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Characterisation of the Regulation of Growth by Nitric Oxide Signalling in *Drosophila melanogaster*

A thesis submitted to the University of Sussex for the degree of
Doctor of Philosophy
September 2009
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.

Anna Scott
September 2009

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Abstract

'Characterisation of the Regulation of Growth by Nitric Oxide Signalling in *Drosophila melanogaster'*
A thesis submitted to the University of Sussex for the degree of Doctor of Philosophy
By Anna Scott
School of Life Sciences
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The molecular mechanisms that control growth appear to be conserved across the animal kingdom, with nitric oxide regulation of cell proliferation and growth being found to be very significant. Indeed, in *Drosophila* larval development and mammalian systems NO has been shown to be particularly important in these processes, and previous work in our laboratory has identified the *Drosophila* forkhead transcription factor dFOXO as a critical target through which NO signalling exerts its regulatory effects on growth, although little is currently known concerning the precise mechanisms involved.

Accordingly, in this thesis, we investigate the processes through which NO may modulate growth and demonstrate that targeted expression of a constitutively active NO Synthase to whole larval salivary glands or clones of cells within the glands, results in reduced endoreplication and growth as measured by nuclear size. Targeted over expression of dFOXO itself is shown to result in similar phenotypes, and subsequent molecular analysis of potential signalling targets required for this inhibition of growth reveals that dFOXO, Thor and Myc expression are regulated *in vivo* by NO.

To elucidate if NO acts directly on dFOXO, the genetic interaction of components of the insulin signalling pathway is analysed, exploiting RNA interference to assay what components are necessary for the NO signal to be effectively transduced, and it is demonstrated that NO control of growth is not through sGC, one of the most significant known targets for NO-mediated regulation in other organisms

We subsequently investigated the roles of Thor, a *Drosophila* 4E-binding protein, and the kinase, Lk6, homologues of which are known to be important in growth regulation in other organisms, and thus potential effectors of NO and dFOXO. However our data demonstrated that neither Thor nor Lk6 are required for the inhibition of growth by NO.

Interestingly a potential anti-oncogenic effect of NO signalling was also revealed following analysis of interactions between NO and Ras or Myc induced growth in which NO was able reduce the overgrowth produce by both these oncogenes.

Overall this research confirms dFOXO as an essential target for NO induced inhibition of growth. The work also eliminates two dFOXO transcription targets, *Thor* and *Lk6,* as necessary for NO to regulate growth.

Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Cdk	Cyclin dependent kinase
cGMP	cyclic GMP
CO_2	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DILPS	*Drosophila* insulin like peptides
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
Dpp	Decapentaplegic
dsDNA	Double stranded DNA
FAD	Flavin adenine dinucleotide
FBS	Fetal Bovine Serum
FLP	Flippase
FRT	Flippase recognition target site
FMN	Flavin mononucleotide
FOXO	Forkhead-related transcription factors
GAL4	Yeast protein that binds DNA
GFP	Green fluorescent protein
IGF	Insulin growth factor
InR	Insulin receptor
IRS	Insulin receptor substrate
L-NAME	Nω-nitro-L-arginine methyl ester
MAC	Macrophage
NADPH	Nicotinamide adenine dinucleotide phosphate
NGF	Nerve growth factor
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PBS	Phosphate buffered saline
PBT	Phosphate Buffered plus Triton

PI3K	Phospho-inositide 3–kinase
PIP_2	Phosphatidylinositol-4,5-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-triposphate
PTEN	Phosphatase and tensin homolog on chromosome 10
RNA	Ribonucleic Acid
RNAi	RNA interference
SD	Standard Deviation
SG	Salivary gland
sGC	Soluble Guanylate Cyclases
siRISC	siRNA-induced silencing complex
siRNA	Small interfering RNAs
SNAP	S-nitroso-N-acetyl-penicillamine
TOR	Target of rapamycin
TSC	Tuberous sclerosis complex
UAS	Upstream activator sequence
wt	Wild type

Chapter 1: General Introduction

1.1. Nitric Oxide

Nitric oxide (NO) is a reactive free radical gas, which is known to have many important physiological roles (Bruckdorfer 2005). NO acts as a short-lived intracellular and transcellular messenger, and has been seen to have many functions within vertebrates as well as invertebrates, acting as a major regulator in nervous, immune and cardiovascular systems (Schmidt & Walter 1994).

In this chapter I will give a basic introduction to NO focusing on its role in cell signaling within the fruit fly *Drosophila melanogaster*.

1.1.2. Nitric Oxide Synthase

In living systems, NO is synthesized from L-arginine in a reaction catalysed by nitric oxide syntheses (NOS) (Fig. 1.1) (Stuehr 1997). NOS acts by oxidising the guanidine group of L-arginine in a process which uses several cofactors and consumes five electrons, resulting in the formation of NO and L-citrulline (Bredt & Snyder 1994). Three different isoforms of NOS have been identified in mammals: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) (Bruckdorfer 2005). Although these nomenclature is used for the 3 isoforms, most mammalian cell types and tissues express one or more isoforms of NOS (Bruckdorfer 2005).

