‘Investigating the Regulation of Growth by Nitric Oxide Signalling in *Drosophila melanogaster*’

A thesis submitted to the University of Sussex for the degree of
Master of Philosophy
September 2011
By
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Declaration

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to this or any other University for the award of any other degree.

Mona Khosravi

September 2011

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Abstract

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Mechanisms associated with growth regulation have been shown to be highly conserved in mammals and *Drosophila*, especially when examining the insulin signalling pathway. Previous studies suggest that nitric oxide (NO) signalling can inhibit growth and cell proliferation in a *Drosophila* forkhead box O (dFOXO)- dependent manner. dFOXO is a component of the insulin signalling pathway and has also been demonstrated to inhibit growth via its interactions with components in this pathway; however, the mechanism by which dFOXO and NO interact is unclear.

Since inhibition of growth by NO is dependent on dFOXO, this thesis examines the effect of co-expressing nitric oxide synthase II (NOS2) with three dFOXO alleles (*dFOXO*^{25}, *dFOXO*^{21} and *dFOXO*^{BG01018}) in *Drosophila* salivary glands taken from third instar larvae. It concludes that the *dFOXO*^{25} null allele appeared to be the strongest deletion of *dFOXO* given that salivary gland nuclei appear most similar in size to the wild type. This indicates that NO-induced growth inhibition only occurred to a very small degree as a result of a powerful loss-of-function of dFOXO exhibited in *dFOXO*^{25} homozygotes.

This thesis also investigates the effects of NO on salivary glands taken from the same developmental stage when co-expressed with overexpressed oncogenes, dMyc and Ras^{V12}. Nuclei measurements were larger than the NOS2-only expressing line and smaller than the lines expressing only each of the oncogenes. However, TEM analysis revealed that co-expression might induce endoplasmic reticulum (ER) stress in the glands. Research shows that NO and these oncogenes can provide the reactants necessary to generate peroxynitrite, which is associated with the generation of ER stress.

When examining the effects of these growth regulators on mitochondria and Golgi, this thesis reports that dFOXO, NOS2 and dMyc can increase mitochondrial biogenesis. Golgi was unaffected by expression of the growth regulators

Abbreviations

ATP	Adenosine triphosphate
bHLHZ	Basic helix-loop-helix zipper
BrdUTP	5-bromo-2'- deoxyuridine 5'-triphosphate
Cdk	Cyclin dependent kinase
cGMP	Cyclic guanosine monophosphate
CNS	Central Nervous System
CR	Caloric restriction
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DILPS	*Drosophila* insulin like peptides
dInR	*Drosophila* insulin receptor
DNA	Deoxyribonucleic Acid
ES	Embryonic stem
EYFP	Enhanced yellow fluorescent protein
FHREs	Forkhead-response elements
FLP	Flippase
FOX	*Forkhead* box
FOXO	Forkhead-related transcription factors
FRT	Flippase recognition target site
GAL4	Yeast protein that binds DNA
GAPs	GTPase activating proteins
GFP	Green fluorescent protein
IGF	Insulin growth factor
InR	Insulin receptor
IPCs	Insulin-producing cells
iPS	Induced pluripotent stem
IRS	Insulin receptor substrate
MAC	Macrophage
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
$O2^-$	Superoxide
$ONOO^-$	Peroxynitrite

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDK1	phosophoinositide-dependent kinase 1
PI3K	Phospho-inositide 3–kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triposphate
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog on chromosome 10
RNA	Ribonucleic Acid
RNAi	RNA interference
ROS	Reactive oxygen species
RR	Ribonucleotide reductase
rRNA	Ribosomal RNA
SG	Salivary gland
sGC	Soluble Guanylate Cyclases
SNAP	S-nitroso-N-acetyl-penicillamine
SpNO	Spermine nitric oxide
TEM	Transmission Electron Microscopy
TOR	Target of rapamycin
TORC1	Target of rapamycin complex 1
TSC	Tuberous sclerosis complex
UAS	Upstream activator sequence
VEGF	Vascular endothelial growth factor
wt	Wild type
YFP	Yellow fluorescent protein

Chapter 1

General Introduction

1.1. Nitric Oxide

NO acts as a diffusible, intercellular messenger in signalling pathways and is highly reactive with a biological half-life of less than a second *in vivo* (Pacher *et al*., 2007). NO has crucial roles in immunity, the nervous system and vasodilation; however, it can also lead to pathophysiology when present at inappropriate levels in certain tissues.

This chapter will briefly outline various signalling mechanisms of NO in different organ systems and address the effects that these have on growth, metabolism and age-related diseases.

1.1.1 Nitric Oxide Biosynthesis

The amino acid, L-arginine, reacts with nitric oxide synthases (NOSs) in the process of NO biosynthesis (Palmer *et al*., 1988). This is an oxidative process during which L-arginine is converted into L-citrulline as illustrated below:

Fig 1.1. Biosynthesis of NO

Overall reaction

$$\textit{L-arginine} + 2\ \textit{NADPH} + O_2 \rightarrow \textit{L-citrulline} + \textit{NO} + 2\textit{NADP}^+$$

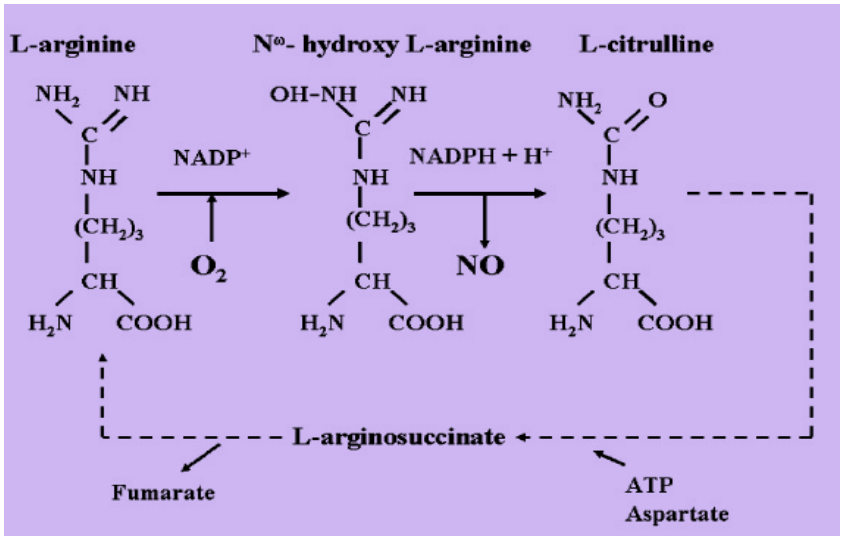


Fig. 1.1 **Biosynthesis of NO from L-arginine.** (Bruckdorfer, 2005)

Therefore, NOS enzymes catalyse the formation of NO and require the following obligatory cofactors: calcium-calmodulin, HEME and flavins (Lacza, 2006). NO can diffuse from the source of synthesis to a neighbouring cell where it has several cytoplasmic targets. Given its short half-life, this range is approximately 100μm (Lancaster, 1997) qualifying NO as a short-range signalling molecule; nonetheless, it has many diverse targets, modifying the function of a wide range of molecular processes. NO can target ribonucleotide reductase, cyclooxygenases and several mitochondrial enzymes (Dawson and Dawson, 1995; Garthwaite and Boulton, 1995). Nakaya *et al*. (2000) also showed that NO can regulate cell-cycle proteins, such as p53, to induce cell cycle arrest via phosphorylation in signalling pathways.

1.1.2. NOS isoforms: nNOS, eNOS, iNOS, mtNOS and dNOS

The three main NOS isoforms in mammals are well documented; they are: neuronal NOS (nNOS/NOS1), inducible NOS (iNOS/NOS2) and endothelial NOS (eNOS/NOS3) (Knott and Bossy-Wetzel, 2009). Furthermore, *Drosophila* expresses a variant of the NOS gene known as dNOS (Kuzin *et al*., 1996). More recently, research addresses another classification of NOS, mitochondrial NOS (mtNOS) (Ghafourifar and Cadenas, 2005). Each isoform is located in different tissue types and expressed over different periods at varying levels; these distinctions occur as a result of their specific physiological roles.

1.1.3. nNOS

nNOS acts in neurons specific to both the central and peripheral nervous systems. NO is required as a neurotransmitter in the nervous system for neuronal plasticity and intercellular communication (Knott and Bossy-Wetzel, 2009). NO biosynthesis is relatively low in this system, but is constantly generated by constitutively active nNOS. NO acts in several nervous system processes to help coordinate important neural signals such as relaxation of the alimentary canal to facilitate digestion and signalling to neural blood vessels allowing dilation in penile and cerebral arteries (Bredt *et al*., 1991; Burnett *et al*., 1992).

1.1.4. eNOS

Similarly, NO production via eNOS is comparable as this isoform is also constitutively active, producing NO from L-arginine at a low, consistent rate to maintain physiological

function (Moncada *et al*., 2006). However, this biosynthesis takes place in endothelial tissue in the vascular system, in which NO acts to relax the surrounding tissue and promote vasodilation and vascular smooth muscle cells are common sites of eNOS activity.

eNOS-produced NO can bind with the protoporphyrin ring in the heme group of soluble guanylate cyclase (sGC) to generate the second messenger molecule, 3', 5'-cyclic guanosine monophosphate (cGMP) (Bruckdorfer, 2005). As a result, cGMP can then interact with phosophodiesterases (Hartzel and Fishmeister, 1986), protein kinases (Paupardin-Tritsch *et al*., 1986), and ion channels (Nawy and Jahr, 1990). These pathways have been found to generate signals to the nucleus, modifying gene transcription and thus, produce a sustained or permanent change in the cell (Gudi *et al*., 1999). In the endothelium NO presents as a relaxation-inducing molecule that regulates vascular tone (Ignarro *et al*., 1987).

1.1.5. iNOS and macrophage function

In contrast, iNOS synthesises NO over a relatively shorter period of time and at a higher level. This production occurs in the immune system, specifically glia and macrophages, upon activation via pathogen recognition (Merrill *et al*., 1997). NO biosynthesis in this system is, therefore, induced and the isoform acts to trigger cell death by toxic levels of NO production. Additionally, NO can react with superoxide anions to form the toxic oxidant, peroxynitrite, which can destroy pathogens by targeting their nucleic acids, proteins and oxidising lipids (Szabó, 2003).

1.1.6. mtNOS

The most recently discovered isoform, mtNOS, is thought to produce NO that functions to buffer Ca^{2+} and control energy production in the inner membrane of mitochondria (Ghafourifar and Cadenas, 2005).

Lacza *et al*. (2006) discussed other studies that challenge the existence of mtNOS. Such studies argued that a NOS-like sequence is not contained in the mitochondrial genome, meaning that NOS has to be imported into the mitochondria, appropriately folded and supplied with the obligatory cofactors previously mentioned (Brookes, 2004). Lacza *et al*. (2006) acknowledges these arguments and suggests that eNOS may be mistaken for a novel, mtNOS isoform. Additionally, at least three different sources of NO can

explain its presence in mitochondria: nitrosothiols acting as NO donors, eNOS attachment to the outer mitochondrial membrane and nitrate reductase activity demonstrated in the electron transport chain. Yet, investigating synthesis of NO in mitochondria is problematic. Mitochondria act as NO-sinks, making it difficult to measure intramitochondrial levels of NO even if it is produced there (Pearce *et al*., 2002). Furthermore, when preparing mitochondria for analysis, contamination is always present between 1-4% and may influence results if contaminants contain extramitochondrial NOS (Sims, 1990).

Nevertheless, several studies confirm the existence of mtNOS (or at least eNOS in mitochondria) and characterise its function as being essential for mitochondrial biogenesis via eNOS null mutations (Nisoli *et al*., 2003).

1.1.7. dNOS

Recently, *Drosophila* has been demonstrated to express a solitary NOS enzyme (dNOS) which has functions in pathways corresponding to development, immunity and behaviour (Kuzin *et al*., 1996; Wingrove and O'Farrell, 1999; Gibbs, 2003). Regulski and Tully (1995) first characterised *dNOS* by cloning the gene and screening the *Drosophila* genome with a phage library utilising a section of *nNOS* found in rats. The study identified the location of *dNOS* at position 32B on the second chromosome. *dNOS* comprises 19 exons and spans 34 kilobases of genomic DNA (Stasiv *et al*., 2001). *dNOS* encodes at least ten different transcripts expressed during *Drosophila* development and encodes for approximately seven different proteins (Stasiv *et al*., 2001). However, only one of these proteins, dNOS1, is enzymatically active.

dNOS1 is the only full length protein and its enzymatic activity is due to its carboxyl terminal reductase domain that the other truncated proteins lack. Additionally, Stasiv *et al*. (2004) showed that one of the truncated proteins, dNOS4, can inhibit the antiproliferative activity of dNOS1 in *Drosophila* larvae through ectopic dNOS4 expression in the imaginal discs. Adult flies have a hyper-proliferative phenotype, indicating that dNOS1 function has been disrupted. The primary protein produced by *dNOS*, dNOS1, will be referred to as dNOS herein.

Similar to nNOS and eNOS, generation of NO by dNOS occurs in a Ca^{2+}- and calmodulin- dependent manner (Sengupta *et al*., 2003). In a biochemical analysis Ray *et*

al. (2007a) compared the oxygen domain of dNOS (dNOSoxy) with mammalian isoforms and concluded that dNOSoxy is most similar to the oxygen domain found in nNOS, except that they possess different kinetic properties. This similarity is corroborated in Regulski and Tully (1995) which reported a high degree of homology in protein structure between dNOS and the mammalian constitutively expressed NOS isoforms, nNOS and eNOS, with the former being the most similar in structure (43% sequence similarity).

Interestingly, Ray *et al*. (2007a) also reported that computer models indicate this difference in kinetic properties enables dNOS to function more efficiently and actively in NO release when compared with mammalian isoforms. This may allow for increased diversity of signalling in *Drosophila*. Further biochemical comparison of the dNOS reductase domain and its associated calmodulin binding site also confirms the most significant similarity with those structures in nNOS (Ray *et al*., 2007b).

Recently, Yakubovich *et al*. (2010) reported that NO is not required for normal development of *Drosophila.* This is in contrast to Regulski *et al*. (2004) which previously characterised a mutation in a conserved residue that the researchers believed completely abrogated NO activity. Regulski *et al*. (2004) reported that this lesion (NOS^{c}), believed to be a NO null mutation, produced lethality. Upon further investigation, Yakubovich *et al*. (2010) stated that this is incorrect due to two factors: the mutation was not rescued once NO is reintroduced and the failure of Regulski *et al*. (2004) to generate deletions for all of the seventeen alleles identified in the *NOS* complementation group. Yakubovich *et al*. (2010) modified the knockout design, which included a lethal lesion, and found that a more accurate deletion of *dNOS wa*s not lethal in *Drosophila*; in fact, the flies appeared healthy, despite the deletion.

1.1.8. Localisation of NO activity using the NADPH-diaphorase method

The NADPH-diaphorase (NADPHd) staining method has been validated in many invertebrates, such as *Drosophila*, by purifying host NOS and demonstrating co-localisation for NOS and NAPDHd activities (Müller, 1994). This technique is based on detecting an insoluble formazan precipitate produced by NADPHd and levels of staining correlate with NOS expression. In *Drosophila* Kuzin *et al*. (1996) used this technique to monitor dNOS activity and expression, which increased as development progressed in the developing imaginal discs. Initially, these increasing levels were

demonstrated in the eye, haltere, wing, and genital discs during the third instar; however, staining decreased in a specific spatial pattern once pupal development commenced.

1.1.9. NO regulates growth and proliferation

Stuehr and Nathan (1989) demonstrated that NO is a product of activated macrophages and has a cytostatic effect on its cellular targets while inhibiting respiration by damaging mitochondria. In an effort to further characterise this cytostatic effect, Kwon *et al.* (1991) identified ribonucleotide reductase (RR) as a potential target for NO, given its chemical properties. RR is a rate limiting step in DNA synthesis and NO is found to inactivate this enzyme in tumour cells, producing the cytostatic effect and reversibly inhibiting DNA synthesis. Thus, NO can ultimately slow growth by reducing rates of cellular division.

In the developing *Drosophila* eye, NO is upregulated and acts in conjunction with RBF (Retinoblastoma-family protein) to promote antiproliferation by restricting entry into the S phase of the cell cycle (Kuzin *et al*., 1999). Wanga *et al.* (2007) also confirmed NO action in blocking cell cycle progression by restricting the G_1-to-S phase transition, which resulted in G_1 arrest.

In *Drosophila* it has been shown that NO acts an antiproliferative agent during larval development (Kuzin *et al*., 1996). The adult fly is characterised by the size, shape and structure of its organs as a result of developmental processes that take place in the imaginal discs. Due to its role in limiting cell growth, it is thought that dNOS in *Drosophila* plays a pivotal role in development from a larval stage (Kuzin *et al*., 1996). The study suggested that NO expression is high in the developing imaginal discs. Ectopic expression of NO in larvae results in hypotrophy of the organs and limbs while inhibition of NO produces hypertrophy in these anatomical features. Kuzin *et al*. (1996) concluded that antiproliferation caused by NO action regulates the balance between cell proliferation and differentiation. In a study within our own lab, it was shown that for NO to have an inhibitory effect on growth in *Drosophila* the transcription factor dFOXO must be present (Kimber *et al*.).

1.1.10. NO donors

Using the [^{3}H]-thymidine incorporation technique, Magalhães *et al*. (2006) investigated the rate of DNA synthesis in chick retinal cells in culture. [^{3}H]-thymidine is a radioactive nucleoside that is integrated into new DNA strands during mitosis. By measuring the levels of radioactivity in the DNA using a scintillation beta counter, Magalhães *et al*. (2006) were able to gauge the levels of proliferation that occurs in response to NO. Two NO donors, S-nitroso-*N*-acetyl-penicillamine (SNAP) and Spermine nitric oxide (SpNO) complex, were separately incubated with these cells and [^{3}H]-thymidine incorporation observed. The study reported that this incorporation decreased by ≈ 70% in the presence of both donors and is thought to occur via S-nitrosylation as dithiotreitol reverses [^{3}H]-thymidine incorporation inhibition. SNAP also caused antiproliferation in purified glial cell cultures.

However, Hu *et al*. (2002) demonstrated that using the [^{3}H]-thymidine incorporation method as a measure of the effect of NO on DNA replication is inappropriate, since radioactivity is shown to promote apoptosis in cells. This study showed that in the murine fibroblasts 3T3 cell line, dose-dependent inhibition of proliferation and DNA synthesis is caused by the radioactive isotope and not thymidine itself. Using a stable isotope, it demonstrated that experiments can use thymidine in conjunction with mass spectrometry as a non-radioactive indicator of DNA replication and cell proliferation. Ironically, the radioactive form of thymidine modifies the very parameter it is intended to measure. Orlov *et al*. (2003) supported this conclusion by confirming that [^{3}H]-thymidine-DNA labelling inhibits proliferation and induces apoptosis on its own in canine kidney epithelial cells and porcine aorta endothelial cells. Therefore, studies using this method can generate more accurate data by not using thymidine in its radioactive form.

There are several alternatives to using [^{3}H]-thymidine incorporation in order to measure the effect of NO on DNA synthesis. Flow cytometry has been used to measure the effect of SNAP in this lab by Kimber (2005) on *Drosophila* S2 cells as an alternative to radioisotopes. This study also demonstrates the antiproliferative effect of NO on cells. Additionally, 5-bromo-2'- deoxyuridine 5'-triphosphate (BrdUTP) can be used as a marker of DNA strand breaks and thus, replication.

1.1.11. NO function in Energy Expenditure and Metabolism

Given that NO has been shown to increase mitochondrial biogenesis physiologically and pathologically in leukemia (Carew, 2004), researchers should therefore investigate its role in regulating mitochondria-associated processes such as energy expenditure and metabolism. These two processes are heavily associated with preserving good health in mammals.

Deviations from normal functionality of these processes are shown to accompany medical conditions such as obesity, a result of caloric intake being greater than energy expenditure (Knott and Bossy-Wetzel, 2010). Additional conditions such as age-related disorders (cancer, cardiovascular disease, neurodegeneration and diabetes) are also associated with dysfunction of these two processes. Exercise and caloric restriction (CR) dieting increase fat and glucose metabolism, and mitochondrial function, which have been shown to negate these disorders, a fact that one would attribute to the benefits these two practices have on regulating energy expenditure and metabolism (Knott and Bossy-Wetzel, 2010). As a result of both of these therapies, increased longevity and decreased risk of age-related disorders are observed. Although the role of NO in exercise has not yet been fully uncovered, it increases mitochondria numbers and energy production, indirectly regulating fat cell differentiation, CR and obesity.

1.1.12. Role of NO in Pathophysiology

NO production can also be associated with pathophysiology when formed in excess. As mentioned above, high levels of NO production can prove toxic, a fact that is harnessed by cells belonging to the immune system to fight pathogens. This potential for toxicity indicates that NO could be involved in pathological processes and conditions. Research has found that NO can combine with superoxide anions (O_2^-) to yield peroxynitrite ($ONOO^-$). In mitochondria, for example, this product stimulates the release of cytochrome complex (*c*) from the inner mitochondrial membrane, a process normally initiated in response to pro-apoptotic stimuli (Ghafourifar *et al*., 1999). Therefore, this NO-derived product can induce cell death and mitochondrial stress in several cell types, blocking Complexes I and IV in the respiratory electron transport chain (Knott and Bossy-Wetzel, 2009). Pacher *et al*. (2007) provides a comprehensive discussion of the role of NO in generating peroxynitrite and gives evidence to support its association in

many pathological conditions such as cardiac and vascular diseases, circulatory shock, inflammation, cancer, stroke, neurodegenerative disorders and diabetes.

Specifically, NO has been known to react with protein-based cysteine residues to induce S-nitrosylation which can impair protein function (Stamler *et al*., 2001). Interestingly, in research concerning age-related neurodegenerative diseases, S-nitrosylation of this nature with a variety of proteins has been demonstrated to accompany stroke, Alzheimer's and Parkinson's (Knott and Bossy-Wetzel, 2009).

1.1.13. Role of NO and peroxynitrite in causing cancer

Ironically, NO is demonstrated to have both a causative and preventive effect in cancer research. There is substantial evidence that its causative properties are a result of its ability to form peroxynitrite, the primary agent linked with DNA damage which can lead to cancer.

NO is shown to stimulate tumour angiogenesis through promoting angiogenic and lymphangiogenic factor expression, specifically vascular endothelial growth factor (VEGF) (Jenkins *et al*., 1995). NO accomplishes these processes by decreasing the expression of thrombospondin-1, an endogenous antiangiogenic factor (Ridnour *et al*., 2005), and also by promoting the maturation of blood vessels by recruiting pericytes (Yu *et al*., 2005).

Furthermore, NO has been linked with breast and colon cancer. It is strongly associated with enhanced migration and invasion observed in tumour cells via sGC- and MAPK-dependent signalling mechanisms (Orucevic, *et al*., 1999; Siegert *et al*., 2002; Jadeski *et al*., 2003).

NO has also been associated with DNA damage, resulting in clonal transformations and mutations in the DNA (Pacher *et al*., 2007). Formation of peroxynitrite can trigger oxidative modifications in guanine (Niles *et al*., 2006) and guanine nitration (Sawa and Ohshima, 2006). In addition to chemically modified guanine, the DNA base has been shown to mutate to all of the other bases as a result of peroxynitrite reactions in mammals (Suzuki *et al*., 2002) and viruses (Neeley *et al*., 2004). Peroxynitrite also acts indirectly to damage DNA as it can target DNA repair enzymes such as 8-oxoguanine DNA glycosylase which is responsible for excising mutated 8-oxoguanine and is inactivated by peroxynitrite (Jaiswal *et al*., 2001).

There are also many studies which contradict these reports and identify the tumour-suppressing activity of NO. In mice, iNOS knockouts are demonstrated to induce lymphomagenesis (Scott *et al*., 2001), tumourigenesis in the intestines (Dhar *et al*., 2003) and sarcomagenesis (Hussain *et al*., 2004). Fukumura *et al*. (2006) reported that these contradictions can be explained by the activity of p53 (tumour suppressor) in cells containing NO, cellular sensitivity to NO, types of iNOS cells implicated, and the intensity and duration of NO exposure.

Mutant mice with a modified adenomatous polyposis coli gene, normally demonstrate spontaneous polyp generation in the intestines. However, this phenotype can be rescued if combined with iNOS knockout genes or if iNOS is inhibited (Ahn and Ohshima, 2001), suggesting a pathological role for NO-producing iNOS. Similarly, Kisley *et al*. (2002) showed that genetic ablation of iNOS in mice results in an 80% reduction of lung tumour formation induced via treatment with urethane.

Both the physiological and pathophysiological roles of NO are thus well documented and key to investigating the role of this messenger molecule in cancer, age-related diseases, metabolism and mitochondrial function.

1.1.14. Role of NO in treating cancer

In spite of a great number of studies that have been conducted to licence NO-based treatments, only two types of NO donor drugs have been approved for clinical usage and neither of them have been applied to cancer. For reasons mentioned in the previous section, it is clear that clinical applications of NO must be carefully designed in order to avoid pathology. Its beneficial effects are often only possible in extremely small concentrations of NO (at the picomolar level) to avoid cytotoxicity (Miller and Megson, 2007).

Diazeniumdiolates (NONOates) are a class of NO drugs that several studies show to be promising anti-cancer treatments (Wu *et al*., 2001; Cai *et al*., 2003; Chen *et al*., 2006). JS-K is a terminal oxygen-protected NONOate generated by the US National Cancer Institute, and has been demonstrated by Shami *et al*. (2003) to impede tumourigenesis in a great variety of cancer cells while leaving healthy cells unaffected. NONOates are very promising for cancer therapy since they allow researchers to easily predict the rates

of NO-release. However, further research is necessary to ensure that the application of future NONOate-based NO donor drugs can be safely conducted.

1.1.15. Using MAC-NOS to overexpress NO in *Drosophila*

This study will use the mouse macrophage *iNOS* gene (NOS2) characterised in a UAS-MAC-NOS construct to examine the function of NO *in vivo*. NOS2 will be expressed under the control of the GAL4-UAS system which allows for tissue-specific expression of NOS2 during a specific developmental stage in order to generate the appropriate phenotypes that can be subsequently analysed. Specifically, the subject tissue in this case is the salivary glands and the developmental stage analysed is the third instar (Brand and Perrimon, 1993).

Lowenstein *et al*. (1992) stated that NOS2 produces a high NO output and does not require binding of Ca^{2+} or calmodulin in order to function. This is in contrast to the other isoforms (including dNOS) justifying the use of a UAS-MAC-NOS construct for analysing *in vivo* overexpression of NO without Ca^{2+} and calmodulin being required.

1.2. Significance of using *Drosophila* as a model for growth and cell proliferation

The *Drosophila* genome has been fully sequenced and now a great deal of genetic and physiological data has been collected (Buckingham *et al*., 2004). Therefore, the *Drosophila* model has a well-developed genetic toolkit. In addition, flies can be easily and cheaply cultured and possess a short generation time which can be utilised to quickly investigate the effects of genetic manipulation on cell proliferation and growth during development.

In development, DNA replication is necessary for cells to grow and differentiate into the different organ systems. In *Drosophila* the larval stage is specialised for growth and feeding. Prior to pupariation, the 3-4 days that larvae spend feeding is characterised by an increase in body mass of approximately 200 times (Church and Robertson, 1966). This is primarily a result of an increase in cell size as opposed to nutritional intake. Larval development salivary glands undergo endoreplication, a process characterised by DNA replication occurring in the absence of cytokinesis (Smith and Orr-Weaver, 1991).

Endoreplication occurs for approximately ten rounds, resulting in polyploidy and also an increase in large nuclei contained in giant polytene chromosomes in third instar

larvae (Yao *et al*., 2008). This process leads to an increase in cell size, but not cell number. In this crucial stage of development, once staining procedures are conducted, the size of these salivary glands greatly facilitates investigation of their cellular structure and genetic content via different microscopes. Given that the scale of observation is also increased, any alterations to these components induced through genetic modifications can be more easily compared with the wild type.

In *Drosophila,* growth is regulated by NO signalling and many components of the insulin signalling pathway, characterised by interactions between oncogenes and tumour suppressors.

1.3. Insulin

In mammals, the hormone insulin is synthesised and secreted by β-cells within the islets of Langerhans in response to increased levels of glucose and amino acids. It functions to regulate blood glucose homeostasis following a meal via signalling cells to uptake glucose in the blood and also in insulin-responsive tissues such as muscle, adipose and liver (Géminard *et al*., 2006).

1.3.1. Insulin and diabetes

Diabetes mellitus is a pathological condition characterised either by failure to synthesise insulin (type 1 diabetes) or the more commonly inheritable form characterised by reduced insulin sensitivity (type 2 diabetes); the latter is a multifactorial syndrome caused by a combination of genetic predisposition and environmental factors such as: aging, obesity and physical inactivity. In 2005 the prevalence of diabetes in the United States was estimated at 20 million people with 1.5 million new cases reported that year alone. Additionally, 90-95% of these cases are due to type 2 diabetes (Deshpande *et al*., 2008).

Diabetes is globally approaching epidemic proportions due to changes in human behaviour and lifestyle (Zimmet *et al*., 2001). As a result it is crucial to understand the insulin pathway as genetic defects that disrupt this molecular mechanism and its components can generate insulin resistance which account for the vast majority of diabetes cases. The insulin pathway is highly conserved in *Drosophila* and has been the focus of much research. This qualifies *Drosophila* as a suitable model organism in which the mechanism of the insulin signalling pathway can be manipulated and

characterised. A more well-defined insulin pathway can assist clinical therapeutic methods in humans, fostering better treatment or preventative measures in light of the prevalence of diabetes.

The insulin/insulin-like growth factor (IGF) system (IIS) is split into two subsystems which complement and interact with each other to regulate growth, reproduction, metabolism and longevity (Nakae *et al*., 2001). IGF, a known downstream target of growth hormone, functions to regulate growth and cell size at both prenatal and postnatal levels. Additionally, both insulin and IGF are reported to bind and regulate physiological processes via the insulin receptor (Géminard *et al*., 2006).

Géminard *et al*. (2006) also identified seven *Drosophila* insulin-like peptides (DILPs). These are expressed in separate tissues in *Drosophila* in insulin-producing cells (IPCs) and larval tissues such as the gut, imaginal discs and ventral nerve chord cells (Brogiolo *et al*., 2001). Brogiolo *et al*. (2001) suggests that DILPs do not have redundant functions, given that they localise in different tissues; however, Rulifson *et al*. (2002) suggests that there might be some redundancy in their function by using gain of function experiments. Genetic ablation of the IPCs is documented to result in larval growth defects and increased longevity (Ikeya *et al*., 2002; Broughton *et al*., 2005) since *DILP1*, *-2*, *-3* and *-5* are produced in these cells. Yet, expressing DILP2 successfully rescues the phenotypes associated with the genetic ablation, which implies that redundancy is possible. Furthermore, Brogiolo *et al*. (2001) reported genetic interaction between *dInR* and *DILP2* which is the most similar to insulin out of the seven DILPs.

