Evaluation of *foxO* modulation in modelling Parkinson Disease in *Drosophila melanogaster*

By

Mahin S. Chavoshi Jolfaei

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ABSTRACT

Symptoms of Parkinson Disease (PD), the second most common neurodegenerative disease, emerge due to degeneration of dopaminergic neurons. Approximately 10% of PD is familial with a number of genes that have been recognized to play a role. In 2012, a genome wide study revealed a role for the *foxO* transcription factor in PD. To more fully understand human diseases, model organisms such as *Drosophila melanogaster* are widely used. In the present study, I have attempted to model Parkinson Disease in Drosophila by *foxO* modulation using RNAi transgenes. To achieve this goal, I conducted longevity assays and locomotion measurements along with supportive experiments that target expression in the developing eye. Results suggest that under certain conditions, slight elevation of *foxO* by down-regulation of one of *foxO'*s inhibitors, the kinase *minibrain* (*mnb*), can model PD in flies. Results are presented here showing that expression of *mnb-RNAi* (and predicted subsequent slight elevation of *foxO*) in dopaminergic neurons results in significant loss of climbing ability: the defining feature of PD models in fruit flies. Other results suggest that slight decrease of *foxO* by *foxO-RNAi* decreases life span significantly when expressed under the control of *TH-Gal4(Tyrosine Hydroxylase-Gal4)* . In addition, results show that *GFP-RNAi* expression under the control of *TH-Gal4* reduces life span significantly.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ab42	Amyloid β Peptide 42
α-synuclein	Alpha-synuclein
ATXN1	Ataxin 1
BLAST	Basic Local Alignment Search Tool
cDNA	complementary DNA
CI	Confidence Interval
Cy0	Curly O
DA neurons	Dopaminergic neurons
ddc	Dopa decarboxylase
º C	Degree Celsius
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
DYRK1A	Dual-specificity tyrosine-phosphorylation regulated kinase
EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1
foxO	Fork-head box O
G	Gram
GAL4	Yeast transcriptional activator for galactose-inducible genes

GFP	Green fluorescent protein
HTT	Huntingtin
JNK	c-Jun N-terminal kinases
L	Litre
mnb	*minibrain*
ML	Millilitre
mRNA	Messenger ribonucleic acid
NES	Nuclear Export Signal
NLS	Nuclear Localization sequence
PARK	Parkinson disease, familial
PD	Parkinson's disease
PK	Protein Kinase
RISC	RNA-induced silencing complex
RNAi	RNA interference
SEM	Scanning Electron Microscope
Ser/Thr kinase	Serine/Threonine kinase
SGK	Serine/threonine-protein kinase
siRNAs	Small interfering RNA
SV40	Simian vacuolating virus 40
TBP	TATA-binding protein

TH	Tyrosine hydroxylase
UAS	Upstream Activating Sequence
VPS35	Vacuolar protein sorting-associated protein 35

INTRODUCTION

Parkinson Disease

Parkinson Disease (PD), after Alzheimer Disease, is the second most common neurodegenerative health concern and the most prevalent neurodegenerative movement disorder (Schiesling *et al*., 2008). Pathologically, PD for the most part, is characterized by the loss of dopaminergic neurons (DA neurons) in the ventral mesencephalic *substantia nigra pars compacta*. Another hallmark seen in most cases of PD, is the formation of Lewy bodies (aggregation of proteins including α-synuclein) in the neurons of ventral midbrain and some other regions such as prefrontal cortex. For a long time after its initial description by James Parkinson in 1817, PD was considered a non-genetic condition. It is only for a few decades that we have begun to understand that inherited forms (autosomal-dominant and autosomal-recessive) of the disease account for 5 to 15% of all PD cases. What causes sporadic PD is not completely clear and it might vary in different forms of the disease, but it is thought that emergence of the disease is most likely the result of interplay between genetic susceptibility factors and the environmental agents which act on the background of an ageing brain (de Lau and Breteler, 2006). Regardless, it has been found that compared to the general population, life span in PD patients is reduced.

Sporadic or idiopathic PD as a consequence of loss of DA neurons which are vulnerable to degeneration (Levine *et al*., 1994) and subsequent impairment of innervation of the putamen affects motor abilities and cognitive function. The associated impairments often include resting tremor, rigidity, bradykinesia and postural instability; PD affects more than 1% of the population by the age of 65 years and approximately 4 to 5% by the age of 85 years and it seems to be increasing in occurrence with the increased life expectancy (Trinh *et al*., 2014). Affected cognitive functions, particularly in advanced stages of PD, include dementia, depression, fatigue, mood disorders, sleep problems, olfactory changes and anxiety. Despite the reality that the definite cause of PD is not known, a number of studies attribute the cumulative effects of oxidative stress, inflammation, mitochondrial dysfunction, and impaired proteasomal degradation to cause sporadic PD.

Depending upon the circumstances, the occurrence of sporadic PD and familial PD may vary. The discovery of genes implicated in heritable forms of PD has provided new insights into the molecular events leading to neurodegeneration, which, in turn, can be applicable to both categories of the disease. Mutations in the genes *PARK1*, *PARK3*, *PARK4*, *PARK5*, *PARK8*, *PARK11*,*VPS35* and *EIF4G1* are associated with autosomal dominant forms of PD and mutations in *PARK2*, *PARK6*, *PARK7* and *PARK9* are associated with autosomal recessively inherited PD. The latter is characterized by selective dopaminergic neural cell death and the absence of the Lewy body (Shimura *et al*, 2000). In addition, mutations of polyglutamine disease genes are shown to contribute to sporadic PD symptoms.

These genes include: *HTT*, *ATXN1*, *ATXN2*, *ATXN3*, *CACNA1A* and *TBP* (Yamashita *et al*., 2014). It is worth mentioning that most of these genes normally regulate the various enzymes involved in protein degradation by the proteasomal pathway.

To name some functions associated with familial PD genes, it can be said that the *α-synuclein* gene encodes a soluble 140 amino acid protein that is the main component of lewy bodies (Olanow and Brundin, 2013). The *α-synuclein* gene may act to integrate presynaptic signalling and membrane trafficking, and defects in this gene have been implicated in the pathogenesis of PD. The *LRRK 2* gene encodes a protein that associates with the mitochondrial outer membrane, and mutations within this gene lead to an autosomal dominant form of the disease (Bonifati, 2006). The *PARK2* gene encodes a multiprotein E3 ubiquitin ligase complex that mediates the targeting of substrate proteins for proteasomal degradation, and its mutations has been found to be linked with the autosomal recessive form of PD (Shimura *et al.*, 2000). The *PARK 7* gene encodes a redox-sensitive chaperone that functions as a sensor for oxidative stress, which apparently protects neurons against oxidative stress and cell death. In the treatment of this progressive disease, a number of approaches and therapeutic steps are among the most common and reveal the ongoing research trends in the area of PD treatment. These include 1) Drugs that mostly are intended to re-establish striatal dopamine levels in the patients; 2) Viral-vector mediated '*in vivo'* gene therapy; 3) Transplantation of embryonic stem cell, fetal midbrain dopaminergic neuron and encapsulated cells, and 4) Continuous infusion of trophic factors.

However, as there is no absolute curative treatment currently existing for PD, the importance of investigation into the cellular /molecular basis of PD is evident.

Model Organisms

Model organisms can be defined as '*in vivo'* media for experiments that in humans would be unfeasible or unethical. Research on model organisms can focus on a wide variety of experimental techniques and goals from different areas of biology—from ecology, behaviour, and biomechanics, to the functional scale of tissues, organelles, and proteins (Meunier *et al.*, 2012). There are many model organisms among viruses, prokaryotes and eukaryotes, unicellular and multicellular organisms; in plant and animal phyla each probably apt for certain studies (Bernards and Hariharan, 2001). Generally, when researchers look for an organism to employ in their studies, they look for several features, most notably size, generation time, accessibility, ease of manipulation, genetic wealth of knowledge, conservation of mechanisms, and potential economic benefits. In the same spirit, it can be proposed that multiple model systems can be employed in cross-genomic analysis of human disease genes to address different health issues. For instance, *Drosophila melanogaster* and *Caenorhabditis elegans* are excellent models for examining the coordinated actions of genes that function as components of a signal-transduction pathway.

Drosophila melanogaster

Extensive use of *Drosophila melanogaster* as a model system to answer questions in human genetics arises from the fact that Drosophila is indeed a well-studied organism, and in fact many landmark discoveries in the field of Biology have been made possible by this tiny insect, some of which resulted in the awarding of the Nobel Prize. To name some of these discoveries, I should mention great findings by T.H. Morgan on sex-linked traits and independent segregation of traits on separate chromosomes and linkage of traits on the same chromosome in 1910 and 1911, X-ray induced mutagenesis by H.J. Muller in 1930, the concept that developmental genes act in spatially localized manner by E. Lewis in 1978, and the use of a saturation screen for developmental patterning genes by E. Wieschaus, G. Jürgens, C. Nüsslein-Volhard, in 1980 (Ahmed *et al*., 2014). Aspects that facilitate extensive studies on Drosophila include but are not limited to having conserved developmental processes (typical examples: *HOX* Genes and neural induction) and shared mechanisms for human developmental disorders.

Merits of *Drosophila melanogaster* include the following: 1) it has well-established genetics, and the molecular tools for precise genetic manipulations are available; 2) Drosophila has 14400 genes on their four chromosomes of which are 61% similar to human genes, the fact that makes it a better model organism than other systems such as nematode and yeast.

3) it has been found that 77% of human disease genes have fly counterparts (Reiter *et al*., 2001) and there are matches to diseases in categories as diverse as cancer, cardiac diseases, neurological diseases, immune dysfunction, metabolic and developmental disorders. 4) Drosophila has a short generation time (~10 days) and a high number of progeny (around 100 flies); and 5) it has a completely sequenced.

Drosophila eye development

The adult Drosophila eye is made of 750 to 800 multicellular units called ommatidia (Perry, 1968). Each ommatidium is a cluster of 20 cells that contain 8 photoreceptors neurons, 8 pigment cells, and 4 lens secreting cone cells. Mechanoreceptory bristles are located in regular distances from ommatidia and are composed of four cells with one of them being a neuron. Thus, the developing eye is one neuron-rich tissue in which eye distortion caused by cell death or developmental defects can be detected.

Gal4/UAS System

Drosophila researchers owe much of their knowledge of biological events to ongoing progression in analytic and detection tools and techniques.

As our tool-box expands, we are more and more able to gain closer, more accurate insight into what biological processes are like. One very practical example of facility development is the invention of the *Gal4/UAS* system for targeted gene expression in Drosophila. This system enables us to investigate the function of genes implicated in a wide variety of biological processes. *Gal4* encodes a protein of 881 amino acids, identified in the yeast *Saccharomyces cerevisiae* as a regulator of genes (e.g., *Gal10* and *Gal1*) induced by galactose (Laughon *et al.*, 1984). Gal4 regulates the transcription of *Gal10* and *Gal1* genes by directly binding to four related 17 basepair (bp) sites located between these loci (Giniger *et al.*, 1985). The Upstream Activating Sequences (*UAS*) to which *Gal4* binds is CGG-N11-CCG, where N can be any base (Campbell *et al.*, 2008). *UAS* is essential for the transcriptional activation of these *Gal4*-regulated genes and has the same function as an enhancer element found in multicellular eukaryotes.

Expression of *Gal4* in Drosophila initially appeared to have no adverse effect on phenotypes and an important article describing the development of the *Gal4/UAS* system for targeted gene expression in Drosophila was published (Brand and Perrimon, 1993). However, later on some researchers reported that expression of *Gal4* in the dopaminergic neurons can significantly decrease life span (Haywood *et al.*, 2002) and that directed expression of this regulator in the eye region can lead to cell death (Kramer and Staveley, 2003). Although some care must be exercised in experimental design and interpretation of the phenotype, the *Gal4/UAS* system has become invaluable in Drosophila research.