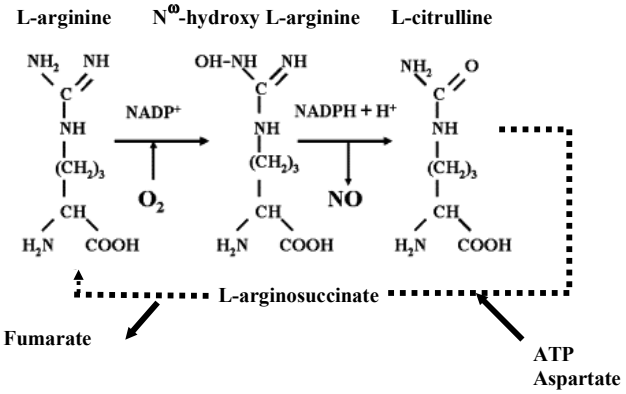


Figure 1.1. NO biosynthesis from L-arginine. The intermediate in the formation of NO, the enzyme bound N-hydroxy-L-arginine is shown. The dotted line shows recycling of L-citrulline back to L-arginine. Figure from (Bruckdorfer 2005).

1.1.3. Nitric Oxide function in vertebrate systems

In mammalian systems NO is known to modulate a range of physiological functions. These include: control of vascular tone (Palmer et al 1987), the functionality of neurons (Meffert et al 1994) reviewed in (Prast & Philippu 2001) and the immune response (Nathan & Hibbs 1991) reviewed in (Bogdan 2001). However, although NO has many roles as a biological messenger for the purpose of this thesis I intend to focus on its role in cell proliferation.

NO has been shown to inhibit the proliferation of many cell types, tumours and tumour cell lines in a wide variety of organisms. This action of NO on cell proliferation is reviewed in Villalobo 2006. Thirty nine cell types of differing tissue origin have demonstrated sensitivity to the inhibitory effect of exogenous NO. These tissues include muscle, endothelial, epithelial, lymphocyte and neural progenitor cells (Villalobo 2006). As well as these normal cell types, exogenous NO can also inhibit the proliferation of tumor cell lines that include those derived from breast cancer (Pervin et al 2001), colon cancer (Jarry et al 2004) and neuroblastoma cells (Murillo-Carretero et al 2002).

The inhibitory effect on proliferation by NO has been shown many times but the mechanism(s) by which NO is inducing this effect is not clearly understood. To date, many studies have focused on how NO blocks the progression of the cell cycle. In many cell types NO has been shown to inhibit the G_1 to S transition there by resulting in G_1 arrest (Fig. 2.) (Gansauge et al 1998, Wanga et al 2007)

However, previous work has demonstrated that NO does not solely control proliferation, but that it also acts to balance cell proliferation and differentiation events (Kuzin et al 1996) (Peunova & Enikolopov 1995). For example, in brain development in the African clawed toad, *Xenopus laevis*, transitions of neural precursor cells from proliferation to differentiation determine the distinct anatomical features of the brain. It is thought that NO may mediate this transition as an area of NOS-expressing cells lies adjacent to the zone of dividing neuronal precursors, and when NOS is inhibited, larger brains which are not correctly organized, are produced (Peunova et al 2001).

