *Hip1*, $Hip1^{L-2}$ and $Hip1^{L-6}$, the truncated versions of *Hip1*, $Hip1\Delta ANTH^{5.2}$ and $Hip1\Delta ANTH^{11.2}$, 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*, $\boldsymbol{Hip1^{L-2}}$ $Hip1^{L-2}$*/+; GMRGal4UASfoxo1/+*, $\boldsymbol{Hip1^{L-6}}$ $Hip1^{L-6}$*/+; GMRGal4UASfoxo1/+*, $\boldsymbol{Hip1\Delta ANTH^{5.2}}$ $Hip1^{5.2}$*/GMRGal4UASfoxo1*, $\boldsymbol{Hip1\Delta ANTH^{11.2}}$ $Hip1^{11.2}$*/+; GMRGal4UASfoxo1/+*, ***mnb*** $UASmnb^{EY14320}$*/+; GMRGal4UASfoxo1/+*. Bars with dissimilar superscripts indicate groups that differ significantly.

A



B


Average area of ommatidia (μm²)
200
150
100
50
0
a
a b
b
a
a
a
a
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2
mnb


Bristle Number
140
120
100
80
60
40
20
0
a
a
b
b
a
a
b
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2
mnb

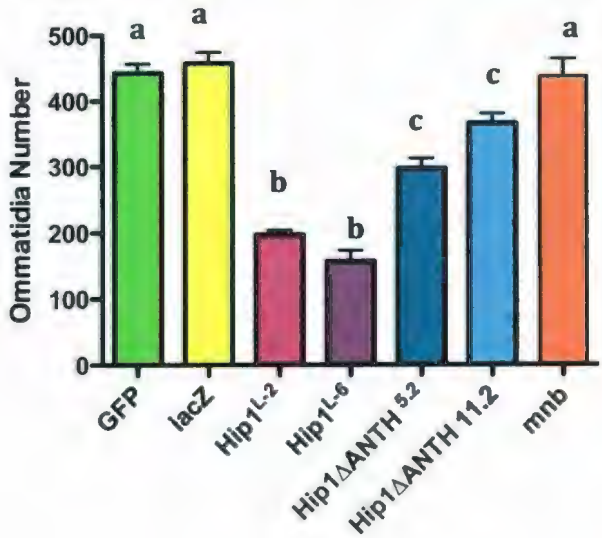

Ommatidia Number
500
400
300
200
100
0
a
a
b
b
c
c
a
GFP
lacZ
Hip1L-2
Hip1L-6
Hip1ΔANTH 5.2
Hip1ΔANTH 11.2
mnb

Figure 20. Biometric analysis of a potential role of *Hip1*, *Hip1ΔANTH* and *mnb* on eye development along with the *GMRGal4* and *UASfoxo1* transgenes in males. Directed expression of both full length versions of *Hip1*, $Hip1^{L\text{-}2}$ and $Hip1^{L\text{-}6}$, the truncated versions of *Hip1*, $Hip1\Delta ANTH^{5.2}$ and $Hip1\Delta ANTH^{11.2}$, 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/GMRGal4UASfoxo1*, ***lacZ*** *UASlacZ/GMRGal4UASfoxo1*, $\boldsymbol{Hip1^{L\text{-}2}}$ $Hip1^{L\text{-}2}$*/+; GMRGal4UASfoxo1/+*, $\boldsymbol{Hip1^{L\text{-}6}}$ $Hip1^{L\text{-}6}$*/+; GMRGal4UASfoxo1/+*, $\boldsymbol{Hip1\Delta ANTH^{5.2}}$ $Hip1^{5.2}$*/GMRGal4UASfoxo1*, $\boldsymbol{Hip1\Delta ANTH^{11.2}}$ $Hip1^{11.2}$*/+; GMRGal4UASfoxo1/+*, ***mnb*** $UASmnb^{EY14320}$*/¬; GMRGal4UASfoxo1/+*. Bars with dissimilar superscripts indicate groups that differ significantly.

Table 4. Comparison of differences in ommatidia area. bristle number and ommaditia number when *mnb* or *Hip1/Hip1ΔANTH* is expressed along with *foxo1* via the *GMRGal4* transgene.