In the *Gal4/UAS* system, expression of the gene of interest is controlled by the *UAS* element and as *GAL4* is still absent in this line, the responder line remains silent. To activate their transcription, responder lines are crossed to flies expressing *Gal4* in a particular spatiotemporal pattern. The resulting progeny then express the gene of interest in a transcriptional pattern that reflects the Gal4 pattern of the corresponding driver (Duffy, 2002). It has been found that Gal4 activity in Drosophila is dependent upon temperature and it is minimal at 16°C and reaches its maximum at 29°C providing a wide range of target gene expression levels and albeit affecting growth in turn. As a general rule, it is crucial to ensure that the biological process of study is not affected by the presence of either the Gal4 protein or the responder. Plus in selecting between maternal or paternal use of driver and responder lines, one might want to keep in mind the drastic developmental difference existing between Drosophila egg and sperm, potential effect of large bulk of maternal factors deposited inside the egg only (Giordano *et al*., 2002). The *Gal4/UAS* system can be employed to study both gain-of-function and loss of function phenotypes (Figure 1).

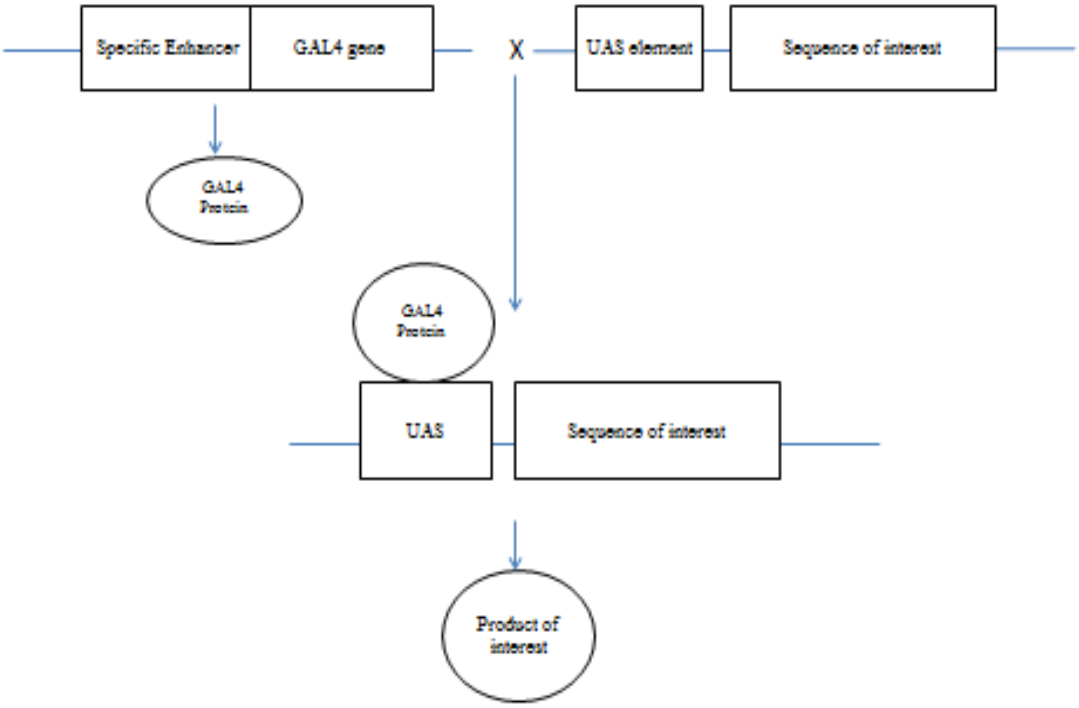

Figure 1. Gal4/UAS system. Tissue specific targeted gene expression is made possible by Gal4/UAS system: transcription factor Gal4 binds its *cis*-acting element Upstream Activating Sequesnce (UAS) and promotes the expression of target gene or sequence of interest.

Transgenic RNAi in Drosophila

The *Gal4/UAS* system is used to direct the expression of a hairpin double-stranded RNA which will be processed by dicer enzymes into siRNAs to direct sequence-specific degradation of the target mRNA. These constructs are made by cloning short gene fragments (300-400 bp) as inverted repeats in the antisense-sense orientation into a modified pUAST vector with 10 copies of UAS sites. To generate 300-400 bp gene fragments, primers are designed to PCR amplify DNA from any predicted protein-coding gene in the Drosophila genome sequence (Fire *et al*., 1998). RNAi libraries are constructed as genetic screens in model organisms and have provided remarkable insights into numerous aspects of development, physiology and pathology. With the availability of complete genome sequences and the introduction of RNA-mediated gene interference (RNAi), systematic reverse genetic screens have become possible. This powerful tool can be applied in a tissue-specific manner able to the conditional inactivation of gene function in specific tissues of the intact organism (Dietzl *et al*., 2007). To date, approximately 22,270 transgenic lines are generated, covering 88% of the predicted protein-coding genes in the Drosophila genome. Molecular and phenotypic assays indicate that most of these transgenes are functional. The transgenic RNAi libraries open the prospect of systematically analyzing gene functions in any tissue and at any stage of the Drosophila lifespan.

fork-head box O (foxO) Transcription Factor

forkhead genes encode a subgroup of the helix-turn-helix class of proteins that act as transcription factors or regulators. A defining feature of the foxO protein is the forkhead box, a sequence of 80 to 100 amino acids forming a motif (called Winged Helix motif due to the butterfly-like appearance of the loops) that bind to DNA (Tuteja *et al.*, 2007), and as their function suggests, they contain both Nuclear Localization sequence (NLS) and Nuclear Export Signal (NES) which are the main sites of foxO modulation. Originally, *foxO* genes were given very different names (such as *HFH*, *FREAC*, and *fkh*), but in the year 2000 a unified nomenclature was used that grouped the *fox* genes into 19 subclasses (*foxA-foxS*) based on sequence conservation. Protein multiple alignment results suggest that they present high level of conservation within different species. There are 4 mammalian *foxO* members *foxO1/FKHR/foxO1a, foxO3/FKHRL1/foxO3a, foxO4/AFX* and *FoxO6* (Calnan and Brunet, 2008), one homologue in *C. elegans* (*DAF-16*) (Perens and Shaham, 2005) and one homologue in *Drosophila melanogaster* (*dfoxO*) (Kramer *et al*., 2003). foxO proteins are conserved in pathways that play a role in coordination of cellular responses to changes in inner or outside environment and seem to do so by transcriptional regulation of many target genes and interactions with a vast range of transcriptional regulators that affect cell functions such as cell cycle, apoptosis and metabolism.

foxO modifiers

The foxO protein can be modified through different post transcriptional modifications including acetylation, ubiquitination and phosphorylation. *Akt* is a kinase that can phosphorylate foxO both in the cytoplasm and the nucleus on the three sites of T32, S253 and S315 both '*in vitro'* and '*in vivo'* (all mammalian foxO proteins contain three Akt phosphorylation sites except for foxO6), and this phosphorylation leads to nuclear exclusion of foxO3 (Brunet *et al*., 1999). Akt-mediated nuclear exclusion of *foxO* is facilitated by 14-3-3 proteins (Morrison, 2009), a family of conserved modulator proteins that regulate different signaling pathways in the cell by binding to specific Ser/Thr-phosphorylated motifs on target proteins and affect their function by means of altering the enzymatic activity of the target protein, protein stability or cellular localization. The foxO protein is one main component of the insulin signalling pathway and it plays a role in nutrient constraint conditions; it is involved in stress resistance response and inhibits growth, through the action of target genes such as *d4E-BP* (Jünger *et al*., 2003). It has been shown that in *C. elegans,* an absence of Akt signalling activates *DAF-16* (the *foxO* homologue) and causes dauer formation (Kops *et al*., 2002) promoting cell entering into quiescence and/or protecting these cells against oxidative stress.

It has been found that foxO proteins can be phosphorylated on multiple sites besides Akt sites such as at Ser329, which has been found to utilize another kinase, dyrk1a (*dual-specificity tyrosine-phosphorylation regulated kinase 1a* (Woods *et al.*, 2001)). dryk1a is located within the Down Syndrome Critical Region of human chromosome 21 and the *minibrain* gene, the Drosophila homologue of human *dyrk1a*, is located on first chromosome (chromosome X) of Drosophila. There are several isoforms of minibrain protein, a potentially nuclear protein, with the one closest to human homologue being mnb isoform E. *mnb* genes encode kinases being able to phosphorylate serine/threonine residues on other proteins as well as auto-phosphorylation of tyrosine residues on the mnb protein itself (Hong *et al.*, 2012). The minibrain protein isoform E consists of 908 amino acids and contains a distinct amino-terminal domain, a kinase core domain, and a variable C-terminal. Studies done on mice reveal that increased *dyrk1a* expression leads to Down Syndrome-like phenotypes (Arron *et al*., 2006) and mnb has been shown to play role in postembryonic neurogenesis. Mutations within the *mnb* gene cause a reduced optic lobe in adult flies and abnormal spacing of neuroblasts in the larval brain (Tejedor *et al*., 1995). mnb has been shown to share 85% amino acid similarity within the catalytic site which consists of a conserved motif of YQY (dyrk1a) and YHY (mnb) (Kentrup *et al*., 1996). Moreover, mnb contains a nuclear translocation signal a tyrosine-rich hydrophilic region, responsible for nuclear localization, a 13- histidine repeat region possibly for metal binding and a motif of 17 clustered serine/threonine residues that may be putative phosphorylation sites.

One target of dyrk1a/mnb is the foxO1 transcription factor, and phosphorylation of foxO by mnb on serine 329 sequesters it from the nucleus (Figure 2) . In our lab (Inpken and Staveley, unpublished), it has been observed that when *mnb* and *foxO* were both expressed in the eye, *mnb* appears to partially rescue the *foxO* phenotype. Therefore, *mnb* seems to act as an inhibitor of *foxO* and it exerts its effects on *foxO* protein by post translationally phosphorylating *foxO*. Other than phosphorylation which in most cases leads to *foxO* nucleus sequestration and thus inactivation, there are other mechanisms that each might act independently or with cross talk to the Akt pathway, namely reversible acetylation, methylation and ubiquitination, some of the effect of which is quite controversial (Daitoku, *et al*., 2004). For example, the exact effect of acetylation on foxO activity according to some results is increased foxO activity while other results suggest quite the reverse (Huang and Tindall, 2011). As for foxO regulation by ubiquitination, it is important that if foxO protein has already been induced by phosphorylation (by Akt, Erk-1/2 and Ikk) or is it undergoing mono-ubiquitination or polyubiquitination which targets foxO for degradation.