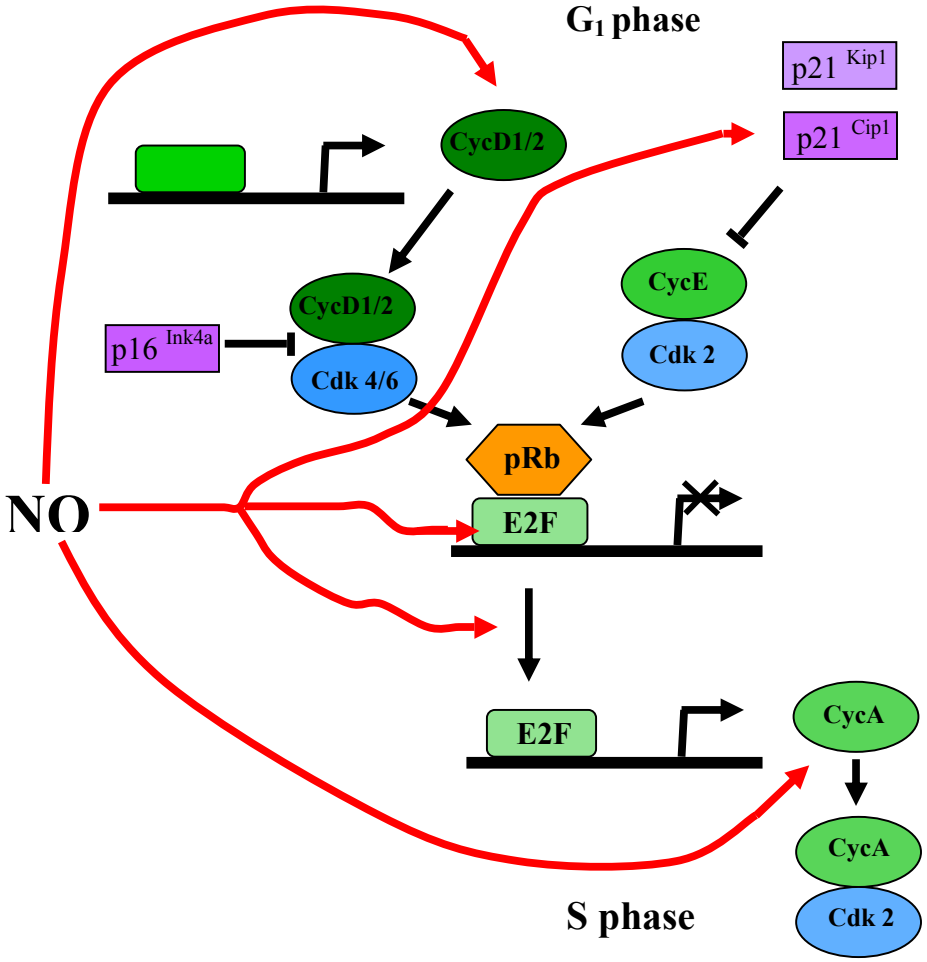


Figure 1.2. Major effects of NO inhibition of the G_1/S transition of the cell cycle (adapted from Villalobo 2006). During normal cell cycle progression, up-regulation of cyclins D1 and D2 (CycD1/2) prepares the stage for activation of Cdk4/6 during the G1 phase. Cdk4/6, activated by CycD1/2, plus Cdk2, in turn activated by cyclin E (CycE), phosphorylate the retinoblastoma protein (pRb) at multiple sites, releasing the repressor activity exerted on transcription factors of the E2F family. These transcription factors control, among others, the expression of cyclin A (CycA), which by activating Cdk2 is required for the progression of the cell cycle to its S phase. The inhibitory action of p16Ink4a, acting on the CycD1/2–Cdk4/6 complexes, plus p27Kip1 and p21Cip1/Waf1, both acting on the CycE–Cdk2 complex, are also indicated. Major targets of NO relevant to inhibition of cell cycle progression at the G1/S transition, including the down-regulation of cyclin D and cyclin A expression, the up-regulation of the expression of both the inhibitory protein p21Cip1/Waf1 and the transcription factor E2F, and the dephosphorylation of pRb, are also shown (Villalobo 2006).

1.1.4. NO activity and soluble guanylate cyclase

Some of the molecular actions of NO are known to be due to the formation of complexes between NO and the metallo prosthetic groups of various proteins/enzymes, for example, with the haem group of soluble guanylate cyclase (sGC) (Stamler 1994). sGC is responsible for the formation of cyclic GMP from the nucleotide GTP. cGMP acts a secondary messenger, which

goes on to signal through cGMP-dependent protein kinases or PKGs. Active forms of PKG can phosphorylate many substrates including the IP3 receptor and subunits of myosin light chain phosphatase (Krumenacker et al 2004). The basal activity of sGC can be increased up to 200-fold by the binding of NO, but the NO-heme complex has a half-life around 0.2s (Bruckdorfer 2005).

1.1.5. NOS in *Drosophila melanogaster*

The *Drosophila melanogaster* NOS gene was cloned and characterised by screening a phage library of the *Drosophila* genome using a fragment of rat nNOS gene (Regulski & Tully 1995). In *Drosophila*, NOS is encoded by a single *dNOS* gene, found on the second chromosome at the position 32B (Regulski & Tully 1995). The major product of the gene is the dNOS1protein, which bears extensive homology to all three of the mammalian NOS isoforms but shows highest homology with the neuronal NOS (nNOS) with 43% amino acid sequence identity (Regulski & Tully 1995). As with all mammalian NOS enzymes, the dNOS protein contains putative binding sites for calmodulin, FMN, FAD, and NADPH, and dNOS activity is Ca2+/calmodulin dependent when expressed in cell culture (Regulski & Tully 1995).

1.1.6. NOS expression in *Drosophila*

Across a variety of insect species the NADPH-diaphorase reaction has been widely used as a histochemical marker for NOS. The basis of this assay is the detection of an insoluble formazan precipitate formed by the NADPH diaphorase enzyme. Using NADPH-diaphorase, the activity of NOS has been followed through *Drosophila* larval development (Kuzin et al 1996). NOS activity is seen in high levels in the developing imaginal discs with staining becoming more intense as development proceeds. The wing, eye, haltere, and genital discs in the third instar show distinct and reproducible patterns of intense staining which then gradually decreases in a specific spatial pattern during early pupal development (Kuzin et al 1996).

1.1.7. NO function in development

NO has also been shown to inhibit cell proliferation in other organisms, as well as in mammalian systems. In 1995, Peunova and Enikolopov (Peunova & Enikolopov 1995) observed that the growth arrest and differentiative effect of nerve growth factor (NGF) on

PC12 cells was mediated by NO. It was shown that NOS expression was induced by NGF along side differentiation, that NO acted as a cytostatic agent in neural cells and that if NOS was inhibited it stopped the NGF-induced proliferation decrease, and in turn prevented differentiation of cells towards neuronal phenotype (Peunova & Enikolopov 1995). This finding indicated that NO act as a negative regulator of proliferating neuroblasts, with a role in neuronal differentiation. This role results in neuroblasts being able to escape from their state of proliferation, thereby allowing their differentiation towards a neuronal phenotype (Contestabile & Ciani 2004). The antiproliferative action of NO has not only been demonstrated in neuronal precursor cells. Kuzin *et al*. (1996) showed that in *Drosophila*, NO negatively regulates cell proliferation of imaginal discs in late larval stages, with an increase in NOS activity levels, coinciding with temporal cytostasis. In this instance NOS inhibition results in excess cell proliferation and hypertrophy of organs. In contrast, ectopic expression of NOS was seen to have the opposite effect, causing hypotrophy (Kuzin *et al* 1996). These results together have shown NO acts as an antiproliferative agent, playing an important role in development, allowing cell differentiation to occur by halting proliferation. This function of NO is further supported by its action on DNA replication. NO has been identified as acting on DNA synthesis by Garg and Hassid 1989, with NO-generating vasodilators effectively inhibiting DNA synthesis and proliferation of rat aortic smooth muscle cells. Similarly, Kuzin *et al* saw that regions of developing imaginal tissue with high levels of NOS, showed reduced DNA synthesis as determined by BrdU incorporation (Kuzin *et al* 1996). Kuzin *et al* also found that manipulation of endogenous or transgenic NOS activity in imaginal discs during eye disc development was able to enhance or suppress the effects of RBF and E2F on development of the eye. This suggested a role for NO in the developing imaginal eye disc via interaction with the retinoblastoma (Rb) pathway (Kuzin et al 2000).