Comparison	Sex	Ommatidia Area	Bristle Number	Ommatidia Number
Hip1 $^{L-2}$	F	NS	↓ $p < 0.0001$	↓ $p < 0.0001$
vs. *GFP*	M	↓ $p < 0.001$	↓ $p < 0.0001$	↓ $p < 0.0001$
Hip1 $^{L-6}$	F	NS	↓ $p < 0.0001$	↓ $p < 0.0001$
vs. *GFP*	M	NS	↓ $p < 0.0001$	↓ $p < 0.0001$
Hip1ΔANTH$^{5.2}$	F	NS	NS	↓ $p < 0.0001$
vs. *GFP*	M	NS	NS	↓ $p < 0.0001$
Hip1ΔANTH$^{11.2}$	F	NS	NS	↓ $p < 0.01$
vs. *GFP*	M	NS	NS	↓ $p < 0.01$
mnb	F	NS	↓ $p < 0.0001$	↓ $p < 0.01$
vs. *GFP*	M	NS	↓ $p < 0.0001$	NS
Hip1 $^{L-2}$	F	NS	↓ $p < 0.001$	↓ $p < 0.0001$
vs. lacZ	M	NS	NS	↓ $p < 0.0001$
Hip1 $^{L-6}$	F	NS	↓ $p < 0.0001$	↓ $p < 0.0001$
vs. lacZ	M	↓ $p < 0.0001$	↓ $p < 0.0001$	↓ $p < 0.0001$
Hip1ΔANTH$^{5.2}$	F	NS	↑ $p < 0.01$	↓ $p < 0.0001$
vs. *lacZ*	M	NS	NS	↓ $p < 0.0001$
Hip1ΔANTH$^{11.2}$	F	NS	NS	NS
vs. *lacZ*	M	NS	NS	↓ $p < 0.001$
mnb	F	NS	↓ $p < 0.001$	NS
vs. *lacZ*	M	NS	NS	NS
Hip1^{L-2}	F	NS	NS	NS
vs. *Hip1*$^{L-6}$	M	↑ $p < 0.0001$	NS	NS
Hip1ΔANTH$^{5.2}$	F	NS	NS	↓ $p < 0.01$
vs. *Hip1ΔANTH*$^{11.2}$	M	NS	NS	↓ $p < 0.01$
Hip1^{L-2}	F	NS	↓ $p < 0.0001$	↓ $p < 0.0001$
vs. *Hip1ΔANTH*$^{5.2}$	M	NS	↓ $p < 0.01$	↓ $p < 0.0001$
Hip1^{L-6}	F	NS	↓ $p < 0.0001$	↓ $p < 0.0001$
vs. *Hip1ΔANTH*$^{5.2}$	M	↓ $p < 0.0001$	↓ $p < 0.0001$	↓ $p < 0.0001$
Hip1^{L-2}	F	NS	↓ $p < 0.0001$	↓ $p < 0.0001$
vs. *Hip1ΔANTH*$^{11.2}$	M	NS	NS	↓ $p < 0.0001$
Hip1^{L-6}	F	NS	↓ $p < 0.0001$	↓ $p < 0.0001$
vs. *Hip1ΔANTH*$^{11.2}$	M	↓ $p < 0.0001$	↓ $p < 0.0001$	↓ $p < 0.0001$
Hip1^{L-2}	F	NS	NS	↓ $p < 0.0001$
vs. *mnb*	M	NS	NS	↓ $p < 0.0001$
Hip1^{L-6}	F	NS	NS	↓ $p < 0.0001$
vs. *mnb*	M	$p < 0.0001$	NS	↓ $p < 0.0001$
Hip1ΔANTH$^{5.2}$	F	NS	↑ $p < 0.0001$	↓ $p < 0.0001$
vs. *mnb*	M	NS	NS	↓ $p < 0.0001$
Hip1ΔANTH$^{11.2}$	F	NS	↑ $p < 0.0001$	NS

vs. *mnb*	M	NS	NS	↓ $p < 0.001$
GFP	F	NS	NS	NS
vs. *lacZ*	M	NS	NS	NS

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 foxo1 sensitized eye development when compared to mnb with foxo1 and Hip1 with foxo1