1.4. Insulin signalling pathway in *Drosophila*

1.4.1. The *Drosophila* insulin receptor

The insulin pathway commences when insulin and ILPs bind the InR. Components required in this pathway in mammals are reportedly conserved in *Drosophila*, with respect to the mechanism of signalling and regulation (Kozma and Thomas, 2002; Leevers, 2001). This level of conservation is astonishing when considering the evolutionary distance between these organisms.

The *Drosophila* insulin receptor (dInR) has a 368-amino-acid COOH-terminal extension which comprises multiple tyrosine phosphorylation sites (Yenush *et al*., 1996); dInR is also similar to human IR, but is larger in structure as a result of the

extension. These two receptors both have two α and β subunits and given the sequence similarities between them, it is perhaps unsurprising that dInR is demonstrated to bind mammalian insulin with substantial affinity (Petruzzelli *et al*., 1986).

Additionally, dInR is required for development in *Drosophila*. Mutations to *dInR* are normally recessive embryonic lethal, confirming that this receptor is necessary for normal development in addition to growth (Fernandez *et al*., 1995). This is expected as binding dInR is the initial step in the insulin signalling pathway and also suggests that the receptor has multiple outputs.

1.4.2. Chico recruits PI3K via dInR phosphorylation

As the *Drosophila* homologue for vertebrate insulin receptor substrate (IRS) 1-4, Chico has a vital role in regulating growth and cell size autonomously. Similar to *dInR* mutants, null mutations for *chico* result in a decrease in size of over 50% in flies, when compared with the wild-type proportions (Böhnin *et al*., 1999). The study argues that this phenotype is due to the loss of *chico* function as a regulator of cell size and growth in *Drosophila*. Clancy *et al*. (2001) also confirmed that chico regulates lifespan by reporting an average increase of ≈ 48% in homozygotes. In the insulin signalling pathway, once dInR is bound by DILP, chico becomes phosphorylated and subsequently recruits phospho-inositide 3-kinase (PI3K).

1.4.3. PI3K comprises Dp110 and p60 subunits

PI3K activity is normally dependent on the availability of dietary protein/amino acids (Britton *et al*., 2002). *Dp110* codes for the *Drosophila* homologue of mammalian class 1a p110 that encodes the catalytic subunit of PI3K. Mutations of this gene produce larvae that are reduced in size and do not continue to grow once they have entered the third instar (Weinkove, 1999). Dp110 is recruited through the SH2 domain found on its adaptor, p60. PI3K recruits and activates several downstream targets in the insulin pathway via the generation of phosphatidylinositol-3, 4, 5-triphosphate (PIP_3).

1.4.4. PIP_3 generation via PI3K

When Chico binds to PI3K, this results in the stimulation of its kinase activity. PI3K subsequently phosphorylates the 3-position belonging to the inositol ring of phosphatidylinositol 4, 5-diphosphate (PIP_2) which is also associated with the plasma

membrane. PIP_3 is the result of this chemical reaction. Edgar (1999) noted that Chico is not required for PI3K activation, since dInR has several dPI3K-SH2 docking sites on its COOH-terminal extension.

Serine-threonine kinase Akt (also known as protein kinase B (PKB)) and phosophoinositide-dependent kinase 1 (PDK1) are recruited to the plasma membrane as a result of PI3K activation. This association occurs via their pleckstrin-homology domains binding to PIP_3 (Yang and Xu, 2011); therefore, PIP_3 also recruits Akt to the plasma membrane. Lawlor and Alessi (2001) reported that its association with PIP_3 enables the phosphorylation and consequently, the activation of Akt via PDK1.

1.4.5. PTEN antagonises PI3K to negatively regulate growth

PTEN is a dual protein/lipid tensin and phosphatase homologue. It is the primary PIP_3 substrate and antagonises PI3K function by converting PIP_3 to PIP_2 (Huang *et al.*, 1999). Therefore, PTEN activity negatively regulates growth, characterising the phosphatase as a tumour suppressor. In spite of a relatively high number of activating mutations demonstrated in p110α, loss of PTEN lipid phosphatase is associated with far more cases of activation of the PI3K pathway observed in many human cancers (Shaw and Cantley, 2006). *PTEN* mutations are most commonly observed in the phosphatase domain and are often germline or sporadic in nature (Myers and Tonks, 1997).

1.4.6. AKT inactivates and destabilises dFOXO and TSC2 via phosphorylation

Akt phosphorylation of FOXO generates a 14-3-3 protein binding site which causes dFOXO to become inactivated by sequestration in the cytoplasm. Essentially, the insulin pathway negatively regulates FOXO transcriptional activity as a tumour suppressor via Akt.

Additional targets of Akt include the tumour suppressor tuberous sclerosis complex 2 (TSC2). Similar to dFOXO, Akt binds to and phosphorylates TSC2 to control growth and cell size (Manning *et al*., 2002). When Akt phosphorylates TSC2, the TSC1-TSC2 complex becomes unstable which results in the inability of TSC to inhibit the phosphorylation of d4E-BP and S6K by suppressing Rheb activity.

1.4.7. S6K and d4E-BP phosphorylation results in global translation initiation

Once d4E-BP and S6K become phosphorylated, this catalyses global translation through inactivation and activation of the two molecular targets respectively. In its inactive form the translation initiator, eIF4E, is normally bound to d4E-BP which results in translational inhibition; however, once phosphorylated, d4E-BP undergoes a change in conformation, preventing its binding and inactivation of eIF4E. Target of rapamycin (TOR) is activated via Rheb and directly phosphorylates d4E-BP and S6K. Once phosphorylated, activated S6K further phosphorylates its target, S6, which is a ribosomal protein. This action results in the translation initiation of 5' terminal oligopyrimidine tract mRNAs which facilitate protein synthesis required for cell size (Yang and Xu, 2011).

Furthermore, dFOXO sequestration in the cytoplasm inhibits transcription of d4E-BP, which normally occurs when dFOXO is unphosphorylated (Junger *et al*., 2003). This results in the negative regulation of growth, previously mentioned. As a result, insulin signalling functions to promote growth and cell size through stimulating protein synthesis and by inhibiting tumour suppressors, dFOXO and TSC2. **Fig.1.2.** illustrates the interplay and relationships required for growth control in the insulin signalling pathway.

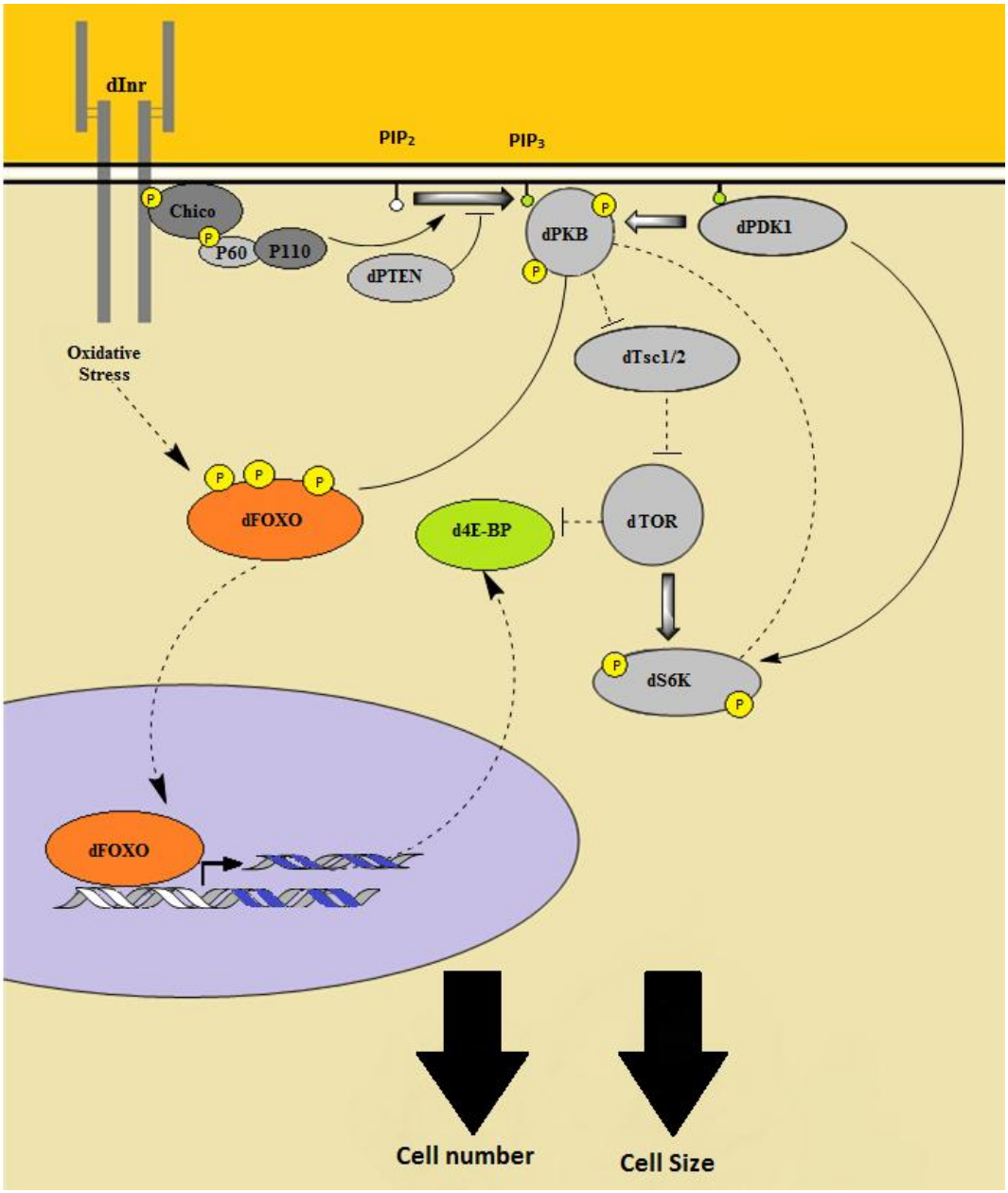


Fig.1.2. Insulin signaling pathway in *Drosophila* (adapted from (Jünger *et al*., 2003)).
Here dFOXO protein activity is characterised in the insulin signalling pathway. When this pathway is activated, several phosphorylation events occur and dFOXO is subsequently inactivated by Akt via phosphorylation. When insulin levels decrease, dFOXO becomes dephosphorylated and is able to enter the nucleus and increase transcription of dInR and d4E-BP. Increased expression of dInR enables the cell to become more sensitive to insulin and prepares the organism to take full advantage of insulin present in the next meal. In the meantime, d4E-BP upregulation acts to decrease protein synthesis via its inhibitory binding action with eIF4E. The specifics of the interactions between other relevant components of the insulin signalling pathway are previously discussed in this chapter.

1.5. dFOXO

The FOXO family of transcription factors have been identified as tumour suppressors in many species and regulate growth under the control of insulin/insulin-like signalling. In mammals four primary FOXO members have been identified: FOXO1, FOXO3, FOXO4 and FOXO6. *Caenorhabditis elegans* has only one member, DAF-16. In *Drosophila*, a homologue of DAF-16 and FOXO4 has been identified as simply dFOXO (Puig *et al*., 2003).

Recently research has shown that dFOXO is sensitive to insulin signalling and acts as a transcription factor, regulating a significant array of downstream targets. dFOXO is also regulated by Akt signalling which, when activated in the presence of insulin, can sequester dFOXO in the cytoplasm, negating its activity as a transcription factor in the nucleus (Puig *et al.*, 2003). This is turn inhibits RNA transcription of normal dFOXO gene targets which code for proteins that have many regulatory functions, including cell cycle progression, metabolism, apoptosis and growth. However, when insulin levels are low and Akt is inactive, dFOXO is able to perform its transcriptional functions which have been shown to affect over 200 genes. One of these targets is the gene coding for dInR, which is upregulated by dFOXO once insulin levels are low, increasing sensitivity to insulin in preparation for the next meal (Puig *et al*., 2005).

Another target of dFOXO that is upregulated by the transcription factor is d4E-BP which inhibits the translation initiation factor, eIF4E (Eukaryotic Translation Initiation Factor 4E). As a negative regulator of growth, dFOXO promotes the expression of d4E-BP which binds to eIF4E, preventing it from binding to the mRNA 5' cap of many different mRNAs (Gingras *et al*., 1999). Thus the translation of proteins essential for promoting growth is inhibited via dFOXO action. In addition, dFOXO signalling via d4E-BP induces resistance to oxidative stress and starvation.

Previous experiments in our laboratory investigated the relationship between dFOXO and NO. When NOS2 is expressed in third instar larval salivary glands, the organs become reduced in size and d4E-BP-LacZ staining is increased, suggesting that dFOXO has a role in this process due to the increase of d4E-BP expression (Kimber, 2005). In order to further elucidate this putative role, dFOXO mutants were generated along with NOS2 expression. The results show that the NOS2-induced size reduction is then suppressed along with a decrease in d4E-BP-LacZ staining, which suggests that NO

signalling is dependent on the presence of dFOXO. d4E-BP expression is also demonstrated to be reduced when using RNAi-NOS. When using RNAi-NOS third instar larvae also demonstrate hampered development as they are unable to progress to the pupal stage. Experiments that use NOS2 as a form of NO overexpression show that protein translation is inhibited; this is most likely as a result of NO signalling through dFOXO to increase d4E-BP and inhibit eIF4E action.

1.6. dMyc

The oncogenic Myc family of proteins operates as transcription factors which regulate growth, metabolism, proliferation, apoptosis and immortalisation in *Drosophila* and mammals. *Drosophila* Myc (dMyc) is encoded by the *dimunitive* (*dm*) gene and its function has been investigated by producing *dm* null mutants. These mutants exhibit a decrease in body size when compared with the wild type. Myc is thought to heterodimerise with Max in order to form stable compounds capable of binding DNA sequences which code for proteins associated with promoting growth (Gallant *et al*., 1996).

Additionally, Myc is thought to be a target of FOXO in the nutrient sensing pathway. During fasting, FOXO null mutants are characterised by low levels of Myc protein, implicating FOXO as a regulator of Myc expression (Teleman *et al*., 2008). Studies show that Ras, another oncogene, also exerts control over Myc expression.

1.7. Ras

Ras proteins are well documented in tumourigenesis and mutant forms are heavily associated with approximately 30% of human cancers (Giehl, 2005). Therefore, much research has attempted to characterise the oncogenic effects of Ras proteins and their effectors. Ras activation occurs once tyrosine kinase receptors bind growth factors and has GTPase activity. It can be inactivated once the GTP is hydrolysed, a process which can also be accelerated via GTPase activating proteins (GAPs) (Koh, 2006).

When analysing the results of this experiment it will also be important to note that endogenous Ras and activated Ras mutations (such as Ras^{V12835}) have been demonstrated to stabilise (Sears *et al.*, 2000) and promote expression of Myc (Prober and Edgar, 2002), which would suggest that Ras overexpression experiments may also include effects from increased Myc expression.

1.8. Aim

The aim of this project was to investigate growth regulation within *Drosophila melanogaster* salivary gland cells. Using *Drosophila* the role of NO and its inhibitory effects on growth regulation were compared with the wild type. In addition, the role of oncogenes, Myc and Ras, in regulating growth in these cells were also examined. Genetic manipulation techniques were utilised in order to elucidate the genetic and molecular mechanisms of growth inhibition via the tumour suppressor, dFOXO, and overexpressed NO using NOS2, and their potential interactions with the oncogenes. Three experimental procedures were be used in order to accomplish this; these methods are Axiophot microscopy, confocal microscopy and Transmission Electron Microscopy (TEM).

Nuclei measurements were compared in order to measure the effects of NO and dFOXO on growth in the salivary gland cells using Zeiss Axiophot microscopy. Comparative analysis was conducted between null alleles relative to the wild type using homozygous and transheterozygous dFOXO null allele combinations. The null alleles were also used in combination with NOS2 to confirm the interaction described in previous work in the laboratory.

Golgi and mitochondria were examined in wild type control lines. The effects of dFOXO, NOS2 and the oncogenes on the structure of Golgi and mitchondria were characterised using confocal microscopy to visualise the cellular structures.

Using TEM microscopy wild type salivary gland cells were visualised in order to observe the normal structure and size of cellular components such as: endoplasmic reticulum, chromosomes, secretory vesicles and Golgi. The effects of NO were examined and compared with the wild type controller line. Myc and Ras were introduced separately and the effect of NOS2 was also examined in conjunction with each oncogene.

Chapter 2

Materials and Methods

2.1. General

2.1.1 Fly Husbandry

Drosophila stocks and crosses were cultivated on D+ food in plastic vials measuring 8cm x 2.5cm and flies were contained by rayon balls or cotton wool. Developing flies were maintained at either 18°C or 25°C on a 12 hour light-dark cycle. Adults were anaesthetised using CO_2. 3rd instar larval salivary glands and adults were observed using a Nikon SM2645 dissecting microscope Microtec MFO-90 light source.

D+ Glocuse Food Media

Agar 40g

D+ Glucose anhydrous 551g (Fisher Scientific)

Yeast 143g

Sucrose 185g

Maize meal 236g

Nipagen 10%w/v 82mls

Propionic acid 25mls

Water 5500mls

Method

Anhydrous D+ Glucose and yeast were mixed into a paste by adding a small volume of water. Similarly, the maize and agar were also mixed with water and then brought to the boil in order to dissolve. Once dissolved, the paste was added and the combination was brought to the boil. The mixture was allowed to cool prior to being poured into glass bottles or plastic vials.

2.1.2. The GAL4-UAS system allows targeted gene expression in *Drosophila*

Genetic manipulation can be achieved using a variety of methods; however, the GAL4-UAS system has proved to be one of the most versatile and useful techniques as shown in Duffy (2002) who compared it to a swiss army knife. In Brand and Perrimon (1993) this bipartite system was used to overexpress genes in *Drosophila*. The gene of interest is regulated by an upstream activating sequence (UAS). This UAS element contains five GAL4 binding sites and once GAL4 is expressed and binds with the UAS sites, transcription of the gene of interest occurs in a pattern that correlates with the specific GAL4 pattern of expression. Therefore, the expression of the gene of interest via UAS is termed the responder line and the GAL4 line is called the driver, both of which correspond to parental lines in *Drosophila*.

2.1.3. *dFOXO* ^{BG01018}

Experiments also analysed the effect of the FOXO hypomorph, $dFOXO^{BG01018}$, described in Dionne *et al*. (2006). The study identified a transposon insertion, BG01018, while conducting a genetic screen for *Drosophila* mutants that differ in mortality when compared to the wild-type when infected with *Mycobacterium marinum*. The insertion is located ≈ 130 nucleotides upstream of the start codon for *dFOXO* and is reported to induce a mild loss of function for the gene. $dFOXO^{BG01018}$ mutants were provided by Marc Dionne (Kings College London).

2.2. Nuclei Staining and Measuring

Materials

1xPBS (10xPBS: 1.37M NaCl, 0.1M Na_2HPO_4, 0.01M NaH_2PO_4; pH7.4)
4% Paraformaldehyde in 1xPBS
DAPI : 1:20,000 in 1x PBS with 0.1% Triton X-100
Aquamount (Polysciences)

Method

Wandering third instar larvae were dissected for their salivary glands via the inversion technique and fixed in 4% paraformaldehyde for 20 minutes. Glands were subsequently washed in 2x 5minutes in 1x PBS. Then they were incubated in diluted DAPI solution for 15 minutes. The glands were subsequently washed 2x 5minutes in 1xPBS prior to dissection. Finally, they were mounted on slides using Aquamount and observed on a Zeiss Axiophot microscope at 40x.

2.3. Preparation of Larvae for Transmission Electron Microscopy

All larvae were grown at 25ºC and picked as wandering third instars. Salivary glands were dissected in Tissue Culture Medium (TCM) and then immediately transferred to fixative and processed for transmission electron microscopy (TEM) as follows:

- Primary fixation in 2.5% (v/v) glutaraldehyde in Na Cacodylate/HCl buffer, 0.1M, pH7.4 for a few hours at room temperature and then overnight at 4º C
- Rinsed in Na Cacodylate/HCl buffer, 0.1M pH7.4 (X 4) over several hours
- Salivary glands pipetted into clear eppendorfs Secondary fixation in 1% (w/v) osmium tetroxide (OsO4) in Na Cacodylate/HCl buffer, 0.1M, pH7.4 for 2h at room temperature
- Rinsed thoroughly in d/w (X 5), including an overnight rinse at 4º C
- Dehydrated in an ethanol series: 50%, 75% and 3 X dried, absolute EtOH for 20min each
- Propylene oxide (PO; 2 X 20min) (a 'transition' solvent)
- Into 50:50 PO/TLV (Taab Low Viscosity) resin overnight
- Into complete TLV resin the next day
- TLV resin changed several times over 3-4 days
- Larvae oriented within plastic embedding moulds and polymerised overnight (16h) at 60deg C

The following stages were completed with the assistance of Dr. Julian R. Thorpe.

- Thin (100nm) sections were cut with an Leica Ultracut Ultramicrotome and collected upon TEM support grids
- Sections were post-stained in 2% (w/v) aqueous, 0.22μm-filtered uranyl acetate for 1h, followed by 15min in lead citrate
- Sections were examined in a Hitachi-7100 TEM at 100kV and images acquired digitally with an axially-mounted (2K X 2K pixel) Gatan Ultrascan 1000 CCD camera

Chapter 3

Investigating potential interactions between NO and oncogenes

3.1. Introduction

NO functions to inhibit proliferation and evidence suggests it signals through FOXO (Kimber, 2005), a known tumour suppressor. Therefore, this suggests that NO may have tumour suppressive activity, possibly negating the action of oncogenes. In this chapter potential interactions between NO and oncogenes, Myc and Ras, were investigated as they relate to growth and development of cellular structures in the salivary glands.

3.1.1. Myc

The Myc family of proto-oncogenes codes for transcription factors which comprise a basic helix-loop-helix zipper (bHLHZ) protein structure (Oskarsson and Trumpp, 2005). This family has been exhaustively researched; in recent years genome binding, genetic profiling and genetic analyses have revealed the scope of Myc action in *Drosophila* and mammals. Myc functions in normal cells to incorporate environmental signals into varied processes such as: growth, immortalisation, metabolism, differentiation, proliferation and apoptosis (Eilers and Eisenman, 2008).

Myc is a well-established transcription factor with the ability to both activate and repress transcription of target genes (Oskarsson and Trumpp, 2005). The majority of genes that are stimulated by Myc are transcribed by RNA polymerase II; however, RNA polymerase I which encodes rRNA (Grewal *et al.*, 2005) and RNA polymerase III encoding tRNA (Steiger *et al*., 2008) also have Myc-induced transcriptional roles. Additionally, the mechanism of activation of gene transcription by dMyc is conserved in vertebrates (Gallant *et al*., 1996).

The control of gene expression by *Drosophila melanogaster* Myc (dMyc) is particularly important in ribosomal RNA (rRNA) synthesis especially during larval development. In ribosome biogenesis, rRNA synthesis is demonstrated to be a rate limiting step which varies depending on cellular growth status (Grewal *et al*., 2005). Myc induces gene

expression to promote cellular growth via ribosome biogenesis, protein synthesis and metabolism (Eilers and Eisenman, 2008).

In *Drosophila* dMyc is encoded by the dimunitive (*dm*) gene. Flies with null *dm* mutations have phenotypes characterised by decreased body size and sterility in females as a result of degenerated ovaries (Gallant *et al*., 1996). A study by Pierce *et al.* (2004) investigating overexpression of dMyc revealed that the protein controls growth-regulated gene expression. The study concluded that cellular growth within *Drosophila* is dependent on the function of dMyc, and overexpression of dMyc results in an increase in nucleolar sizes and significant elevation in the level of nuclear DNA.

dMyc is able to heterodimerise with a similar helix-loop-zipper protein known as Max (Eilers and Eisenman, 2008). The heterodimers formed between Myc and Max are stable and are able to recognise and bind to specific DNA sequences. The particular sequence recognised by these stable heterodimers is known as the E-box sequence (coding-sequence CACGTG). This binding leads to the transcriptional activation of down-stream target genes (Gallant *et al*., 1996).

Teleman *et al*. (2008) suggested that Myc is an effector of the target of rapamycin complex 1 (TORC1) by regulating transcription of TORC1 targets. Using a MEME motif search, the study investigated which motifs matched for sequences immediately upstream and downstream of genes regulated by rapamycin and discovered that the E box motif that scores the highest belongs to the Myc/Max transcription factors. Further investigation using the RNAi technique to deplete Myc reduces the expression levels of TORC1 targets, suggesting that Myc is required to control the genetic expression of TORC1 targets.

Additionally, Teleman *et al.* (2008) established dMyc as a target of dFOXO in the nutrient-sensing mechanism. In wild-type *Drosophila* muscle tissue, *dMyc* mRNA levels decrease by 50% with normal expression of FOXO when fasting; however, in *FOXO* null mutations, *dmyc* mRNA was unaffected. This suggests that dFOXO inhibits dMyc expression levels to conserve energy in *Drosophila* muscle during fasting associated with low insulin levels. In contrast, dFOXO mutants express lower levels of dMyc protein in adipose tissue when compared with the wild-type; therefore, dFOXO is required for constant levels of dMyc in adipose tissue while fasting, but inhibits dMyc expression in muscle.

Furthermore, Bouchard *et al*. (2007) reported that FOXO impairs Myc-driven lymphomagenesis by directly binding to the *Arf* locus in Eμ-*myc* transgenic hematopoietic stem cells. In addition, Delpuech (2007) found that FOXO3a activation represses the expression of several previously identified Myc target genes in mammals.

3.1.2. Myc, Stem Cells and Cancer

The scope of Myc targets extends further to include embryonic stem (ES) cells (Kim *et al*., 2008). Takahashi and Yamanaka (2006) reported that c-Myc is included in a 4-factor reprogramming set which enables somatic cells to become induced pluripotent stem (iPS) cells. Numerous studies indicate that although Myc is not absolutely required, it amplifies the capacity of the other 3 transcription factors of the reprogramming set (Sox2, Oct4 and Klf4) by a factor of 2-10 fold in stimulating the formation of iPS cells. Research has established that the magnitude of this augmentation depends on the cell type in which this occurs and analyses have been conducted for human and mouse fibroblasts, liver cells and mature B cells (Okita *et al*., 2007; Takahashi *et al*., 2007; Wernig *et al*., 2007; Hanna *et al*., 2008). Studies show that Myc is only briefly required for augmenting this reprogramming since ectopic expression of Myc does not occur once the iPS cells have been generated. These results suggest that utilising delivery vectors such as the adenovirus, which can transiently increase Myc expression, should be preferred instead of Myc overexpression when seeking to induce pluripotency in cells, since the overexpression can promote tumourigenesis in iPS cells (Eilers and Eisenman, 2008).

3.1.3. Myc and NO

NO has been demonstrated to inhibit proliferation in several cell types and promotes differentiation in neural cell precursors. In contrast N-Myc, a member of the Myc family, promotes proliferation and is negatively regulated when these neural cells are induced to differentiate via retinoic acid. Ciani *et al*. (2004) reported that NO inhibits the proliferation of neuronal cell precursors, instead promoting their differentiation by negatively regulating N-Myc in the presence of retinoic acid. This proliferative inhibition occurs both when using nNOS and/or an exogenous source of NO and could

possibly be used in cancer therapeutic strategies for treating neuroblastomas expressing N-Myc.

Given that NO signals through FOXO, and dMyc expression is demonstrated to be regulated by dFOXO in a tissue-dependent manner (Teleman *et al*., 2008), this chapter investigates if NO has a similar effect on Myc expression by comparing the effect of Myc on cellular structures with co-expressed Myc and NO via NOS2.

Scott (2009) investigated the potential interaction between dMyc and NOS2 by co-expressing the two growth regulators in third instar larval salivary gland cells. dMyc and NOS2 were also expressed separately and a wild type line was used as a control. The results from this experiment are included below in **Fig.3.1**.

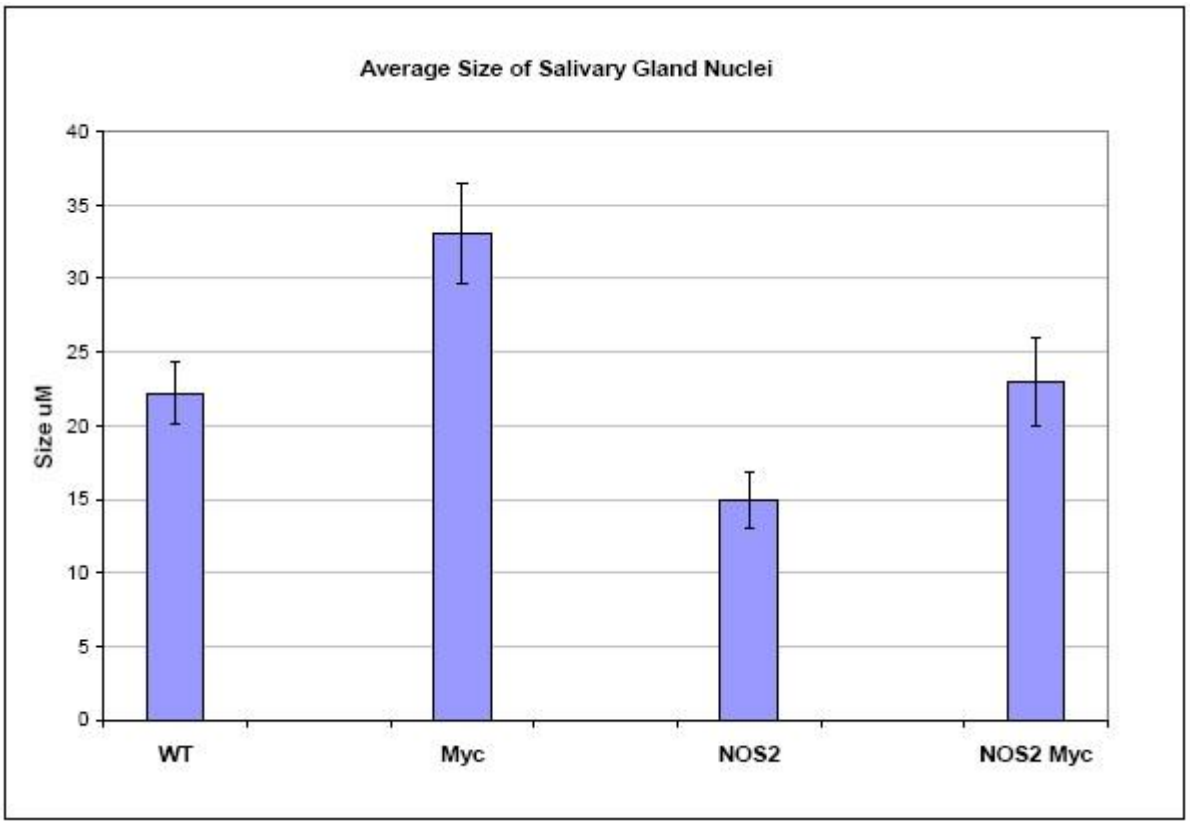


Fig 3.1. Average size of salivary gland nuclei expressing dMyc and NOS2 (Scott, 2009). Flies with dMyc expressed alone were shown to have the largest nuclei measurements at approximately 33.0µm. Co-expression of NOS2 and dMyc was remarkably similar in size to the wild type at 22.9 µm; however, nuclei sizes were larger than the line expressing NOS2 alone. These results suggest that NOS2 has an inhibitory effect on the Myc-induced overgrowth phenotype (Scott, 2009).