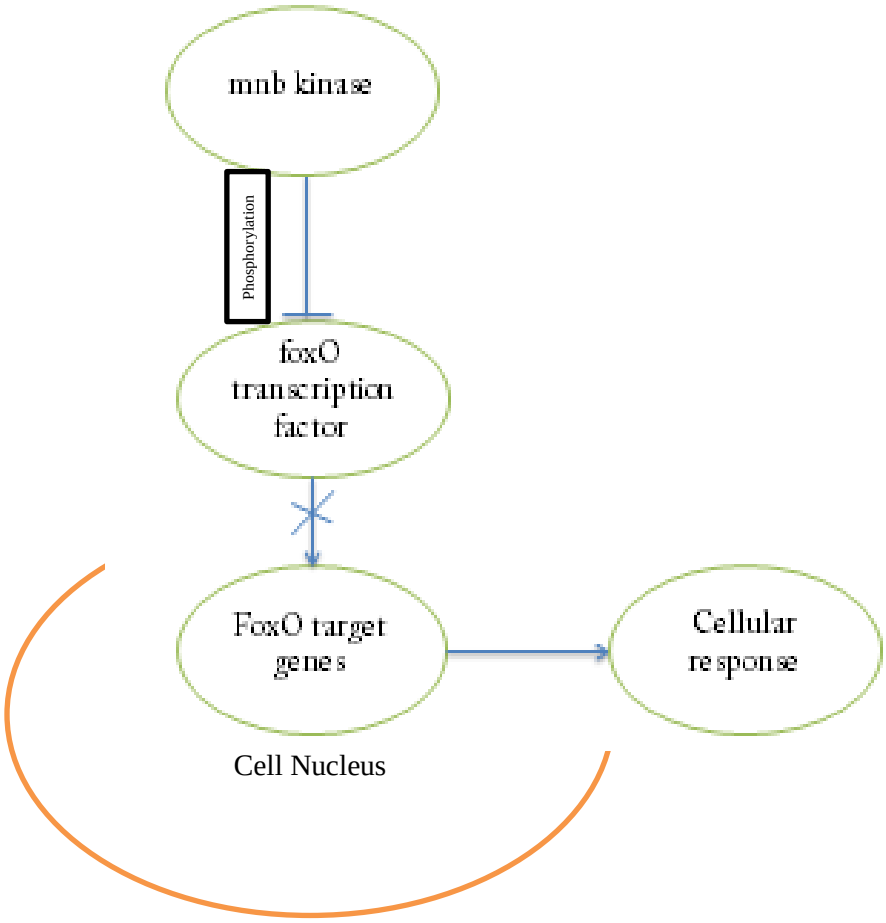

Figure 2. Proposed relationship between mnb kinase and foxO. It is proposed that mnb kinase exerts its effects on foxO transcription factor by phosphorylation and inhibiting its nuclear translocation thus inhibiting its effect on target genes.

Research Statement:

Considering the importance of studies on degenerative diseases to increase the wealth of knowledge and since the recent publication on the implication of *foxO1* in PD patients (Dumitriu *et al*., 2012), I decided to have a closer look at *foxO* modulation in terms of its potential in modeling PD. In study carried out by Dumitriu and colleagues in 2012, they produced and analyzed expression data from the prefrontal cortex Brodmann Area 9 (one of the brain regions that become affected in PD besides *substantia nigra pars compacta*). They found 507 out of the 39,122 analyzed expression probes different between PD and control samples. One of the genes with significantly increased expression in PD was the *forkhead box O1* (*FOXO1*) transcription factor. To investigate the role of foxO transcription factor in Drosophila models of PD, I employed an RNAi approach for its subtle nature of elevating/decreasing gene expression and *Drosophila melanogaster* for its convenience as a model organism. As the direct overexpression of *foxO* in dopaminergic neurons did not generate viable progeny (Staveley unpublished), I used *mnb-RNAi* to indirectly elevate foxO transcription factor levels in the nucleus and *foxO-RNAi* for the direct decrease of *foxO.*

I designed a set of experiments to obtain a better insight into the role of *foxO* in inducing cell degeneration and I hypothesized that elevation of *foxO* in dopaminergic neurons would provide a Drosophila model of PD in some aspects. Therefore, I employed locomotion assay and also carried out longevity assay and eye morphology experiments. With the literature reporting on the inhibitory effect of mnb on foxO and the implication of *foxO* in PD, my initial prediction was that expression of *mnb-RNAi* thus slightly elevating *foxO* activity in dopaminergic neurons using *Tyrosine Hydroxylase-Gal4* (*TH-Gal4*) driven expression and/ or *dopa-decarboxylase-Gal4* (*ddc-Gal4*) will result in diminished climbing ability in flies while both experiment and control group of flies would exhibit a similar life span.

MATERIALS AND METHODS

Drosophila Culture and Stocks

Drosophila melanogaster stocks were kept at room temperature and all the experiments were maintained in a 25 ºC incubator. All stocks and crosses were raised in a medium containing 65 g/L cornmeal, 50 ml/L fancy grade molasses, 10 g/L yeast, 5.5 g/L agar and ~ 900 ml water and stocks solid media was changed for new fresh food every two to three weeks. Drosophila media was prepared by Dr. Staveley and treated with 2.5 ml/L propionic acid and 5 ml/L of 10% methylparaben in ethanol to prevent mold growth, and then 7 ml of media was poured into each vial and stored in refrigerator at 4 to 6ºC after cooling at room temperature and becoming solid.As shown in Table 1, a series of ten fly lines was utilized in the experiments. *TH-Gal4* along with the *GMR-Gal4* (Freeman, 1996), *UAS-lacZ* (Brand and Perrimon, 1993), *UAS- mnb-RNAi and UAS-foxO-RNAi* obtained from Bloomington Stock Center at Indiana University. *ddc-Gal4* (Li *et al*., 2000) was kindly provided by Dr. Jay Hirsh (University of Virginia) and w^{118}*; GMR-Gal4/CyO;UAS-foxO/TM3* compound line was generated by Dr. Staveley. Directed expression of the transgenes in the eye region was accomplished by crossing responder lines and *GMR-Gal4* females whereas directed expression of transgenes in DA neurons was achieved by crossing responders to *TH-Gal4* and *ddc-Gal4* females. All crosses were done as per standard methods and only male flies of each critical class were collected to avoid possible affected longevity in females.

Table 1. Genotype and related information of Drosophila stocks.

Genotype and stock numbers	Chromosome(s) affected	Comments
TH-Gal4 (8848)	1,3	Expresses *GAL4* in dopaminergic neurons
Ddc-GAL4	2	Expresses *GAL4* in dopaminergic neurons (Li *et al*., 1997) plus serotonergic neurons (Alic *et al*., 2012)
GMR-Gal4 (b1104)	1, 2	Expresses *GAL4* in the eye disc, provides strong expression in all cells behind the morphogenetic furrow (Freeman, 1996)
UAS-lacZ $^{4\text{-}2\text{-}1}$ (b2128)	2	Expresses *lacZ* under *UAS* control (Brand and Perrimon, 1993)
UAS-GFP-RNAi (b9330)	1,3	Expresses a dsRNA of *GFP* under *UAS* control
UAS-mnb-RNAi (b35222)	1,3	Expresses dsRNA for RNAi of *mnb* under *UAS* control
UAS-foxO-RNAi (v106097)	2	Expresses dsRNA for RNAi of *foxO* under *UAS* control
w118; GMR-Gal4 /CyO;UAS-foxO/TM3		Two balancer chromosomes used. Expresses both *Gal4* and *foxO* in the eye

Scanning Electron Microscopy of the Drosophila eye

Approximately ten males from each cross were collected and aged upon standard media at 25 º C for three to five days to allow for phenotypic stabilization before storing at -80º C. Frozen flies were examined under a dissecting microscope then mounted on aluminum studs and allowed to undergo desiccation for approximately 48 hours (gold-coating was not required). Samples were visualized by using an MLA 650F Scanning Electron Microscope chamber (Memorial University of Newfoundland CREAIT Facility) and 10 pictures (597 times magnification) per genotype were captured for further analysis. The area of the eye was measured based on the presence of Ommatidia. The area of a single ommatidium was determined by measuring the average area of a "floret" of ommatidia consisting of a central unit surrounded by six others then dividing by 7. Counts and measurements were conducted using software ImageJ 1.42q (Abramoff *et al*., 2004) and the data were transferred to Graph Pad Prism 5 (Graphpad Software, San Diego California USA, *www.graphpad.com*) for drawing bar graphs and analysis to detect any significant difference between the numerical values.

Longevity Assay

To perform longevity assays, critical class genotype were collected under carbon dioxide every 24 hours until a minimum of approximately 200 critical class (males of desired genotype) flies were obtained. They were then transferred into upright standard plastic vials containing standard media and kept in numbers of no more than 20 to prevent overcrowding. As flies aged they are transferred to new food without anaesthesia and monitored for viability until all have perished (Staveley *et al*., 1990). The longevity of the flies were recorded manually on ageing sheets and then transferred to Prism 5 software (Graphpad Software, San Diego California USA, *www.graphpad.com*) for analysis. Survival fractions were calculated using the product limit (Kaplan-Meier) method and the statistical test of Mantel-Cox was carried out to detect any significant difference between survival curves (longevity assay was done three times overall).

Locomotor Activity Assay

Locomotor activity of flies was assessed using a series of climbing experiments (Todd and Staveley, 2004) over the life of a cohort of flies. In this assay which was done three times overall, seventy or so males of critical class of each cross were collected within 24 hours post eclosion and placed into vials in groups of ten.

They were maintained on standard media and were assayed for climbing ability in intervals of seven days starting at day four or five after eclosion. A funnel was used to transfer flies to the apparatus and then sponges were put into both ends of the tube to prevent flies from escaping and allow gas exchange to occur. Each time, flies from each tube were put in a climbing apparatus which is a 30 cm glass tube with a diameter of 1.5 cm divided into five 2 cm sections graduated from 1 to 5 (remaining 20 cm of the tube acts as a buffer zone that limits the interference between flies during climbing). Following the natural negative geotaxis response, flies walk up the tube after being tapped on a surface and their ability to climb was evaluated at 10 seconds 10 trials per day per cohort. The section each fly climbed to was recorded and the numbers put in the "Climbing index" formula: Climbing index = Σ (nm)/N where n is the number of flies, m is the section (1-5) and N is the total number of flies in that trial. The data then were transferred to Prism 5 (Graphpad Software, San Diego California USA, *www.graphpad.com*) for analysis. Essentially, the climbing experiment was terminated when either there was no or very few flies left alive or their climbing ability was so diminished that is near to complete loss. To compare climbing ability, climbing indices were subtracted from 5 to follow the inverting the y-axis of the graphs and it was determined via a non-linear regression curve fit within a 95% confidence interval (CI). Unpaired t-test was carried out to detect any significant differences between means of groups and the slopes of the curves with non-overlapping 95% CI were considered significantly different.

RESULTS

GFP-RNAi under the control of GMR-Gal4 does not alter development in D. melanogaster eyes raised at 25 ºC.

The *Glass Multiple Reporter gene* (*GMR*)-*Gal4* transgene (Freeman, 1996), causes high levels of expression in Drosophila eye imaginal discs. Flies heterozygous for *GMR-Gal4* do not show an apparent abnormal phenotype at 25º C (Kramer and Staveley, 2003). Figure 3A and 3B display SEM images of *D. melanogaster* eyes with targeted expression of *lacZ* or *GFP- RNAi* at 25 º C. *GFP-RNAi* construct expresses dsRNA to target non-existent in fly GFP mRNA molecule. The rationale for applying such line is to determine if *GFP-RNAi* can act as control in RNAi experiments. The Rationale for using GMR-Gal4/UAS-lacZ as opposed to w1118 as a control line is to try to compensate the effect of Gal4 transcription factor in the experiment. As lacZ system is not operating in flies at all and exogenous expression of lacZ has not proved any adverse effect, it is now widely used in fly experiments as a reliable control in UAS-Gal4 system. Results show that in both genotypes eye morphology appears normal and statistical analysis does not reveal any significant difference in their ommatidia and bristle numbers as well as their ommatidia area, counts and measurements are graphed in Figure 3C, 3D and 3E.