As *mnb* and *Hip1* have been found to interact with *foxo1* 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 *foxo1*-sensitized eye development. To determine if *Hip1* and *mnb* interact, *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. A summary of these results can be seen in Table 5. Notably, when compared to *Hip1* with *foxo1* controls, *Hip1* and *mnb* together with *foxo1* increase ommatidia area for both full length versions, $Hip1^{L-2}$ and $Hip1^{L-6}$ and one truncated version of *Hip1*, $Hip1\Delta ANTH^{1.2}$ (Figures 21 and 22). Compared to *mnb* and *foxo1* alone, *Hip1* and *mnb* together with *foxo1* increase ommatidia area for one full length version, $Hip1^{L-2}$, and one truncated version of *Hip1*, $Hip1\Delta ANTH^{1.2}$ (Figures 21 and 22). Differences in ommatidia 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 *foxo1* decreased bristle number for all versions of *Hip1* compared to the *Hip1* with *foxo1* controls (Figures 21 and 22). When compared to the *mnb* with *foxo1* 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*,

Hip1^{L-2} and *Hip1*$^{L-6}$, 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*$^{1.2}$ in males (Figures 21 and 22).

There was no significant difference in ommatidia number between the full length versions of *Hip1* with *mnb* and *foxo1* compared to *Hip1* with *foxo1* controls. (Figures 21 and 22) Ommatidia number was increased in flies over-expressing the two truncated versions of *Hip1*, *Hip1ΔANTH*$^{5.2}$ and *Hip1ΔANTH*$^{1.2}$compared to Hip1 with *foxo1* controls (Figures 21 and 22). When compared to mnb with *foxo1* controls, both the full length versions and one truncated version of Hip1 increased ommatidia number, while *Hip1ΔANTH*$^{1.2}$ decreased ommatidia area (Figures 21 and 22). There was no difference between the two full length versions of Hip1 with mnb and *foxo1*, 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 *foxo1*-sensitized eye development to alter the ommatidia area, bristle number and ommatidia number compared to controls.

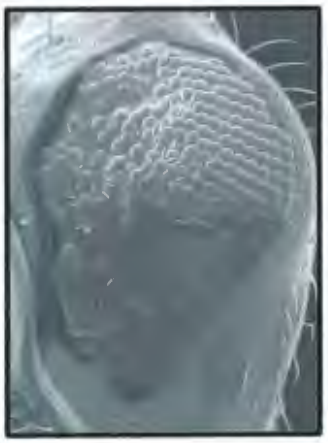
A *GMRGal4UAS foxo1* plus ...

mnb

Hip1^{L-2}

mnb; Hip1^{L-2}

Hip1^{L-6}



mnb; Hip1^{L-6}

A *GMRGal4UAS foxo1* plus ...