3.1.4. Ras

Ras proteins are essential components of many signalling pathways within cells, and mediate a wide range of cellular events.

For the purpose of this project I will be briefly exploring the effect Ras has on cell growth regulation.

It is well known that Ras is a proto-oncogene and that certain mutations in the Ras gene cause the normally functioning gene to become an oncogene. Mammalian Ras family members K-Ras, H-Ras and N-Ras are often reported to demonstrate such mutations in leukemia, for example, and account for 30% of human cancers (Giehl, 2005; Repasky *et al*., 2004). H-ras mutations are commonly associated with kidney and thyroid carcinomas; N-ras mutations are present in melanoma and hepatocellular carcinomas; K-ras mutations are frequently seen in pancreatic and colorectal carcinomas (Adjei, 2001).

3.1.5. Ras regulates cellular growth

It has been shown that Ras is involved in cell growth, survival and differentiation in *Drosophila* and that mutant forms of Ras can be the basis for tumourigenesis within cells (Oskarsson and Trumpp, 2005). Halfar *et al*. (2001) tested the function of Ras in the *Drosophila* eye by inducing clones of ommatidial cells homozygous for the Ras^{x7b} null mutations. Indeed these clones couldn't be recovered in the *Drosophila* adult eye, implying that Ras is necessary for growth, proliferation and survival. However, in the imaginal discs the study discovered that proliferation is possible in $Ras^{-/-}$ cells, but with decreased growth rate. The study concluded that in addition to growth, Ras is essential for survival and differentiation in postmitotic cells in eye imaginal discs.

Growth control by Ras in *Drosophila* is believed to be due to the inherent growth deficit in $Ras^{-/-}$ clones and also via an inability to compete with the faster growing wild-type cells. Research has verified this conclusion in the *Drosophila* eye imaginal disc and the wing disc (Halfar *et al*., 2001; Prober and Edgar, 2000).

3.1.6. Ras increases dMyc expression

Research suggests that Ras can stabilise Myc protein (Sears *et al.*, 2000), yet until recently there was no evidence to suggest that Ras regulates Myc on a genetic level. In addition to promoting cell growth, Ras has been demonstrated to control cell fate specification in *Drosophila* and *Caenorhabditis elegans* (Rommel and Hafen, 1998). dMyc has not been shown to influence differentiation, but the fact that it is stabilised by Ras warranted further investigation into the interaction between the two oncogenes.

Prober and Edgar (2002) utilised Ras^{V12}, an activated form of Ras, to investigate the effects on dMyc expression. Using the Flp/Gal4 technique (Neufeld *et al*., 1998), the study generated clones of cells that express Ras^{V12} in the developing wing disc in order to discover which effector pathway the two oncogenes interact in. Additionally, Prober and Edgar (2002) used dMyc-specific antibody staining to gauge the expression of dMyc in the clones. Although expression of a Ras^{V12} mutant, Ras^{V12G37}, fails to upregulate dMyc expression, Ras^{V12S35} accomplishes this. Increased expression of dMyc is observed throughout the wing disc, although it was difficult to determine this in the wing pouch, which inherently has significant levels of endogenous dMyc. Ras^{V12S35} (also known as Raf^{GOF}) is a Ras^{V12} effector loop mutant demonstrated to specifically activate MAPK; thus, the study concludes that Ras^{V12835} acts through the Raf/MAPK pathway to activate dMyc.

While showing that these Ras mutants can regulate dMyc expression, it was imperative to discover if this also occurs in endogenous Ras during development. Using FLP/FRT-mediated mitotic recombination, Prober and Edgar (2002) produce *ras* mutant clones ($ras^{-/-}$) in $ras^{+/-}$ tissues via the ras^{c40b} allele which does not have an open reading frame (Schnorr and Berg, 1996). In order to determine if *ras* is required for normal dMyc expression the study used dMyc-specific antibody staining to observe expression levels. Staining intensity is substantially reduced in cells containing $ras^{-/-}$ in the entire wing disc, even in the wing pouch. Therefore, this confirms that normal dMyc expression is Ras-dependent in the developing *Drosophila* wing disc.

3.1.7. Ras and NO

While investigating the role of MAPK pathways in nNOS induction, Schonhoff *et al.* (2001) demonstrated that Ras must function correctly if NO is to be produced in differentiating PC12 cells. The study infected these cells with a dominant negative Ras adenovirus and introduced nerve growth factor which is shown to increase nNOS expression. However, nNOS does not show activity under these circumstances, which suggests that Ras regulates nNOS production in these cells.

Similarly, this present study will also examine the potential interaction between Ras and NO in salivary gland cells. This experiment will generate flies expressing activated Ras using Ras^{V12} separately and then co-express Ras^{V12} with NOS2 under the control of the GAL4 driver, c147. Any potential effects on cellular structures will be compared with the wild-type. Third instar larvae will be dissected for their salivary glands and cellular structures visualised using TEM.

The incentive for conducting this experiment is derived from combining the findings of research examining the effect of NO and Ras^{V12} on gene expression separately. Previous work in the laboratory reported by Kimber (2005) described microarray data from *Drosophila* S2 cells treated with a NO donor and compared the results to untreated S2 cells. Asha *et al*. (2003) analysed microarray data of Ras^{V12} expression compared with wild-type Ras expression in haemocytes. Scott (2009) describes an inverse correlation documented in these two studies regarding the promotion and suppression of genes regulated by Ras and NO that were identified. Ras^{V12} upregulates 1286 genes in haemocytes (Asha *et al*., 2003); however, 83 of these genes are downregulated by the NO donor (Kimber, 2005). The magnitude of the upregulation of these common genes by Ras^{V12} is indirectly proportional to their downregulation via the action of the NO donor (Scott, 2009), suggesting a proportionately opposite effect between the two transcriptional regulators.

Scott (2009) investigated the potential NOS2 suppression of Ras^{V12}-induced growth in salivary gland nuclei. The study used an average of late third instar, wild type salivary nuclei sizes as a control and compares the measurements with NOS2-induced growth inhibition and Ras^{V12}-induced growth promotion separately, in addition to co-expression of Ras^{V12} and NOS2. The results are illustrated in **Fig. 3.2.** below:

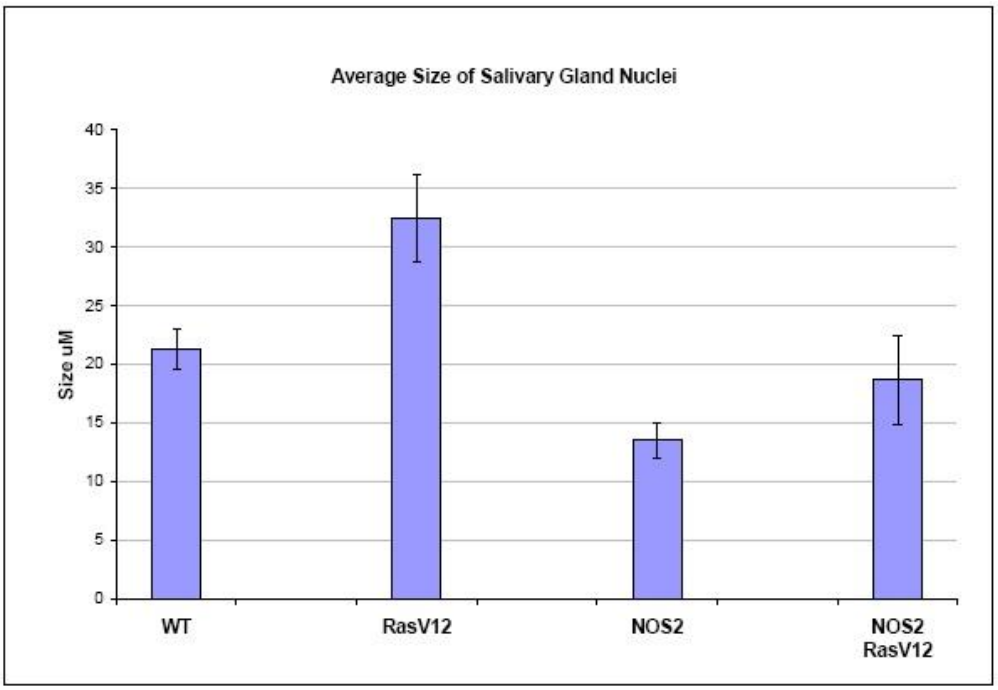


Fig. 3.2.Average size of salivary gland nuclei expressing RasV12 and NOS2 (Scott, 2009).

Fig.3.2. illustrates that the Ras^{V12} line had the largest nuclei measurements when compared with the other lines. Co-expressed Ras^{V12} and NOS2 produced nuclei that were smaller than both the wild-type and Ras^{V12} line, but larger than the NOS2 line. This showed that NOS2 acts to dilute the overgrowth phenotype demonstrated by Ras^{V12} activity. The results can also be interpreted as Ras^{V12} acting to suppress the growth inhibition phenotype of NOS2. Nevertheless, the opposite effects that both these growth regulators had on nuclei sizes correspond with the findings from Kimber (2005) and Asha *et al*. (2003) (Scott, 2009).

3.2. Results

This chapter intends to complement the findings reported in Scott (2009) by examining the effect of the oncogenes, Myc and Ras, when combined with NOS2. Similarly, this experiment will focus on the salivary gland as a model for this co-expression; however, instead of investigating nuclei growth, the cellular structures in salivary glands were visualised using TEM and compared with the wild type. Effects on the structure of nuclei, polytene chromosomes and secretory vesicles as a result of co-expressing NOS2 and the oncogenes were visualised. Forty five animals from each genetic cross were dissected and then eight glands were selected randomly for TEM visualisation.

3.2.1. Wild type and NOS2

Fig. 3.3 shows that nuclei sizes were reduced in the NOS2 line compared with the wild type. Given that NO is demonstrated to inhibit growth and proliferation, these results were expected. Interestingly, secretory vesicles that were clearly present in the wild type were not visible in lines expressing only NOS2. Whether this was a result of the vesicles becoming too small to visualise or if the action of NOS2 completely inhibited the development of any secretory vesicles is unclear.

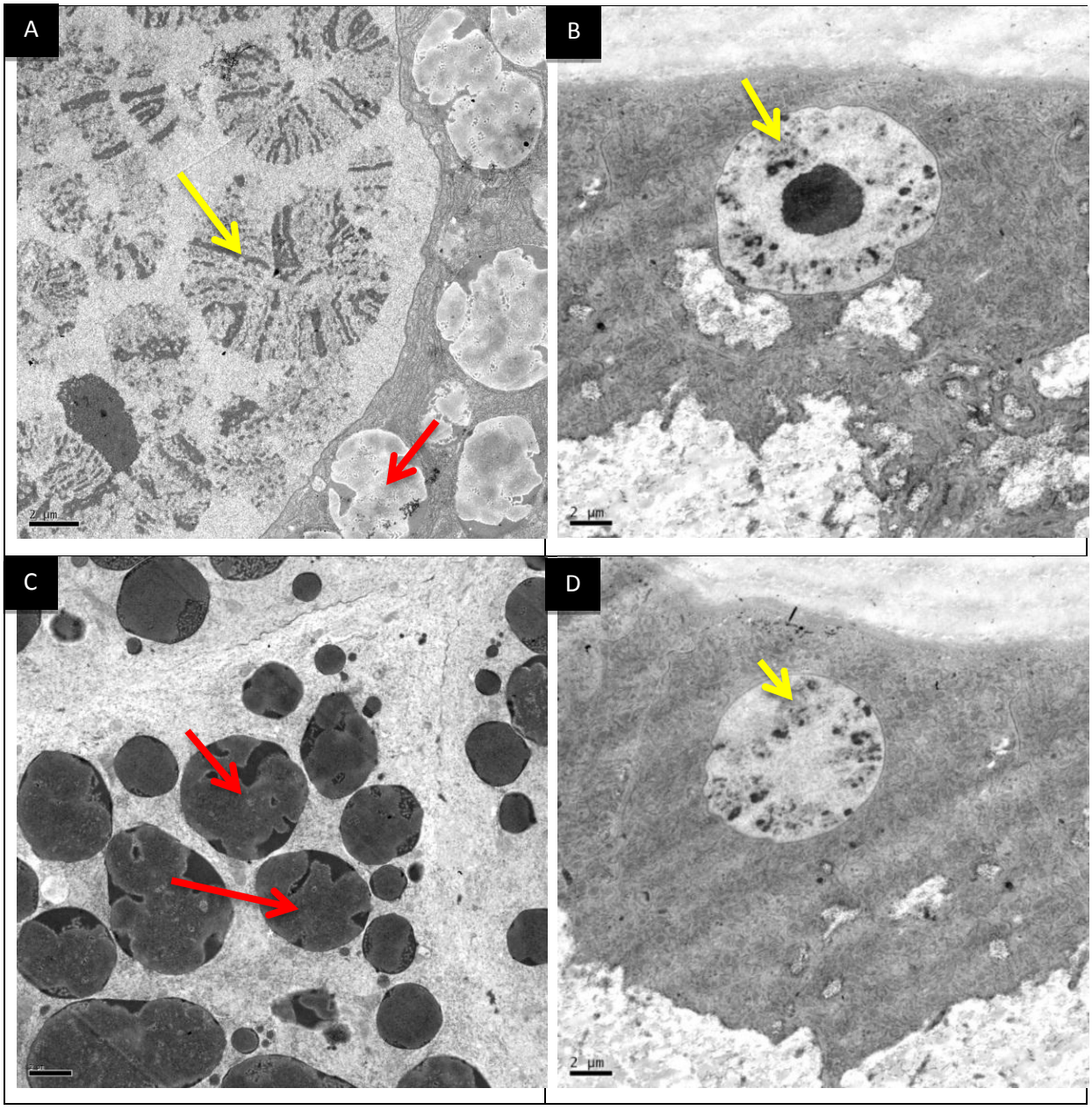


Fig.3.3. Wild type (c147-GAL4/+): (A and C) Wild type nuclei, polytene chromosomes and secretory vesicles were visualised on the left.
Overexpression of NOS2 (*UAS-NOS2*; c147-GAL4): (B and D) Nuclei and polytene chromosomes from flies that overexpressed NO are shown on the right.

*Yellow arrows (→) identify polytene chromosomes; red arrows (→) indicate secretory vesicles. Scale bars 2µm.

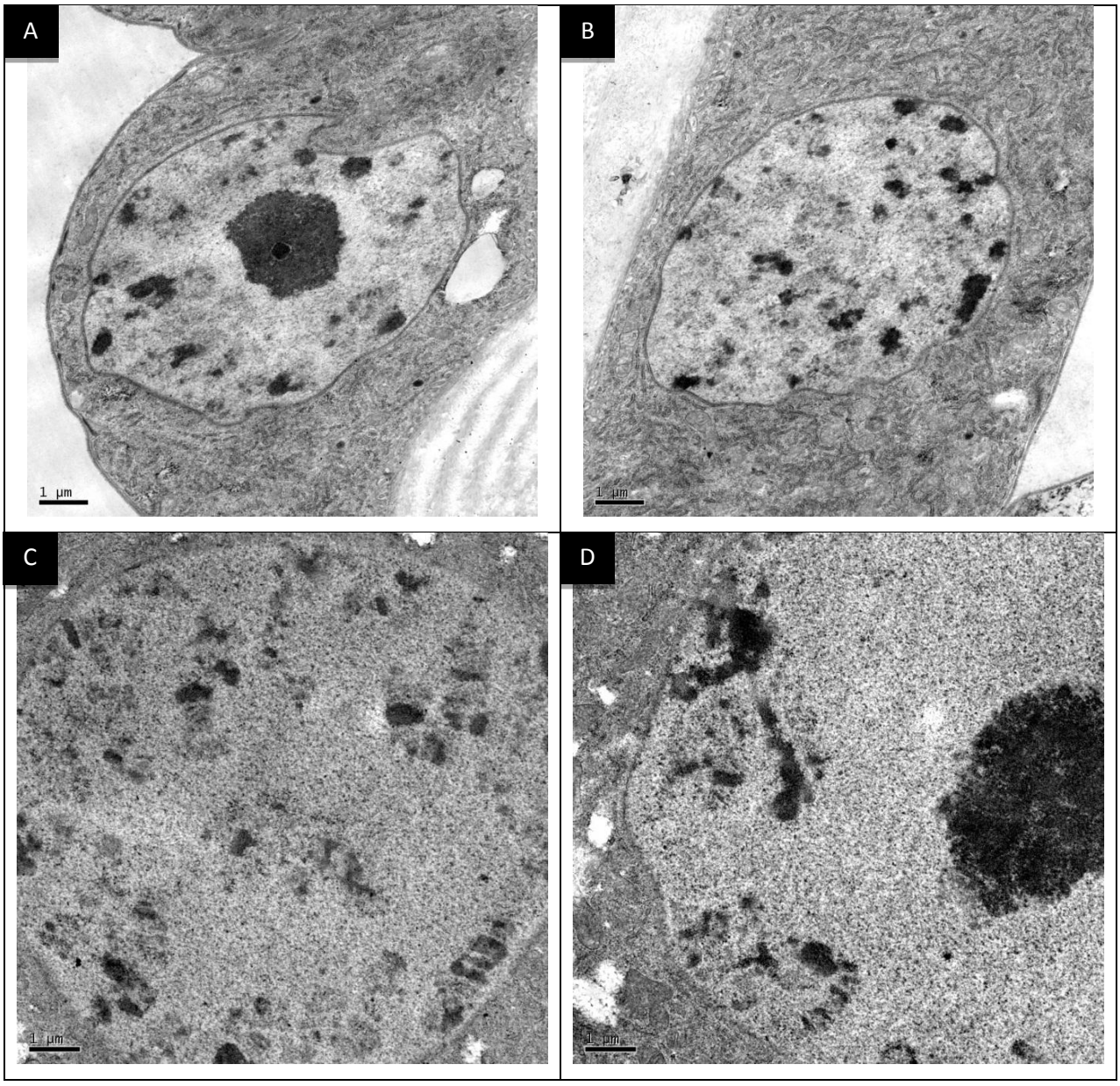


Fig.3.4. Overexpression of NOS2 (*UAS-NOS2*; c147-GAL4): (A-D) Images of the nuclei and polytene chromosomes for flies expressing NOS2 were generated. These images were visualised at a higher magnification (2500X) to provide more detail. Scale bars 1µm.

3.2.2. Ras^{V12}

In order to explore the effect of NOS2 on the overgrowth phenotype documented in Ras^{V12} activity, this experiment sought to co-express these two growth regulators. The images produced by introducing Ras^{V12} co-expression with NOS2 (Fig.3.5.) were similar to the results found with dMyc and NOS2 (Fig.3.6). When expressed on its own, Ras^{V12} induced an overgrowth phenotype in nuclei, chromosomes and secretory vesicles (Fig.3.5.).

Co-expression of Ras^{V12} and NOS2 (Fig.3.5.) produced smaller cellular structures when compared with the wild type and Ras^{V12} alone, as seen in Scott (2009) which measured salivary gland nuclei size; however, structures were larger than those observed in lines expressing only NOS2 (Fig.3.4.). Unexpectedly, the ER in this co-expression experiment appears to be largely absent when compared with the wild type. Small portions of ER in proximity of the nucleus are visible, but in much smaller quantities. The outer membrane of the nucleus also appears to be budding (Fig.3.7.).

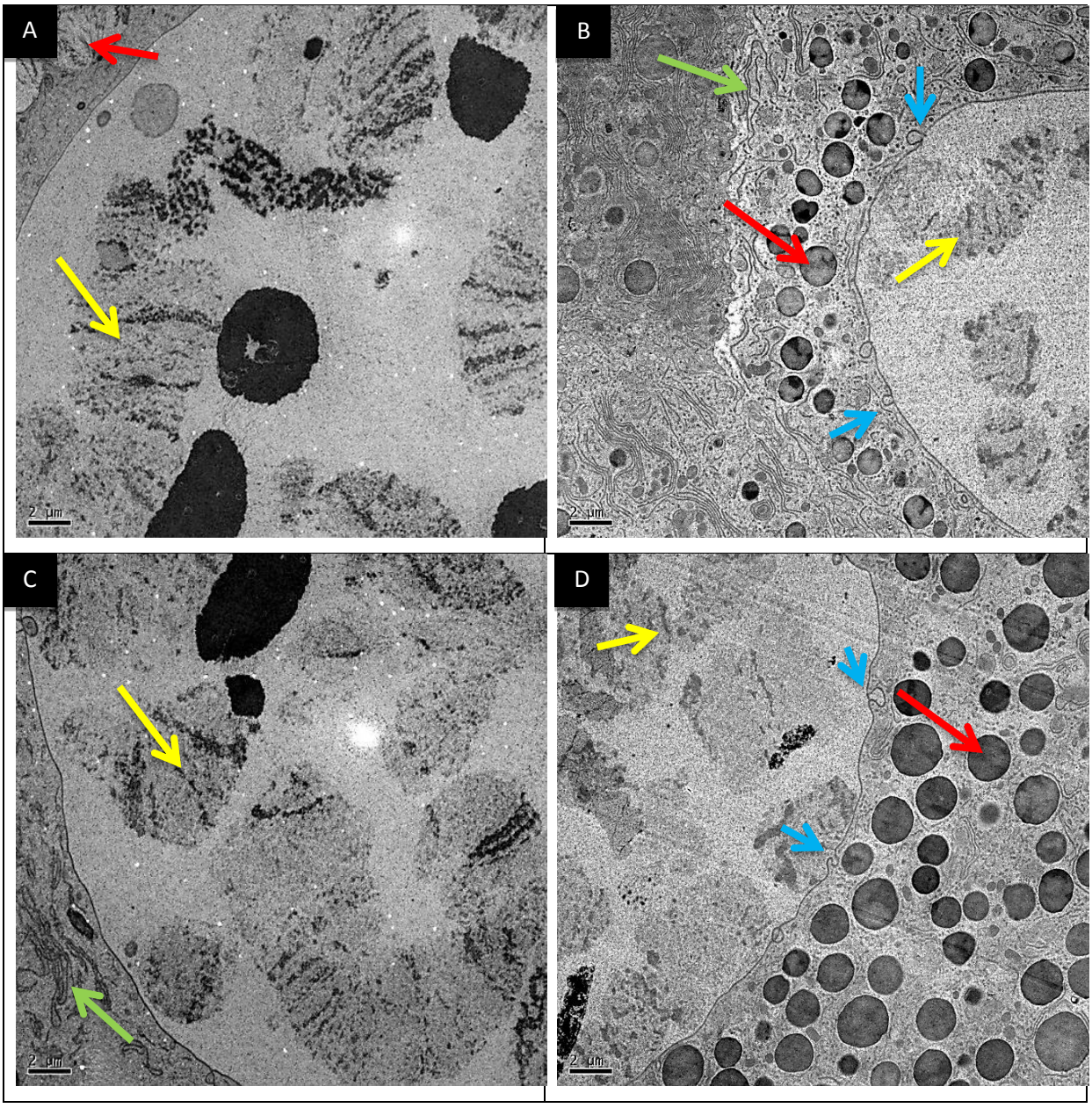


Fig. 3.5. Activated Ras^{V12} (c147-GAL4/*UAS-Ras^{V12}*): (A and C) Nuclei, chromosomes and secretory vesicles from animals expressing activated Ras are shown on the left.**Coexpression of Ras^{V12} + NOS2 (NOS2/+; c147-GAL4/*UAS-Ras^{V12}*):** (B and D) Chromosomes, nuclei and secretory vesicles are illustrated from animals expressing both Ras^{V12} and NOS2.

*Yellow arrows ( ) identify polytene chromosomes; red arrows ( ) indicate secretory vesicles; blue arrows ( ) represent a malformation in the outer nuclear membrane; green arrows ( ) represent the ER. Scale bars 2µm.

3.2.3. dMyc

As dMyc expression has been reported to increase cellular growth, it was expected that lines expressing only dMyc only would have the largest cellular structures. Furthermore, lines expressing only NOS2 had the smallest structures, which was also expected given that NO induces growth arrest (Gibbs, 2003).

When salivary gland cellular structures from flies co-expressing NOS2 and dMyc were visualised (Fig.3.6.), it was noted that the average structural sizes of cellular components appeared to be smaller than the wild type (Fig.3.3.). Apart from a reduction in size, nuclei, secretory vesicles and chromosomes appeared to be normal. It is interesting to note that ER formation was very similar to the lines co-expressing Ras^{V12} and NOS2 (Fig. 3.5). The connection between the outer nuclear membrane and the ER membrane was poorly defined and resembles budding from the outer membrane of the nucleus. ER structures are mostly absent and appear to have either been degraded or not well developed (Fig.3.8.).

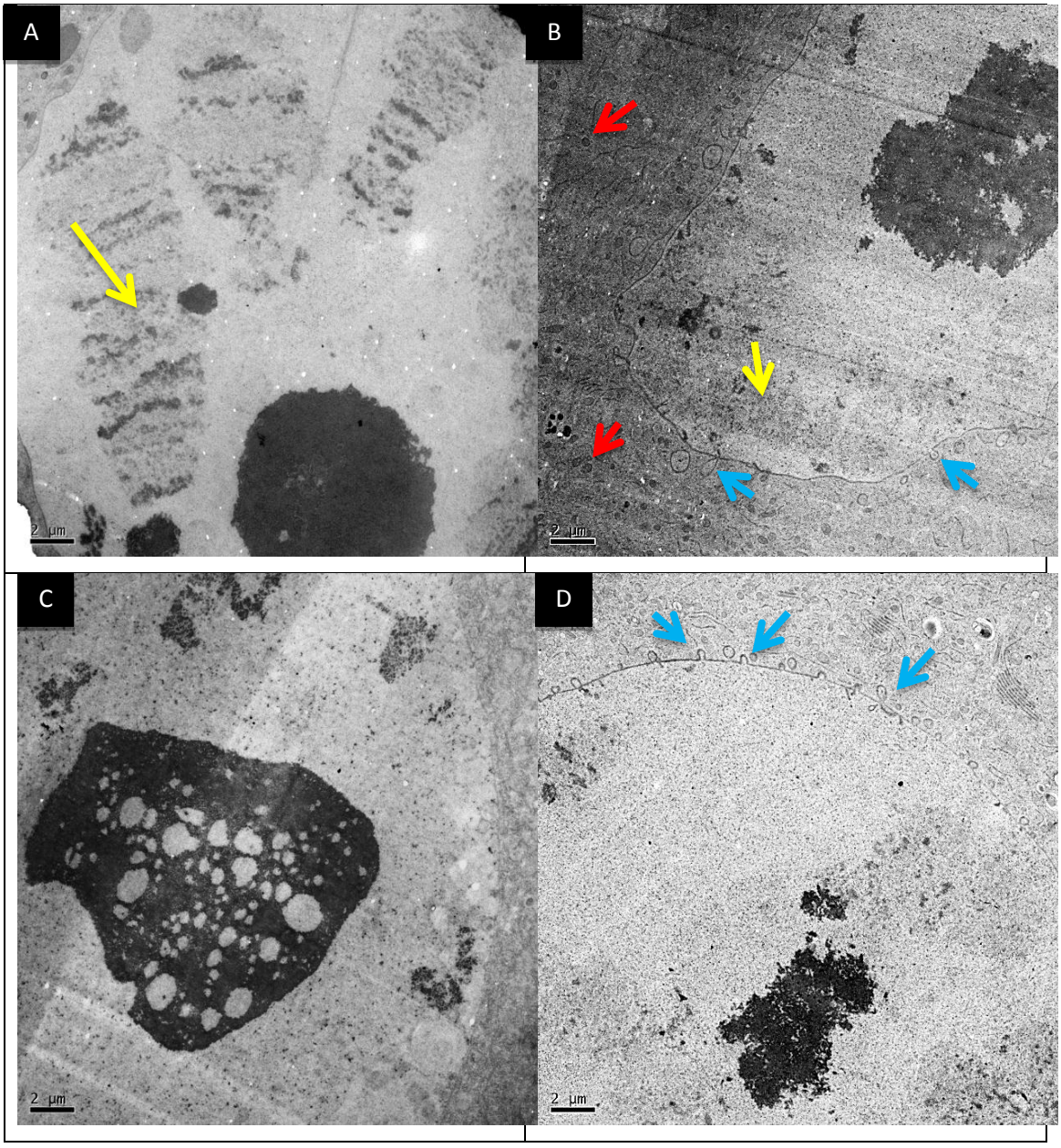


Fig. 3.6. Overexpression of dMyc (c147-GAL4/+; *UAS-dMyc*/+): (A and C) Polytene chromosomes, nuclei and were visualised in flies overexpressing dMyc on the left.

Coexpression of dMyc and NOS2 (NOS2/+; c147-GAL4/+; *UAS-dMyc*/+): (B and D) These structures were also visualised in lines co-expressing dMyc and NOS2 shown in the images on the right.

*Yellow arrows (→) identify polytene chromosomes; red arrows (→) indicate secretory vesicles; blue arrows (→) represent a malformation in the outer nuclear membrane. Scale bars 2µm.