Drosophila NOS nulls created have been reported to be homozygous late embryonic and larval lethal, showing the essential role of NOS within *Drosophila* development (Regulski *et al* 2004).

NO has been also been identified as playing a role in visual system development through another pathway (Gibbs *et al* 2001). During the development of photoreceptors within *Drosophila,* there is a period of NO-sensitive soluble guanylate cyclase (sGC) production (just before functional connections with the optic lobe interneurons are made), which in turn stimulates production of cGMP that can then interact with other molecular targets (Gibbs *et al*

2001). The inhibition of either NO or sGC during this time results in disorganisation in retinal projection pattern, and leads to the overgrowth of the retinal axons.
NO has also been shown to be involved in development in other insect species. In the moth *Manduca sexta* the suppression or the removal of NO effects cell proliferation and optic lobe development (Champlin & Truman 2000).

1.2. Cell Proliferation and Growth Control

Although much is known about NO and its mediation of cell proliferation, little is known about the mechanism involved (Villalobo 2006).
In this thesis the molecular and cellular mechanisms by which NO induces growth inhibition will be investigated. Accordingly, the nature of cell proliferation and growth control within the context of *Drosophila* will first be introduced.

It can be seen that body plans between different species can vary considerably, but growth control is an evolutionarily conserved process with many similarities across different organisms. An understanding of growth control is important, not only to help better understand how genes are able to control animal size, but also in the context of disease, as when regulation of growth goes wrong it can result in numerous diseases including cancer (Weinkove & Leevers 2000).
A simple definition of growth is as an accumulation of mass which can be either an increase in cell number or size or both. Cell proliferation is the increase in cell number, whereas increase in cell size requires biosynthesis. Cells are able to couple both the control of growth and cell proliferation, in order for tissues to develop. Cells multiply under the control of the cell cycle, which acts to ensure DNA is replicated before mitosis. Usually an accumulation of mass is followed by subsequent cell division, with animal and organ growth being generally accompanied by increases in cell number, not cell size (Weinkove & Leevers 2000). However, this is not the case with certain larval tissues of *Drosophila.*

Drosophila is useful model organism for the study of growth. *Drosophila* has a short generation time, as well as being easily and inexpensively cultured. As a consequence much work has been done with *Drosophila* which has considerably contributed to our knowledge on

growth regulation and elucidation of many of the cell-signalling components required. *Drosophila* larvae contain imaginal discs, which are epithelia organs that reorganize to generate adult epidermal structures, these include wings, eyes and legs (Demerec 1950). The imaginal discs have been utilised to study growth control providing a model of proliferating tissue for research. *Drosophila* imaginal discs accumulate mass and cell number and can be studied during the larval instar phases before metamorphosis. Imaginal discs not only provide a cellular model system but also a model which incorporates the types of cell-cell communication networks that occur in other tissues. This results in a significantly more sophisticated model than would be provided by a homogenous population of tissue culture cells. Using *Drosophila* imaginal wing discs, proliferation and growth have been extensively studied. An example of such work is that of Neufeld *et al* where stimulatory and inhibitory components of the cell cycle were over-expressed. They then found that within the wing disc tissue, compensatory mechanisms exist to keep growth and cell proliferation regulated (Neufeld et al 1998).

Growth and cell cycle progression can also be studied using endoreplicating cells in tissues whose patterning has already been established. Endoreplicating cells undergo a modified cell cycle, in which cellular DNA synthesis occurs without subsequent mitosis. The continued DNA replication increases nuclear size and cell size throughout larval development. Endoreplicated cells in which the sister chromatids remain closely associated are referred to as polytene (Edgar & Orr-Weaver 2001). In *Drosophila* larvae, salivary gland cells are endoreplicating which results in large cells with polytene chromosomes which have up to 2048 copies of the euchromatic genome neatly aligned in parallel arrays (Edgar & Orr-Weaver 2001). Endoreplicating cells have been shown to be sensitive to nutritional control with numerous genes required for protein synthesis being vital for endocycling (Britton & Edgar 1998, Galloni & Edgar 1999). Salivary glands can be used to study growth in *Drosophila* as due to the large cell size, any change in protein expression and change in size is relatively easily determined.

In order to properly consider Nitric Oxide signaling in the context of growth control, an understanding of the components of cellular signaling pathways known to regulate growth is important. These are discussed below.

1.2.1. Ras

Ras is a proto-oncogene, and it has been estimated that 30% of all human tumors contain an activating mutation in Ras (Vojtek & Der 1998). Ras is a guanine nucleotide binding protein, a GTPase. As with all GTPases, it is controlled by the GDP/GTP cycle. Ras is inactive when bound to GDP but becomes active when bound to GTP. Ras in the active GTP bound form acts in signal transduction pathways to transmit extracellular signals from receptor tyrosine kinases to downstream serine/threonine kinase targets. These serine/threonine kinases include Raf (c-Raf-1, A-Raf, and B-Raf), MEK (MAPK/ERK kinases 1 and 2), and ERK1/2 (Vojtek & Der 1998).

In *Drosophila,* Ras 85D has been found to promote growth in several tissues. Clones of cells in wing discs expressing activated Ras ($Ras^{V12)}$ have increased cell and clone size when compared to wild type controls (Prober & Edgar 2000). In the *Drosophila* eye disc Ras has been shown to mediate growth, survival and differentiation through MAPK activity (Halfar et al 2001). Ras is also capable of controlling levels of other growth effectors such as dMyc, another proto-oncogene (Prober & Edgar 2000). The transcription factor dMyc has similar effects on growth to Ras, and wing disc cells expressing Ras^{V12}, an activated form of Ras, show increased levels of dMyc protein (Prober & Edgar 2000). Ras has effects on multiple pathways, including those involving phosphoinositide 3-kinase (PI3K) (Prober & Edgar 2000). However, Ras is not required for dPI3K signaling although it is required to maintain normal levels of dMyc protein. In salivary glands Ras^{V12} has also been seen to cause significant cell growth, which results in large over-grown salivary glands (Berry & Baehrecke 2007).