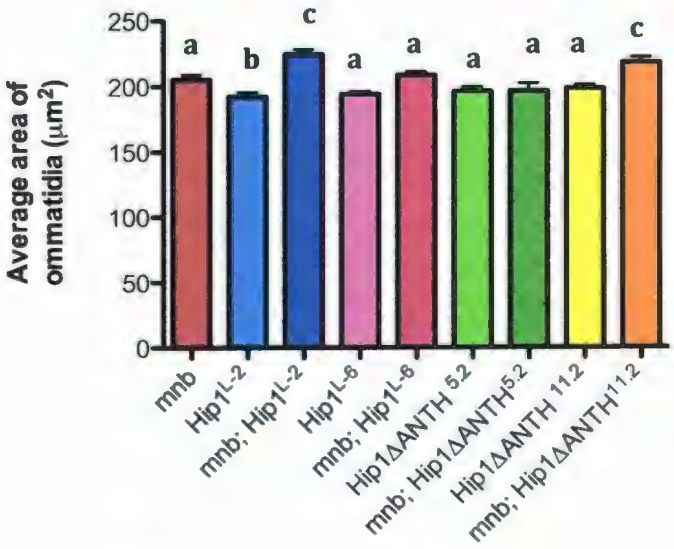
Hip1ΔANTH$^{5.2}$

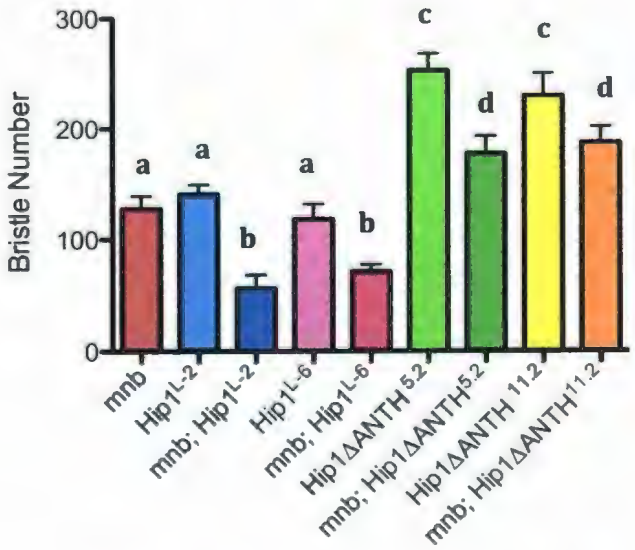
mnb; Hip1ΔANTH$^{5.2}$

Hip1ΔANTH$^{11.2}$

mnb; Hip1ΔANTH$^{11.2}$

B


Average area of ommatidia (μm²)
250
200
150
100
50
0
a
b
c
a
a
a
a
a
c
mnb
Hip1L-2
mnb; Hip1L-2
Hip1L-6
mnb; Hip1L-6
Hip1ΔANTH 5.2
mnb; Hip1ΔANTH5.2
Hip1ΔANTH 11.2
mnb; Hip1ΔANTH11.2


Bristle Number
300
200
100
0
a
a
b
a
b
c
d
c
d
mnb
Hip1L-2
mnb; Hip1L-2
Hip1L-6
mnb; Hip1L-6
Hip1ΔANTH 5.2
mnb; Hip1ΔANTH5.2
Hip1ΔANTH 11.2
mnb; Hip1ΔANTH11.2

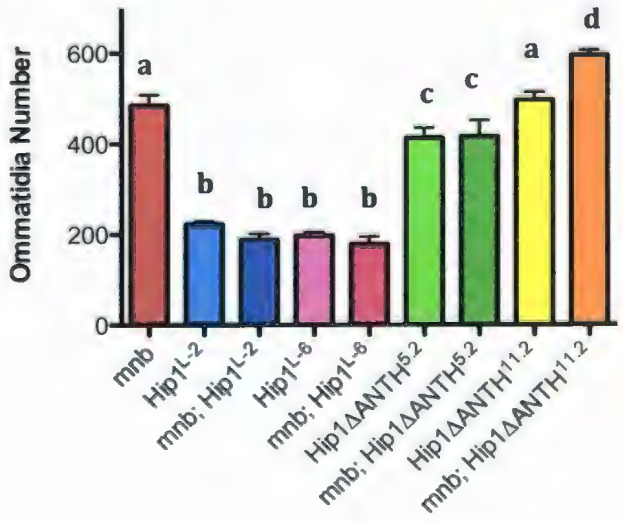

Ommatidia Number
600
400
200
0
a
b
b
b
b
c
c
a
d
mnb
Hip1L-2
mnb; Hip1L-2
Hip1L-6
mnb; Hip1L-6
Hip1ΔANTH5.2
mnb; Hip1ΔANTH5.2
Hip1ΔANTH11.2
mnb; Hip1ΔANTH11.2

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*, *Hip1*$^{L-2}$ and *Hip1*$^{L-6}$, the truncated versions of *Hip1*, *Hip1ΔANTH*$^{5.2}$ and *Hip1ΔANTH*$^{11.2}$, 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*^{EY14320}*/+; GMRGal4UASfoxo1/+*, ***Hip1***$^{L-2}$ *UASHip1*$^{L-2}$*/+; GMRGal4UASfoxo1/+*, ***mnb; Hip1***$^{L-2}$ *UASmnb*^{EY14320}*/+; UASHip1*$^{L-2}$*/+; GMRGal4UASfoxo1/+*, ***Hip1***$^{L-6}$ *UASHip1*$^{L-6}$*/+; GMRGal4UASfoxo1/+*, ***mnb; Hip1***$^{L-6}$ *UASmnb*^{EY14320}*/+; UASHip1*$^{L-6}$*/+; GMRGal4UASfoxo1/+*, ***Hip1ΔANTH***$^{5.2}$ *UASHip1*$^{5.2}$*/GMRGal4UASfoxo1*, ***mnb; Hip1ΔANTH***$^{5.2}$ *UASmnb*^{EY14320}*/+; UASHip1*$^{5.2}$*/GMRGal4UASfoxo1*, ***Hip1ΔANTH***$^{11.2}$ *UASHip1*$^{11.2}$*/+; GMRGal4UASfoxo1/+*, ***mnb; Hip1ΔANTH***$^{11.2}$ *UASmnb*^{EY14320}*/+; UASHip1*$^{11.2}$*/+; GMRGal4UASfoxo1/+*. Bars with dissimilar superscripts indicate groups that differ significantly.