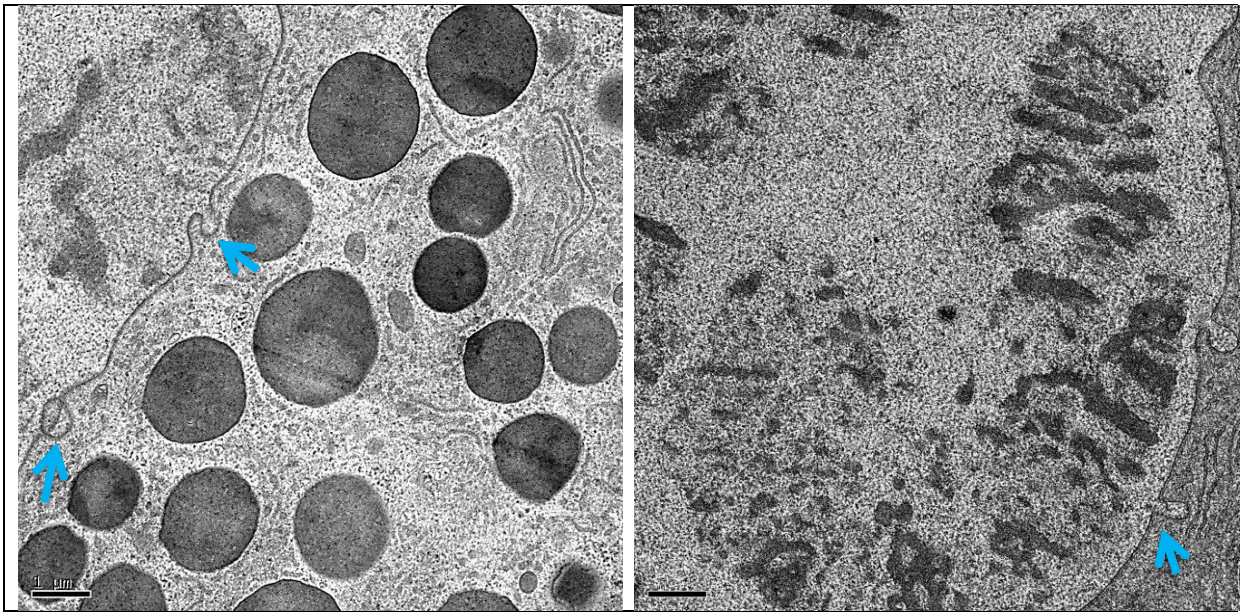


Fig. 3.7. Coexpression of Ras^{V12} and NOS2 (NOS2/+; c147-GAL4/*UAS-Ras^{V12}*). These images were visualised at a higher magnification (2500X) to provide more detail.
Blue arrows (⟶) represent a malformation in the outer nuclear membrane. Scale bars 1µm.

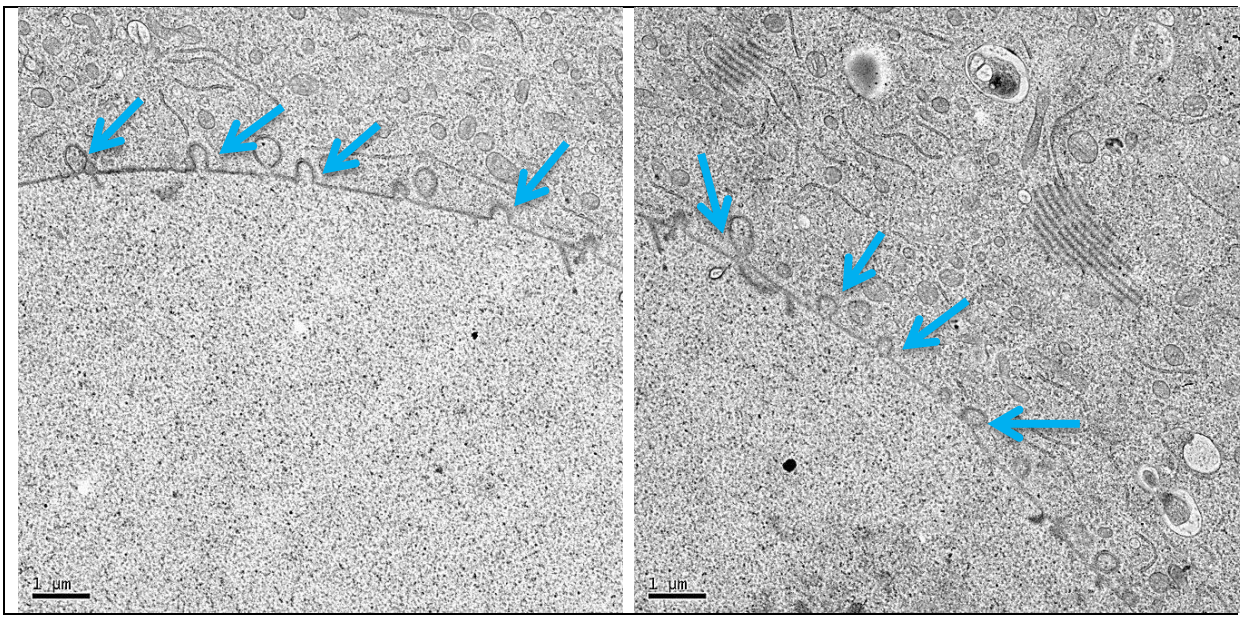


Fig. 3.8. Coexpression of dMyc and NOS2 (NOS2/+; c147-GAL4/+; *UAS-dMyc*/+): These images were visualised at a higher magnification (2500X) to provide more detail.

Blue arrows (⟶) represent a malformation in the outer nuclear membrane. Scale bars 1µm.

- Note that ER formation was very similar to the lines co-expressing Ras^{V12} and NOS2

3.3. Discussion

3.3.1. dMyc

The mechanism behind the interaction between NO and dMyc is currently not clearly defined; however, the results indicate that NO expression can act to almost completely reduce the overgrowth phenotype associated with dMyc. In neuronal cell precursors Ciani *et al*. (2004) demonstrated that NO inhibits proliferation to instead promote differentiation through negatively regulating N-Myc expression when retinoic acid is present. This demonstrates that NO can act as an antiproliferative agent. This study suggests that NO signalling can negatively regulate dMyc-induced growth through FOXO.

Other studies have highlighted FOXO inhibition of dMyc expression which may explain the results seen in dMyc and NOS2 co-expression here, given that NO is demonstrated to signal through dFOXO (Kimber *et al*.). In Eμ-*myc* transgenic hematopoietic stem cells, Bouchard (2007) reported that FOXO acts to negate the oncogenic action of Myc by binding to the *Arf* locus. Delpuech (2007) also explored the interaction between the two and found that FOXO3a activation negates Myc activity by inhibiting the expression of several Myc target genes. Moreover, Teleman *et al*. (2008) reported that *dMyc* mRNA levels are reduced by 50% when FOXO is normally expressed during fasting in *Drosophila* muscle tissue, which is also confirmed in Demontis and Perrimon (2009).

3.3.2. Ras

As previously mentioned Ras^{V12} and NO both regulate the expression of 83 common genes, demonstrating an opposite effect on the transcription of these genes (Asha *et al*., 2003; Kimber, 2005). Scott (2009) also examined the co-expression of Ras^{V12} and NOS2 by measuring the salivary gland nuclei sizes and finds that co-expression of the two produces nuclei sizes that lie in between values measured when they are expressed separately. From these studies, it can be concluded that co-expression mutes the more extreme effects of both growth regulators.

As demonstrated in **Figs. 3.7. and 3.8.,** which show co-expression of NOS2, and Myc and Ras^{V12} separately, the endoplasmic reticulum, which can be observed in proximity to the nucleus in the wild type, NOS2 and oncogene lines, is largely absent when NOS2 is co-expressed with each oncogene. Yang *et al*. (1997) described normal development

of the nuclear envelope as a specialised subcompartment of the ER. In the cell this would normally consist of nuclear pore complexes, the nuclear lamina, and outer and inner membranes. In fact the outer nuclear membrane is normally continuous with smooth and rough ER located in proximity to the nucleus, forming an interconnected boundary (Subramanian and Meyer, 1997). However, in this experiment, the ER membrane connecting the ER and the nuclear envelope appears to be partially formed, resembling budding from the outer nuclear membrane.

Given that only co-expression of these oncogenes and NOS2 show this phenotype, this study aims to investigate potential interactions between these compounds that would affect the ER in such a manner. Using the available literature this study concludes that ER stress may be manifested by the combination of these oncogenes and NO as alternatives are not available.

As previously mentioned in Chapter 1, the toxic oxidant, peroxynitrite, is formed when NO and superoxide react at physiological pH. Dickhout *et al*. (2005) reported that peroxynitrite can cause endoplasmic reticulum stress and apoptosis in human vascular endothelial cells. Peroxynitrite is also implicated in human atherosclerosis, since its marker, 3-nitrotyrosine is upregulated in atherosclerotic lesions.

In this experiment, since NO was generated in abundance by NOS2, this paper investigates whether NO, Ras^{V12} and/or dMyc can produce superoxide, a ROS, in order to drive peroxynitrite formation that can cause ER stress.

3.3.3. Myc increases production of ROS

Vafa *et al*. (2002) reported that c-Myc can increase ROS in normal human fibroblasts, which can induce DNA damage. Given that Myc has many targets, it is difficult to pinpoint a mechanism through which it can increase ROS production. The study states that it is likely that ROS is produced as a result of biochemical imbalances that occur due to the effect of oncogenic Myc inducing unusual synthesis of a large number of gene products.

The dMyc gene used in this experiment has only 26% amino acid sequence similarity to c-Myc (Gallant *et al*., 1996). However, when conducting a protein database search, dMyc shows significant homology with the mammalian oncogene regarding its functional regions. Its NH_2-terminus shows 57% similarity with c-Myc, and mutations

in the region usually abolish Myc action. 57% similarity is also seen in its acidic region and 40% in its COOH-terminal sequence (Gallant *et al*., 1996). In addition, dMyc is capable of restoring the proliferative function of c-Myc null mutants and co-transforming primary mammalian cells (Trump *et al*., 2001). Therefore, there are sufficient molecular similarities that qualify *Drosophila* as a good model when comparing the two Myc proteins (Orian *et al*., 2003).

3.3.4. Ras^{V12} is involved in generation of ROS necessary for Ras transformation

Oncogenic Ras is believed to produce ROS during Ras transformation, but little is known about the mechanism through which this occurs. Oncogenic Ras is shown to be linked to superoxide generation in transformed fibroblasts (Mitsushita *et al*., 2004) and Choi *et al*. (2008) reported that a BLT-Nox1-linked cascade may be the mechanism through which this occurs via H-Ras^{V12}. Ras transformation can be inhibited via antioxidants (Irani *et al*., 1997); therefore, it is necessary for Ras to depend on super oxide production in order to undergo transformation and escape inhibition by antioxidants.

To conclude, in this Chapter the results indicate that NO decreased the size of cellular structures and polytene chromosomes. Additionally, secretory vesicles were not clearly visible in these lines expressing NOS2. When NO was co-expressed with each oncogene (Myc and Ras^{V12}), cellular structures were also smaller than the wild type. Furthermore, secretory vesicles were visible, but reduced in size. Interestingly, combining NO with the oncogenes appeared to have an effect on the formation of ER which suggests that ER stress was induced. This study suggests that the combination of NO and the oncogenes acts to induce ER stress through the formation of ROS which combines with NO to produce peroxynitrite, a toxic oxidant known to generate ER stress.

Chapter 4

dFOXO Mediates NO Signalling in the Regulation of Cellular Growth

4.1. Introduction

4.1.1. Conservation of the *FOX* family: an introduction to FOXO

The *forkhead* box (FOX) gene family of transcription factors is characterised based on a conserved DNA-binding domain, consisting of a 110-amino acid motif. X-ray crystallography examining this domain has defined a three-dimensional structure containing three α-helices which are flanked by two distinctive loops resembling butterfly wings (Friedman and Kaestner, 2006). Consequently, the highly conserved DNA-binding motif is often described as a 'winged-helix' structure.

In humans, the FOX family has over 100 members, ranging from FOXA-R, with the nomenclature based on sequence similarity. The term 'forkhead' is originally coined in *Drosophila* as the name of a gene that, when mutated, produces a phenotype of ectopic head structures with a characteristic spiked head appearance resembling a fork. This gene that was originally discovered is now characterised as FOXA (Myatt and Lam, 2007).

However, this study will focus on the 'O' class of FOX proteins, members of which share the distinction of being regulated by the insulin/PI3K/Akt signalling pathway. Structurally, this class is distinguished from the other classes by an insert of five amino acids present in the DNA-binding domain that play an important role in sequence-specific interaction with binding sites. Most of the other FOX proteins bind consensus sequences that share the core sequence (A/C)AA(C/T) whereas FOXO proteins bind (T/C)(G/A)AAACAA (Myatt and Lam, 2007). This distinction gives the FOXO class a mechanism for preferentially binding with a specific group of target sites in a genome.

Drosophila FOXO (dFOXO) is the singular FOXO gene in *Drosophila* as opposed to mammals which have three highly related FOXO genes (1,3 and 4) (Barthel *et al*.,

2005). Furthermore, dFOXO has significant sequence similarity with the mammalian members of the FOXO family in the forkhead domain (Jünger *et al*., 2003).

There is substantial evidence indicating that FOXO proteins have an evolutionarily conserved role as mediators of the effects of insulin and growth factor signalling on a variety of physiological functions. These include apoptosis, cell proliferation, oxidative stress resistance and metabolism.

4.1.2. FOXO is a downstream component of the insulin signalling pathway

The insulin signalling cascade is initiated via binding at the insulin receptor (InR). The downstream events are numerous; however, this study will concentrate on one aspect of this cycle that modulates gene expression via the FOXO protein discussed below (Puig and Tijan, 2005). Signalling from the insulin receptor is mediated by two pathways: either the PI3K/AKT cascade or the Ras/MAP cascade (Puig *et al.*, 2003). It is via the PI3K/AKT pathway that the FOXO proteins are utilised in modulating gene expression. (Puig and Tijan, 2005).

4.1.3. AKT regulates FOXO by inhibiting its transcriptional activity

Once insulin binds to the InR, PI3K is subsequently activated; this triggers a cascade of signals and events within the cell which will ultimately lead to growth regulation (Puig *et al.*, 2003). Activated PI3K leads to an increase in the amounts of PIP3 (phosphoinositide lipids) available in the cell, acting as relay molecules or second messenger molecules, to propagate the signal along the cascade. As a result of the increase in PIP3 production, Akt is localised to the plasma membrane of the cell and is subsequently phosphorylated. This phosphorylation leads to Akt becoming activated and enables it to phosphorylate other components of the signalling cascade which lie downstream, one of which is FOXO (Puig *et al.*, 2003).

Akt phosphorylates FOXO at three conserved serine/threonine residues, which causes FOXO to be kept within the cytoplasm of the cell, thus inhibiting DNA binding via its forkhead box and decreasing RNA synthesis (Puig *et al.*, 2003). As a result Akt is able to regulate gene expression by negatively regulating the action of FOXO (Puig and Tijan, 2005).

The result of this cascade is that many cellular events and components are affected; these include: cell cycle progression, metabolism, apoptosis, growth and general

survival of the cell. Puig *et al.* (2003) concluded that within *Drosophila*, this cascade can control cell size, life span and overall body size.

4.1.4. Activated FOXO functions as a tumour suppressor

Additionally; FOXO has been associated in immunology and diseases such as cancer. In mammals, Paik *et al*. (2007) demonstrated the importance of FOXO transcription factors in preventing cancer. When performing broad somatic deletions for several FOXO alleles (1, 3a and 4), mice developed lymphomas and haemangiomas. Yet when performing these deletions for each allele separately, neoplasia is significantly reduced. The study suggests that although FOXOs can negatively regulate tumour growth, this function is redundant among FOXO subtypes.

Furthermore, FOXO inactivation has been linked to breast cancer, the second leading cause of cancer death in women. Kong *et al*. (2010) reported that microRNA-155 (miR-155), often expressed at high levels in breast cancer, targets and inhibits FOXO3a. This regulation of FOXO3a by miR-155 is determined by overexpressing miR-155 which then represses FOXO3a protein action. The reverse proves to be true as miR-155 knockdown increases FOXO3a activity. miR-155 appears to directly inhibit FOXO3a through binding to it rather than via phosphorylation. Kong *et al*. (2010) showed that ectopic expression of miR-155 acts to promote cell survival and increase chemoresistance. miR-155 knockdowns increase apoptosis (as a consequence of FOXO3a being uninhibited) and chemical sensitivity in cells (Kong *et al*., 2010). Therefore, miR-155 should be considered a target of breast cancer therapy.

FOXO proteins have been associated with processes that inhibit growth and even contribute to cell death via apoptosis (Barthel *et al*., 2005). Puig *et al.* (2003) demonstrated that dFOXO acts to both stimulate and inhibit components of the insulin pathway in which insulin acts as an indicator of nutritional load.

4.1.5. FOXO regulates InR transcription in a feedback mechanism and is a sensor for insulin

Puig *et al.* (2005) showed that the insulin signalling pathway includes a feedback mechanism in which InR synthesis is regulated by dFOXO in flies and FOXO1 in mammals. These transcription factors function to initiate transcription of InR during fasting. In *Drosophila* this was demonstrated by initiating starvation which

consequently increased mRNA expression levels of dInR in normal flies; however, dInR mRNA levels were unaffected in starved *dFOXO* null mutant flies. Similarly, InR mRNA levels in mammals are increased via FOXO1 DNA-binding action once starvation is induced. In addition to the dInR mRNA increasing during starvation, there is a parallel increase in dInR protein levels (Puig *et al*., 2005).

These corresponding results strongly support the theory that the FOXO transcription factors act in mammals and *Drosophila* as 'insulin sensors' to prepare the insulin signalling pathway. Insulin sensitivity is greatly increased during fasting simply because of the increase in its receptors by dFOXO and in the PI3K/AKT pathway. Upon feeding, the presence of insulin triggers Akt to phosphorylate dFOXO which sequesters it in the cytoplasm. dFOXO is unable to enter the nucleus to bind with the dInR promoter and dInR mRNA and protein levels fall, reducing insulin sensitivity. This is perhaps an adaption to permit a rapid response to insulin in fasting organisms so that they can take full advantage once a meal has been consumed (Puig *et al*., 2005).

Similarly, dFOXO has also been found to upregulate the transcription of *d4E-BP* (also known as Thor) which is a protein which binds to the translation initiator, eIF4E.

4.1.6. dFOXO controls d4E-BP transcription

A component of the eIF4F protein complex, eIF4E binds to the mRNA 5' cap of many eukaryotic mRNAs to initiate translation of proteins (Gingras *et al*., 1999). eIF4E activity is in turn negatively regulated by the binding action of eIFE-binding protein (4E-BP), one of the binding partners of eIF4E (Haghighat *et al*., 1995). As a result, in the presence of unbound eIF4E, abundant 4E-BP expression results in decreased translation initiation of many mRNAs via its negative regulatory binding action on eIF4E. 4E-BP is further characterised as a downstream effector of the TOR/PI3K signalling pathway. Once flies are subjected to oxidative stress, such as hydrogen peroxide exposure, their survival can be maintained by adequate 4E-BP activity; therefore, this protein can heavily influence lifespan under such stress (Zinke *et al*., 2002). Dietary restriction has a similar effect since a lack of nutrients inhibits the target of rapamycin (TOR) pathway that would otherwise phosphorylate 4E-BP. In this case since 4E-BP is unphosphorylated, it is free to bind with eIF4E and negatively regulate translation.

In *Drosophila d4E-BP* transcription is activated by dFOXO. However, once activated, the PI3K pathway promotes Akt action which in turn, phosphorylates dFOXO, preventing it from entering the nucleus and upregulating transcription of *d4E-BP* (Puig *et al.*, 2003). This shows the regulatory effect that Akt activity can have on d4E-BP by controlling it via dFOXO at the levels of gene transcription and translation.

The evidence of this relationship between dFOXO and d4E-BP led Tettweiler *et al*. (2005) to further investigate how critical the presence of d4E-BP is to survival once flies are subjected to poor nutrition and oxidative stress. Indeed the study indicated that ectopic expression of d4E-BP, induced in flies with *dFOXO* null mutations, can overcome oxidative stress and fully rescue oxidative stress sensitivity in the absence of dFOXO. This provides further evidence that d4E-BP is important for survival under stressful conditions and can act independently of dFOXO. Additionally, given that dFOXO overexpression correlates with increased longevity when subjected to oxidative stress (Giannakou *et al.*, 2004), Tettweiler *et al*. (2005) suggested that d4E-BP expression can also control lifespan independently of dFOXO, even though dFOXO increases its expression.

4.1.7. FOXO regulates the immune system

Recently, FOXOs and other FOX classes have been demonstrated to function crucially in several aspects of immunity. This study will focus on the FOXO class for the purposes of this discussion, investigating the current understanding of the role of FOXO in immunity.

Coffer and Burgering (2004) examined the connection between FOXO and the immune system in the mammalian model by inducing FOXO3a null mutations in mice. The FOXO3a$^{-/-}$ mice exhibit "spontaneous lymphoproliferation, mild multi-system, non-lethal inflammation, T_H1- and/or T_H2-cell hyperactivation, NF-κB hyperactivation and cytokine overproduction". These results indicate that the role of FOXO3a in immunity is to control lymphocyte proliferation and apoptosis, suppress T-cell activation and negate autoimmunity (Coffer and Burgering, 2004).

An additional role of FOXO in the immune system is described in Becker *et al*. (2010) which showed an evolutionarily-conserved role of FOXO in regulating antimicrobial peptides (AMPs). These immune effector molecules are important in fighting infection

in animals and plants (Bulet *et al.*, 2004). In *Drosophila* FOXO is shown to activate AMPs independently of their normal immunoregulatory pathways. This was demonstrated by testing *FOXO* null mutants and FOXO overexpression for AMP induction. Null mutants exhibit a loss of AMP induction while the overexpression studies show that AMP is greatly enhanced in this case. In *Drosophila* and humans, FOXO-dependent regulation of AMPs that combat infection is thought to be evolutionarily conserved (Becker *et al*., 2010).

4.1.8. Previous studies in the laboratory investigating interactions between NO and dFOXO

A previous study in our laboratory conducted by Kimber (2005), investigated the possibility of interaction between nitric oxide (NO) and dFOXO in the insulin signalling pathway using dFOXO loss-of-function mutants (null alleles), $dFOXO^{21}$ and $dFOXO^{25}$. These mutations were also co-expressed with UAS-RNAi NOS and UAS-MAC-NOS separately. The alleles were induced by the mutagen ethyl methanesulfonate (EMS) which generates point mutations in $dFOXO^{21}$ and $dFOXO^{25}$ and converted codons W95 and W124, respectively, to stop codons, disrupting dFOXO function (Jünger *et al*., 2003).

With the purpose of evaluating the effects of expressing UAS-RNAi NOS and UAS-MAC-NOS on Thor expression in a null dFOXO background, the experiment used *Thor* reporter staining to visualise this expression. The c147-GAL4 line was used to drive expression of the transgenes only in the salivary glands.

Observing the progeny of these crosses led to the conclusion that development does not appear to be significantly disrupted in the transgenic flies. When compared with the wild-type, both crosses show no extended wandering and larvae develop normally, progressing to the puparium stage. Transgenic flies with the MAC-NOS construct, however, appear to demonstrate increased lethality, with approximately half of the progeny not surviving past eclosion.

General reduced *Thor* reporter staining is observed with little variation in the MAC-NOS construct. Additionally, $dFOXO^{21}$ and $dFOXO^{25}$ mutants demonstrate different levels of *Thor* staining with less staining seen in $dFOXO^{25}$ mutants. This is in contrast to

what would be expected as the two homozygotic lines both function as *dFOXO* null mutations and, consequently, should essentially display very similar levels of staining.

Further investigation into the relationship between NO signalling via dFOXO was conducted by examining the effect that NOS2 expression had on *Thor* expression in the salivary glands. When expressed with a functional *dFOXO* copy, NOS2 expressing larvae have salivary glands that are reduced in size and increased Thor-LacZ staining. This phenotype and *Thor* staining levels do not occur in $dFOXO^{25}$ homozygotes, showing complete suppression of these NOS2 induced phenotypes, but are incompletely suppressed in $dFOXO^{21}$homozygotes. This difference in suppression is significant and implies that the mutations in both alleles have different effects that influence the strength of the *dFOXO* mutation. This effect might be due their different locations with the mutations generating stop codons at marginally different positions which could account for the different levels of suppression documented in Kimber (2005).

Given that Kimber (2005) conducted these experiments on whole salivary glands, Scott (2009) opted to observe the effects of NO signalling on dFOXO in single cells located in the salivary glands during the third instar. Scott (2009) reported that the nuclear to cytoplasmic ratio of dFOXO protein expression in a UAS-NOS2 background is statistically significantly different compared with the wild type, with dFOXO nuclear expression being higher than levels in the cytoplasm. This analysis was conducted in whole salivary glands and UAS-NOS2-expressing clones. However, dFOXO protein levels are increased in comparison to the wild type in both analyses.

4.1.9. $dFOXO^{21}$ and $dFOXO^{25}$: effective null mutations of *dFOXO*

The variation between two mutations used in *Drosophila* ($dFOXO^{21}$ and $dFOXO^{25}$) is illustrated below in a comparison between the structure of dFOXO, human FOXO and DAF-16 forkhead domains:

Fig.4.1. Multiple sequence alignment indicating point mutations in *dFOXO* mutants

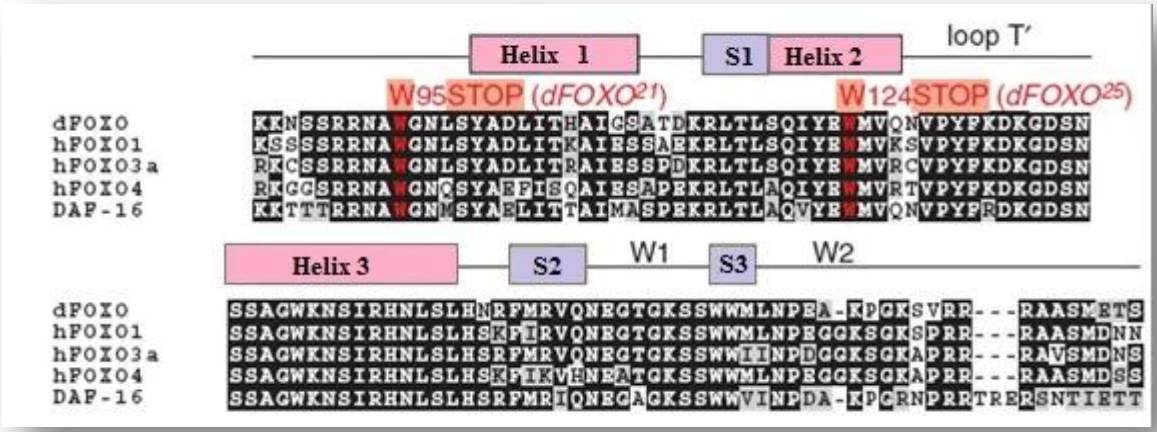


Fig. 4.1. This multiple sequence alignment highlights that the forkhead domain is significantly conserved across the three species in both primary and secondary structure (shown above the sequence). Most relevant to this experiment are the two EMS-induced point mutations shown here in red. The colour coding of the amino acids residues reflects similarities and differences in primary structure. Grey and black show similar and identical sequences respectively (adapted from Junger *et al*., 2003).

4.1.10. *dFOXO*^{BG01018}: a 'mild' *dFOXO* mutant

When conducting a genetic screen for *Drosophila* mutants that differ in lifespan when infected with *M. marinum* compared with the wild type, Dionne *et al*. (2006) identified a specific long-lived mutant, *dFOXO*^{BG01018}. The mutant has a transposon insertion termed BG01018 which is located approximately 130 nucleotides upstream of the *FOXO* start codon. Significantly, the effect of this mutation when compared with the other *dFOXO* alleles is characterised as mild (Dionne *et al*., 2006).

4.1.11. *dFOXO*$^{21,\ 25\ and\ BG01018}$ in immunity

As previously stated, research has shown that FOXO has documented roles in the immune system. Dionne *et al*. (2006) characterised the role of FOXO in *M. marinum* infected *Drosophila* while examining the metabolic consequences of this infection. *M. marinum* is closely related to *M. tuberculosis* which causes tuberculosis (Tønjum *et al.*, 1998). *M. marinum* itself causes lethal tuberculosis-like symptoms in *Drosophila*. Dionne *et al*. (2006) compared the lifespan of *M. marinum* infected wild-type and FOXO null mutants (*dFOXO*^{21}, *dFOXO*^{25} and *dFOXO*^{BG01018}). The *FOXO* mutant

animals all have a longer lifespan relative to the wild-type, which is an interesting discovery in itself; however, the three groups differ between themselves in terms of lifespan. The heterozygotes containing the $dFOXO^{BG01018}$ allele have the least increase in lifespan while flies heterozygous for $dFOXO^{21}$ and $dFOXO^{25}$ alleles survive longer. Studies suggest that differences observed in these mutations might be a result of the different sequences of each of their stop codons (Jünger *et al*., 2003; Kimber, 2005).

A previous study conducted within our lab reveals some findings that point to the relationship between dFOXO and NO. It is shown that NO is dependent on dFOXO for its inhibitory effect on growth; this is explained further in the NO section of the introduction. However, it is also essential to add that in the same study it is also found that NO acts on dFOXO, ultimately promoting growth retardation within cells (Kimber *et al.*).

4.1.12. NO growth inhibition is FOXO-dependent as previously demonstrated in laboratory

Previous research conducted in our lab from Kimber *et al*. demonstrated that NOS-induced growth regulation via NO signalling is dependent on the presence of dFOXO. Using larval salivary gland cells as a model, this was determined by silencing dFOXO through RNAi, which completely eliminates the growth inhibition produced by upregulated NO expression. Furthermore, NO signalling increases dFOXO expression which in turn upregulates *d4E-BP* transcription, resulting in growth inhibition as previously mentioned. This growth inhibition, however, can occur independently of d4E-BP.

Kimber (2005) demonstrated that FOXO and NO share seven transcriptional targets and function to regulate their expression (**Table.4.1**). As a result the expression levels of some of these targets are reduced in response to NO and insulin signalling. Alternative responses to signalling can also be seen in the FOXO targets: *Pepck* and *Thor*, for example, which both demonstrate increased and decreased expression levels via NO and insulin signalling respectively. These results suggest that *Pepck* and *Thor* expression could be controlled through FOXO action influenced by NO signalling.

Table.4.1. A summary of transcripts measured in *Drosophila* S2 tissue culture cells, which are shown to be dFOXO targets through microarray analysis. Transcripts contain forkhead-response elements (FHREs) and are negatively regulated via insulin signalling (Kimber, 2005).