1.2.2. Myc

Myc is part of a network of transcription factors which act in a wide variety of processes including growth and proliferation, differentiation, apoptosis and oncogenesis (Pierce et al 2004). This transcription network is known as the Max transcription factor network, it is made up of a group transcription factors that share two common features. Firstly they contain a basic-helix-loop-helix-leucine zipper (bHLHZ) motif which mediates protein-protein binding and DNA binding (Grandori et al 2000). Secondly, they all form individual heterodimers with Max which is also a bHLHZ protein (Grandori et al 2000). Myc acts in this Max network by binding with Max, and in turn the heterodimer formed binds DNA and activates transcription.

Myc target genes include those involved in cell metabolism, ribosome biogenesis and translation control. Myc can also act to repress transcription by forming an inhibitory complex with Miz-1 (Pierce et al 2004). The Max network also includes the Mxd family of proteins. Mxd proteins can also bind to Max and DNA, acting as antagonists of the Myc function (Grandori et al 2000).

Drosophila has one ortholog to the mammalian Myc transcription factor dMyc. dMyc is encoded by the *diminutive* gene (*dm*) (Secombe et al 2004). In *Drosophila,* as in mammalian cells, dMyc acts as regulator of growth and proliferation. When the expression of dMyc is reduced in *Drosophila* it results in a reduction in the size of both cells and animals (Neufeld et al 1998, Pierce et al 2004). Conversely, when dMyc is over expressed in *Drosophila* it results in an increase in size of cells with both mitotic and endoreplicating cells, including those of the salivary gland, being larger than normal (Grewal et al 2005, Neufeld et al 1998, Pierce et al 2004). dMyc has also been shown to be a regulator of rRNA synthesis and ribosome biogenesis during larval development (Grewal et al 2005).
Myc has been identified as both a direct and indirect target for the transcription factor dFOXO, with *myc* mRNA levels being controlled by dFOXO in a tissue-specific manner. dFOXO can inhibit or increase *myc* expression (Teleman et al 2008). FOXO has been shown to suppress Myc driven proliferation (Bouchard et al 2007) and Myc dependent gene expression in cell culture (Delpuech et al 2007).

1.2.3. Insulin Signaling Pathway

The insulin pathway is one of the most intensively studied growth-regulatory signaling pathways and is conserved from *C. elegans* to mammals (Nelson & Padgett 2003). Insulin is categorised as an IGF-1 growth factor. The binding of insulin to the insulin receptor activates the insulin-signaling pathway. The binding of insulin initiates a cascade of events which results in phosphorylation of several adaptor proteins, including the insulin receptor substrates (IRS) (Casas-Tintoa et al 2007).
Reduction in the insulin signal in *Drosophila* tissues has been shown to result in reduced disc growth, disc size and reduce size and DNA content of endoreplicating cell. Also mutants in the insulin signaling pathway have both reduced larval and adult fly size (Britton et al 2002). In contrast, if the pathways activity is enhanced, growth and cell size are increased.

Unsurprisingly, it is easier to get a clear idea of how each individual step works by breaking the insulin pathway down into individual components.

The components of insulin signaling in the context of *Drosophila* are shown in Figure 1.3. and will be outlined below. It is important to note that the insulin pathway is highly conserved between *Drosophila* and mammals.