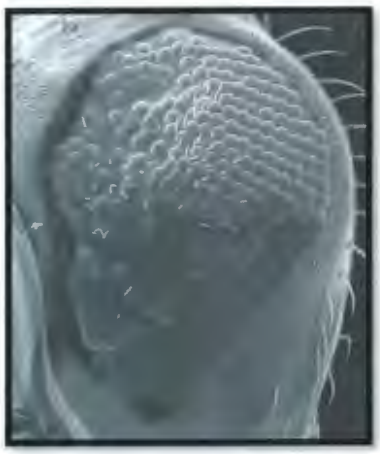
A *GMRGal4UAS foxo1* plus ...

mnb

Hip1^{L-2}

mnb; Hip1^{L-2}

Hip1^{L-6}



mnb; Hip1^{L-6}

A *GMRGal4UAS foxo1* plus ...

Hip1ΔANTH$^{5.2}$

mnb; Hip1ΔANTH$^{5.2}$

Hip1ΔANTH$^{11.2}$

mnb; Hip1ΔANTH$^{11.2}$

B

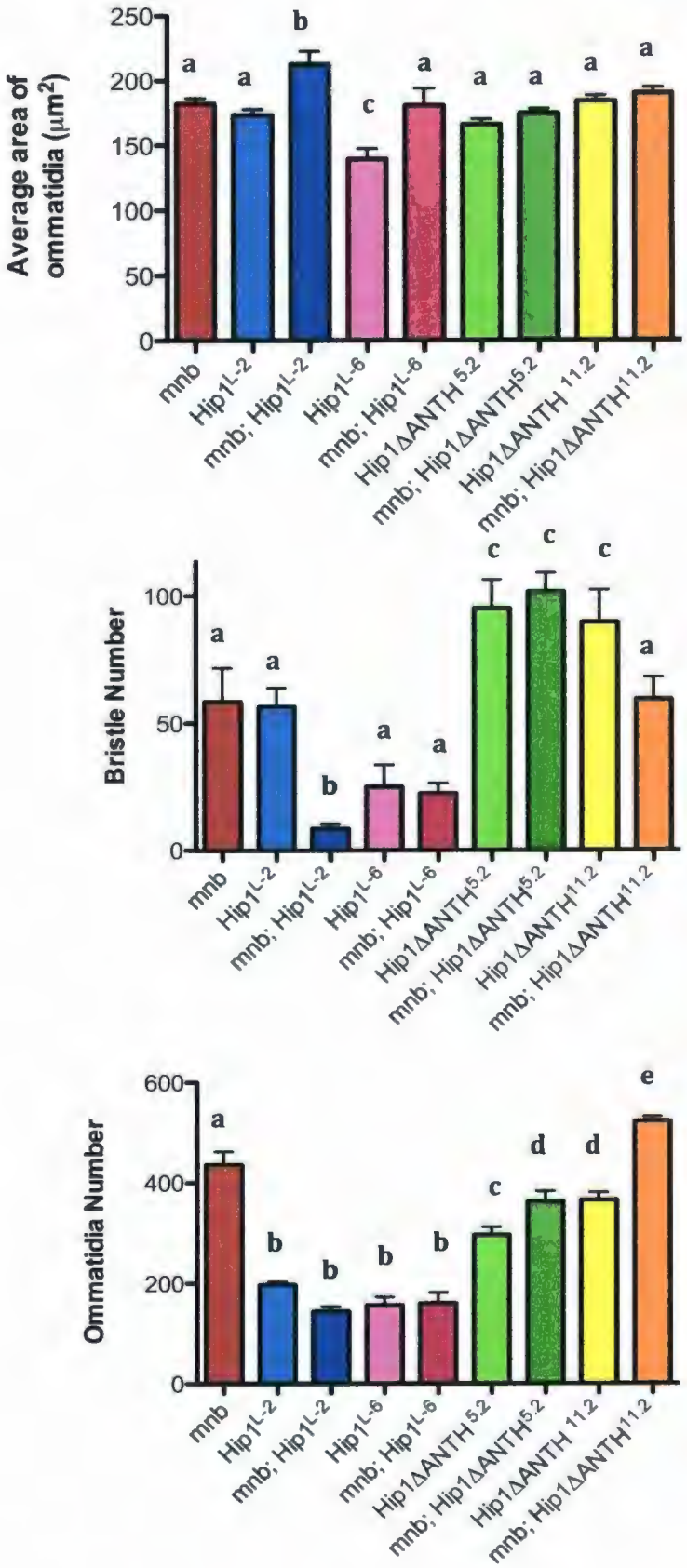

Average area of ommatidia (μm²)
250
200
150
100
50
0
a a b c a a a a a
mnb
Hip1L-2
mnb; Hip1L-2
Hip1L-6
mnb; Hip1L-6
Hip1ΔANTH5.2
mnb; Hip1ΔANTH5.2
Hip1ΔANTH11.2
mnb; Hip1ΔANTH11.2
Bristle Number
100
50
0
a a b a a c c c a
mnb
Hip1L-2
mnb; Hip1L-2
Hip1L-6
mnb; Hip1L-6
Hip1ΔANTH5.2
mnb; Hip1ΔANTH5.2
Hip1ΔANTH11.2
mnb; Hip1ΔANTH11.2
Ommatidia Number
600
400
200
0
a b b b b c d d e
mnb
Hip1L-2
mnb; Hip1L-2
Hip1L-6
mnb; Hip1L-6
Hip1ΔANTH5.2
mnb; Hip1ΔANTH5.2
Hip1ΔANTH11.2
mnb; Hip1ΔANTH11.2

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*, $Hip1^{L-2}$ and $Hip1^{L-6}$, the truncated versions of *Hip1*, $Hip1\Delta ANTH^{5.2}$ and $Hip1\Delta ANTH^{11.2}$, 