Affy ID	Common	Repressed Insulin FHREs	Normalized 0hr Genespring	Welch t test GenespringP-value	Normalized 4hr Genespring	Welch t test Genespring	Normalized 8hr Genespring	P-value Welch t test	Normalized 12hr Genespring
141780_at	Cyp9c1 Cytochrome P450	4.1	1	0.73	1.04	0.16	1.18	0.34	1.11
143299_at	Pepck	4.6	1	0.08	1.86	0.23	2.82	0.02	2.62
151885_at	Long-chain-fatty-acid-CoA-Ligase	2.7	1	0.75	-1.05	0.62	1.07	0.13	-1.75
153081_at	Phosphorylase Kinase Gamma	2.4	1	0.4	-1.14	0.5	1.1	0.93	1.01
153432_at	Thor	3.3	1	0.23	2.86	0.26	8.16	0.05	5.95
154078_at	Cyp4e2 Cytochrome P450	2.9	1	0.41	1.27	0.12	1.89	0.04	1.91
154586_at	CPTI (mitochondrial carnitine palmitoyltransferase)	4.5	1	0.78	-1.09	0.67	-1.17	0.25	-1.58

In Kimber *et al.* NOS is shown to increase the expression of both dFOXO and d4E-BP, which results in growth inhibition. However, this growth inhibition and upregulated d4E-BP expression is FOXO-dependent, while growth inhibition via NO signalling is not d4E-BP-dependent. NO regulation of d4E-BP is analysed here through microarray analysis of the tissue culture cells and also by measuring d4E-BP-LacZ transcript and protein levels in transgenic flies. Additionally, the study has shown that overexpression of dFOXO and NOS separately, results in similar reduced growth phenotypes.

Scott (2009) targeted murine NOS2 expression in single salivary gland cells in order to analyse the effects of increasing NO expression locally. The salivary gland cells

exhibited growth inhibition. On the cellular level it was shown that NO is able to diffuse between cells, and trigger increased dFOXO protein levels in both adjacent wild-type cells and NO-expressing cells. As previously mentioned, NO has a very short half-life of a few seconds which explains the results seen here. NO was only able to signal to increase in dFOXO levels at a short-range, only affecting neighbouring cells. Antibody staining using anti-dFOXO also showed that increased dFOXO expression is localised both in the cytoplasm (1.7 fold increase) and nucleus (1.6 fold increase).

When measuring the effects of NOS2 on d4E-BP signalling, Kimber *et al*. showed that NO increases d4E-BP expression. In order to verify that this was not a result of natural, physiological variation between the flies sampled, Scott (2009) targeted NOS2 expression to single cells and the promoter activity and protein expression levels of d4E-BP was compared with non-adjacent cells in the same salivary gland. These analytical methods show that d4E-BP increased 1.6 fold in both the targeted cells when compared with the wild-type, non-adjacent cells.

In order to test whether NO-induced growth inhibition is dFOXO-dependent, Kimber *et al*. measured NOS2 in flies homozygous for the null allele $dFOXO^{25}$. In this case, the flies were viable and growth normal, which supports the theory that the growth inhibition is dFOXO-dependent. Similarly, the $dFOXO^{25}$ allele was used again when determining if dFOXO is required for increased d4E-BP expression via NO signalling. Kimber (2005) analysed d4E-BP expression in these mutants, while also expressing NOS2. The removal of dFOXO resulted in d4E-BP expression that was similar to the wild-type expression levels, highlighting that NO functions to increase d4E-BP in a dFOXO-dependent process.

When investigating the effects of overexpressing NO and dFOXO separately, Kimber *et al.* measured nuclei taken from larval salivary glands. Overexpression of both of these genes resulted in reduced nuclear sizes. On average, the wild-type nuclei measure at 21μm and was reduced to under 13μm in flies overexpressing NO and 8μm in those overexpressing dFOXO.

4.1.13. Analysis of nuclei sizes when *dFOXO* mutants and NOS2 are co-expressed

This chapter investigates the effect on nuclear sizes when NOS2 is expressed in animals carrying the *dFOXO* alleles: $dFOXO^{21}$, $dFOXO^{25}$ and $dFOXO^{BG01018}$. Using both trans-heterozygous and homozygous genotypic combinations of the alleles, this study will compare the nuclear sizes and determine the levels of suppression induced by the different alleles.

Given that NO expression in a wild type dFOXO background decreases growth, the strength of mutation examined in these alleles will be indicated by the nuclear sizes. The change in nuclear size when NOS2 is expressed in a *dFOXO* mutant animal compared to NOS2 expression in a wild type background reveals the strength of that *dFOXO* allele. Additionally, Kimber *et al*., Scott (2009) and this present study use nuclear size measurements in order to estimate growth within the salivary glands, but this present study tests three *dFOXO* alleles instead of overexpressed dFOXO.

In order to investigate if overexpressed NO can inhibit salivary gland growth in different dFOXO backgrounds, the three alleles ($dFOXO^{21}$, $dFOXO^{25}$ and $dFOXO^{BG01018}$) were each co-expressed in homozygous and trans-heterozygous combinations with UAS-NOS2. In the trans-heterozygous combinations of the alleles, the parental cross was constructed using females from the first line annotated and males from the second line; for example, in the $dFOXO^{21}/dFOXO^{25}$ line, females were taken from the $dFOXO^{21}$ line and crossed with males from the $dFOXO^{25}$ line. In lines $dFOXO^{25}/dFOXO^{21}$ and $dFOXO^{25}/dFOXO^{BG01018}$, this method of gender selection was also conducted. Wild type c147-GAL4/+ was used as a control line. A NOS2-only expressing line was also used as a reference. 20 female third instar larvae from each line were dissected for their salivary glands. The glands were prepared, stained with DAPI and mounted in Aquamount (see Materials and Methods chapter). Using Zeiss Axiophot microscopy 1050 nuclie from 132 salivary glands were visualised and measured at 40x magnification.

4.2. Results

4.2.1. Statistical analysis of salivary gland nuclei measured in the different lines visualised using DAPI staining

The control line c147/+ had the largest average nuclei size, measuring 23.8 μm, while the smallest nuclei sizes were found in the UAS-NOS2/+;c147/+ line with an average of 10.0μm. The other averages were taken from nuclei of the trans-heterozygous and homozygous *dFOXO* mutants (*dFOXO*$^{21,\ 25 and\ BG01018}$) expressing NOS2. Out of these *dFOXO* mutants, the homozygous *dFOXO*^{25}/*dFOXO*^{25} animals were the most similar to the wild-type nuclear size, with an average size of 22.3μm. The lowest of these were nuclei of trans-heterozygous *dFOXO*^{21}/*dFOXO*^{BG01018} mutants with an average of 13.80μm.

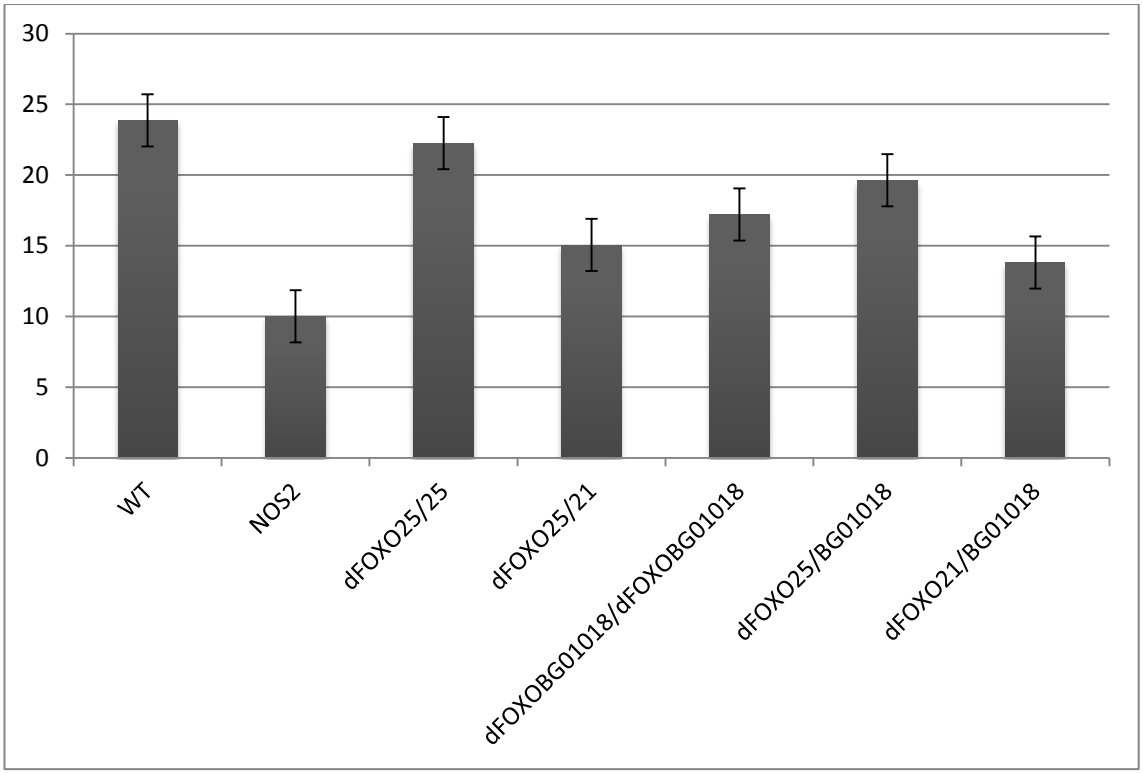


Fig.4.2. is a graph of the average salivary gland nuclei sizes measured from the animals of the genotypes shown. All the lines expressed NOS2 except for the wild type. Error bars represent standard deviation.

WT (c147-GAL4/+), **NOS2** (*UAS-NOS2/*+; c147-GAL4/+), **NOS2 *dFOXO*^{25}/*dFOXO*^{25}** (UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO*^{25}/ *dFOXO*^{25}), **NOS2 *dFOXO*^{25}/*dFOXO*^{21}** (UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO*^{25}/ *dFOXO*^{21}), **NOS2 *dFOXO* ^{BG01018}/*dFOXO* ^{BG01018} (**UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO*^{BG01018}/ *dFOXO*^{BG01018}), **NOS2 *dFOXO*^{25}/ *dFOXO*^{BG01018} (**UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO*^{25}/ *dFOXO*^{BG01018}), **NOS2 *dFOXO*^{21}/*dFOXO* ^{BG01018}** (UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO*^{21}/ *dFOXO*^{BG01018}).

4.2.2. Ultrastructural visualisation of the salivary glands expressing NOS, dFOXO, Myc and Ras via TEM

In order to observe any ultrastructural changes to the cells of the salivary glands resulting from the expression of NOS, dFOXO, Myc and Ras, preparations were observed using the TEM. Three types of cellular structures could be easily observed; these include: nuclei, chromosomes and secretory vesicles. Comparisons were made between animals expressing these genes as well as comparisons to wild type animals. All images were visualised at 1000x magnification.

When compared with the *yw* control line, the NOS2-expressing animals produced cells that were reduced in size. Additionally, the nuclei and polytene chromosomes were also decreased in size, indicating reduced endoreplication. Secretory vesicles could not be observed, suggesting that they were absent in the cell. Therefore, NOS2-expressing cells had smaller nuclei and polytene chromosomes compared to wild types, and the secretory vesicles were not visible (Fig.4.4.).

Expression of NOS2 in $dFOXO^{25/25}$ homozygotes generated cellular structures similar to the wild type. Cellular structures visualised from glands extracted from these animals were almost identical to wild type proportions observed in the nuclei, polytene chromosomes and secretory vesicles (Fig.4.5.).

Moreover, introducing NOS2 in $dFOXO^{25}/dFOXO^{BG01018}$ transheterozygotes generated smaller nuclei and chromosomes, but secretory vesicles were similar to wild type proportions. These animals also expressed NOS2 and displayed smaller nuclei and polytene chromosomes than the wild type and the $dFOXO^{25/25}$ animals, indicating a decrease in endoreplication relative to these lines. Secretory vesicles appeared normal (Fig.4.6.).

In addition, expression of NOS2 in $dFOXO^{BG01018}/dFOXO^{BG01018}$ homozygotes generated smaller nuclei and chromosomes, but secretory vesicles were quite similar in comparison to the wild type. Animals that expressed NOS2, and were homozygous for $dFOXO^{BG01018}$, produced nuclei and chromosomes that appeared slightly smaller than the previously mentioned lines. Secretory vesicles remained normal (Fig.4.7.).

Expression of NOS2 in $dFOXO^{25}/dFOXO^{21}$ transheterozygotes generated smaller nuclei, chromosomes and secretory vesicles. Salivary gland cells contained nuclei,

chromosomes and secretory vesicles that were all slightly reduced in size when compared with the previously mentioned lines (Fig.4.8.).

Expressing NOS2 in *dFOXO*^{21}/*dFOXO*^{BG01018} transheterozygotes generated small nuclei, chromosomes and secretory vesicles. This line had small nuclei, polytene chromosomes and secretory vesicles when compared with the wild type. It also had the smallest nuclei measurements on average when using DAPI staining (with the exception of the *UAS-NOS2* line) (Fig.4.9.).

Note that *dFOXO*^{21} homozygote animals did not survive to third instar larvae when expressing NOS2. Unfortunately, this prevented a direct comparison with all three of the *dFOXO* homozygotes.

Fig.4.3.Control Genotype (c147-GAL4/+): Wild type nuclei, polytene chromosomes and secretory vesicles.

*Yellow arrows (→) identify polytene chromosomes inside the nucleus; red arrows (→) indicate secretory vesicles. Scale bars 2µm.

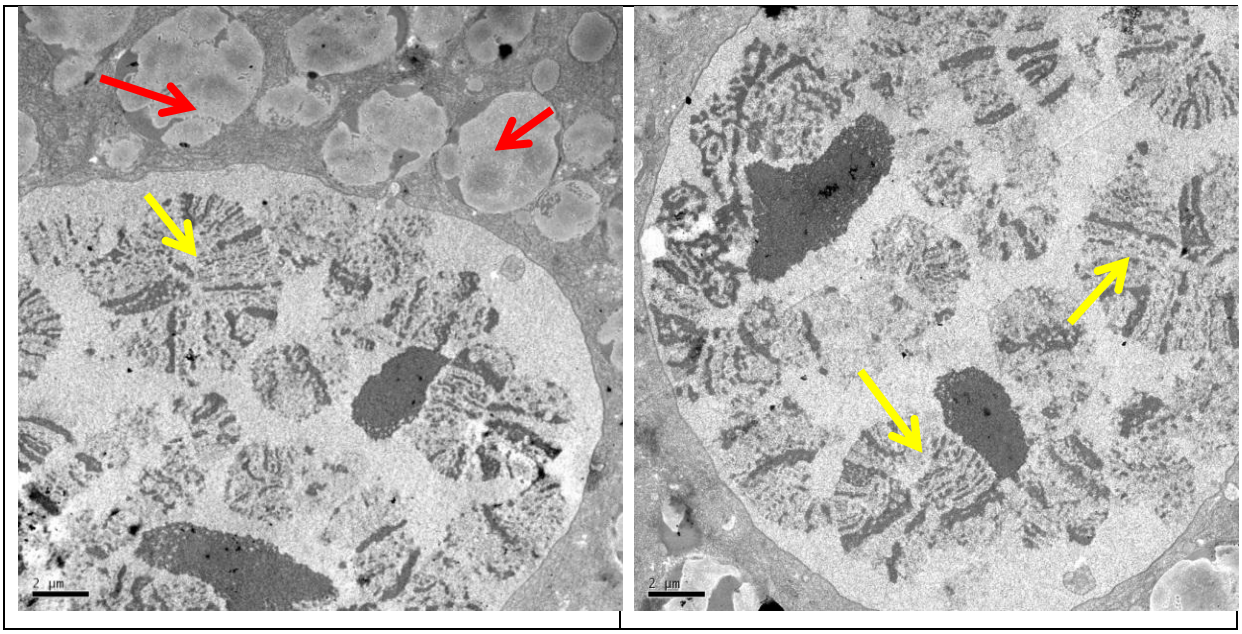


Fig.4.4.Overexpression of NOS2 (*UAS-NOS2*/+; c147-GAL4/+): Nuclei and polytene chromosomes from animals overexpressed NO are shown.

*Yellow arrows (→) identify polytene chromosomes inside the nucleus; red arrows (→) indicate secretory vesicles. Scale bars 2µm.

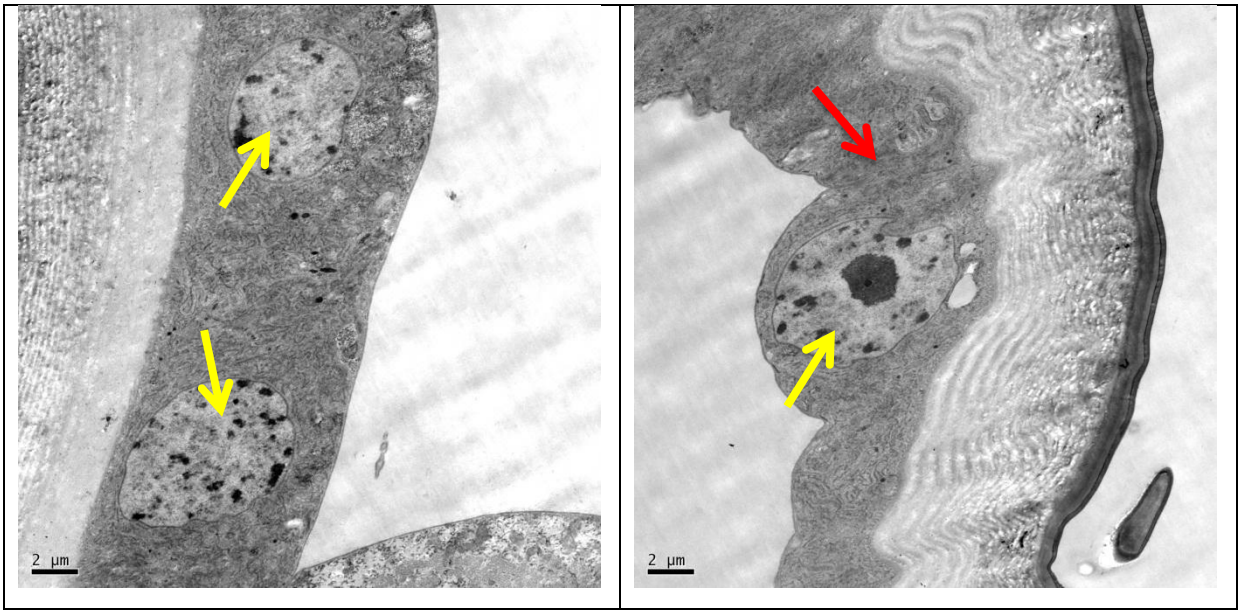


Fig.4.5. UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO*^{25}/ *dFOXO*^{25}

*Yellow arrows (→) identify polytene chromosomes inside the nucleus; red arrows (→) indicate secretory vesicles. Scale bars 2μm.

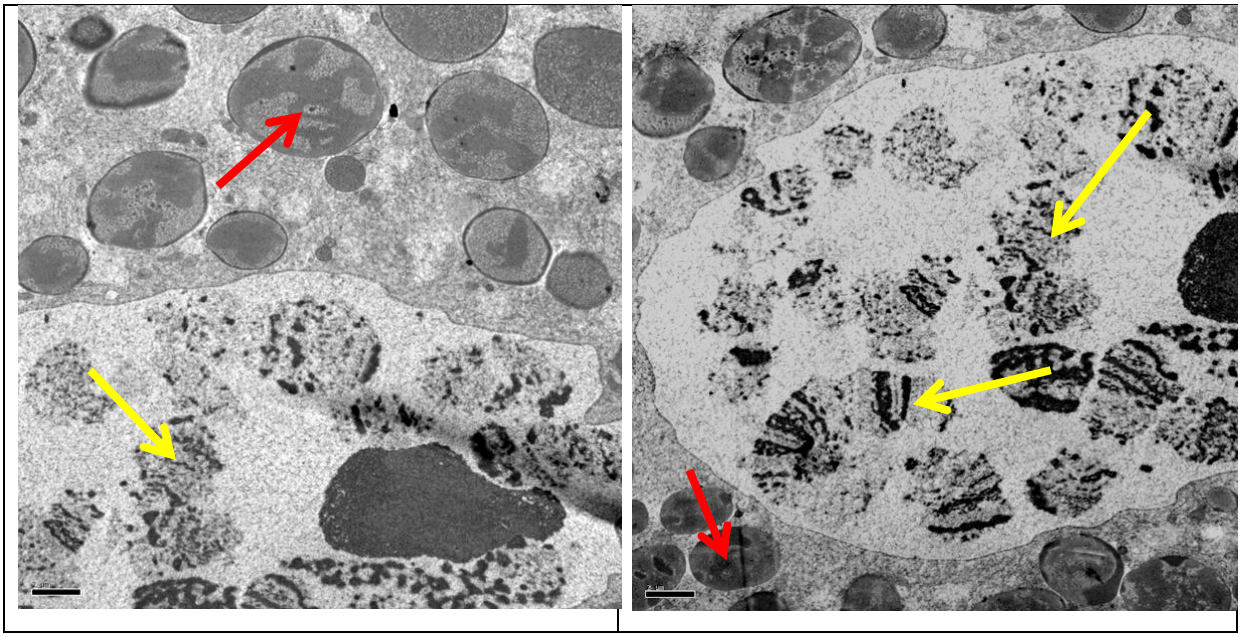


Fig.4.6.UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO*^{25}/ *dFOXO*^{BG01018}

*Yellow arrows (→) identify polytene chromosomes inside the nucleus; red arrows (→) indicate secretory vesicles. Scale bars 2µm.

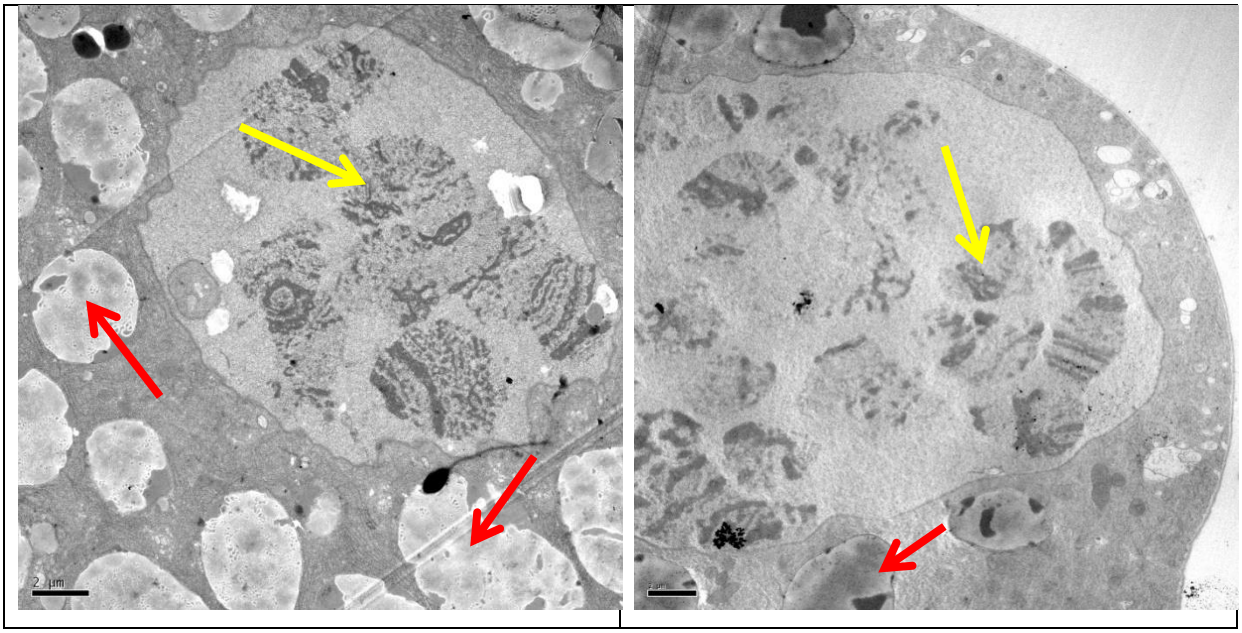


Fig.4.7.UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO*^{BG01018}/ *dFOXO*^{BG01018}

*Yellow arrows (→) identify polytene chromosomes inside the nucleus; red arrows (→) indicate secretory vesicles. Scale bars 2μm.

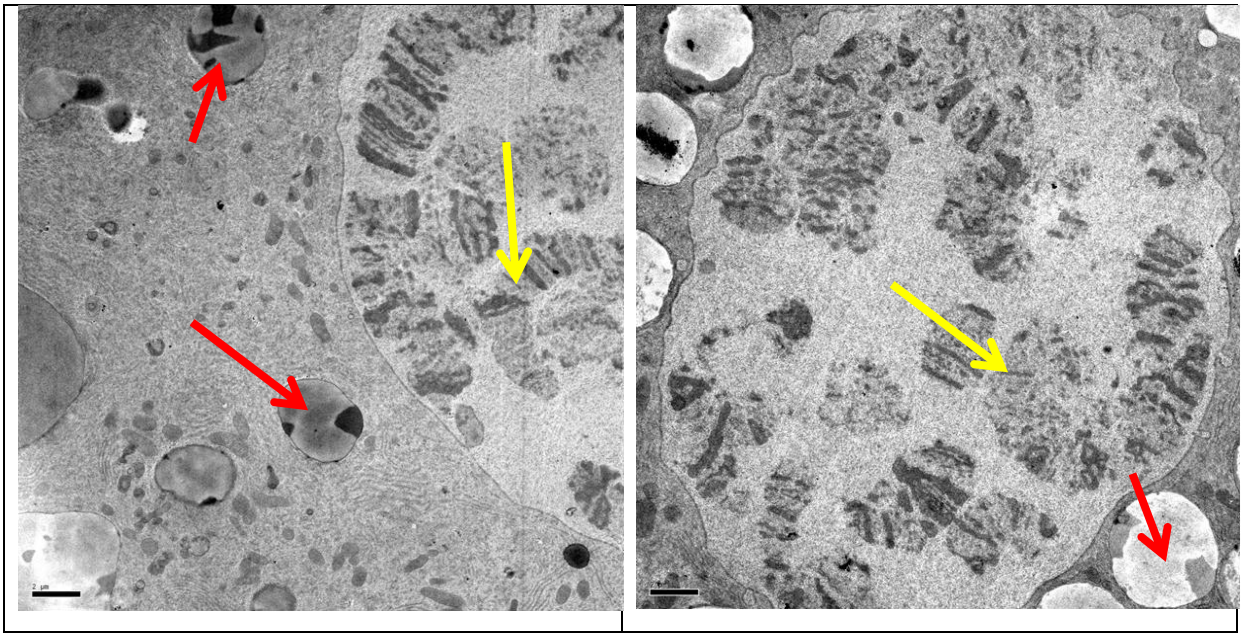


Fig.4.8.UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO^{25}/ dFOXO^{21}*

*Yellow arrows (→) identify polytene chromosomes inside the nucleus; red arrows (→) indicate secretory vesicles. Scale bars 2μm.

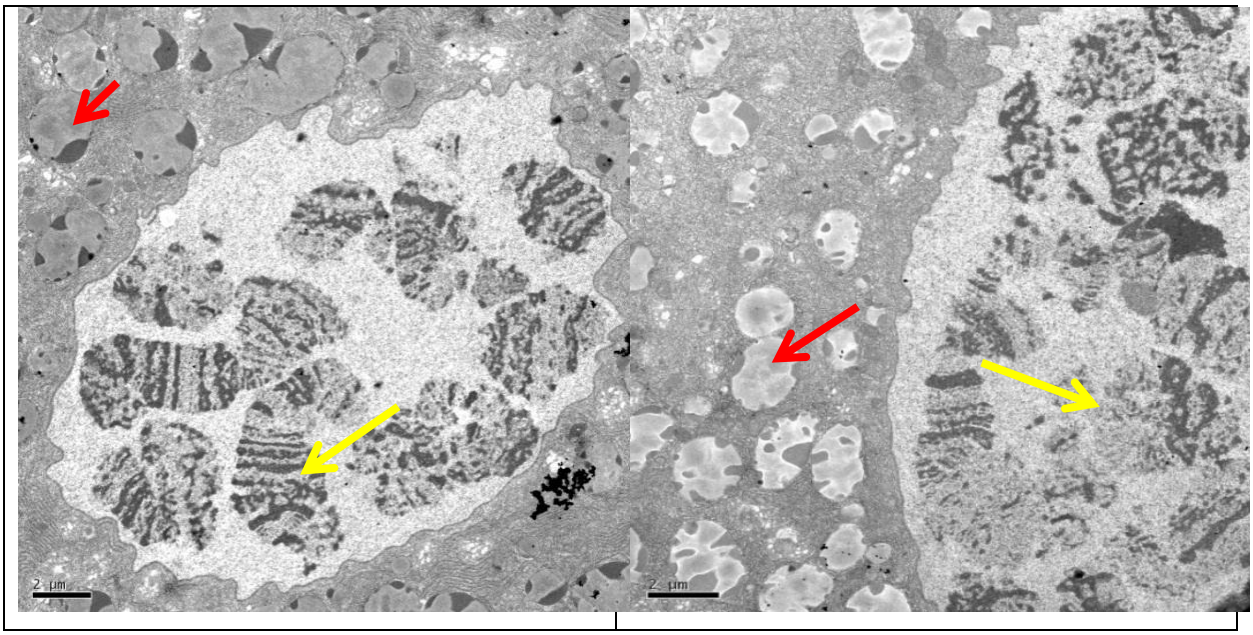


Fig.4.9. UAS-NOS2,UAS-GFP/+;c147/+;*dFOXO*^{21}/ *dFOXO*^{BG01018}

*Yellow arrows (→) identify polytene chromosomes inside the nucleus; red arrows (→) indicate secretory vesicles. Scale bars 2μm.