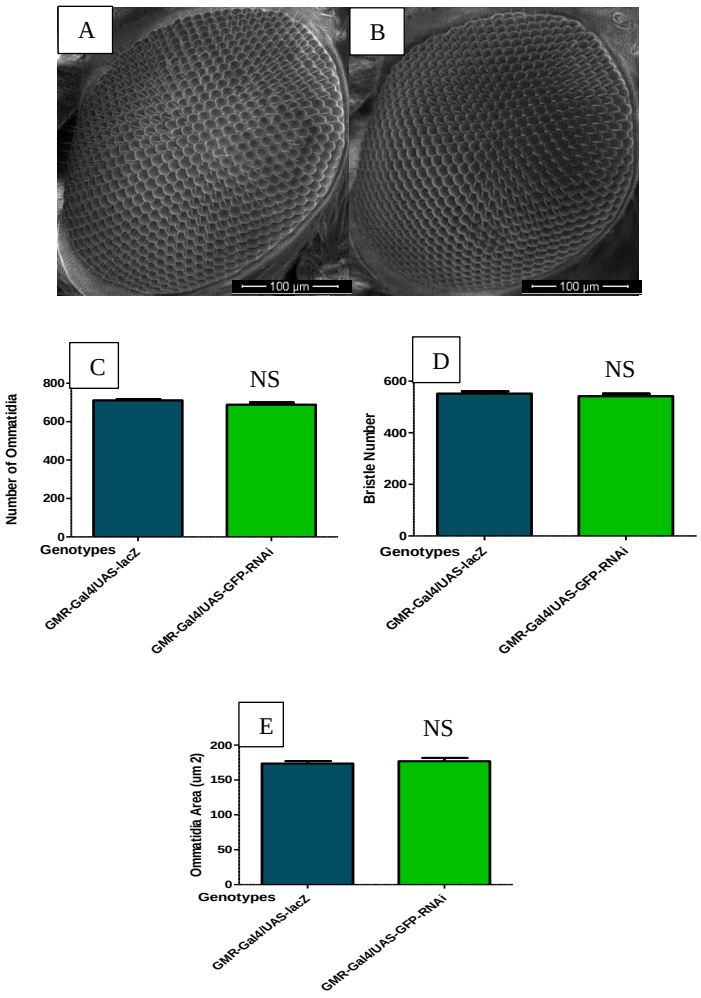

Figure 3. The expression of *GFP-RNAi* has no effect on *D. melanogaster* eyes at 25º C. A and B scanning electron micrographs of *D. melanogaster* eyes with the directed expression of *lacZ* (**A**) and *GFP-RNAi* under the control of *GMR-Gal4* (**B**). Genotypes are *GMR-Gal4/UAS-lacZ* (**A**) and *GMR-Gal4/UAS-GFP-RNAi* (**B**). *GFP-RNAi* does not alter ommatidia number compared to *GMR-Gal4/UAS-lacZ* (**C**). *GFP RNAi* does not influence the number of ommatidia (**D**) plus ommatidia area is not altered by *GFP-RNAi* (**E**). (N=10 p-value<0.05 is considered significant). Error bars represent standard error of the mean and NS indicates Not significant.

GFP-RNAi under the control of GMR-Gal4 alters the consequences of foxO overexpression in D. melanogaster eyes raised at 25º C.

The expression of foxO in the Drosophila eye, directed by *GMR-Gal4*, produces an abnormal rough eye phenotype characterized by reduced number of ommatidia, partial absence of bristles and distorted eye periphery (Kramer *et al*., 2003). *GFP-RNAi* enhances the phenotype induced by *GMR-Gal4/UAS-foxO*. The *GMR-Gal4/UAS-foxO* phenotype becomes more severe when *GFP-RNAi* (construct used to target non-existent GFP mRNA in Drosophila) is used (Figure 4B). Although ommatidia area is not significantly altered and bristles are absent in both *GMR-Gal4,UAS-foxO*/*UAS-lacZ* and *GMR-Gal4,UAS-foxO*/*UAS-GFP-RNAi* genotypes, biometric analysis revealed a significant reduction in ommatidia number in *GMR-Gal4,UAS-foxO*/*UAS-GFP-RNAi* compared to the control. Figure 4A and 4B display SEM images of *D. melanogaster* eye with *GFP-RNAi* and with expression of *lacZ* in 25 º C and Figures 4C, 4D and 4E illustrate the related graph.

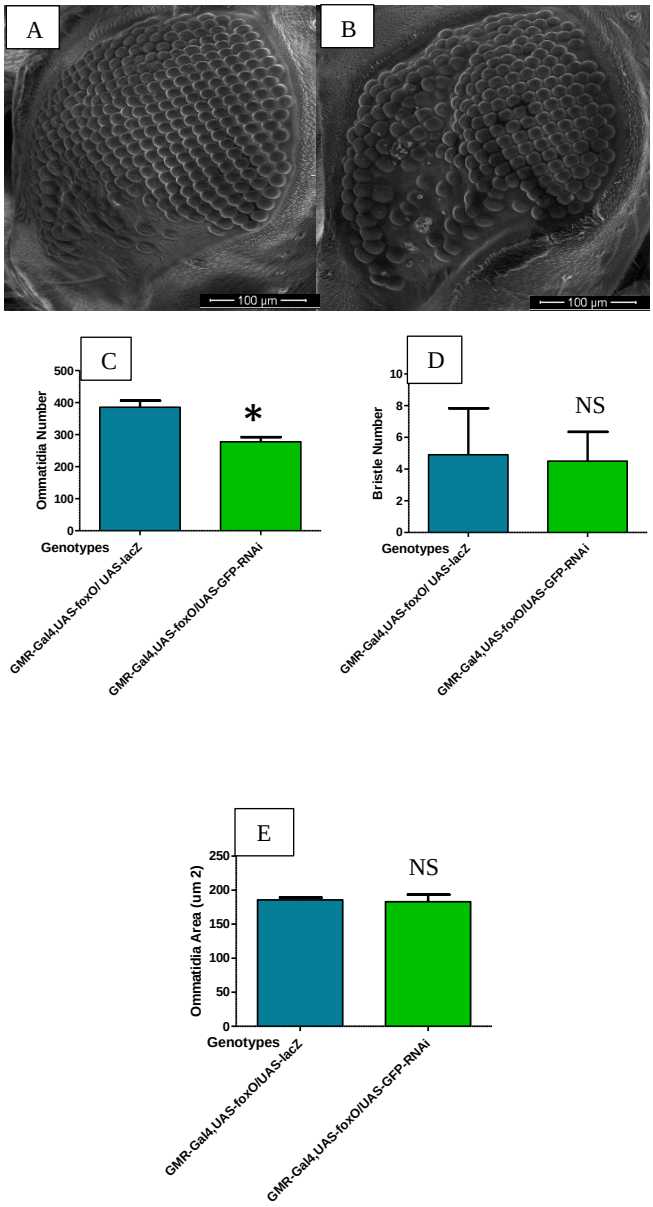

Figure 4. The expression of *GFP-RNAi* enhances the phenotype induced by *GMR-Gal/UAS-foxO* in *D. melanogaster* eye raised at 25 º C. Scanning electron micrographs of *GMR-Gal4,UAS-foxO/UAS-lacZ* (**A)** and *GMR-Gal4,UAS-foxO/UAS-GFP-RNAi***(B)**.*GFP-RNAi* reduces ommatidia number (p-value=0.0007) (**C**) but does not alter ommatidia area (**D**) or bristle number (**E**). N-value is 10, error bars represent standard error of mean, NS indicates Not Significant and asterisk indicates significant difference.

GFP-RNAi expression under the control of ddc-Gal4 reduces life span but not climbing ability in D. melanogaster raised at 25º C.

GFP-RNAi in dopaminergic neurons plus some other cells using *ddc-Gal4* - driven causes a decrease in life span compared to *ddc-Gal4/UAS-lacZ* flies (Figure 3A). Median survival of *ddc-Gal4/UAS-lacZ* flies is 47 days and 38 days for *ddc-Gal4/UAS GFP-RNAi* flies. Flies present slightly but not significantly decreased median life span when *GFP-RNAi* is expressed under the control of *ddc-Gal4* transgene. Mantel-Cox test was carried out to detect significant differences between two genotypes and the N-value is approximately 200. Figure 5A illustrates the related survival curve and Figure 5B displays climbing curves of the two genotypes: *ddc-Gal4/UAS-lacZ* and *ddc-Gal4/UAS-GFP-RNAi*. One of the hallmark phenotypes of the Drosophila PD models is the premature loss of climbing ability and climbing ability over time of 70 males of critical class of two genotypes were evaluated. Climbing was continued until minimum of five alive flies were left. Statistical tests suggest that no significant difference existed among the genotypes. Figure 5B shows the fitted non-linear regression curves in *ddc-Gal4/UAS-lacZ* and *ddc-Gal4 /UAS-GFP-RNAi* genotypes. The 95% confidence interval of the slopes were evaluated and found to overlap indicating that any observed differences are likely due to chance.

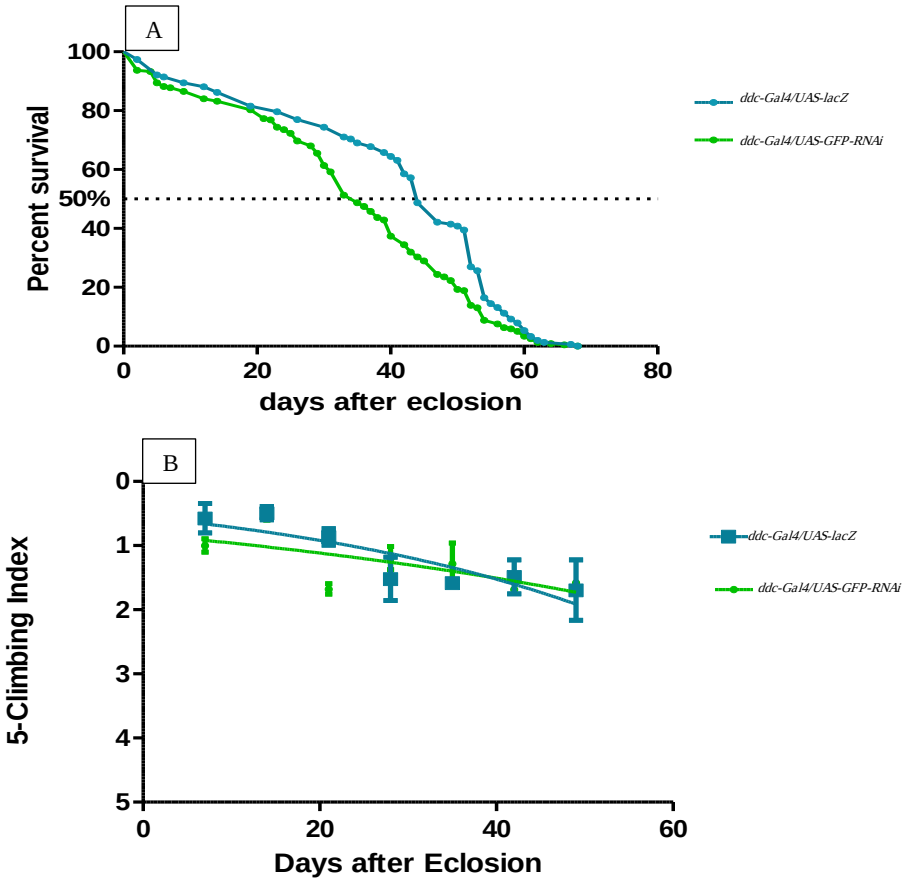

Figure 5. The expression of *GFP-RNAi* directed by *ddc-Gal4* reduces life span but not climbing ability in *D. melanogaster* raised at 25 º C. *GFP-RNAi* directed by *ddc-Gal4* in dopaminergic neurons slightly decreases life span compared to *ddc-Gal4/UAS-lacZ* control, N-value is 200 (**A**) Climbing ability (on 70 flies initially) is not significantly altered by *GFP-RNAi* directed by *ddc-Gal4* in dopaminergic neurons of *D. melanogaster* (**B**).

GFP-RNAi expression under the control of TH-Gal4 reduces life span but not climbing ability.

The *TH-Gal4* transgene is used to direct the expression of a targeted gene exclusively in dopaminergic neurons. Here, the *TH-Gal4* transgene was combined with the *UAS-GFP-RNAi* responder to induce *GFP-RNAi* expression in dopaminergic neurons and *TH-Gal4/UAS-lacZ* was selected as the control. 200 males of each critical class genotype were selected and their life span was compared. Survival curves displayed in Figure 6A shows that there is a significant decrease in life span when *GFP-RNAi* is expressed in dopaminergic neurons with *TH-Gal4* transgene. The climbing ability over time however, was not significantly different between the genotypes of *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-GFP-RNAi.* Figure 6B illustrates the climbing curves for the two genotypes. 70 males of each genotype of *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-GFP-RNAi* were selected to perform locomotor assay and the assay was continued until a minimum of five flies were left alive. Statistical analysis results did not reveal any significant difference in the two genotypes. Non-linear regression curves were fitted with a 95% confidence interval, curve slopes where compared and seen to overlap thus indicating that any observed differences are due to chance rather than a certain pattern.