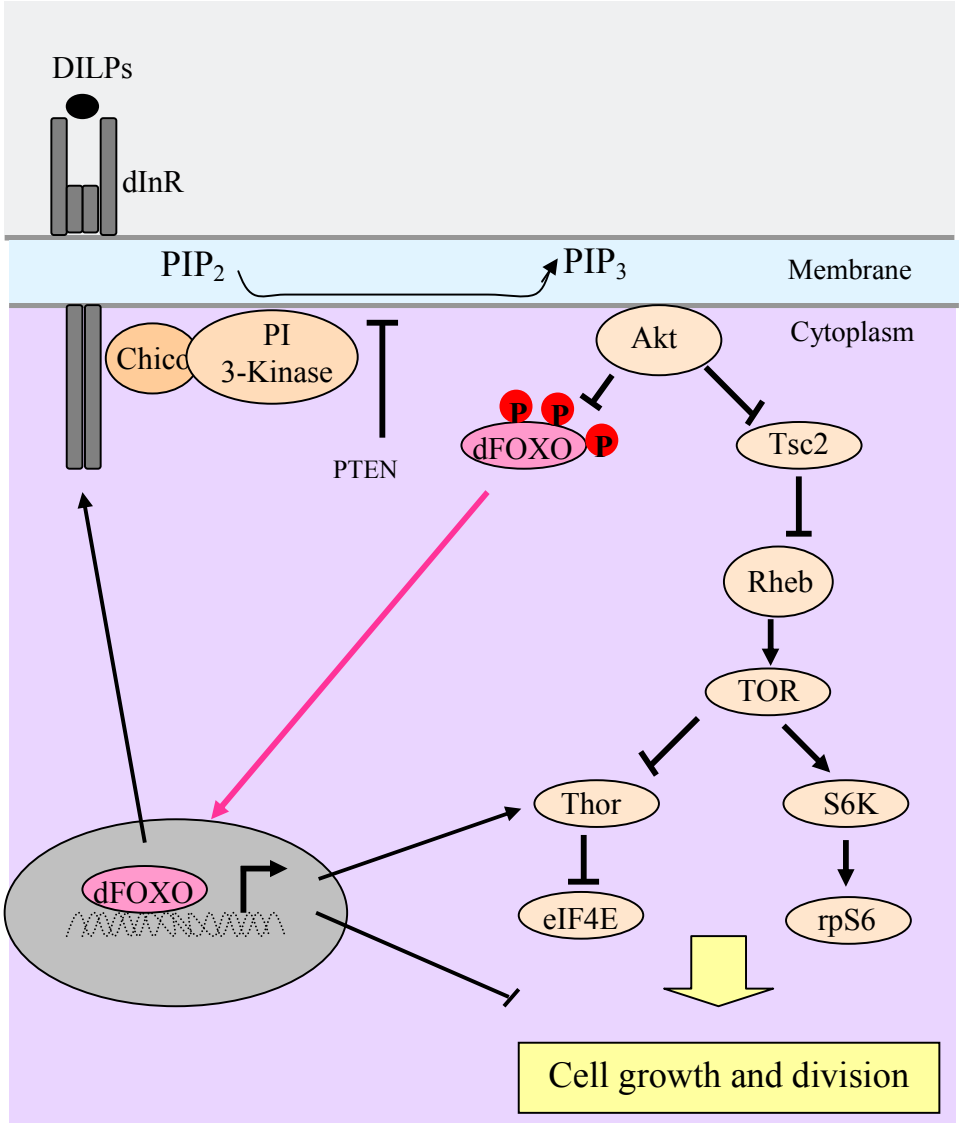


Figure 1.3. Insulin signaling pathway in *Drosophila* (adapted from(Neufeld 2003)). The dFOXO protein mediates a transcriptional response to insulin signaling. Under conditions of abundant nutrients, dFOXO is phosphorylation by Akt and retained in an inactive state in the cytoplasm. When insulin levels fall, dFOXO is dephosphorylated and translocated into the nucleus (pink arrow), where it stimulates transcription of Thor and presumably other negative regulators of growth. In addition, active

dFOXO increases expression of the insulin receptor gene, which may result in increased insulin sensitivity under low insulin conditions (Neufeld 2003).

1.2.3.1. *Drosophila* Insulin receptor and Peptides

The *Drosophila* homolog of the insulin/IGF1 receptor (dInR) is essential for normal growth (Brogiolo et al 2001, Fernandez et al 1995). dInR is a tyrosine kinase receptor which, on binding with *Drosophila* insulin-like peptides (DILPs), results in the autophosphorylation of the dInR which then phosphorylates insulin receptor substrate (IRS) proteins. *Drosophila* has seven insulin like peptides, dilp1 to 5 show the most significant homology to mouse and human insulins (Rulifson et al 2002). Dilp2 has been demonstrated to have a genetic interaction with *dInR* (Brogiolo et al 2001).

The sequence similarity between the human and *Drosophila* receptors is also high, and dInR is able to bind mammalian insulin with high affinity (Garofalo 2002). All described alleles of *dInR* are recessive, and homozygous embryonic or early larval lethal. It has been shown only weak heteroallelic combinations of *dInR* alleles were found to be viable and yield adults, although these animals are developmentally delayed, with small body size, and female sterility (Brogiolo et al 2001, Fernandez et al 1995). *dInR* expression is regulated by a feedback mechanism whereby the transcription factor, dFOXO, which lies downstream of dInR in the insulin signaling pathway can stimulate its expression (Casas-Tintoa et al 2007, Puig et al 2003, Puig & Tjian 2005).

A *Drosophila* IRS protein, called Chico, has been described by Böhni et al, 1999, and *Chico* mutant flies, like dInR mutants, are smaller in size due to a reduction in cell size and cell number (Böhni et al 1999).

Targeted ablation of the *dilp* expressing neurons in the larval brain results in an undergrowth phenotype similar to that found with *Chico* mutants (Rulifson et al 2002). Similarly overexpression of *dilps* results in significant increase in body size (Ikeya et al 2002).

1.2.3.2. PI3K and PTEN

Binding of DILPs to dInR induces phosphorylation of the insulin receptor substrates, activating phospho-inositide 3–kinase (P13K). Activated PI3Ks phosphorylate inositol lipids at the 3′ position, generating a variety of secondary messengers. The most critical secondary messenger produced by PI3K is generated when phosphatidylinositol-4,5-P_2 (PIP_2, mostly found in lipid

membranes) is phosphorylated in the third position, producing phosphatidylinositol-3,4,5-P_3 (PIP_3) (Britton et al 2002).

PI3K is a heterodimer made up of a catalytic subunit (Dp110) and an adaptor subunit (Dp60) (Leevers et al 1996). Dp110 protein is homologous to class I mammalian PI3Ks, p110α and p110β. Overexpression of Dp110 in wing or eye imaginal discs of *Drosophila* larvae results in flies with enlarged wings or eyes respectively. Overexpression of mutated Dp110 containing a mutation predicted to result in the loss of catalytic activity results in smaller wings and eyes (Leevers et al 1996).

A fusion protein called tGPH (tubulin-GPH) was developed during a study looking at PI3K activity *in vivo* in *Drosophila*. The fusion protein contains the pleckstrin homology (PH) domain of the *Drosophila* homolog of the general receptor for phosphoinositides-1 (GRP1) fused to green fluorescent protein (GFP), generating a protein called GPH (**G**FP-**PH** domain) (Britton et al 2002). PH domains bind specifically the secondary messenger, PIP_3. PIP_3 generally resides in lipid membranes, particularly the plasma membrane, GRP1 is recruited to membranes when PI3-kinase activity raises cellular levels of PIP_3. Accordingly high PI3K activity typically results in movement of GPH to the cell membranes (Britton et al 2002).