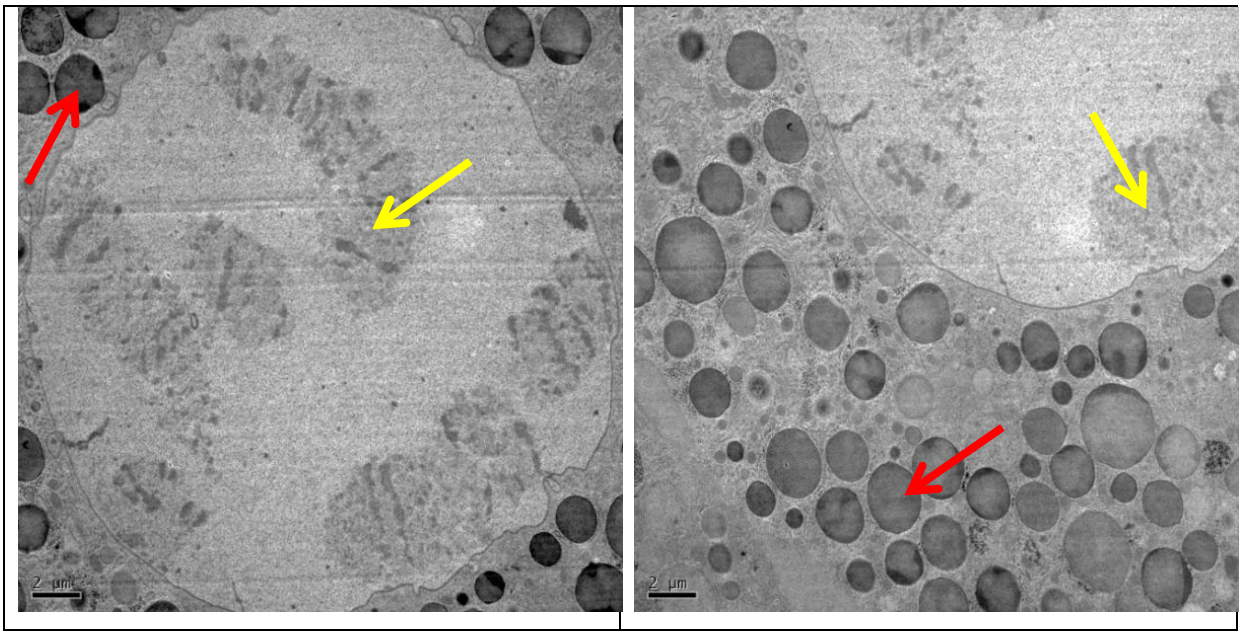


Table 4.2.Comparative analysis of cellular ultrastructure observed in the different lines

Figure Reference	Genotype	NOS2 Expression	Nuclei	Chromosomes	Secretory Vesicles
Fig.4.3.	Control Genotype (c147-GAL4/+)	No	Wild type proportions	Wild type proportions	Wild type proportions
Fig.4.4.	Overexpression of NOS2 (*UAS-NOS2*/+; c147-GAL4/+)	Yes	Greatly decreased in size	Greatly decreased in size	Could not be observed
Fig.4.5.	UAS-NOS2,UAS-GFP/+;c147/+;$dFOXO^{25}$/ $dFOXO^{25}$	Yes	Very similar to wild type	Very similar to wild type	Very similar to wild type
Fig.4.6.	UAS-NOS2,UAS-GFP/+;c147/+;$dFOXO^{25}$/ $dFOXO^{BG01018}$	Yes	Decreased in size	Decreased in size	Similar to wild type
Fig.4.7.	UAS-NOS2,UAS-GFP/+;c147/+;$dFOXO^{BG01018}$/ $dFOXO^{BG01018}$	Yes	Decreased in size	Decreased in size	Similar to wild type
Fig.4.8.	UAS-NOS2,UAS-GFP/+;c147/+;$dFOXO^{25}$/ $dFOXO^{21}$	Yes	Decreased in size	Decreased in size	Decreased in size
Fig.4.9.	UAS-NOS2,UAS-GFP/+;c147/+;$dFOXO^{21}$/ $dFOXO^{BG01018}$	Yes	Strongly decreased in size	Strongly decreased in size	Strongly decreased in size

Table.4.2. This table compares the differences observed in cellular ultrastructure for each genotype. Wild type proportions were used as a control line and sample tissues were all third instar salivary glands. The homozygous $dFOXO^{25}$ (expressing NOS2) animals were most similar to the wild type in cellular ultrastructural proportions. The $dFOXO^{21}/dFOXO^{BG01018}$ line (expressing NOS2) showed the least similarity when compared with the wild type, considering all three cellular structures (nuclei, polytene chromosomes and secretory vesicles) and were significantly reduced in cell size.

4.3. Discussion

4.3.1. *dFOXO*^{25} is the strongest loss of function *dFOXO* allele in this experiment

As the *dFOXO* mutant line with the largest average size of nuclei, measurements from the *dFOXO*^{25}/*dFOXO*^{25} line expressing NOS2 suggested that NO signalling was more impaired when compared with the other *dFOXO* genotypes. This is supported by Dionne *et al*. (2006) which described *dFOXO*^{BG01018} as a mild *dFOXO* null allele. When examining Thor reporter activity in *dFOXO* mutant backgrounds, Kimber (2005) reported that *dFOXO*^{25} homozygotes mutants showed more Thor LacZ staining than *dFOXO*^{21} homozygotes. However, the study is inconclusive as to why this variation might have occured. Since dFOXO is demonstrated to increase Thor expression (Jünger *et al*., 2003), a strong *dFOXO* mutation, such as homozygous *dFOXO*^{25} would decrease Thor expression more significantly than a weaker *dFOXO* mutation, such as homozygous *dFOXO*^{21}.

The three alleles appeared to demonstrate different strengths of *dFOXO* activity; as a result, NO-induced growth inhibition was muted at different levels. The difference in nuclear sizes between the three alleles when combined with NOS2 could be due to the variations in the positions of the stop codons as previously mentioned (Kimber, 2005) in the cases of *dFOXO*^{21} and *dFOXO*^{25}. *dFOXO*^{BG01018} is characterised as a mild mutation containing a transposon insertion upstream of the *dFOXO* gene (Dionne *et al*., 2006); therefore, *dFOXO* transcription is decreased as a consequence of this insertion which reduces dFOXO function to a lesser extent than *dFOXO*^{25}. However, the results from this experiment suggest that *dFOXO*^{BG01018} may have a stronger deleterious effect on *dFOXO* activity than the *dFOXO*^{21} allele.

4.3.2. Using TEM to visualise the ultrastructure of salivary gland cells

When salivary glands from all three *dFOXO* mutant lines were analysed using TEM, alterations to cellular structures such as nuclei, chromosomes and secretory vesicles mostly correlated with changes to the nuclei measurements. When compared with the control line, the ultrastructure visualised in $dFOXO^{25}/dFOXO^{25}$ animals showed the highest degree of similarity to the wild type.

TEM is generally considered a poor indicator of size in cells given that the sections are 100nm thick and the structures measured are several times thicker. However, aberrations in cellular structure and the presence of different organelles can be visualised using TEM, validating its use here. This project reports that no aberrations in organelles' structures were documented here, although the NOS2 expressing salivary gland cells did not contain any secretory vesicles. There is currently no evidence to explain the disappearance of the secretory vesicles in the available literature. The results seen in this Chapter suggest that NOS2 acts to impair the formation of these structures.

To conclude, this Chapter has identified the $dFOXO^{25}$ allele as the strongest reduction in dFOXO activity of the three *dFOXO* mutant alleles discussed here. Futhermore, the $dFOXO^{21}/dFOXO^{BG01018}$ mutants have the weakest impairment of dFOXO activity. This analysis of the different strengths of *dFOXO* mutation was determined by measuring nuclear sizes taken from each line and also using TEM to observe cellular ultrastructure. In addition, this study demonstrates that the inhibition of both growth and secretory vesicle formation by NO can both be suppressed by inhibition of dFOXO activity.

Chapter 5

Investigating The Effects of Nitric Oxide on the Mitochondria and Golgi of Salivary Glands

5.1. Introduction

5.1.1. Mitochondria

Mitochondria are present in significant numbers in metabolically active cells and can account for approximately 40% of volume in the cytoplasm. Their numbers can range from the hundreds to the thousands in cells from organs such as the kidneys, liver and brain. While metabolically-inactive organs such as the skin contain far fewer mitochondria, studies indicate that an adult human might have as much as 10% of their body weight attributed to the mass from mitochondria with the entire body containing approximately 10 million billion mitochondria at a given time (Nisoli and Carruba, 2006).

In addition to the scale of their numbers, mitochondrial function in cellular respiration has fascinated biologists for half a century. More recently, it has been discovered that these organelles never experience stasis. They are always in motion, undergoing fission and fusion within cells, events which lead to changes in numbers, mass and the size of mitochondria (Meeusen *et al.*, 2004; Okamoto and Shaw, 2005). This constant, dynamic activity is regulated by differentiation states and different stimuli.

The role of mitochondria in energy production is crucial to the theories of the original development of complex eukaryotes. Additionally, this function is attributed to diseases associated with aging, cell death and birth (Harman, 1972).

5.1.2. Mitochondrial NOS is a posttranslationally-modifed form of nNOS

Bates *et al*., (1995) was the first study to report the presence of nitric oxide in mitochondria, mitochondrial NOS (mtNOS), via immunocytochemistry in the rat brain and liver mitochondria. Following this discovery several studies demonstrated the characteristics of this NOS isoform which include: localisation to the inner

mitochondrial membrane and Ca^{2+} dependency (Giulivi *et al*., 1998; Carreras *et al*., 2001; Ghafourifar and Cadenas, 2005).

In order to understand how mtNOS is regulated and expressed relative to the other NOS isoforms, Elfering *et al*. (2002) attempted to identify the chemical composition of mtNOS through several techniques. Using amino acid analysis, mass spectrometry of proteolytic fragments, PCR analysis and molecular weight, the study identified mtNOS as nNOSα, eliminating the possibility of a novel isoform. mtNOS differs from nNOS by two posttranscriptional modifications: myristoylation at the N-terminal and phosphorylation of the C-terminal, alterations which may confer enhanced membrane binding and enzymatic regulation respectively (Elfering *et al*., 2002).

5.1.3. Role of NO in regulating mitochondrial function

The function of NO as a vasodilator regulates mitochondrial function. Vasodilation is characterised by blood being directed to tissues; therefore, it presents additional substrates for mitochondria involved with cellular respiration and indirectly aids in spreading heat generated by actively respiring mitochondria. O_2 is also supplied to mitochondria for energy production via the action of NO in releasing O_2 from haemoglobin (Woltz *et al.*, 1999).

NO can compete with O_2 in the electron-transport chain by binding to the terminal protein in the chain, cytochrome *c* oxidase. The function of this terminal enzyme is disrupted through NO binding and serves to negatively regulate oxidative phosphorylation that would otherwise occur through binding with O_2 (Brown and Cooper, 1994; Clementi *et al*., 1998).

Mitochondrial activity is essential for processes such as the glycolytic to oxidative metabolism conversion seen in skeletal muscle fibres (Lin *et al*., 2002). Furthermore, mitochondria are involved in skeletal and cardiac muscle regeneration (Lehman *et al*., 2000; Stamler and Meissner, 2001).

Studies with eNOS null mutations indicate that mice require eNOS for mitochondrial biogenesis, identifying NO as a crucial biogenetic stimulus. Pathology associated with mitochondrial dysfunction includes neurodegenerative diseases, diabetes (type II), liver and heart failure, and neuromuscular disorders (Lehman *et al.*, 2000; Patti *et al*., 2003; Mootha *et al*., 2003).

5.1.4. eNOS mutants inhibit mitochondrial activity in obesity and diabetes

Obesity and diabetes stand out as two pathological conditions that are especially influenced by eNOS null mutations. $eNOS^{-/-}$ mice that consume the same amount of food as the wild type reference gain and retain more weight. This is likely a result of defective energy expenditure (Nisoli *et al*., 2003). Additionally, this is supported as these mutant mice display low O_2 consumption which demonstrates decreased metabolic activity. Therefore, this underlines the importance of functional eNOS in mitochondrial action. The study undertook an *in vitro* experiment by introducing the NO donor, SNAP, to HeLa, 3T3-L1 and U937 cells, and to brown adipocytes and concluded that NO triggers mitochondrial biogenesis in these cells. This process is dependent on guanosine 3', 5'- monophosphate (cGMP) and induces peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), which is known to promote mitochondrial biogenesis (Nisoli *et al*., 2003).

Effective treatment for such impaired energy expenditure could possibly include generating functional mitochondria to increase energy expenditure which would in turn decrease obesity and the likelihood of generating diabetes. However, some studies suggest a relationship between cell dysfunction and mitochondrial biogenesis induced by NO (Carew *et al.,* 2004; Nagy *et al*., 2004).

Mitochondrial biogenesis through NO action may therefore be involved in regulating metabolism and signal transduction. These organelles are crucial for maintaining cell function and survival, and signalling compounds that regulate their production should be carefully investigated.

5.1.5. dFOXO regulates mitochondrial biogenesis via Spargel inhibition

In order to examine the mechanism by which nutrition induces transcriptional modifications, Gersham *et al*. (2007) compared feeding-induced transcriptional changes with dFOXO targets. Remarkably, dFOXO regulated 28% of the genes that respond to feeding. These included genes which are associated with mitochondrial biogenesis, such as a *Drosophila* orthologue of mammalian PGC-1, CG9809, which codes for the protein, Spargel.

In mammals, activation of PGC-1 is reported to increase mitochondrial gene expression and is a 'master regulator of mitochondrial biogenesis' (Nisoli *et al*., 2003). Spargel is

made up of 1088 amino acids and exhibits 68% similarity with PGC-1 in the COOH-terminal motif that binds to RNA. Interestingly, Tiefenbock *et al*. (2010) reported that "Spargel is not a master regulator of mitochondrial biogenesis".

In contrast to the greatly increased mitochondrial biogenesis produced by PGC-1 expression, ectopic expression of Spargel does not increase mitochondrial abundance (Tiefenbock *et al*., 2010). Gersham *et al*. (2007) reported that dFOXO is a vital regulator of the feeding-induced transcriptional response downstream of insulin and suggests that dFOXO mediates repression of Spargel in the insulin signalling pathway which is linked to mitochondrial biogenesis. Therefore, the effects of overexpressed dFOXO on mitochondrial biogenesis in the experiment conducted in this paper will be interesting to note as they may reflect repression of Spargel by dFOXO.

5.1.6. dMyc regulates mitochondrial biogenesis structure and function

In order to investigate dMyc targets, Orian *et al*. (2003) utilised the DamID technique to map dMyc targets in the *Drosophila* genome. This study demonstrated that dMyc binds to six genes associated with mitochondrial biogenesis, function and structure (CG3476, mge, mRpL10, *mRpS7, TFAM* and *Tim10*). Expanding upon this research, Li *et al*. (2005) examined the role of Myc in regulating mitochondrial biogenesis. Ectopic Myc was expressed in P493-6 rat cells and increased mitochondrial growth and function was observed. Furthermore, Myc null alleles were generated in rat fibroblast cells which resulted in decreased mitochondrial mass and number of normal mitochondria. The ability of Myc to rescue this null allelic condition was tested by reintroducing Myc into the fibroblasts. Mitochondrial mass and function were almost completely restored and mitochondria appeared normal. These results support the evidence described in the previous study and demonstrate that Myc is a vital regulator of mitochondrial biogenesis, structure and function.

Larval *Drosophila* cells overexpressing dMyc show increased endoreplication which reflects increased growth. By staining *Drosophila* embryos with MitoTracker red which measures mitochondrial activity, Frei *et al*. (2005) observed that mitochondrial activity is unaffected by overexpressed dMyc. Thus, dMyc can stimulate growth without increased levels of ATP which is unexpected since one would normally predict that mitochondrial activity and energy production would be elevated along with increased growth.

5.1.7. Ras and Mitochondria

The Ras isoform, Kirsten Ras (K-Ras), has been linked to human cancer (Bos, 1989) and differs from the other isoforms by its membrane-binding sequence. Mutated K-Ras is implicated in 90% of human pancreatic cancers, for example (Almoguera *et al.*, 1988). Inactive K-Ras is normally associated with the plasma membrane via farnesylation. It has a polybasic sequence which can be phosphorylated by protein kinase C (PKC). Biovona *et al*. (2006) showed that PKC can regulate the function and location of K-Ras. Once K-Ras is phosphorylated, it disassociates from the plasma membrane and associates with numerous intracellular membranes. The outer mitochondrial membrane is included among these membranes with which K-Ras has been found to associate. Specifically, K-Ras binds with the mitochondrial transmembrane molecule, Bcl-X_L, a member of the Bcl family that regulates autophagy and apoptosis. Phospho-K-Ras then upregulates apoptosis via Bcl-X_L. This association between activated K-Ras and apoptosis might at first appear contradictory since Ras is well documented to induce cell growth and survival. However, Ras and its various isoforms are also known to be associated with proapoptotic processes (Cox and Der, 2003).

5.1.8. Golgi

First discovered by Camillo Golgi in 1898, the Golgi had been the subject of dispute as it was first thought to be an artefact. Its roles in packaging and transporting proteins are well-documented. Proteins can be trafficked to and from the Golgi via vesicular transport. The Golgi is also involved in: secretion granules packaging, glycosylation of glycoproteins and glycolipids on the posttranslational level via glycolysation, sulfation, proteolytic processing of proproteins, packaging lipoprotein and multi-directional trafficking of molecules (Farquhar and Palade, 1981). This study analysed the relationships between Golgi and growth regulators NO and FOXO, in addition to oncogenes Ras and Myc.

5.1.9. Localisation of eNOS on the Golgi is required for NO production in the endothelial cells

In endothelial cells, NO is generated by eNOS and functions to maintain vascular tone and contribute to angiogenesis (Papapetropoulos *et al.,* 1997). Sessa *et al*. (1995) characterised its localisation in endothelial cells and blood vessels as a protein associated with the Golgi complex and plasma membrane. In order for eNOS to efficiently produce NO it must be activated by signalling processes, specifically, Akt-dependent phosphorylation by vascular endothelial growth factor (VEGF). Fulton *et al*. (2002) demonstrated that this phosphorylation occurs on serine 1179 of eNOS; the study investigated this interaction by cotransfecting eNOS and Akt into endothelial cells and stimulating these cells with VEGF. This raised the levels of phosphorylated eNOS (P-eNOS) within the cell relative to total eNOS, but did not change the distribution of these enzymes. These results indicate that phosphorylation and activation of eNOS via Akt and VEGF signalling are critical to produce NO, since an increase in P-eNOS means that more eNOS is activated and produces more NO.

The association between eNOS and the Golgi apparatus and plasma membrane must be correctly localised in order for the agonist VGEF to contribute to optimal NO production (Fulton *et al*., 2002). The requirement of proper localisation is verified by transfecting endothelial cells with a modified form of eNOS which is mistargeted. VGEF stimulation did not increase P-eNOS in this case, signifying that NO production does not increase if eNOS is not localised in the Golgi complex or plasma membrane.

5.1.10. PKBi sequestration in the Golgi regulates FOXO localisation and function

Current literature available does not describe any potential binding, post-translational modification or transport occurring between FOXO and the Golgi apparatus. However, the Golgi apparatus can indirectly affect FOXO localisation and function as a transcription factor by sequestering PKB/Akt inhibitors (PKBis). Maiuri *et al*. (2010) restricted these inhibitors to the Golgi apparatus to elucidate the context-specific physiological outcomes associated with the mechanisms involved in the PKB/Akt pathway, which is comparable to creating gene knockouts with the purpose of examining their function. Since this pathway functions to phosphorylate FOXO, preventing it from localising in the nucleus and regulating transcription (Brunet *et al*., 1999), PKBis can in turn inhibit FOXO sequestration in the cytoplasm through

PKB/Akt pathway inhibition. Maiuri *et al*. (2010) confirmed this action by reporting that PKBis sequestration to the Golgi apparatus hinders its ability to inhibit the PKB/Akt pathway, thus resulting in FOXO localisation in the cytoplasm.

5.1.11. Modification of the CAAX motif targets Ras to the Golgi

Ras proteins are targeted from the cytoplasm to the plasma membrane via posttranslational modification at the C-terminal CAAX motif (Clarke, 1992). A series of enzymes bind this structural motif sequentially, modifying the terminal in order to target it to the plasma membrane (Reiss *et al*., 1990). Additional studies indicate that these enzymes are localised in the endomembrane system (Dai *et al*., 1998; Romano *et al*., 1998; Schmidt *et al*., 1998). This localisation suggests that Ras travels to the cytoplasmic face of the endomembrane system prior to visiting the plasma membrane.

Choy *et al*. (1999) investigated this theory by tagging a catalogue of CAAX proteins (including Ras) with GFP. Using high-resolution digital epifluorescence microscopy to determine the localisation of these proteins, the study demonstrated that Ras is expressed in the Golgi and peri-Golgi vesicles before localising to the plasma membrane. Thus far, three Ras isoforms, neuroblastoma- (N-) Harvey- (H) and K-Ras-4A are documented to transit to the Golgi (Karnoub and Weinberg, 2008).

Choy *et al*. (1999) determined that prenylation via two prenyltransferases is the only posttranslational modification to the CAAX motif that is required for association with the Golgi, while further modifications are required for association with the plasma membrane. Furthermore, the three Ras proteins are also known to traffic to the Golgi from the plasma membrane in a retrograde manner following additional chemical modifications (Rocks *et al*., 2005; Fivaz and Meyer, 2005); this bi-directional, recycling mechanism for Ras proteins suggests a regulatory role in signalling (Rocks *et al.,* 2005).

In T lymphocytes, PLCγ and RasGRP1 are demonstrated to mediate Ras activation on the Golgi. This differs from Ras activation on the plasma membrane which is mediated by the Grb2/SOS pathway (Mor and Philips, 2006). Activation in compartmentalised signalling is thought to serve the purpose of increasing the signal complexity of Ras; however, further investigation is required in order to determine if this type of signalling produces differential signalling outputs. Research suggests that Golgi is coupled to N-Ras signalling because this isoform is the most abundant of the three associated with the Golgi (Omerovic and Prior, 2009).

The available literature does not contain any evidence of chemical interaction and/or localisation between Myc and Golgi. Therefore, for the purposes of this experiment, this paper assumes that there are none.

This chapter discusses an experiment used in this study aimed at investigating possible effects of FOXO, NOS2, Myc and Ras overexpression on expression of mitochondria and golgi. By investigating the expression of mitochondria and Golgi in these different genetic backgrounds, any effects of these growth regulators on the expression of these intracellular compartments can be observed.

This experiment uses mitoYFP and GolgiYFP tagging to observe the expression of mitochondria and Golgi in third instar salivary glands, which can give an approximation of the number of these two organelles. Although mitochondria and Golgi are too small to observe changes in their size in this experiment, these parameter in the larger salivary gland cells will be discussed.

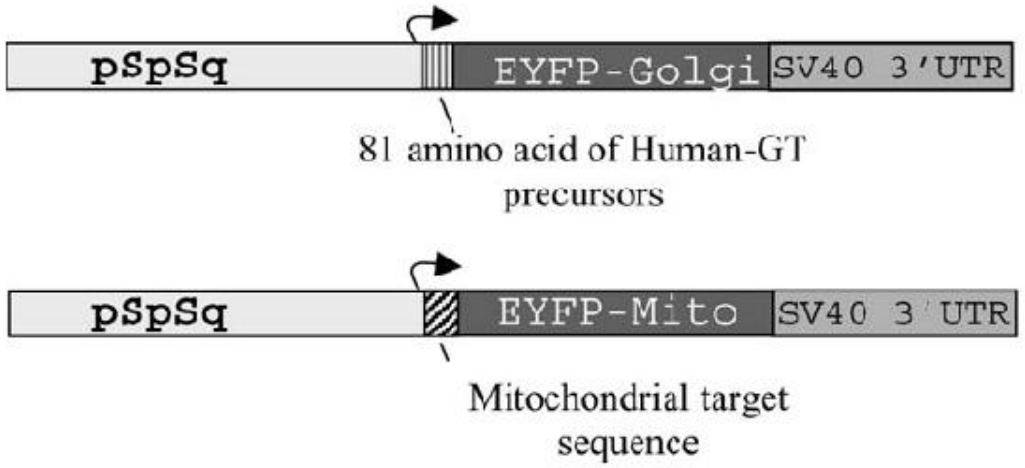
5.1.12. YFP constructs

Green fluorescent protein (GFP) was first discovered in the jellyfish *Aequorea Victoria* and has since been often used as a reporter of gene expression. As a reporter it is useful in biological applications because once mature it is highly stable in many diverse environments and its spectrum can also be manipulated by mutagenesis. However, maturation of GFP is inefficient at 37°C, restricting its use in some experimental designs and prompting researchers to find alternatives. Yellow fluorescent protein (YFP) is one such variant and is often preferred given that it has displayed an improved maturation rate, produces increased brightness, and is less dependent on constant pH and halide levels (Rekas *et al*., 2002).

LaJeunesse *et al*. (2004) described two different transgenes specific for *Drosophila* that ubiquitously expressed enhanced YFP (EYFP) targeted to intracellular membrane-bound compartments: the Golgi and mitochondria. In these constructs, the moieties targeted to each type of membrane protein were amplified using PCR from expression vectors found in mammals and were subsequently cloned into a *Drosophila spaghetti squash* (sqh) CASPER 4 P-element transformation vector (La Jeunesse *et al*., 2004). The sqh promoter is ubiquitously expressed, allowing the study to examine expression of these constructs in several tissues. Given that these expression vectors were derived

from mammals, LaJeunesse *et al*. (2004) sought to observe these constructs in *Drosophila* in order to verify that this method was viable. Salivary glands from third instar larvae expressing these constructs were preferentially examined given the ease of visualisation of these relatively large cells and demonstrated successful uptake of the EYFP constructs and their moieties shown below in Fig.5.1.

Fig.5.1. *Drosophila* sqh was utilised to enhance the expression of the mammalian expression vectors pEYFP-Golgi and pEYFP-Mito for Golgi and mitochondria, respectively, in Drosophila. UTR – untranslated region. SV40 – simian virus 40. ↷ indicates transcription initiation (taken from LaJeunesse *et al.*, 2004).



The constructs for mitoYFP and GolgiYFP used in this experiment were obtained from the Bloomington and are identical to those described in LaJeunesse *et al*. (2004). P{sqh-EYFP-Mito}3 and P{sqh-EYFP-Golgi}3 are homozygous viable and fertile, third chromosome insertions (Flybase).

5.2. Results

Lines that contained mitoYFP and GolgiYFP constructs were crossed with lines expressing FOXO, NOS2, Myc and Ras. Additionally, these two constructs were crossed with yellow white (YW) lines in order to establish viability in a wild type genetic background. Salivary glands were extracted from lines expressing the two constructs, and the progeny of these crosses. These glands were mounted on slides and were visualised using confocal microscopy. All images were viewed at 63X objective (high power). In each image, the green fluorescence indicates YFP reporter activity.

When compared with the control line (Fig.5.2.), the line expressing NOS2 and the mitoYFP construct showed decreased salivary gland cell growth. Additionally, more mitoYFP reporter activity could be seen in the NOS2 expressing animals, indicating that overexpressed NO may have increased mitochondrial biogenesis (Fig.5.3.).

Animals that overexpressed dFOXO and possessed the mitoYFP construct showed increased mitoYFP reporter activity in the line. Salivary glands were even smaller than lines expressing NOS2 (Fig.5.4).

As shown in Fig.5.4. Myc increased mitoYFP reporter activity when compared with the control line. This indicated an increase in mitochondrial expression. The combination of Myc and mitoYFP expression also generated larger salivary gland cells (Fig.5.5.).

The line expressing Ras^{V12} and mitoYFP generated larger salivary glands. However, mitoYFP reporter activity was not abundant and was more comparable with the control line. This suggests that this genotype did not increase mitochondrial expression (Figs.5.6).

In animals that expressed Golgi YFP in a NOS2 background no major changes were observed other than a reduction in salivary glands sizes from NO growth inhibition (Fig.5.8.).

FOXO overexpression in another line resulted in growth inhibition, producing smaller salivary glands when compared with the wild type, but no significant alterations in GolgiYFP staining were observed (Fig.5.9.).

Animals expressing the GolgiYFP construct and Ras^{V12} exhibited larger salivary glands. In addition, GolgiYFP reporter activity was comparable with the control line (Fig.5.10.).

The line expressing the GolgiYFP construct line and Myc demonstrated a general increase in salivary gland size when compared with the wild type. Furthermore, GolgiYFP reporter expression did not appear to be significantly different (Fig.5.11).

Fig.5.2. c147-Gal4/+; p{w[+mC]=sqh-EYFP-Mito}3/+ (control line)

*Yellow arrows ( ) identify expression of mitoYFP. Scale bars 10µm.

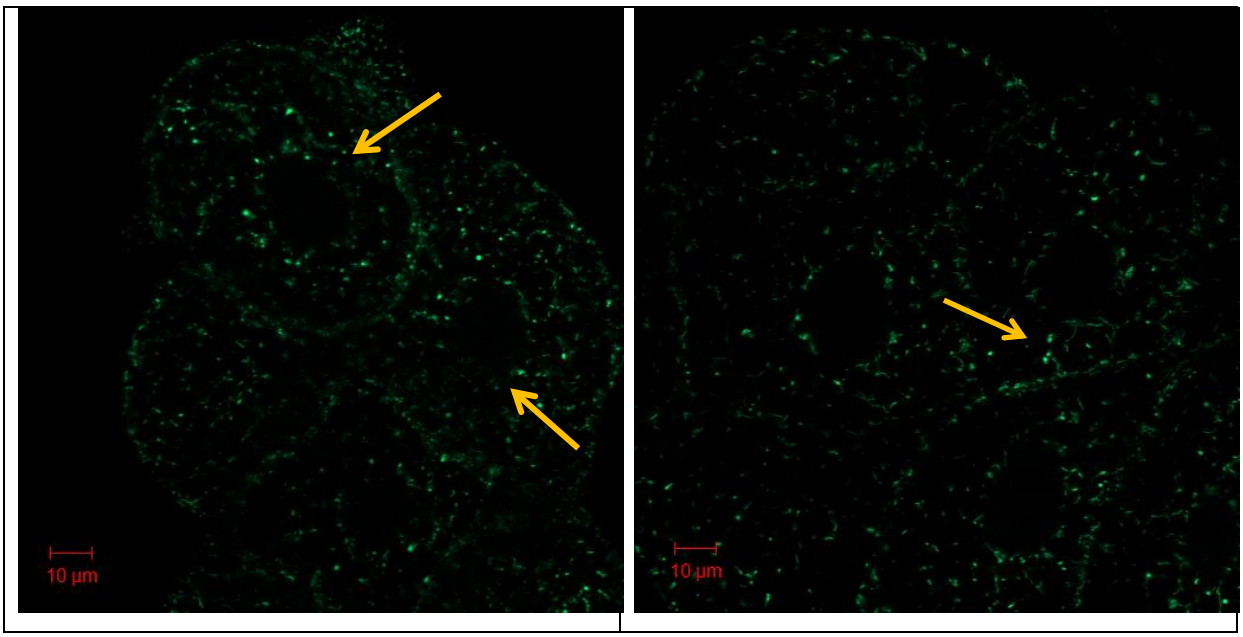


Fig.5.3. NOS2 /+; c147-Gal4/+; p{w[+mC]=sqh-EYFP-Mito}3/+

*Yellow arrows ( ) identify expression of mitoYFP. Scale bars 10µm.