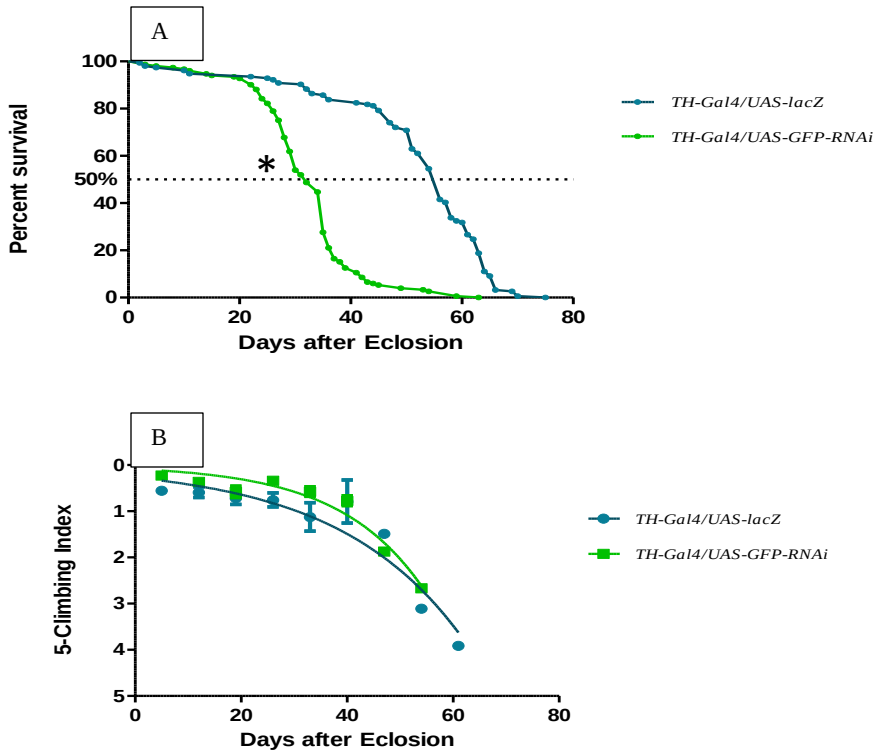

Figure 6. The expression of *GFP-RNAi* directed by *TH-Gal4* reduces life span significantly but does not alter climbing ability in *D. melanogaster* raised at 25 º C. (**A**) 200 male flies of critical class for two genotypes of *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-GFP-RNAi* were collected for longevity assay. *TH-Gal4/UAS-lacZ* flies show a median life span of 38 days whereas TH-*Gal4/UAS-GFP-RNAi* critical class show median life span of 60 days. *GFP-RNAi* shortens life span very significantly according to Mantel-Cox test results. (**B**) 70 flies of critical class were initially chosen for locomotion assay over time in two genotypes of *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-GFP-RNAi*. Non-linear regression fitted curves and statistical analysis show that there is no significant difference in their climbing abilities over time. Error bars represent standard error of mean and asterisk indicates significant difference.

Expression of foxO-RNAi directed by GMR-Gal4 in the eye does not alter eye development in D. melanogaster raised at 25º C.

The *foxO-RNAi* expression directed by *GMR/Gal4* does not significantly alter the eye phenotype. In both genotypes of *GMR-Gal4,UAS-foxO/UAS-lacZ* and *GMR-Gal4,UAS-foxO/UAS-foxO-RNAi,* external area of the eye is smooth and the number of ommatidia is approximatley 700 and the number of bristles is approximately 500. Expression of *foxO-RNAi* in the eye does not make any dramatic difference in the eye morphology or in the number of ommatidia. Figures 7A and 7B show scanning electron micrographs of the two genotypes and Figures 7C, 7D and 7E display the related graphs.

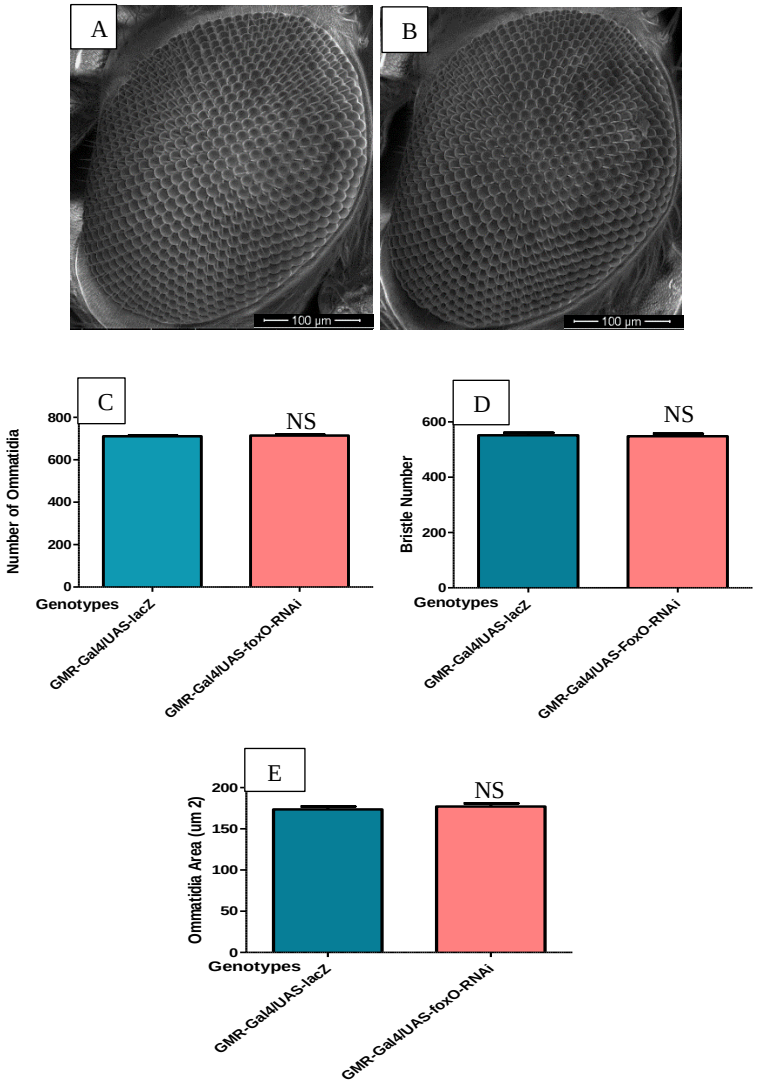

Figure 7: The expression of *foxO-RNAi* directed by *GMR-Gal4* does not alter *D. melanogaster* eye developed at 25º C. *foxO-RNAi* expression does not alter eye morphology (**A** and **B**). Genotypes are *GMR-Gal4*/UAS-*lacZ* (**A**) and *GMR-Gal4/UAS-foxO-RNAi* (**B**). *foxO-RNAi* expression does not make any significant change in the ommatidia number (**C**), bristle number (**D**) and ommatidia area (**E**). P-value less than 0.05 is considered significant. N-value is 10, error bars indicate standard errors of mean and NS means Not Significant.

Expression of foxO-RNAi supresses the severe foxO overexpression-induced phenotype in D. melanogaster eyes raised at 25º C.

Eye development defects caused by overexpression of *foxO* is significantly but not completely suppressed by *foxO-RNAi*. *GMR-Gal4,UAS-foxO/UAS-lacZ* flies have developmental defects when raised at 25ºC. This phenotype improves when *foxO-RNAi* is expressed in the developing eye. Scanning Electron Micrographs for both *GMR-Gal4,UAS-foxO/UAS-lacZ* and *GMR-Gal4,UAS-foxO/UAS-foxO-RNAi* are shown in Figure 8A and 8B. Driving *foxO-RNAi* in the *D. melanogaster* eye using *GMR-Gal4/UAS-foxO* increased the number of ommatidia as well as the number of bristles in 25ºC. Biometric analysis showed that *foxO-RNAi* increased the mean ommatidia number from 400 to 650 (Figure 8C) and the mean number of bristles from 5 to 500 (Figure 8D). *foxO-RNAi* did not alter ommatidia area significantly (Figure 8E).

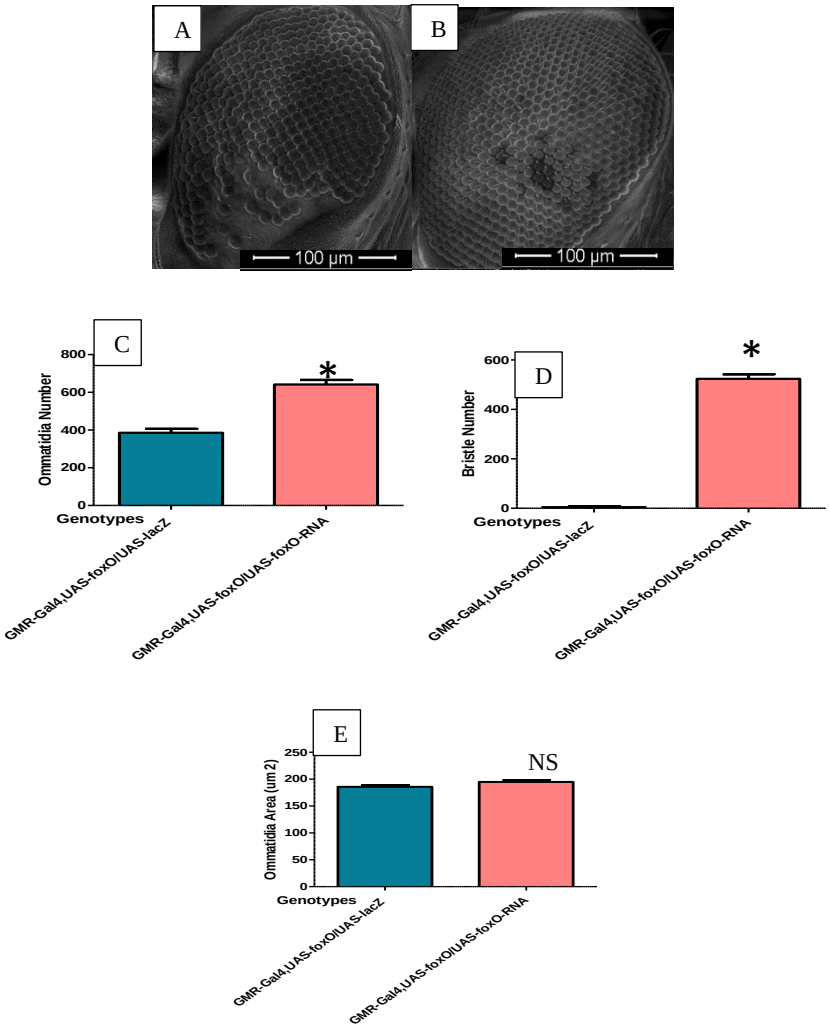

Figure 8. The expression of *foxO-RNAi* by *GMR-Gal4* supresses the *GMR-Gal4/UAS-foxO* eye phenotype in *D. melagaster* raised at 25º C. A and B Scanning electron micrographs of *GMR-Gal4,UAS-foxO/UAS-lacZ* and *GMR-Gal4,UAS-foxO/UAS-foxO-RNAi*. *foxO-RNAi* partially rescued the reduced number of ommatidia (**C**) and *foxO-RNAi* completely rescued the diminished bristle number (P-value < 0.0001 and n=10) but did not alter ommatidia area (E). Error bars represent standard error of the mean. P-values calculated via unpaired t-test, NS means not significant and asterisk indicates significant difference.

foxO-RNAi expression under the direction of ddc-Gal4 does not alter life span or climbing ability in D.melanogaster raised at 25º C.