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*$mnb^{EY14320}$*/+; GMRGal4UASfoxo1/+,* ***Hip1***$^{L-2}$ *UAS*$Hip1^{L-2}$*/+; GMRGal4UASfoxo1/+,* ***mnb; Hip1***$^{L-2}$ *UAS*$mnb^{EY14320}$*/+; UAS*$Hip1^{L-2}$*/+; GMRGal4UASfoxo1/+,* ***Hip1***$^{L-6}$ *UAS*$Hip1^{L-6}$*/+; GMRGal4UASfoxo1/+,* ***mnb; Hip1***$^{L-6}$ *UAS*$mnb^{EY14320}$*/+; UAS*$Hip1^{L-6}$*/+; GMRGal4UASfoxo1/+,* ***Hip1ΔANTH***$^{5.2}$ *UAS*$Hip1^{5.2}$*/GMRGal4UASfoxo1,* ***mnb; Hip1ΔANTH***$^{5.2}$ *UAS*$mnb^{EY14320}$*/+; UAS*$Hip1^{5.2}$*/GMRGal4UASfoxo1,* ***Hip1ΔANTH***$^{11.2}$ *UAS*$Hip1^{11.2}$*/+; GMRGal4UASfoxo1/+,* ***mnb; Hip1ΔANTH***$^{11.2}$ *UAS*$mnb^{EY14320}$*/+; UAS*$Hip1^{11.2}$*/+; GMRGal4UASfoxo1/+*. Bars with dissimilar superscripts indicate groups that differ significantly.

Table 5. Comparison of differences in ommatidia area, bristle number and ommaditia number when *mnb* and/or *Hip1/Hip1ΔANTH* is expressed along with *foxo1* via the *GMRGal4* transgene.