The effects of PI3K activity are counteracted by the action of the tumour suppressor gene PTEN (phosphatase and tensin homolog on chromosome 10) (Gao et al 2000, Goberdhan et al 1999, Huang et al 1999). PTEN acts to regulate the effects of PI3K, as PTEN dephosphorylates PIP_3 back to PIP_2 (Goberdhan et al 1999).

Another regulator of PI3K in *Drosophila,* Susi, has also been identified. Susi directly binds to dP60, the regulatory subunit of dPI3K. Susi has no overt similarity to known inhibitors of PI3K/PKB signaling in other systems, it thus defines a novel mechanism by which this signaling cascade is kept in check, and may be involved in insulin signaling during fasting (Wittwer et al 2005).

1.2.3.3. Akt and dTor

Once the secondary messenger PIP_3 has been produced, it activates Akt. Akt is a serine/threonine kinase (also known as protein kinase B or PKB). Elevated levels of PIP_3 recruit the PH-domain-containing Akt protein to the plasma membrane, and thereby facilitating its further activation by phosphoinositide-dependent kinase 1 (PDK1). *Akt* loss-of-function mutants show reduction in cell size. Conversely, *Akt* overexpression results in an increase in cell size, without altering proliferation (Verdu et al 1999). When Akt is active it

phosophorylates the transcription factor dFOXO, which causes its retention in the cytoplasm. However, when there is low activity of Akt, dFOXO can enter the nucleus (Neufeld 2003). The role and regulation of dFOXO in controlling growth will be discussed in detail below (1.2.3.4.).

TOR (Target of Rapamycin) represents a second branch of the insulin signaling pathway, downstream of Akt. It is a conserved Ser/Thr kinase (Wullschleger et al 2006), and is designated as mTOR/FRAP/RAFT1 in mammals and TOR1 and TOR2 in yeast.. In mammals TOR is capable of promoting cellular proliferation as a response to growth factors and the TOR pathway is known to act as a nutrient-sensitive growth pathway. In *Drosophila,* a homologue dTOR, has been described (Zhang et al 2000). The kinase activity of dTOR is required for growth factor-dependent phosphorylation of S6 kinase. Loss of dTOR produces phenotypes characteristic of starvation, in particular amino acid deprivation. Cell cycle arrest associated with *dTOR* loss of function can be suppressed by the overexpression of cyclin E (Zhang et al 2000). dTOR activity is regulated through TSC1 and TSC2 (tuberous sclerosis complex genes 1 and 2) which together act to inhibit dTOR (Gao et al 2002). TSC2 activity itself is thought to be directly inhibited following phosphorylation by Akt (Potter et al 2002). Although this regulatory mechanism has been questioned as, *Tsc2* mutants in which the Akt phosphorylation sites were changed to nonphosphorylatable or phospho-mimicking residues expressed in *Tsc2*-null mutants could completely rescue the lethality and cell growth defects of *Tsc2*-null mutants (Dong & Pan 2004).

dTOR links to the insulin signalling pathway not only through Akt, but in that dTOR acts to regulate Thor activity. Upon phosphorylation by dTOR, Thor dissociates from the translational regulator, eIF4e, allowing assembly of the initiation complex at the mRNA cap structure, ribosome recruitment, and subsequent translation (Lachance et al 2002). Thor and its regulation will be discussed in more detail below.

1.2.3.4. dFOXO

dFOXO, a downstream target of Akt, is a transcriptional regulator of the Forkhead-box type O (FOXO) class of Forkhead-related factors (Neufeld 2003). Four FOXO species, encoded by four distinct genes, have been identified in mammals: FOXO1 (previously known as FKHR), FOXO3 (previously known as FKHRL1), FOXO4 (previously known as Afx), and FOXO6 (Jacobs et al 2003), whereas in the nematode, *Caenorhabditis elegans,* a single FOXO transcription factor, DAF-16 is found. In *Drosophila,* dFOXO was identified as a homolog of

Caenorhabditis elegans DAF-16 and mammalian FOXO4 (Jünger et al 2003, Puig et al 2003), and the sole FOXO protein present in the species. The DNA binding domain of dFOXO, which lies in the N-terminal region, shows 45% identity with FOXO4, and the amino acid sequence that forms the motif for recognition by Akt (RXRXXS/T) is conserved (Puig et al 2003). The three specific sites at which FOXO4 is regulated by Akt-dependent phosphorylation are also conserved in dFOXO, with the dFOXO residues T44, S190 and S259, corresponding to mammalian FOXO4 T28, S193 and S258 respectively (Puig et al 2003). dFOXO is inhibited by dAkt-mediated phosphorylation when insulin is present, which results in it being retained in the cytoplasm and thereby unable to regulate transcription. However, following a reduction in insulin signalling, dFOXO becomes dephosphorylated and accumulates in the nucleus. where it can act to stimulate transcription of target genes (Neufeld 2003). FOXO proteins may also be regulated at the point of translation or by the stability of the protein (Harvey et al 2008, Mattila et al 2008). In a *Drosophila* cell line expressing a constitutively active dFOXO, 227 genes were seen to be up regulated (Puig et al 2003). Of these 227 genes, two were identified that are also know to be active within the insulin signalling pathway. These were the translational repressor *eukaryotic initiation factor 4E-binding protein* (*d4EBP*) (also known as *Thor*, and discussed below) and *dInR* (Puig et al 2003). The transcriptional activation of *Thor* by dFOXO also corresponds with dAkt activation of dTOR which acts to inhibit Thor activity (Miron 2001). As mentioned above, dFOXO also activates transcription of *dInR* (Puig et al 2003) this provides a feedback loop in which dFOXO can act to increase dInR levels. It has been suggested that this increase in dInR, under conditions where insulin levels are low, means that the system will be able to respond rapidly when nutrients are again available (Puig & Tjian 2005).