When *foxO-RNAi* was expressed under the control of *ddc-Gal4* transgene, it did not alter life span (Figure 9A). 200 males of critical class were selected for longevity assay and the survival pattern illustrated by survival curves was compared. Likewise, climbing ability was not altered when *foxO-RNAi* was expressed in dopaminergic neurons directed by *ddc-Gal4* shown in figure 9B. 70 male of each genotype were selected for testing locomotor ability over time and continued until minimum number of five flies were alive. Both genotypes of *ddc-Gal4/UAS-lacZ* and *ddc-Gal4/UAS-foxO-RNAi* showed a roughly the same pattern of losing their climbing ability over time.

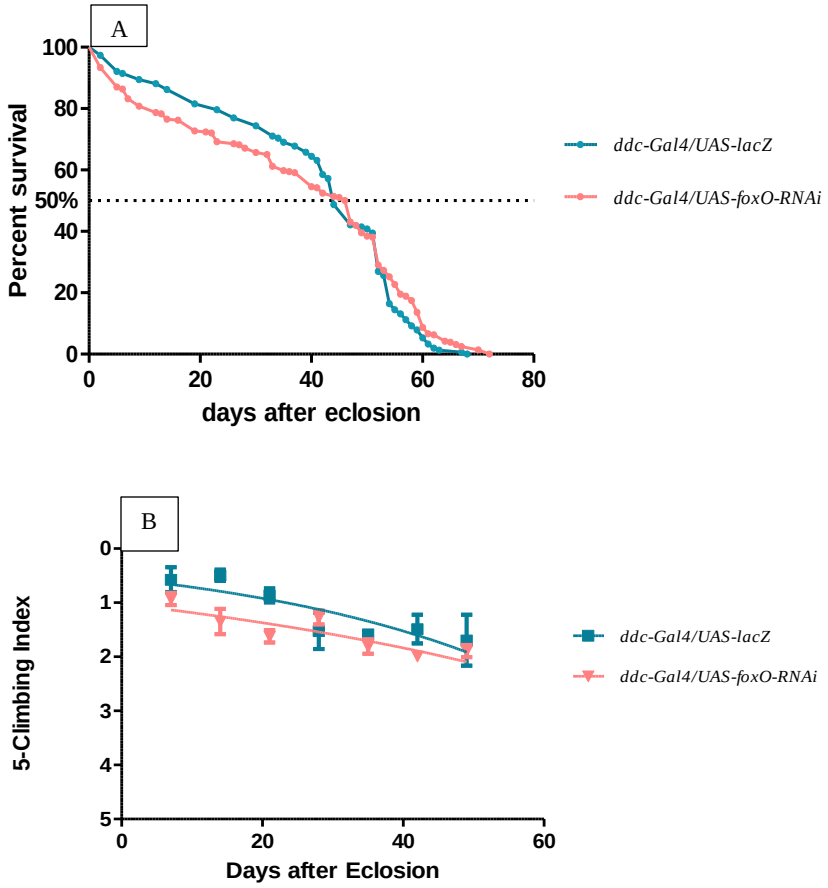

Figure 9. The expression of *foxO-RNAi* under the control of *ddc-Gal4* transgene does not alter life span or climbing ability in *D. melanogaster* raised at 25º C.

Longevity assay was performed on genotypes of *ddc-Gal4/UAS-lacZ* and *ddc-Gal4/UAS-foxO-RNAi* flies and the pairwise comparison does not reveal any significant difference between survival patterns. N value= 200 (**A**). 70 (initially) to 5 (minimum) males of critical class were analysed by a locomotion assay over time and non-linear regression curve was fitted to best demonstrate the climbing over time pattern of two genotypes of *ddc-Gal4/UAS-lacZ* and *ddc-Gal4/UAS-foxO-RNAi*; in 95% CI slopes overlap indicating any difference caused likely by chance (**B**).

The expression of foxO-RNAi directed by TH-Gal4 reduces life span but does not alter climbing ability in D. melanogaster raised at 25º C.

200 males of critical class were selected from each genotype of *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-foxO-RNAi* and a longevity assay was performed. *foxO-RNAi* expressed in dopaminergic neurons under the control of *TH-Gal4* made a slight alteration in climbing ability over time and reduced life span. The survival curves (Figure 10A, P-value <0.001) decrease in life span when *foxO-RNAi* is induced in dopaminergic neurons under the control of *TH-Gal* 4 transgene. The median life span for *TH-Gal4/UAS-lacZ* flies was 58 day whereas the median life span for the *TH-Gal4/UAS-foxO-RNAi* flies was 38 days. Survival curves shown in figure 10A suggest that *TH-Gal4/UAS-foxO-RNAi* flies lived to a maximum of 60 days whereas *TH-Gal4/UAS-lacZ* flies live to a maximum of 80 days. Figure 10B shows the fitted curves of climbing ability over time for two genotypes of *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-foxO-RNAi*. Pair-wise comparison of climbing ability over time for the two genotypes shows that the pattern of their climbing ability is different at days 26 and 40. Climbing ability assay was performed with initially 70 males of critical class until a minimum of 5 flies were left alive.

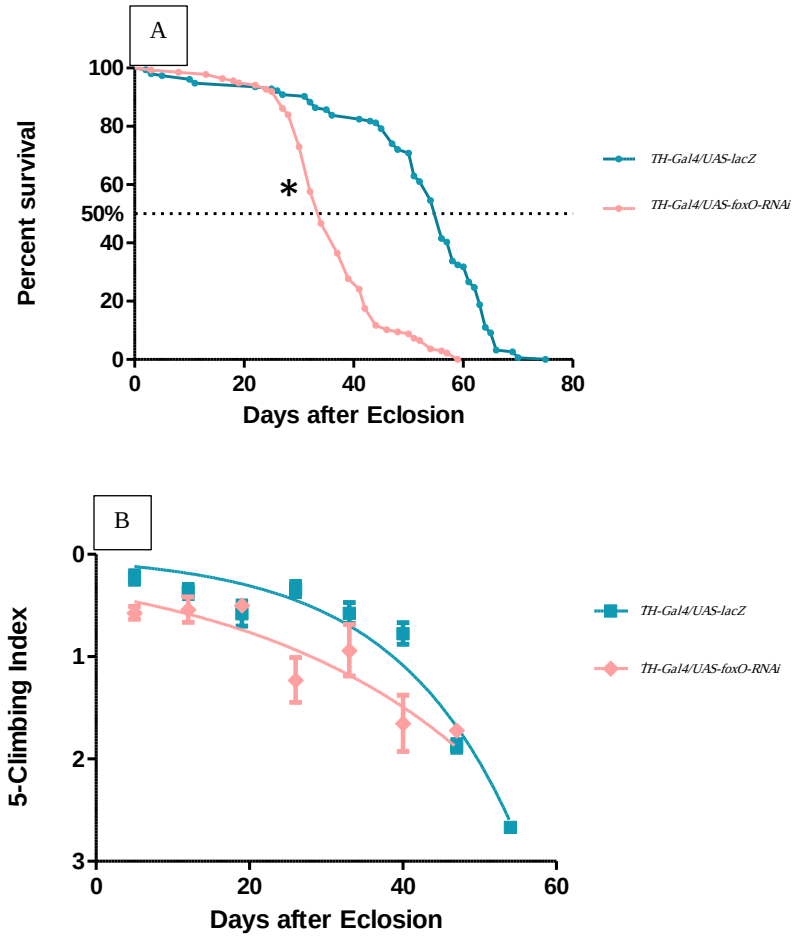

Figure 10. *foxO-RNAi* expression directed by *TH-Gal4* reduces life span significantly but not climbing ability in *D. melanogaster* raised at 25º C. *foxO-RNAi* induced in dopaminergic neurons under the control of *TH-Gal4* transgene shortens life span significantly as supported by Mantel-Cox test results, N-value=200 (**A**). Comparison of climbing ability over time for the two genotypes shows that the pattern of their climbing ability is different at days 26 and 40. N-value is 70 (initially) to 5 (minimum) and genotypes are *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-foxO-RNAi* (**B**) Error bars represent standard error of mean and asterisk indicates significant difference.

Expression of mnb-RNAi under the control of GMR-Gal4 does not alter eye development in D. melanogaster raised at 25º C.

The *mnb-RNAi* transgene under the control of *GMR-Gal4* did not make any significant difference in the number of ommatidia. *mnb-RNAi* in the eye also did not change ommatidia area and bristle numbers dramatically. The number of ommatidia in both genotypes of *GMR-Gal4/UAS-lacZ* and *GMR-Gal4/UAS-mnb-RNAi* was approximately 700 and the number of ommatidia is approximately 500 in *GMR-Gal4/UAS-lacZ* and 480 in *GMR-Gal4/UAS-mnb-RNAi*. Figures 11A and 11B show scanning electron micrographs of *GMR-Gal4/UAS-lacZ* and *GMR-Gal4/UAS-mnb-RNAi* flies respectively and Figure 9C-9E illustrate the related graphs. The number of ommatidia in both genotypes was approximately 700 and bristle number is approximately 500 plus the area of one ommatidium in both genotypes is close to 170 $\mu m^{2.}$ An unpaired t-test was carried out to detect any significant difference between the numerical values of the genotypes where P-value less than 0.05 is considered significant. Statistical analysis did not reveal any significant difference in the two counts and measurements between two genotypes of *GMR-Gal4/UAS-lacZ* and *GMR-Gal4/UAS-mnb-RNAi*. So, eye development was not altered when *mnb-RNAi* is expressed under the control of *GMR-Gal4.*

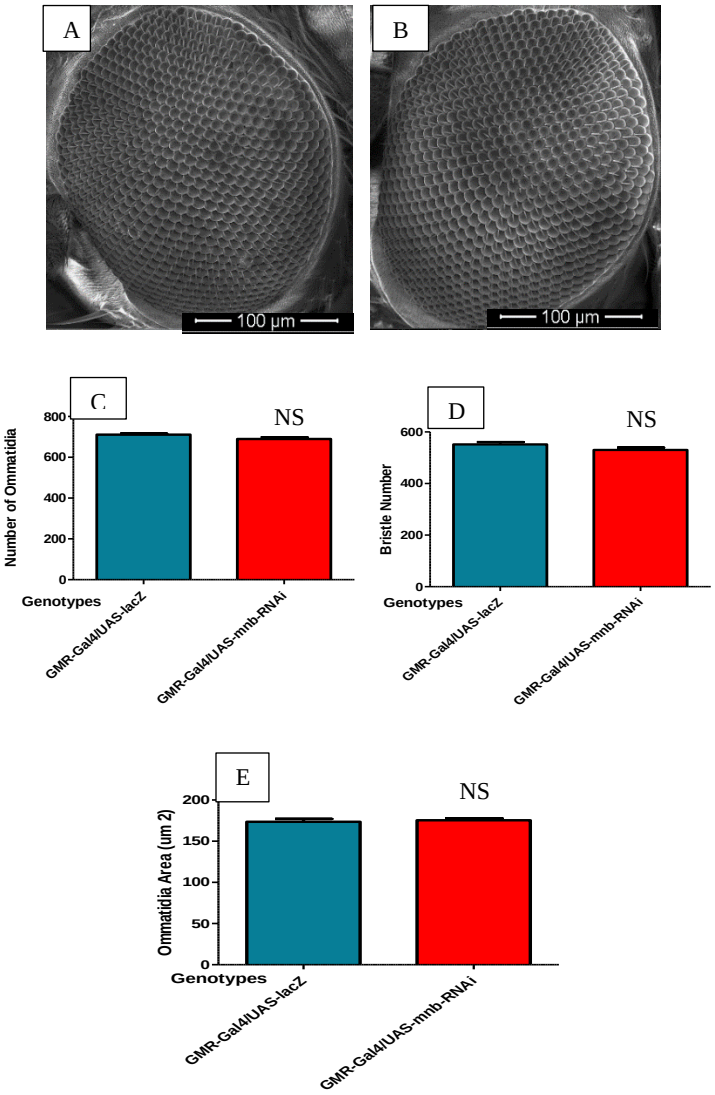

Figure 11. The expression of *mnb-RNAi* under the control of *GMR-Gal4* does not alter eye development in *D. melanogaster* raised at 25 º C. *mnb-RNAi* in the eye does not make any change in eye morphology (**A** and **B**). Genotypes are *GMR-Gal4/UAS-lacZ* (**A**) and *GMR-Gal4UAS-/mnb-RNAi* (**B**) Eye biometric analysis does not show any significant difference in ommatidia number (**C**) and in the number of bristles (**D**) or in the ommatidia area (**E**) (N=10 and NS indicates Not Significant).