Comparison	Sex	Ommatidia Area	Bristle Number	Ommatidia Number
mnb; Hip1^{L-2}	F	↑ p < 0.0001	↓ p < 0.0001	NS
vs. *Hip1^{L-2}*	M	↑ p < 0.0001	↓ p < 0.001	NS
mnb; Hip1^{L-6}	F	NS	↓ p < 0.01	NS
vs. *Hip1^{L-6}*	M	↑ p < 0.0001	NS	NS
mnb; Hip1ΔANTH$^{5.2}$	F	NS	↓ p < 0.001	NS
vs. *Hip1ΔANTH$^{5.2}$*	M	NS	NS	↑ p < 0.001
mnb; Hip1ΔANTH$^{11.2}$	F	↑ p < 0.001	↓ p < 0.01	↑ p < 0.001
vs. *Hip1ΔANTH$^{11.2}$*	M	NS	NS	↑ p < 0.0001
mnb;Hip1^{L-2}	F	↑ p < 0.001	↓ p < 0.001	↓ p < 0.0001
vs. *mnb*	M	↑ p < 0.001	↓ p < 0.001	↓ p < 0.0001
mnb; Hip1^{L-6}	F	NS	↓ p < 0.01	↓ p < 0.0001
vs.*mnb*	M	NS	NS	↓ p < 0.0001
mnb; Hip1ΔANTH$^{5.2}$	F	NS	↑ p < 0.01	↓ p < 0.01
vs. *mnb*	M	NS	↑ p < 0.01	↓ p < 0.001
mnb; Hip1ΔANTH$^{11.2}$	F	↑ p < 0.01	↑ p < 0.01	↑ p < 0.0001
vs. *mnb*	M	NS	NS	↑ p < 0.0001
mnb;Hip1^{L-2}	F	↑ p < 0.01	NS	NS
vs. *mnb; Hip1^{L-6}*	M	↑ p < 0.01	NS	NS
mnb; Hip1ΔANTH$^{5.2}$	F	↓ p < 0.001	NS	↓ p < 0.0001
vs. *mnb; Hip1ΔANTH$^{11.2}$*	M	NS	↑ p < 0.01	↓ p < 0.0001
mnb;Hip1^{L-2}	F	↑ p < 0.0001	↓ p < 0.0001	↓ p < 0.0001
vs. *mnb; Hip1ΔANTH$^{5.2}$*	M	↑ p < 0.001	↓ p < 0.0001	↓ p < 0.0001
mnb; Hip1^{L-6}	F	NS	↓ p < 0.0001	↓ p < 0.0001
vs. *mnb; Hip1ΔANTH$^{5.2}$*	M	NS	↓ p < 0.0001	↓ p < 0.0001
mnb;Hip1^{L-2}	F	NS	↓ p < 0.0001	↓ p < 0.0001
vs. *mnb; Hip1ΔANTH$^{11.2}$*	M	↑ p < 0.01	↓ p < 0.001	↓ p < 0.0001
mnb;Hip1^{L-6}	F	NS	↓ p < 0.0001	↓ p < 0.0001
vs. *mnb; Hip1ΔANTH$^{11.2}$*	M	NS	NS	↓ p < 0.0001
mnb	F	↑ p < 0.01	NS	↑ p < 0.0001
vs. *Hip1^{L-2}*	M	NS	NS	↑ p < 0.0001
mnb	F	NS	NS	↑ p < 0.0001
vs. *Hip1^{L-6}*	M	↑ p < 0.0001	NS	↑ p < 0.0001
mnb	F	NS	↓ p < 0.0001	↑ p < 0.01
vs. *Hip1ΔANTH$^{5.2}$*	M	NS	↓ p < 0.01	↑ p < 0.0001
mnb	F	NS	↓ p < 0.0001	NS
vs. *Hip1ΔANTH$^{11.2}$*	M	NS	NS	↑ p < 0.0001
Hip1^{L-2}	F	N		

Hip1$^{1,-2}$	F		N				
	F		NS		NS		NS
vs. *Hip1*$^{1,-6}$	M	↑	p < 0.0001		NS		NS
Hip1ΔANTH$^{5.2}$	F		NS		NS	↓	p < 0.01
vs. *Hip1ΔANTH*$^{11.2}$	M		NS		NS	↓	p < 0.01
Hip1$^{1,-2}$	F		NS	↓	p < 0.0001	↓	p < 0.0001
vs. *Hip1ΔANTH*$^{5.2}$	M		NS	↓	p < 0.01	↓	p < 0.0001
Hip1$^{1,-6}$	F		NS	↓	p < 0.0001	↓	p < 0.0001
vs. *Hip1ΔANTH*$^{5.2}$	M	↓	p < 0.0001	↓	p < 0.0001	↓	p < 0.0001
Hip1$^{1,-2}$	F		NS	↓	p < 0.0001	↓	p < 0.0001
vs. *Hip1ΔANTH*$^{11.2}$	M		NS		NS	↓	p < 0.0001
Hip1$^{1,-6}$	F		NS	↓	p < 0.0001	↓	p < 0.0001