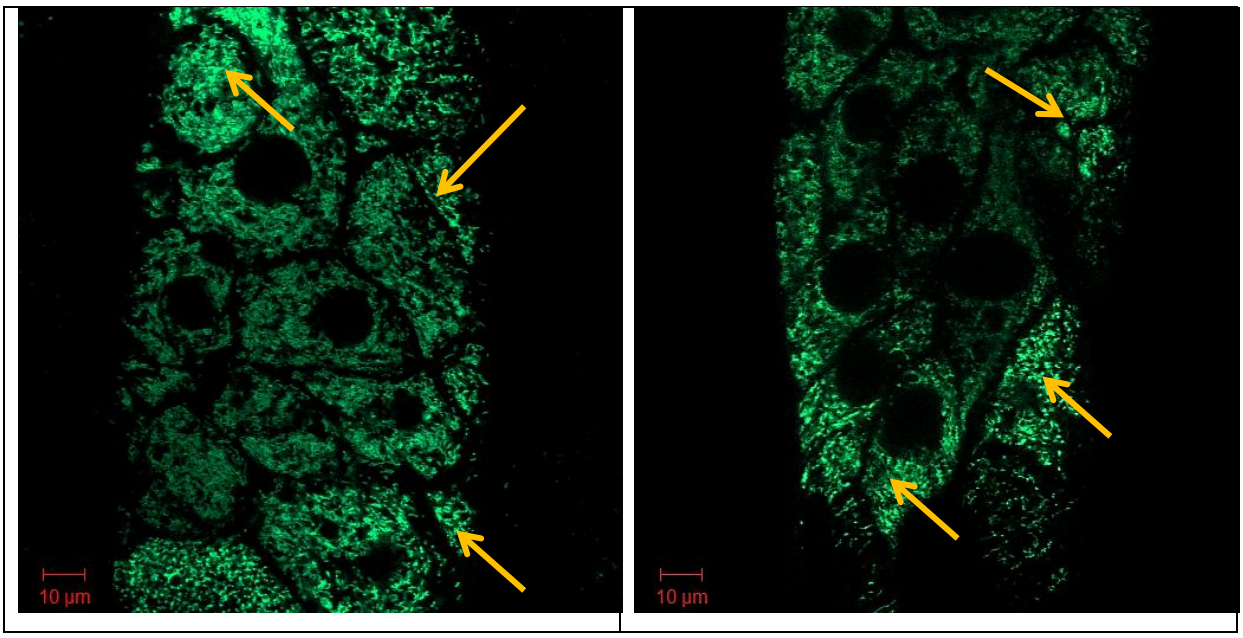


Fig.5.4. c147-Gal4 /UAS-FOXO; p{w[+mC]=sqh-EYFP-Mito}3/+
*Yellow arrows (→) identify expression of mitoYFP. Scale bars 10μm.

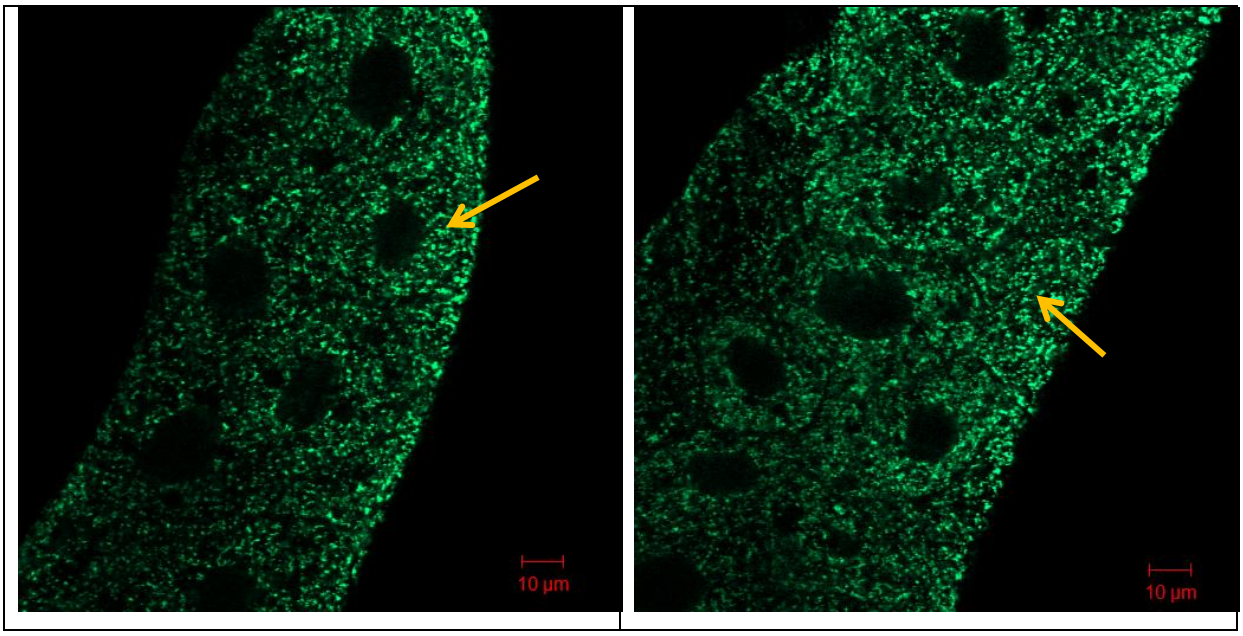


Fig.5.5. c147-Gal4/+; p{w[+mC]=sqh-EYFP-Mito}3/UAS-Myc
*Yellow arrows (→) identify expression of mitoYFP. Scale bars 10μm.

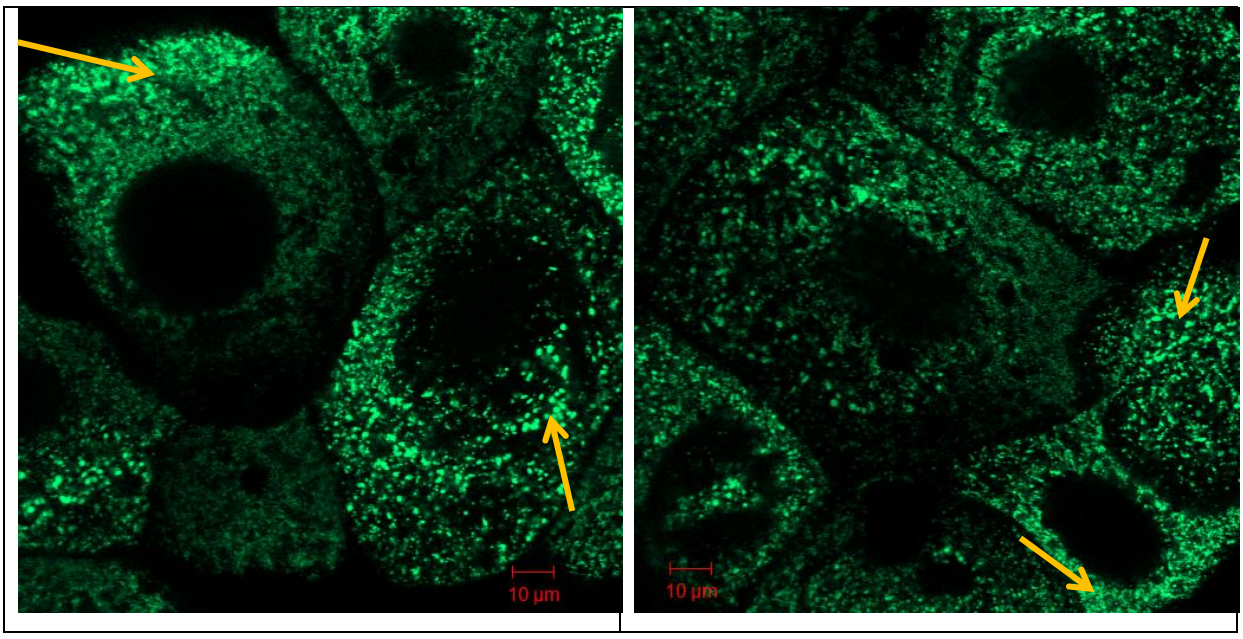


Fig.5.6. c147-Gal4/UAS-Ras^{V12}; p{w[+mC]=sqh-EYFP-Mito}3/+

*Yellow arrows ( ) identify expression of mitoYFP. Scale bars 10µm.

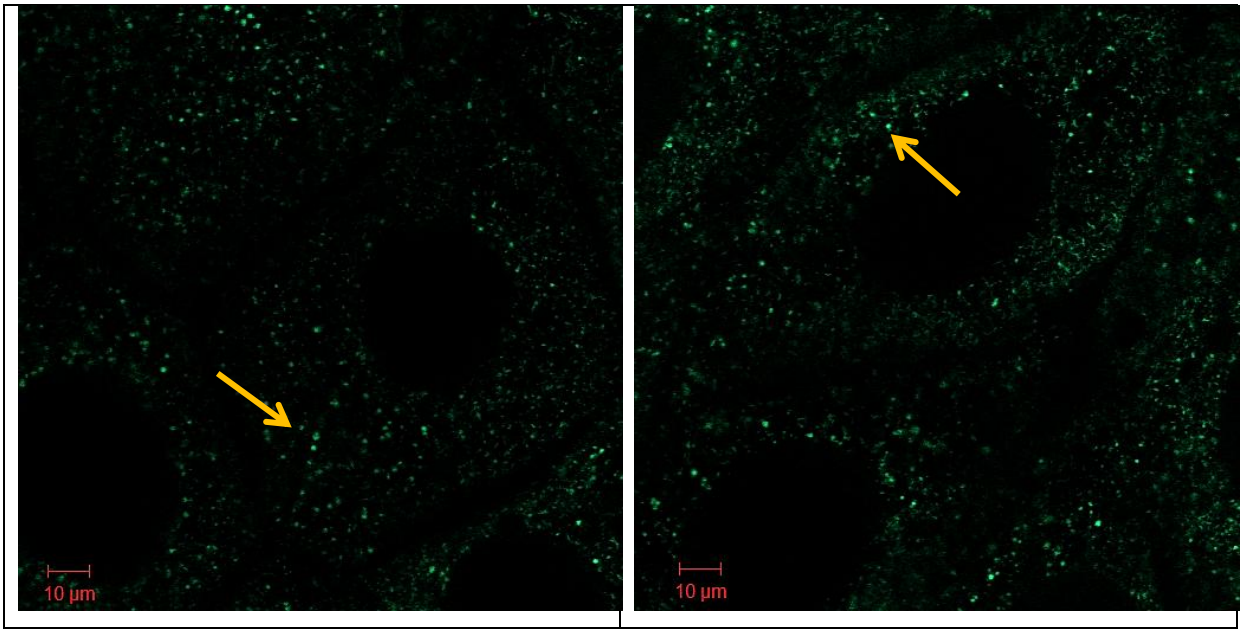


Fig.5.7. c147-Gal4/+; p{w[+mC]=sqh-EYFP-Golgi}3/+ (control line)

*Yellow arrows ( ) identify expression of mitoYFP. Scale bars 10µm.

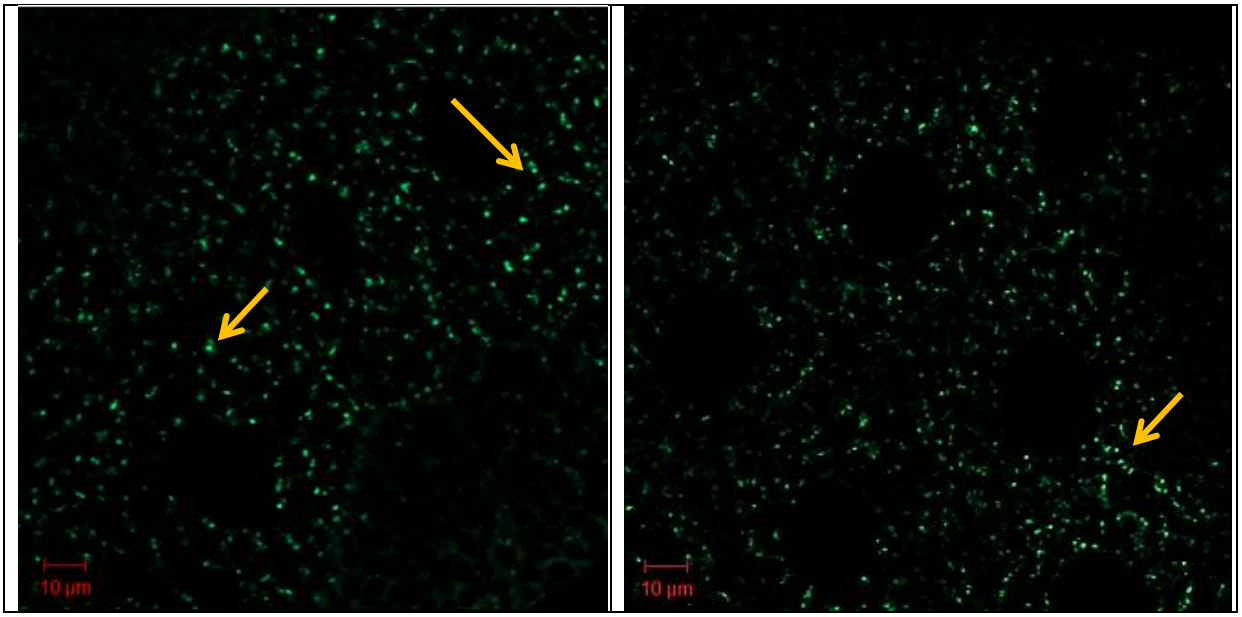


Fig.5.8. NOS2 /+; p{w[+mC]=sqh-EYFP-Golgi}3 /+
*Yellow arrows (→) identify expression of mitoYFP. Scale bars 10μm.

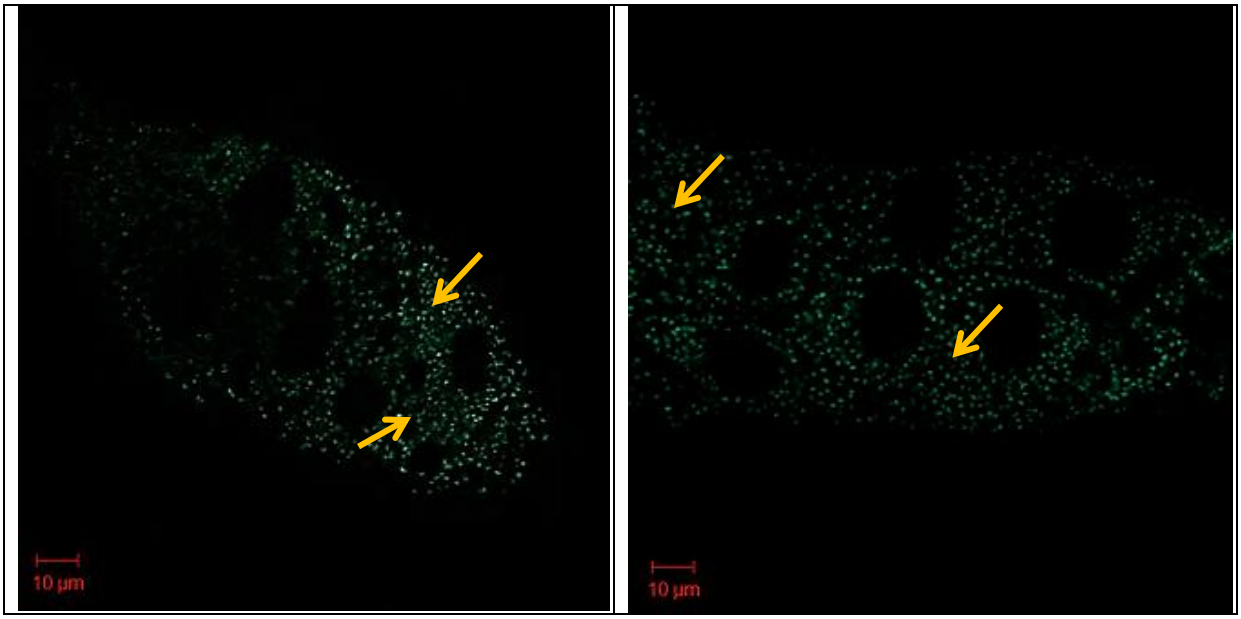


Fig.5.9. c147-Gal4 /UAS-FOXO; p{w[+mC]=sqh-EYFP-Golgi}3 /+
*Yellow arrows (→) identify expression of mitoYFP. Scale bars 10μm.

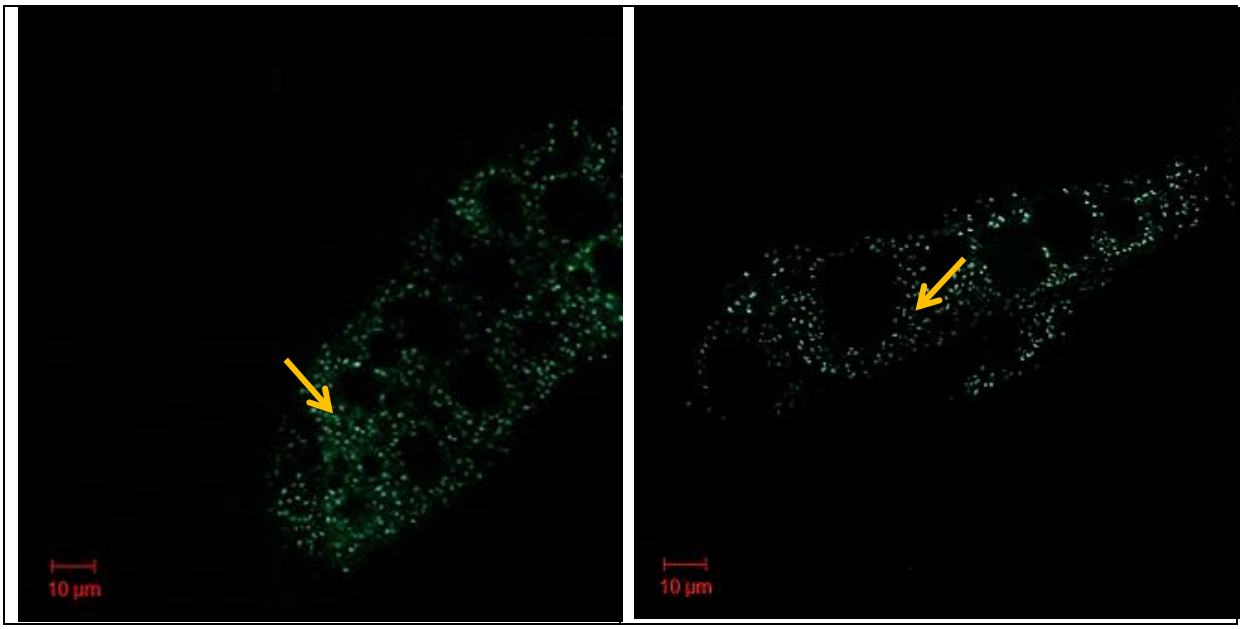


Fig.5.10. c147-Gal4/UAS-Ras^{V12}; p{w[+mC]=sqh-EYFP-Golgi}3 /+

*Yellow arrows (→) identify expression of mitoYFP. Scale bars 10µm.

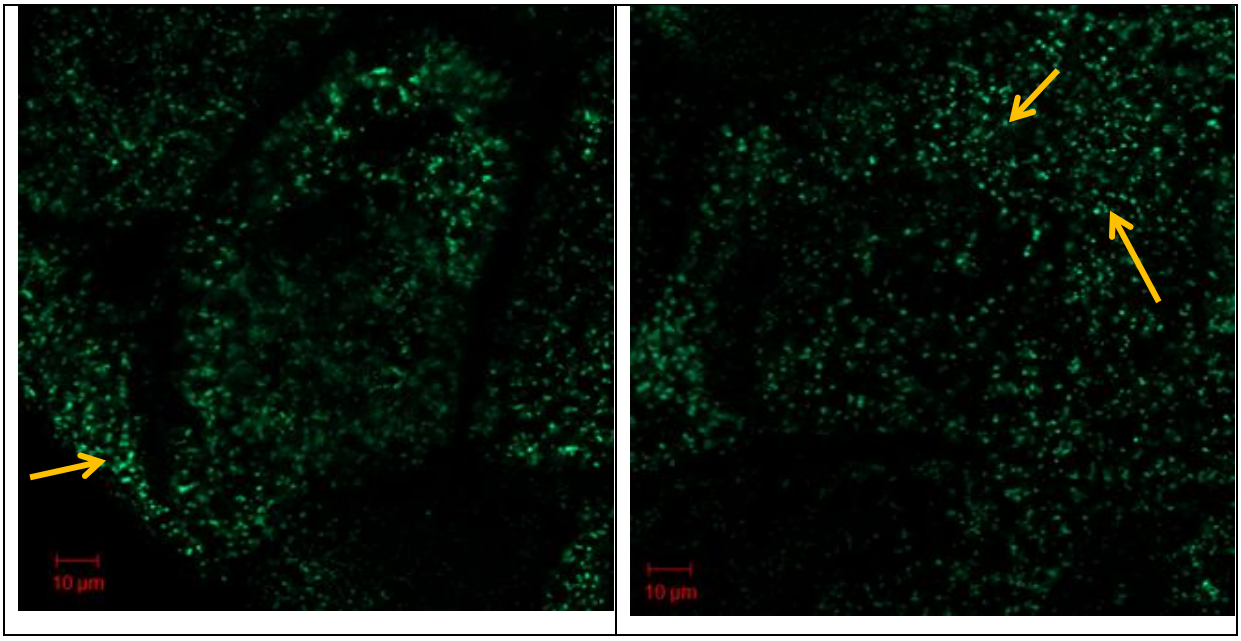
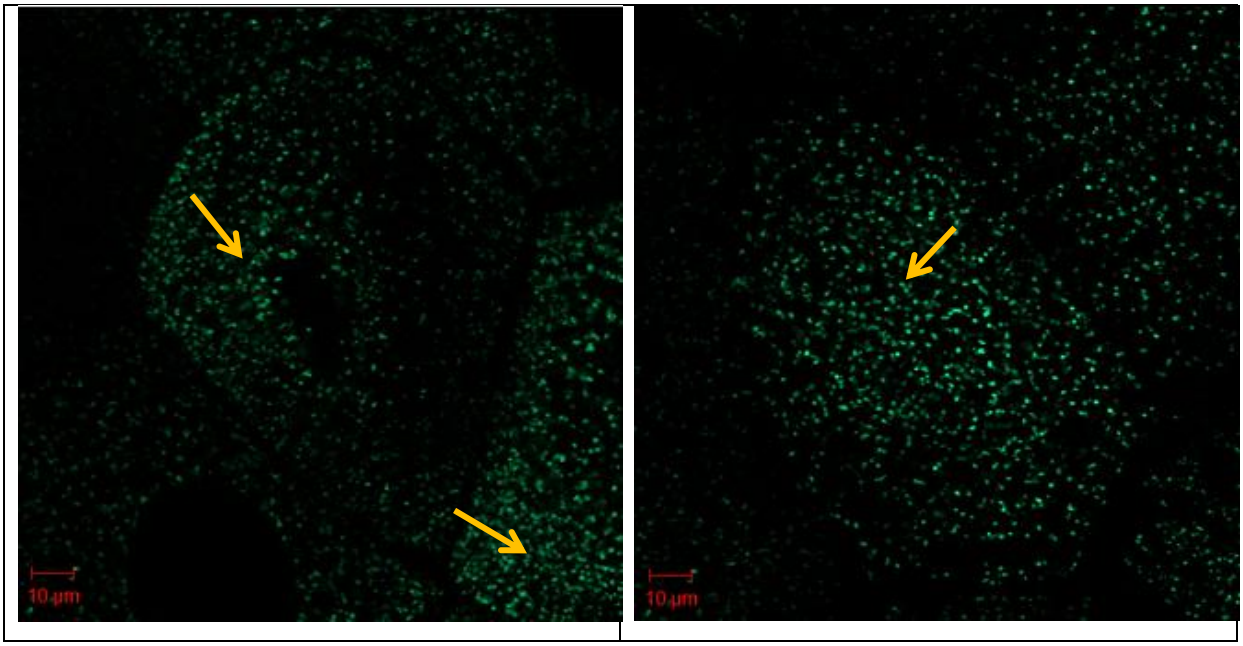


Fig.5.11. c147-Gal4/+; p{w[+mC]=sqh-EYFP-Golgi}3 /UAS-Myc

*Yellow arrows (→) identify expression of mitoYFP. Scale bars 10µm.



5.3. Discussion

5.3.1. NO may increases mitochondrial biogenesis

The results for animals expressing NOS2 showed that the intensity of mitoYFP staining increased, indicating that the number of mitochondria increased (**Figs 5.3.**). eNOS has been demonstrated to regulate mitochondrial biogenesis as demonstrated by decreased biogenesis in eNOS null mutations in mice (Nisoli *et al*., 2003). Nisoli *et al*. (2003) reported that the NO donor SNAP can induce mitochondrial biogenesis in a variety of cells, in a cGMP-dependent mechanism to induce PGC-1 which is a primary regulator of mitochondrial biogenesis. Therefore, the increase in mitoYFP expression indicates that mitochondria biogenesis in *Drosophila* salivary gland cells can also respond to increased levels of NO. This response may also be in a cGMP-dependent manner.

5.3.2. dFOXO may mediates mitochondrial biogenesis

dFOXO overexpression also acted to increase mitoYFP reporter activity, which implies that dFOXO also acts to increase mitochondrial biogenesis. This conclusion is supported by Gersham *et al*. (2007), which argued that the insulin signalling pathway and mitochondrial biogenesis are linked. The study concluded that dFOXO-mediated repression of Spargel drives mitochondrial biogenesis. Although the mammalian homologue of Spargel can increase mitochondrial biogenesis, Spargel does not exhibit this function in *Drosophila* (Tiefenböck *et al*., 2010). The study also noted that Spargel mutants surprisingly do not demonstrate reduced mitochondrial biogenesis in contrast to the mammalian isoform.

Tiefenböck *et al*. (2010) argued that Spargel repression via dFOXO overexpression is responsible for generating the abundant mitochondrial expression. Additionally, since SNAP-induced activation of PGC-1 also acts to generate mitochondrial biogenesis (Nisoli *et al*., 2003) and NO is demonstrated to signal through dFOXO to control growth and proliferation as shown in this Chapter and Kimber (2005), it also possible that NO signals through dFOXO in order to increase mitochondrial biogenesis.

5.3.3. dMyc may increases mitochondrial biogenesis

Fig.5.5. indicates that overexpression of dMyc increased salivary gland size as well as biogenesis of mitochondria. Abundant mitoYFP reporter activity demonstrated here can be explained by several studies. Orian *et al*. (2003) conducted a global genomic mapping analysis of dMyc in *Drosophila* using the DamID method and identified six dMyc targets (*Tim10, mRpL10, mRps7, mge, CG3476 and TFAM*) associated with mitochondrial function, structure and biogenesis. This demonstrates that dMyc has a regulatory role in mitochondria regarding these components.

Subsquently, Li *et al*. (2005) used ectopic expression of Myc in rat P493-6 cells to observe the effects on mitochondria. These cells showed an increase in mitochondrial function and mass, as well as oxygen consumption relative to the wild type. In contrast, *Myc* null rat fibroblasts showed a decrease in mitochondria numbers and mass. The study demonstrated the ability of Myc to partially rescue this phenotype suggesting that the oncogene has a crucial role in mitochondrial biogenesis. However, this role does not extend to mitochondrial activity, as dMyc overexpression has no effect on this characteristic (Frei *et al*., 2005).

These studies account for the increased biogenesis demonstrated in this project, suggesting that dMyc overexpression can drive mitochondrial biogenesis in salivary glands.

5.3.4. Ras^{V12} may not affect expression/biogenesis of mitochondria

As shown in **Fig. 5.6.** salivary gland cells expressing Ras^{V12} showed an increase in salivary gland cell size; however, there did not appear to be an effect on mitochondrial expression when compared with the wild type. This is reflected in the available literature in which there are few studies describing links between Ras and mitochondria. Biovona *et al*. (2006) reported that the Ras isoform, K-Ras, can be activated by PKC and then subsequently associate with several organelles, including mitochondria. The mitochondrial transmembrane molecule, Bcl-X_L, is a K-Ras target and the study found that Phospho-K-Ras upregulated apoptosis via Bcl-X_L. This observation does not relate to the phenotype demonstrated here, as the cells appeared normal despite the increase in size.

5.3.5. NOS2, dFOXO, Ras^{V12} and dMyc might not regulate Golgi expression

The growth regulators used do not alter Golgi expression and only seem to affect the size of the salivary gland cells. Predictably, oncogenes dMyc and Ras^{V12} showed a general increase of salivary gland cell size, also generating larger nuclei relative to the wild type. Growth inhibitors, dFOXO and NOS2 produced decreased salivary gland cells and also smaller nuclei. However, none of these regulators had an impact on Golgi expression which is supported by the absence of studies documenting any effect on Golgi expression when examining the available literature.

Chapter 6

Discussion

6.1. Introduction

Using the model system *Drosophila*, this thesis demonstrates that the *dFOXO*^{25} homozygotes gave the most suppression of NOS2-induced under-proliferation when compared with *dFOXO*^{21} and *dFOXO*^{BG01018}. Additionally, when NOS2 was co-expressed with Ras and Myc, aberrations in ER ultrastructure were visualised. It is suggested that these oncogenes and NOS2 combine to produce peroxynitrite which could explain this phenotype. Furthermore, this thesis argues that overexpression of Myc, dFOXO and NO can induce mitochondrial biogenesis.

6.1.1. NOS2 expression removes the salivary gland secretory vesicles

When TEM was used to visualise the ultrastructure of salivary glands in NOS2 expressing cells, secretory vesicles were not observed. The available literature does not document any previous interaction between nitric oxide and secretory vesicles, even in other organisms. It is interesting to note that this phenotype is also suppressed by inhibition of dFOXO activity.

6.2.Co-expressing NOS2 with dMyc and Ras^{V12} causes ER stress

Using TEM this project reports that when compared with control preparations, nuclei and polytene chromosomes of salivary gland cells from NOS2 expressing appeared much smaller, but retained their structure (Fig. 3.4.). Interestingly, the secretory vesicles were not visible. When co-expression of NOS2 and oncogenes, dMyc and Ras^{V12} separately was conducted, the results show that the ER was affected by the combination of each oncogene with NOS2. ER structures were not clearly visible suggesting that this co-expression can induce ER stress or inhibit development of ER (Fig. 3.7. and Fig. 3.8.). Peroxynitrite can be formed when NO reacts with superoxide (ROS) and has been demonstrated to induce ER stress (Dickhout *et al*., 2005).