The expression of mnb-RNAi directed by GMR-Gal4 does not significantly alter the GMR-Gal4/UAS-foxO phenotypes in D. melanogaster raised at 25º C.

The *mnb-RNAi* in the *GMR-Gal4/UAS-foxO* eye did not supress the *foxO* induced phenotype. Number of ommatidia, bristles and ommatidia area were not significantly altered. Figures 12 A and 12B show scanning electron micrographs of *GMR-Gal4,UAS-foxO/UAS-lacZ* (**A**) and *GMR-Gal4,UAS-foxO/UAS-mnb-RNAi* (**B**). There was not a significant reduction or increase in the number of ommatidia and bristle as well as the ommatidia area. Figures 12C-12E display the related graphs. Medium ommatidia number in *GMR-Gal4,UAS-foxO/UAS-lacZ* was 390 and it was 380 in *GMR-Gal4,UAS-foxO/UAS-mnb-RNAi*. The ommatidia area was close to 180 μm^2 in *GMR-Gal4,UAS-foxO/UAS-lacZ* and 170 μm^2 in *GMR-Gal4,UAS-foxO/UAS-mnb-RNAi*. Unpaired t-test was carried out to detect any significant differences in numerical values where P-value less than 0.05 is considered significant.

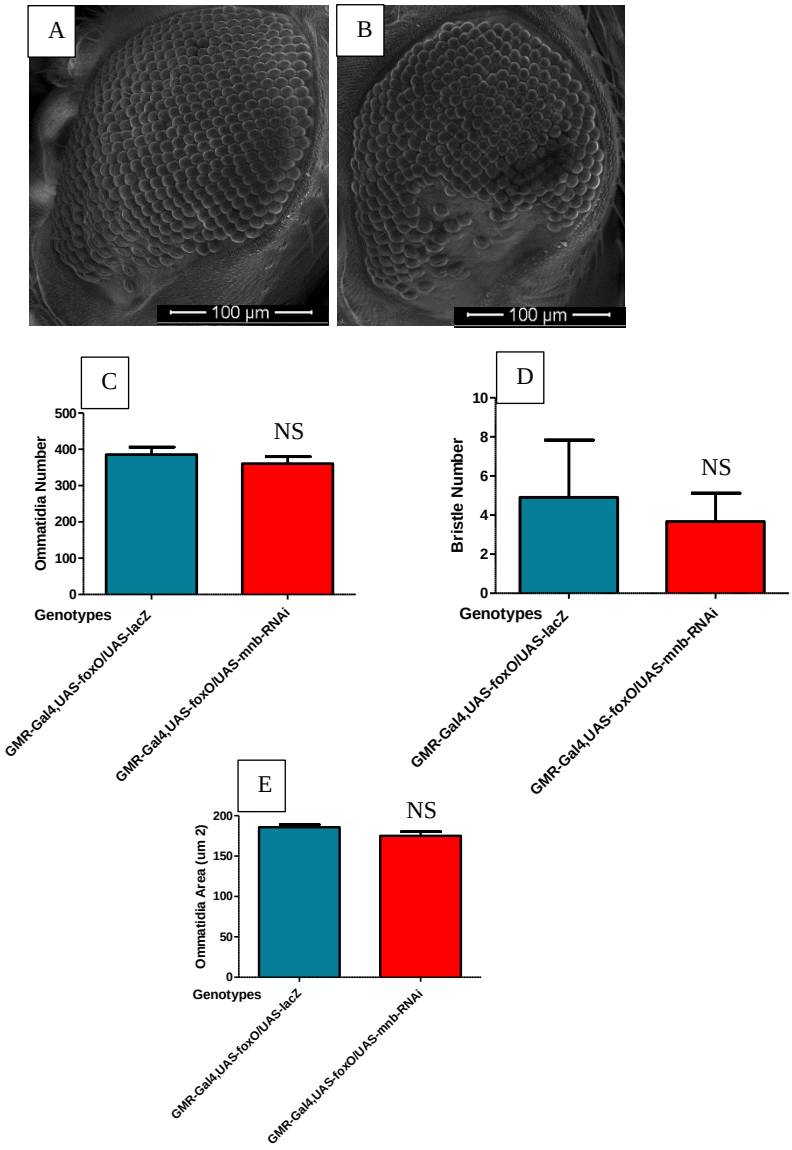

Figure 12. The expression of *mnb-RNAi* in the *GMR-Gal4/UAS-foxO* eye did not significantly alter the *GMR-Gal4/UAS-foxO* phenotype in *D. melanogaster* raised at 25 ºC. Scanning electron micrographs of *GMR-Gal4/UAS-foxO/UAS-lacZ* (**A**) and *GMR-Gal4/UAS-foxO/UAS-mnb-RNAi* (**B**) Expression of mnb-RNAi did not alter ommatidia number (**C**), bristle number (**D**) or ommatidia area (**E**). Error bars represent standard error of mean and NS indicates Not Significant.

Expression of mnb-RNAi directed by ddc-Gal4 does not alter life span or climbing ability in D .melanogaster raised at 25º C.

The *ddc-Gal4* transgene was used to direct the expression of dsRNA for *mnb-RNA* interference in dopaminergic neurons and *ddc-gal4/UAS-lacZ* was used as a control. N-value of 200 males of critical class was selected and the survival curves shown in Figure 13A suggested that both genotypes have median life span of 42 days and both genotypes of *ddc-gal4/UAS-lacZ* and *ddc-Gal4/UAS-mnb-RNAi* live equally well up to close to day 70. Mantel-Cox statistical test did not reveal any significant difference in their survival pattern. Figure 13B displays non-linear regression curves of climbing over time for two genotypes of *ddc-Gal4/UAS-lacZ* and *ddc-Gal4/UAS-mnb-RNAi*. Results of statistical analysis on climbing over time in two genotypes of *ddc-Gal4/UAS-lacZ* and *ddc-Gal4/UAS-mnb-RNAi* did not reveal any significant early loss of climbing ability. 70 flies of critical class were selected initially until the minimum of five flies to assess their climbing ability and the result of comparison shows that slopes of the curves overlap indicating that any seen differences is probably a result of chance.

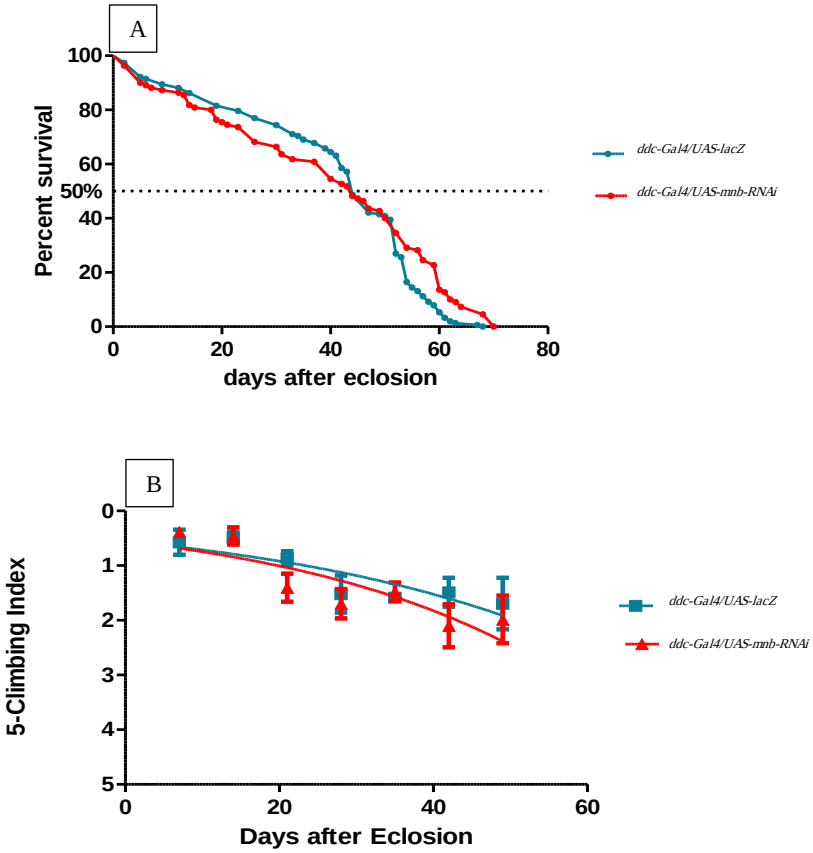

Figure 13. The expression of *mnb-RNAi* directed by *ddc-Gal4* did not alter life span or climbing ability in *D. melanogaster* raised at 25 º C. A Longevity assay results do not reveal any significant difference in life span in genotypes of *ddc-Gal4/UAS-lacZ* and *ddc-Gal4/UAS-mnb-RNAi*. Longevity test was carried out on 200 flies of critical class and the statistical analysis, Mantel-Cox test, was used to detect significant difference in their survival pattern. Both genotypes presented a median life span of 42 days and grew equally well on standard media up to day 70. **B** *mnb-RNAi* directed by *ddc-Gal4* did not change climbing ability over time compared to *ddc-Gal4/UAS-lacZ.*

Expression of mnb-RNAi directed by TH-Gal4 does not alter survival but significantly diminishes climbing ability.

mnb-RNAi expressed by *TH-Gal4* transgene revealed significant decrease in climbing ability but did not alter survival. Figure 14A suggests that there was no significant difference in life span of the two genotypes. *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-mnb-RNAi* both showed a median life span of 58 days and they remained alive until day 78. Their climbing ability over time, however, illustrated in Figure 14B demonstrates a significant loss when *mnb-RNAi* is expressed in dopaminergic neurons directed by *TH-Gal4*. Pairwise, day to day comparison of climbing ability over time for two genotypes of *TH-Gal4/UAS-lacZ* and *TH-Gal4/UAS-mnb-RNAi* flies revealed that the decline in climbing ability in day 26, 40 and 47 is significant. 200 flies of critical class were analysed for longevity assay and 70 flies initially were selected for the locomotor assay and climbing over time until minimum of 5 flies were left alive.