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, *akt* and *foxo1*, in Drosophila neurogenesis. To study this potential novel role for insulin-receptor signalling components on mechanosensory bristle formation, *akt* and *foxo1* were ectopically expressed in the dorsal notum. Considering the important role that *akt* and *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 *akt* causes an increase in the bristle density under the direction of the *pannierGal4* transgene, to indicate a role for *akt* in bristle formation on the back in females. As *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 *et al.* 2008), it is reasonable to believe that *akt* may play a role in mechanosensory bristle formation. To the best of my knowledge this is the first time that a role for *akt* in neurogenesis on the Drosophila dorsal notum has been described. An increase in the level of *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 *foxo1* is expressed in the dorsal notum under the control of the *pannierGal4* transgene, there is no difference in bristle density compared to the controls, suggesting that *foxo1* does not interfere with neurogenesis. In contrast, over-expression of *foxo1* with *apterousGal4* leads to lethality. Further investigation of the ectopic expression of *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 neurogensis. This is reminiscent of the role that *Hip1* plays in Drosophila neurogenesis in the dorsal notum (Moores *et al.* 2008). Compared to the truncated verions 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 $Hip1\Delta ANTH^{5.2}$ increased ommatidia number, while $Hip1\Delta ANTH^{11.2}$ 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.

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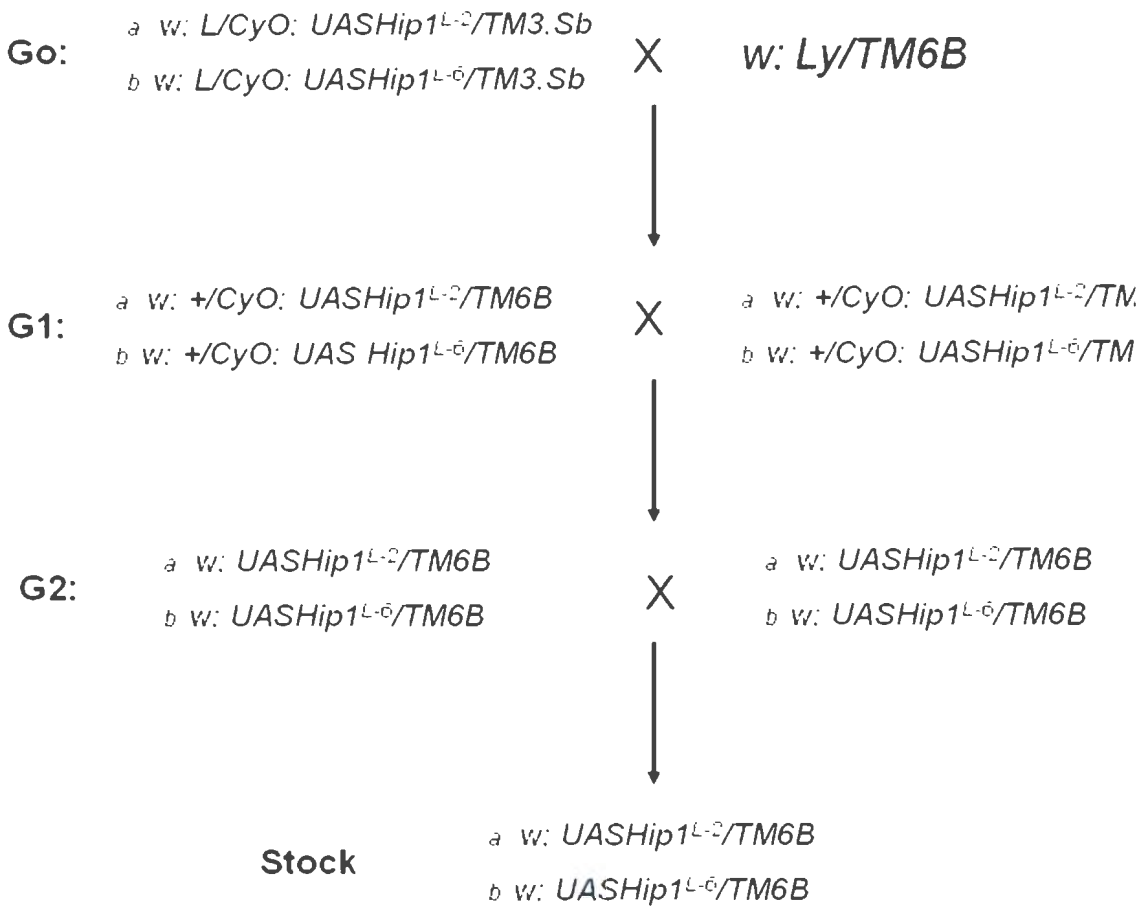
Appendix 1



Schematic diagram for crosses made to eliminate the second chromosome balancer. *w; UASHip1^{L-2}/TM6B* was generated in a and b shows *w; UAS Hip1^{L-6}/TM6B.*