Given that NO is overexpressed here via NOS2 and is therefore available in significant quantities, this project also reports that each oncogene has the potential to produce superoxide. Oncogenic Ras has been shown to produce superoxide in transformed fibroblasts (Mitsushita *et al*., 2004) and H-Ras^{V12} produces ROS via a BLT-Nox-1 linked cascade (Choi

et al., 2008). Since transformation of Ras can be inhibited via the action of antioxidants (Irani *et al*., 1997), ROS production facilitates this process.
c-Myc can also induce the production of ROS, which is demonstrated in human fibroblasts (Vafa *et al*., 2002). Many studies highlight the functional similarities between c-Myc and dMyc (Gallant *et al*., 1996; Trump *et al*., 2001; Orian *et al*, 2003). Therefore, dMyc is suggested here to act in a similar manner to c-Myc in order to produce ROS.
These studies suggest that the combination of overexpressing NOS2 and each of the oncogenes might produce NO and ROS, respectively, in order to induce ER stress via peroxynitrite production.

6.3. *dFOXO*^{25}/*dFOXO*^{25} is the strongest loss of function allele of the dFOXO gene

NO has been previously demonstrated to signal through wild type dFOXO in order to inhibit growth in the nuclei of the salivary glands (Scott, 2009). Here it is demonstrated that the level of suppression of the NO-induced growth phenotype is dependent on the *dFOXO* allele used and, therefore, presumably on the level of dFOXO activity. Expression of NOS2 in a *dFOXO*^{25} homozygous background (a null allele) produced similar salivary gland nuclei sizes (Axiophot) and ultrastructure (TEM) compared to the wild type. Additionally, NOS2-expressing animals that were trans-heterozygous for *dFOXO*^{25} had larger nuclei and more normal ultrastructural components when compared with other combinations of *dFOXO* alleles.
The results show that the *dFOXO*^{25} homozygotes gave the most suppression of NOS2-induced phenotypes when compared with the other allelic combinations, since growth inhibition via NOS2 was greatly reduced. Jünger *et al*. (2003) suggested that the different strengths of mutation are a result of variations in the position of the stop codons generated by the point mutations for *dFOXO*^{21} and *dFOXO*^{25} (**Fig.4.1.**).
Interestingly, the results show that *dFOXO*^{BG01018} gave more suppression than the *dFOXO*^{21} null allele, which is in contrast to Dionne *et al*. (2006), which describes *dFOXO*^{BG01018} as a mild mutation (in terms of life span alteration) in comparison to the other two null alleles. The *dFOXO*^{BG01018} mutation is generated by a transposon insertion that acts upstream of the *dFOXO* transcriptional start site, generating a loss of *dFOXO* function that this present study shows is more severe in respect to growth control than *dFOXO*^{21}, but less severe than *dFOXO*^{25}. Although Dionne *et al*. (2006) analysed the effect of *dFOXO* mutations in terms of lifespan (upon exposure to *M.* marinum) rather than nuclei sizes, the study is similarly comparing the effects of the three *dFOXO* alleles in terms of strength.

6.4. Myc, dFOXO and NOS2 induce mitochondrial biogenesis in *Drosophila* salivary glands

When analysing mitoYFP expression with the oncogenes, and NOS2 and dFOXO, it was demonstrated that Ras^{V12} did not act to increase mitochondrial biogenesis when compared with the control line. However, overexpression of Myc, dFOXO and NOS2 increased mitochondrial biogenesis.

Ectopic expression of Myc can increase mitochondrial biogenesis in rat P493-6 cells and Myc null mutants also show decreased mitochondrial biogenesis (Li *et al*., 2005). This study supports the results demonstrated here.

Nisoli *et al*. (2003) showed that eNOS can act to increase mitochondrial biogenesis, but there is no evidence to suggest that the isoform used here, NOS2, can also induce this. However, the NO donor SNAP has been shown to increase mitochondrial biogenesis in many cells in cGMP-dependent activation of PGC-1, which is known to induce mitochondrial biogenesis (Nisoli *et al*., 2003).

The mammalian homologue of PGC-1, Spargel was demonstrated to be inhibited by dFOXO which increases mitochondrial biogenesis (Gersham *et al*., 2007). Tiefenbock *et al*. (2010) suggested that although these two homologues are both involved in mitochondrial biogenesis in mammals and *Drosophila*, they exhibit opposite effects regarding regulation of this biogenesis.

When investigating the combination of GolgiYFP with these four growth regulators co-expressed separately, the results did not show any change of GolgiYFP expression when compared with wild type controls. This suggests that these growth regulators have no effect on Golgi expression which is supported by a lack of evidence for this in the available literature.

6.5. Future Work

Additional experiments can be conducted in order to confirm that the *dFOXO*^{25} null allele gives the most complete suppression when compared with *dFOXO*^{21} and *dFOXO*^{BG01018}. Previous work has shown that dFOXO acts a crucial mediator of NO signalling
In order to confirm the results that show increased mitochondria expression in cells that overexpressed dFOXO, Myc and NOS2, this thesis suggests that future experiments could generate RNAi lines for the three growth regulators and analyse mitochondrial expression in the salivary glands using mitoYFP. Expression of mitochondria could be analysed and compared with the results seen here. If mitochondria expression is decreased, then that result would support the roles of these growth regulators in mitochondrial biogenesis.

This present study did not investigate mitoYFP expression in *dFOXO* mutants in the presence and absence of NOS2 expression. Further research could be conducted in the future to explore the effects that *dFOXO* mutations would have on mitoYFP expression and NO signalling.

Given that this study suggests a possible involvement of NO-induced peroxynitrite generation in ER stress formation seen in the salivary glands, future research could also compare the levels of peroxynitrite in animals expressing NOS2, and Myc or Ras^{V12} with levels observed in wild type animals. Results from such studies could help quantify the effect of NOS2 and these oncogenes on peroxynitrite production.

Another important observation in this study was the inhibitory effect of NO on secretory vesicle expression. There was a positive correlation between the magnitude of this effect and the deleterious strength of each *dFOXO* allele combination. In order to supplement these results, research could be conducted that overexpress dFOXO instead of using *dFOXO* mutants. Indeed, this was attempted in this present study. However, overexpression of dFOXO yielded very small salivary glands that were difficult to maintain intact during the TEM preparations. As a result, the results were not included. Future experimental designs could attempt to accommodate this limitation and preserve the glands for visualisation.

Appendices

Appendices

Section I. *Drosophila* Fly Stocks Used

Table 1. Bloomington Stock order No. http://flystocks.bio.indiana.edu/

Stock #	Genotype	Comments
4847	w[1118]; P{w[+mC]=UAS-Ras85D.V12}TL1	Expresses activated Ras
6979	w[1118]; P{w[+mW.hs]=GawB}C147	GAL4 expressed in larval brain and salivary glands
9575	y[1] w[*]; P{w[+mC]=UAS-foxo.P}2	Expresses wild type dfoxo under UAS control
7193	w[*]; P{w[+mC]=sqh-EYFP-Golgi}3	Ubiquitously-expressed EYFP tagged with Golgi targeting sequence
7194	w[*]; P{w[+mC]=sqh-EYFP-Mito}3	Ubiquitously-expressed EYFP tagged with mitochondial targeting sequence
9575	y[1] w[*]; P{w[+mC]=UAS-foxo.P}2	Expresses wild type dfoxo under UAS control
9674	w[1118]; P{w[+mC]=UAS-dm.Z}132	Expresses wild type dm (Drosophila myc) under UAS control

Table 2. Fly stocks from M.Dionne.

Genotype	Chromosome	Comments and Reference
y w; ; FRT82[y+] foxo[21] / TM3, Sb[1] Ser[1]	3	foxo nulls, as reported by Junger et al in J Biol 2(3): 20
w[1118]; ; FRT82[y+] foxo[25] / TM6c, Sb[1]	3	foxo nulls, as reported by Junger et al in J Biol 2(3): 20
w[1118]; ; foxo[BG01018]	3	foxo hypomorph found (Dionne et al Curr Biol 16(20): 1977)

Table 3. Fly stocks from other sources.

Genotype	Chromosome	Comments and Reference
UAS-NOS2	1	Expresses the constitutively active mouse NOS2 protein under UAS control (This lab)

Section II. Salivary Gland Nuclei Measurements discussed in Chapter 4 (Raw Data)

The following is a table of the raw data for measurements of salivary gland nuclei visualised by DAPI staining. All glands were obtained from female third instar *Drosophila* larvae. All measurements were recorded at 40x magnification. The genotypes are identified below. (A-G)

A	B	C	D	E	F	G
23.93	7.97	23.12	14.21	16.6	20.36	15.2
23.11	8.55	20.61	16.41	15.89	18.43	13.32
25.54	12.2	20.3	15.76	17.79	19.32	13.96
21.58	9.65	22.8	12.45	17.32	16.56	12.99
24.61	9.27	19.12	14.64	16.98	17.73	13.22
22.34	10.24	21.9	14.98	15.65	20.53	13.88
25.94	9.96	19.45	13.76	19.9	17.43	13.31
23.76	8.99	23.41	15.67	16.53	16.9	12.96
23.76	7.16	24.45	15.2	18.31	17.32	16.28
21.34	9.99	20.94	11.98	20.4	20.52	14.4
24.14	11.32	20.23	14.82	15.42	20.32	15.31
22.36	8.51	21.18	15.32	17.21	18.92	14.18
20.28	10.31	24.9	14.43	16.93	19.93	14.13
22.97	7.78	20.23	15.35	16.65	21.82	13.97
25.21	10.52	20.31	14.21	15.89	18.24	14.65
23.27	11.32	24.15	16.2	16.21	19.89	12.99
21.52	8.93	22.11	15.17	17.31	21.67	13.45
25.87	11.1	22.18	13.77	18.2	18.93	15.52
22.59	13.14	22.5	16.46	19.5	18.52	14.23
22.34	11.8	23.86	14.9	15.95	19.98	14.54
21.95	10.65	18.89	14.29	16.8	21.83	13.33
22.51	8.58	21.32	13.98	17.31	22.46	13.21
20.31	11.58	22.42	13.41	17.23	23.1	12.93
26.63	10.6	23.56	15.76	18.24	19.98	12.22
24.14	10.12	20.49	14.68	18.93	19.58	12.21
24.3	10.88	18.93	13.69	20.43	22.34	15.98
23.18	8.59	25.21	12.79	17.83	22.54	15.54

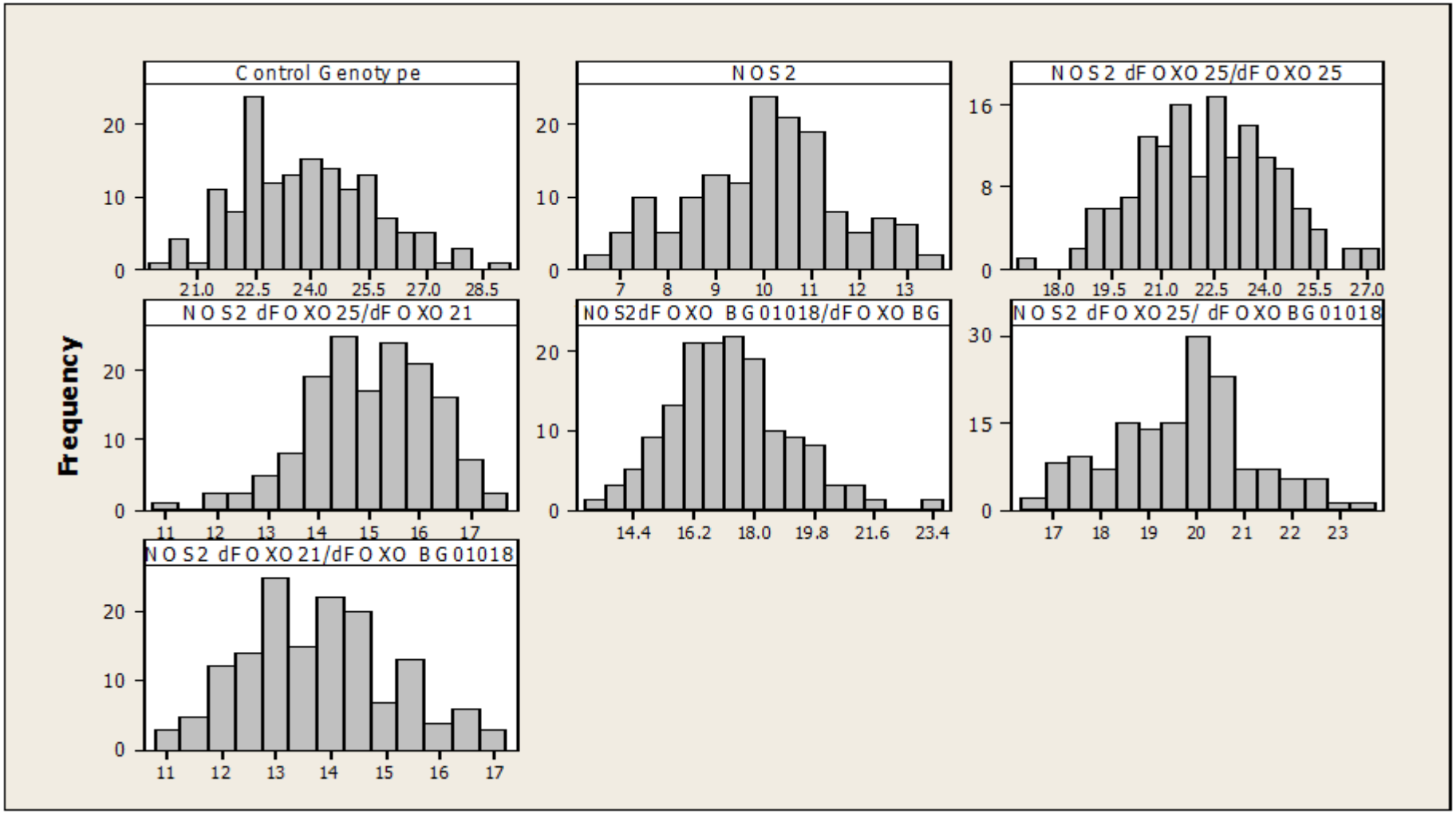
23.98	9.97	18.8	12.88	16.71	20.73	12.24
28.9	10.23	21.1	15.22	15.93	18.77	12.98
27.2	9.74	21.52	13.65	16.87	18.68	15.32
24.51	12.29	19.9	14.13	18.36	19.95	13.37
23.8	7.65	24.3	12.03	17.44	20.38	13.98
25.82	10.3	23.73	14.66	13.95	20.94	12.21
27.1	7.33	21.32	14.27	17.62	19.9	11.31
25.2	12.94	23.89	16.2	13.98	18.84	14.36
23.24	11.15	18.93	13.58	19.21	16.5	13.43
22.37	9.21	20.8	12.63	17.31	19.94	15.36
22.8	11.3	23.86	13.99	18.11	17.93	13.57
25.98	6.77	16.94	14.52	16.84	20.5	14.71
22.51	10.19	19.8	15.87	17.54	18.69	14.46
21.4	10.73	19.54	15.24	16.31	19.91	12.87
22.56	7.88	24.25	12.98	16.99	19.94	12.65
24.45	7.41	24.67	13.76	18.41	19.8	15.32
24.65	7.9	23.51	15.63	17.76	16.9	12.7
22.1	10.55	23.87	13.76	15.31	18.93	14.45
25.12	8.92	22.31	14.17	15.95	19.9	14.21
22.96	9.95	25.2	14.74	16.41	19.68	12.87
25.51	7.65	24.21	15.66	13.86	19.62	11.97
21.56	10.9	20.49	15.11	19.98	17.91	12.31
23.43	9.84	23.3	16.36	19.21	16.93	16.67
25.43	13.47	25.2	12.89	17.79	20.7	12.67
22.4	10.2	22.68	15.12	19.76	19.93	13.34
27.93	10.16	23.63	16.29	17.98	20.73	14.51
23.19	8.79	18.92	15.44	16.26	20.4	13.83
23.41	9.44	22.5	14.87	20.76	21.38	13.21
23.76	10.19	21.81	15.74	14.98	18.74	12.19
27.2	9.5	19.89	14.31	15.21	16.96	14.32
20.68	9.34	19.94	16.47	19.45	19.12	13.21
21.7	10.14	24.2	14.55	18.6	19.32	14.42
21.62	6.65	24.23	15.94	15.23	20.27	14.2
26.7	9.11	22.1	16.24	17.44	20.23	11.98
22.16	11.23	21.96	14.51	14.1	18.9	12.96
22.4	12.2	23.94	13.69	18.81	19.95	14.32
25.59	10.11	22.83	15.31	17.96	19.25	14.43
23.4	12.28	21.1	15.88	18.46	20.93	14.23
22.51	12.7	24.5	14.61	17.25	19.96	15.2
22.61	10.8	20.59	16.72	16.31	21.6	13.21
24.83	10.45	20.84	14.55	19.41	17.43	12.21
21.57	10.2	22.83	12.89	17.11	17.98	13.98
25.13	7.57	18.41	17.12	14.92	21.9	16.7
27.2	10.57	24.56	15.3	18.97	23.45	13.98
21.44	9.4	21.45	15.74	15.65	16.96	12.25

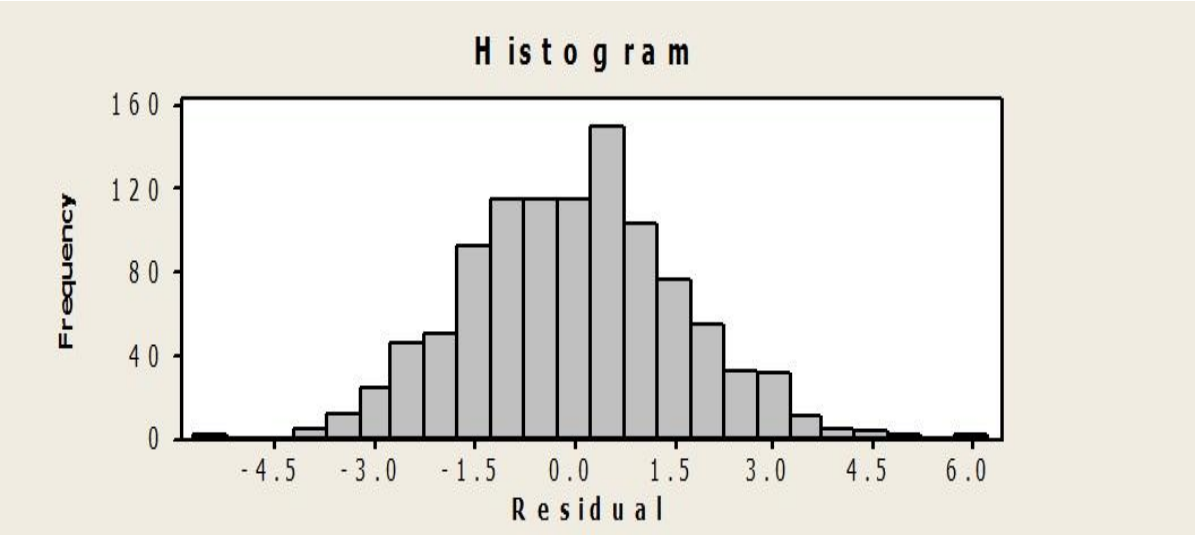
21.59	10.14	22.67	17.54	18.21	18.15	12.31
22.89	11.19	23.3	14.39	16.41	21.43	12.97
25.41	11.1	21.4	16.17	13.22	17.4	13.66
21.76	10.37	23.23	15.41	16.72	19.58	13.98
23.6	7.2	21.67	16.44	15.22	19.7	12.66
23.34	10.3	21.24	13.8	14.21	19.9	15.42
22.67	10.29	20.4	14.98	15.93	18.32	13.52
23.45	8.59	22.38	15.39	15.26	18.45	12.9
21.8	12.34	21.28	17.2	17.12	20.34	16.53
22.51	10.41	24.2	14.9	16.62	17.83	15.21
22.9	9.63	19.32	15.3	16.63	16.93	15.32
23.2	7.65	20.34	16.54	17.98	18.8	14.21
22.2	7.79	19.43	14.15	15.85	17.1	12.98
25.32	9.36	20.32	17.1	18.32	19.43	12.09
24.88	10.81	26.64	15.1	17.54	19.93	13.3
24.5	11.19	21.2	13.35	16.98	18.66	12.02
24.61	8.8	24.3	14.98	15.98	19.93	14.76
25.31	10.1	22.34	16.58	16.79	18.45	16.93
22.8	8.83	24.32	14.31	17.89	19.93	15.95
19.93	6.99	23.1	16.69	15.89	20.8	12.72
22.56	10.31	21.2	16.22	16.6	16.9	15.54
24.5	12.6	25.25	13.99	18.21	18.43	15.74
25.61	9.16	24.45	15.43	17.31	20.72	12.74
20.93	10.44	23.53	14.51	19.54	17.25	13.94
22.32	9.63	21.38	17.55	16.21	18.63	12.56
23.53	9.25	20.63	15.41	16.51	19.63	13.52
24.9	10.79	24.21	14.71	17.98	20.23	15.6
26.2	8.54	23.13	16.98	19.2	18.96	14.43
20.65	8.96	23.43	16.41	18.98	22.58	14.33
24.2	7.61	23.51	13.89	17.71	18.88	13.37
24.97	10.95	22.47	15.78	16.21	18.7	14.55
21.73	6.65	22.53	14.42	18.21	19.93	13.32
22.41	9.95	20.72	14.21	15.96	20.79	15.21
21.88	10.87	19.51	15.76	18.21	20.6	16.98
22.27	10.1	25.51	14.6	19.9	19.42	15.32
23.49	8.62	25.21	15.98	20.96	19.97	12.99
24.12	13.14	22.5	14.67	15.62	21.85	12.94
24.43	11.12	25.51	13.9	17.31	20.34	15.19
23.61	7.21	21.62	14.87	16.32	19.89	14.66
22.7	11.34	21.52	16.21	14.44	21.29	12.39
25.71	12.95	27.14	15.91	15.9	21.8	12.43
25.51	12.3	21.6	13.86	16.31	17.73	12.95
23.87	9.95	23.41	15.46	17.5	18.59	13.21
25.51	8.58	21.23	14.6	18.96	19.93	14.32
24.7	13.1	22.8	16.23	16.89	20.8	13.41

	25.29	12.23	22.61	15.92	19.92	21.7	14.2
	23.66	10.62	26.32	15.27	18.32	19.53	16.32
	24.33	10.54	19.3	15.43	16.32	22.29	12.62
	22.41	8.67	20.59	16.52	17.31	18.87	14.33
	23.9	10.52	22.98	14.59	14.71	18.67	14.21
	24.23	10.43	24.44	15.21	17.65	20.52	11.19
	25.18	11.28	21.9	16.28	17.14	21.16	13.98
	26.12	8.99	22.83	13.63	15.21	20.59	11.49
	22.44	7.63	25.21	14.34	16.21	20.39	15.76
	23.49	10.26	22.73	16.45	14.92	19.44	14.48
	23.76	10.86	21.16	15.92	15.31	17.93	12.97
	28.23	10.1	22.41	15.69	16.41	21.24	12.34
	22.41	12.5	23.73	16.41	15.39	19.94	12.2
	22.1	9.93	21.41	13.98	20.95	21.4	14.21
	27.32	12.9	22.6	13.62	16.21	20.36	13.21
	26.2	10.98	23.41	11.21	15.61	18.98	11.21
	27.9	10.81	18.4	16.13	16.95	19.9	14.11
	23.33	11.98	22.73	15.96	16.93	20.58	16.15
	24.41	13.4	22.61	15.31	15.42	17.66	12.95
	23.77	9.46	21.85	13.98	20.21	20.47	11.34
	24.62	10.93	26.77	15.65	21.4	20.13	15.6
	22.88	7.56	20.21	16.9	17.43	19.63	10.98
	22.26	8.33	25.4	15.97	23.4	18.62	13.85
	24.94	10.21	21.94	15.47	14.41	19.96	12.21
	26.87	8.81	20.83	16.8	17.65	20.55	16.9
	26.41	11.2	21.7	16.89	19.66	19.1	16.38
	22.41	11.32	21.43	14.95	14.42	17.71	11.34
	26.33	7.53	23.13	16.55	17.9	19.47	14.39
	25.57	9.98	23.62	14.54	18.51	20.13	11.32
	26.3	8.9	21.34	14.64	17.97	18.98	15.21
	25.16	10.28	21.62	15.74	16.87	20.59	13.24
	24.39	11.66	20.55	15.36	18.3	20.23	13.21
Average =	**23.86**	**10.02**	**22.27**	**15.05**	**17.21**	**19.63**	**13.81**
Standard deviation =	**1.804617**	**1.573675**	**1.942176**	**1.196942**	**1.707855**	**1.442174**	**1.359224**

A: Control Genotype (c147-GAL4/+)
B: Overexpression of NOS2(*UAS-NOS2/*+; c147-GAL4/+)
C: UAS-NOS2,UAS-GFP/+;c147/+;dFOXO^{25}/ dFOXO^{25}
D: UAS-NOS2,UAS-GFP/+;c147/+;dFOXO^{25}/ dFOXO^{BG01018}
E: UAS-NOS2,UAS-GFP/+;c147/+;dFOXO^{BG01018}/ dFOXO^{BG01018}
F: UAS-NOS2,UAS-GFP/+;c147/+;dFOXO^{25}/ dFOXO^{21}
G: UAS-NOS2,UAS-GFP/+;c147/+;dFOXO^{21}/ dFOXO^{BG01018}

III. Raw Data from Statistical Analysis of Salivary Gland Nuclei measurements





One-way ANOVA: DATA versus GENES

```
Source    DF        SS       MS        F      P
Factor     6  21347.17  3557.86  1394.13  0.000
Error   1036   2643.90     2.55
Total   1042  23991.07

S = 1.598   R-Sq = 88.98%   R-Sq(adj) = 88.92%



Level                          N    Mean  StDev
Control Genotype             149  23.860  1.805
NOS2                         149  10.020  1.579
NOS2 dFOXO25/dFOXO25         149  22.267  1.945
NOS2 dFOXO25/dFOXO21         149  15.052  1.201
NOS2 dFOXO BG01018/dFOXO     149  17.212  1.714
NOS2 dFOXO25/ dFOXOBG010     149  19.625  1.447
NOS2 dFOXO21/dFOXO BG010     149  13.809  1.364

                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level                        ------+---------+---------+---------+---
Control Genotype                                              (*
NOS2                         (*)
NOS2 dFOXO25/dFOXO25                                    (*
NOS2 dFOXO25/dFOXO21                      (*
NOS2 dFOXO BG01018/dFOXO                       (*)
NOS2 dFOXO25/ dFOXOBG010                            (*)
NOS2 dFOXO21/dFOXO BG010            (*
                             ------+---------+---------+---------+---
                                12.0      16.0      20.0      24.0

Pooled StDev = 1.598


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 99.67%
```

Control Genotype subtracted from:

```
                            Lower   Center    Upper
NOS2                       -14.385  -13.839  -13.294
NOS2 dFOXO25/dFOXO25        -2.139   -1.593   -1.047
NOS2 dFOXO25/dFOXO21        -9.354   -8.808   -8.262
NOS2 dFOXO BG01018/dFOXO    -7.193   -6.647   -6.102
NOS2 dFOXO25/ dFOXOBG010    -4.780   -4.235   -3.689
NOS2 dFOXO21/dFOXO BG010   -10.596  -10.051   -9.505

                              -+---------+---------+---------+--------
NOS2                          (*)
NOS2 dFOXO25/dFOXO25                          (*)
NOS2 dFOXO25/dFOXO21                 *)
NOS2 dFOXO BG01018/dFOXO                (*
NOS2 dFOXO25/ dFOXOBG010                   (*)
NOS2 dFOXO21/dFOXO BG010           (*
                              -+---------+---------+---------+--------
                            -14.0      -7.0       0.0       7.0
```

NOS2 subtracted from:

```
                            Lower  Center   Upper
NOS2 dFOXO25/dFOXO25       11.701  12.247  12.792
NOS2 dFOXO25/dFOXO21        4.486   5.031   5.577
NOS2 dFOXO BG01018/dFOXO    6.646   7.192   7.738
NOS2 dFOXO25/ dFOXOBG010    9.059   9.605  10.151
NOS2 dFOXO21/dFOXO BG010    3.243   3.789   4.335

                              -+---------+---------+---------+--------
NOS2 dFOXO25/dFOXO25                                             *)
NOS2 dFOXO25/dFOXO21                                    (*)
NOS2 dFOXO BG01018/dFOXO                                   (*)
NOS2 dFOXO25/ dFOXOBG010                                       (*)
NOS2 dFOXO21/dFOXO BG010                                *)
                              -+---------+---------+---------+--------
                            -14.0      -7.0       0.0       7.0
```

NOS2 dFOXO25/dFOXO25 subtracted from:

```
                            Lower  Center   Upper
NOS2 dFOXO25/dFOXO21       -7.761  -7.215  -6.669
NOS2 dFOXO BG01018/dFOXO   -5.600  -5.055  -4.509
NOS2 dFOXO25/ dFOXOBG010   -3.188  -2.642  -2.096
NOS2 dFOXO21/dFOXO BG010   -9.003  -8.458  -7.912

                              -+---------+---------+---------+--------
NOS2 dFOXO25/dFOXO21                    (*
NOS2 dFOXO BG01018/dFOXO                   (*)
NOS2 dFOXO25/ dFOXOBG010                      (*)
NOS2 dFOXO21/dFOXO BG010              (*)
                              -+---------+---------+---------+--------
                            -14.0      -7.0       0.0       7.0
```

```
NOS2 dFOXO25/dFOXO21 subtracted from:

                            Lower  Center   Upper
NOS2 dFOXO BG01018/dFOXO   1.615   2.161   2.706
NOS2 dFOXO25/ dFOXOBG010   4.028   4.573   5.119
NOS2 dFOXO21/dFOXO BG010  -1.788  -1.243  -0.697

                              -+---------+---------+---------+--------

NOS2 dFOXO BG01018/dFOXO                              (*)
NOS2 dFOXO25/ dFOXOBG010                                 (*
NOS2 dFOXO21/dFOXO BG010                          (*)
                              -+---------+---------+---------+--------
                            -14.0      -7.0       0.0       7.0


NOS2 dFOXO BG01018/dFOXO BG0 subtracted from:

                            Lower  Center   Upper
NOS2 dFOXO25/ dFOXOBG010   1.867   2.413   2.959
NOS2 dFOXO21/dFOXO BG010  -3.949  -3.403  -2.857

                              -+---------+---------+---------+--------
NOS2 dFOXO25/ dFOXOBG010                              *)
NOS2 dFOXO21/dFOXO BG010                       (*)
                              -+---------+---------+---------+--------
                            -14.0      -7.0       0.0       7.0


NOS2 dFOXO25/ dFOXOBG01018 subtracted from:

                            Lower  Center   Upper
NOS2 dFOXO21/dFOXO BG010  -6.362  -5.816  -5.270

                              -+---------+---------+---------+--------
NOS2 dFOXO21/dFOXO BG010                   (*
                              -+---------+---------+---------+--------
                            -14.0      -7.0       0.0       7.0
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