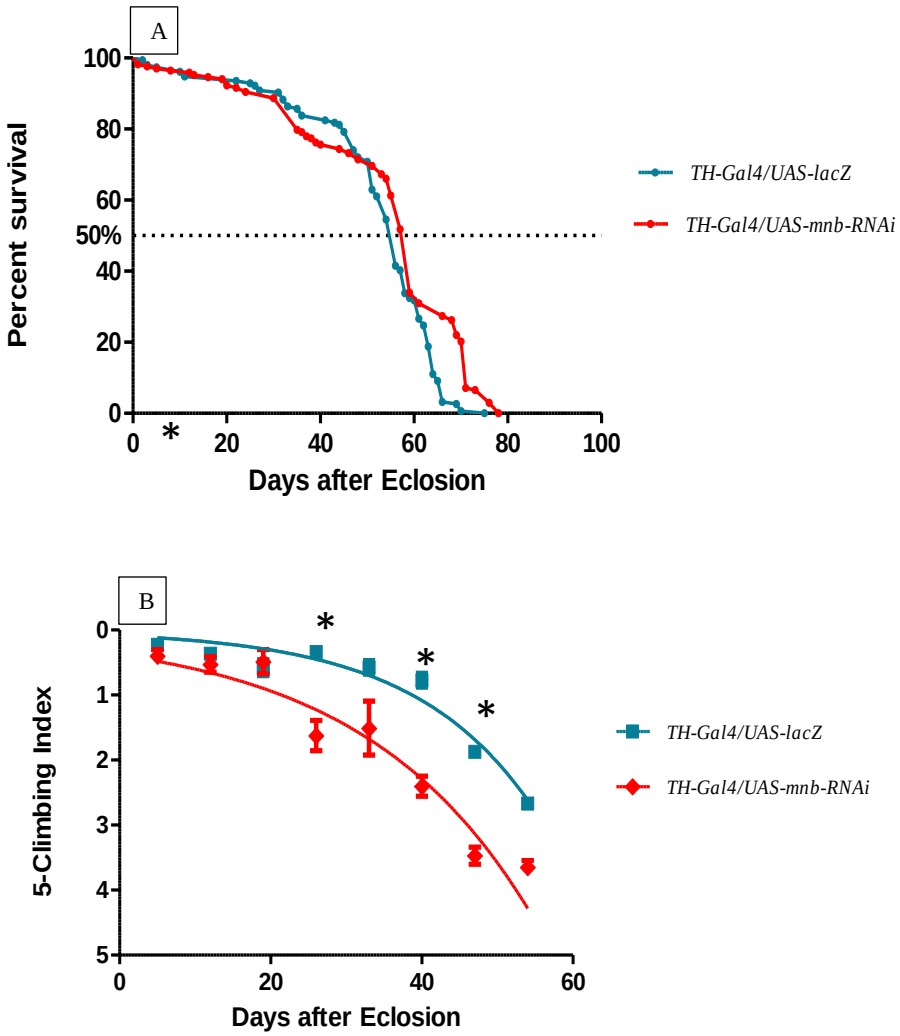

Figure 14. The expression of *mnb-RNAi* in dopaminergic neurons using *TH-Gal4* transgene does not alter life span but decreases climbing ability significantly in *D. melanogaster* raised at 25 ° C. life span was not reduced or increased when *mnb-RNAi* is expressed under the control of *TH-Gal4* transgene, N-value=200 (**A**). Climbing ability was reduced considerably at days 26, 40 and 47 when *mnb-RNAi* was induced in dopaminergic neurons using *TH-Gal4* transgene, N-value =70 (initially) and 5 (minimum) (**B**) Error bars represent standard error of mean and asterisk indicates significant difference.

DISCUSSION

As *foxO1* has been recently implicated as playing a role in Parkinson Disease (Dumitriu *et al*., 2012), I investigated the potential of modelling PD in Drosophila by altering *foxO* activity. This study is in support of evidence from human whole genome microarray implying a role of *foxO1* gene in PD prevalence. Since the previous study showed that *foxO1* genes and the genes under its regulation increase significantly in PD patients, I decided that if we can elevate Drosophila *foxO* in dopaminergic neurons and this mimics PD-like phenotype, I can model this aspect of PD (with increased *foxO*) and provide it for further studies. So, my initial hypothesis was that by elevating *foxO* in dopaminergic neurons I should result in a similar phenotype as in other PD models such as decreased climbing abilities. Since directly elevating foxO in dopaminergic neurons is lethal in Drosophila (Staveley, unpublished), I decided to attempt to increase *foxO* in dopaminergic neurons only to a modest extent and indirectly to avoid detrimental effects of its direct overexpression. To do so, I took advantage of pre-existing conditionally expressed RNAi transgenic lines and I took an approach to indirectly increase *foxO* by inhibiting its inhibitors. There are a number of gene products have been demonstrated to alter the activity of *foxO* (reviewed in Arden, 2006).

Although initially *mnb* and *akt* were considered as potential post-translational modifiers of *foxO*, flies expressing the *akt-RNAi* transgene proved to be not vigorous enough to carry out experiments upon (data not shown). *FKHR* (*foxO1*) is phosphorylated by human *dYRK1A* on Serine 329 (Woods *et al*., 2001), so I decided to evaluate the effects of *foxO* modulation on longevity, locomotion ability and eye development. I utilized *foxO-RNAi* to directly decrease foxO expression and *mnb-RNAi* for slight, indirect elevation in *foxO* activity. Initially, I tested the candidate lines of *UAS-lacZ* and *UAS-GFP-RNAi* to find out which one functioned as a more suitable control for the experiments.

Results suggest that expression of *GFP-RNAi* directed by *TH-Gal4* in dopaminergic neurons results in a significant decrease in life span (Figure 4A). This result is found consistent with a previous study conducted showing that applying *GFP-RNAi* can have detrimental effects (Alic *et al*., 2012). This reduced life span might occur due to targeting of a similar sequence as GFP mRNA thus making a complementary structure with *GPF-RNAi* which eventually leads to some adverse effects on life span. Although locomotion does not seem to be altered when *GFP-RNAi* is expressed in dopaminergic neurons directed by *ddc-Gal4* and *TH-Gal4* (Figures 3B and 4B) and longevity is not influenced when *GFP-RNAi* is expressed directed by *ddc-Gal4* (Figure 3A), it is concluded that *UAS-GFP-RNAi* may not function as a benign control as hypothesized initially. So, I decided to conduct the rest of my experiments with *UAS-lacZ* $^{4\text{-}1\text{-}2}$ as the control line.

The results of *foxO-RNAi* expression in the eye and in dopaminergic neurons suggest that a slight decrease in *foxO* in the eye directed by *GMR-Gal4* transgene does not have any significant effect on eye development as demonstrated by unaffected ommatidia number, bristle number and ommatidia area (Figure 5). The result here suggests that Drosophila eye development is not critically sensitive to the alteration of endogenous *foxO* caused by *foxO-RNAi* and taking out slight amounts of *foxO* in the eye does not alter its development significantly. Overexpression of *foxO* in the eye results in a phenotype characterized by misformed eye shape with reduced number of ommatidia and bristles (Figure 6A). The *foxO-RNAi* expression directed by *GMR-GAL4/UAS-foxO* can suppress the phenotype shown by increased number of ommatidia and bristle (Figures 6C and 6D). The results suggest that directed expression of *foxO-RNAi* can supress the effect of exogenous *foxO* and partially reverse or rescue the phenotype. *foxO-RNAi* expression in dopaminergic neurons gives two distinct results: 1) *foxO-RNAi* directed by *ddc-Gal4* does not alter life span or climbing ability compared to *ddc-Gal4/UAS-lacZ* (Figure 7); 2) *foxO-RNAi* expression directed by *TH-Gal4* decreases life span significantly but does not alter locomotion over time when directed by *ddc-Gal4* transgene (Figure 7). Reduced life span in *foxO-RNAi* flies in their dopaminergic neurons (using *TH-Gal4* as driver line and not with *ddc-Gal4*) compared to *UAS-lacZ* flies suggested a protective role for *foxO* against cell death in dopaminergic neurons (Li *et al*., 2012).

Directed expression of *mnb-RNAi* in the eye by *GMR-Gal4* transgene does not alter eye development significantly as shown by unaffected ommatidia number; ommatidia area and bristle number (Figure 9) and the inhibition of *mnb* using *mnb-RNAi* directed by *GMR-Gal4/UAS-foxO* does not display any significant change in the eye either (Figure 10). From the result obtained, it can be deduced that inhibition of *mnb* in the eye both in the presence of exogenous *foxO* or in its absence is not significant to eye development. This finding is consistent with the result that *mnb* mutants present fairly normal eyes and that decrease in *mnb* levels does not alter eye development significantly (Tejedor *et al*., 1995). Expression of *mnb-RNAi* in dopaminergic neurons exhibit two different results: 1) directed by *ddc-Gal4*, *mnb-RNAi* expression does not alter life span or longevity compared to that of *ddc-Gal4/UAS-lacZ* (Figure 11); 2) expression of *mnb-RNAi* in dopaminergic neurons using *TH-Gal4* transgene, altered climbing ability over time but did not alter life span (Figure 12).The difference in expression array presented by the two transgenes may account for the observed difference in results; the *ddc-Gal4* transgene direct the expression of *Gal4* and hence the gene under the control of *UAS* element in a slightly different and wider area than that of the *TH-Gal4* transgene. In fact, dopa decarboxylase enzyme is synthesized in the 150 dopamine and serotonin neurons and in a subset of glial cells and in the most hypodermal cells whereas TH is expressed in dopamine synthesizing cells (Alic *et al*., 2012). Another possible explanation for the observed difference is that the expression of protein Gal4 may not be as robust in *ddc-Gal4* as it is with *TH-Gal4* transgene.

A significant decrease in climbing ability at days 26, 40 and 47 can be due to slight elevation of *foxO* caused by *mnb-RNAi* in dopaminergic neurons. If so, it is consistent with the results from a genome wide study (Dumitriu *et al*., 2012) indicating the role of *foxO* increase in Parkinson Disease. Nevertheless, as there is a growing body of evidence on the regulatory effects of *dyrk1A/mnb* on *foxO* in different cellular processes including metabolism and cell growth (including Hong *et al*., 2012) and with the experimental set-ups in my project, I can deduce that slight inhibition of *mnb* can mimic some aspects of PD in Drosophila.

The *foxO* transcription factor promotes the expression of different genes including *MnSOD, Cyclin G2, NPY* and *Bim*1 thus playing role in different cellular processes (further experiments can be done to identify which downstream genes are altered in case of up/down regulation of foxO). While many studies including Hossain *et al*., 2013 study) suggest that *foxO* protects against cell death particularly in food reduced conditions and other stress-causing conditions by acting as an innate immunity foster element (Wang *et al.*, 2014), some other papers (including Fu and Tindall, 2008) point to the flip side and indicate that *foxO* plays a role in inducing apoptosis exhibiting as a tumour suppressor in a variety of cancers (by inducing the expression of death receptor ligands such as Fas ligand Bcl-2 family members). So it seems that *foxO* can play roles in several physiological processes at different developmental stages and environmental conditions.

The apparent complexity in verifying results may call for repeating similar experiments to verify these results or trade them for new, more precise findings which can be one way of looking at future studies. Other ways of progressing with studies of this nature will be the same studies with more accurate assessment techniques for example better performing climbing system like the one described in Podratz *et al*., 2013 as the currently used manual system is laborious, time-consuming and subject to human error. One other aspect of continuing and improving this study is to employ new techniques such as immunohistochemistry to evaluate the presence of dopamine in Parkinson Disease models. In addition, for assuring the actual expression of a certain construct in prepared fly lines, one can benefit from the techniques that evaluate the expression or even quantifies them such as qPCR. Other ways of evaluating the role of certain genes in cell death behaviour may involve gene knock-downs in Drosophila embryo and evaluating caused alterations in developing flies with fluorescence detection or other macroscopic measurements such as size measurements or morphological assessments. Plus, foxO activity or modulation of transcriptional targets could also be assessed. To verify actual alteration in levels of mnb or foxO, immunohistochemical staining in brain tissue or the eye (depending on the experiment rationale) can be performed.

Conclusion:

Supported by the results, as the initial prediction was, expressing *mnb-RNAi* in dopaminergic neurons using the *TH-Gal4* transgene seems to partially mimic/model some aspects of Parkinson Disease but does not do so with *ddc-Gal4* tested by climbing test (as described by Todd and Staveley, 2004).

Results do not support the hypothesis that *GFP-RNAi* transgene can function as a suitable control. Expression of *GFP-RNAi* directed by *TH-Gal4* leads into significant reduced life span therefore *GFP-RNAi* may not be operating as a benign control.

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