A recently identified transcriptional target of dFOXO is *Lk6,* a *Drosophila* Mnk-like kinase (Teleman et al 2008) Lk6, like Thor, acts to regulate eukaryotic initiation factor 4E (eIF4E) activity (eIF4E will be discussed below). In this same study *Myc*, itself a transcription factor (section 1.2.2), was also identified as a dFOXO target, although in a tissue specific manner (Teleman et al 2008). Accordingly, it has been suggested that Myc is the convergent point for regulation by dFOXO and TOR branches of the insulin pathway (Teleman et al 2008).

Although dFOXO can act to inhibit growth, *dFOXO* null mutants appear to be wild type, growing to normal size (Jünger et al 2003). However, when *dInR, Chico*, *PI3K* or *Akt* are mutated in a *dFOXO* null mutant background, the reduced growth phenotypes seen in these

mutants in a *dFOXO* $^{+/+}$ background are suppressed. This demonstrates that dFOXO can act to inhibit growth under conditions of reduced insulin signalling (Jünger et al 2003). dFOXO has also been seen to suppress growth when there is increased activity of the TOR pathway (Harvey et al 2008, Jünger et al 2003). dFOXO has also recently been shown to regulate cAMP signalling by inducing expression of an adenylate cyclase, *ac76e,* and this has been shown to increase starvation resistance and limit growth (Mattila et al 2009).
Together all these data point to dFOXO having an important role in growth control. However, under normal laboratory conditions, where food is plentiful, dFOXO seems to be inactive as high levels of insulin mean that dFOXO is phosphorylated and therefore inactive.

1.2.3.5. Thor

Thor is a member of the 4E-binding protein (4E-BP) family, which in mammals have been defined as critical regulators in a pathway that controls initiation of translation through interaction with eukaryotic initiation factor 4E (eIF4E) (Bernal & Kimbrell 2000).
The role of Thor as a regulator is dependent on its binding of eIF4E. The sequestration of eIF4E in this way preventing it from forming the translation initiation complex. However, phosphorylation of Thor then results in the release of eIF4E and translation (Bernal & Kimbrell 2000). The regulation and function of this translation initiation complex and eIF4E will be described in more detail below.
As mentioned above, Thor is a downstream target of the insulin signaling pathway (Miron 2001). As a function of its involvement with this pathway, Thor phosphorylation occurs as a result of insulin treatment, with the subsequent release of eIF4E allowing translation to occur (Lachance et al 2002, Miron 2001). This phosphorylation event is thought to be (as in mammalian systems) regulated by TOR (Miron et al 2003). TOR can also act to phosphorylate S6K but in contrast to the mechanism of Thor phosphorylation, in a PI3K- and Akt-independent manner (Miron et al 2003).
Thor can also be regulated at the transcriptional level, through the insulin signaling pathway via dFOXO (Jünger et al 2003, Puig et al 2003).
Expression within the wing-imaginal disc of a Thor mutant that will bind deIF4E most strongly Thor^{LL}, has been shown to result in a decrease in wing size. However, this reduction in wing size was not seen if wild type Thor was expressed (Miron 2001). Null mutants of *Thor* are viable and do not exhibit increased growth, although their immune response is compromised (Bernal & Kimbrell 2000). Teleman et al (2005) demonstrated that Thor did not function under

normal growth conditions but instead acts as a metabolic 'brake' that is activated under conditions of environmental stress in order to control fat metabolism (Teleman et al 2005). This role for Thor is also supported by the work of Tettweiler et al (2005) where Thor was shown to be critical for survival under conditions of dietary restriction or oxidative stress (Tettweiler et al 2005).

1.2.3.6. Lk6

Mammalian cells respond to a variety of extracellular stimuli via activation of specific mitogen-activated protein (MAP) kinase cascades. A subfamily of murine serine/threonine kinases, whose members bind tightly to the growth factor-regulated MAP kinases are known as MAP kinase-interacting kinases (Mnk) (Waskiewicz et al 1997).The *Drosophila* Lk6 protein is the functional homolog of the mammalian Mnk kinases (Arquier et al 2005). *Lk6* has been identified as a transcriptional target of dFOXO (Teleman et al 2008) and has been shown to regulate the activity of translation initiation factor eIF4E (Fig. 1.4.) (Arquier et al 2005, Parra-Palau et al 2005, Reiling et al 2005). The regulation of eIF4E activity by Lk6 induced phosphorylation has also been demonstrated (Arquier et al 2005, Reiling et al 2005) although the exact role of this phosphorylation event on eIF4E activity is unclear. Lk6 has been shown in one study to be vital for normal growth and development (Arquier et al 2005). In *Lk6* null mutants, slower development and reduced viability and adult size has been observed (Arquier et al 2005). These results were comparable to that seen in animals expressing a non-phosphorylatable form of eIF4E (Lachance et al 2002). These data suggest that Lk6 acts to positively phosphorylate eIF4E which is then able to activate protein synthesis, although results in mammalian studies have been contradictory, with it being shown that phosphorylation of eIF4E reduces its affinity for the capped mRNA (Scheper et al 2002). It should also be noted that even in the studies showing that phosphorylation of eIF4E through Lk6 acted to activate translation (Arquier et al 2005), overexpression of Lk6 in the eye disc resulted in subtle growth impairments (Arquier et al 2005).

So even in studies where Lk6-mediated activation of translation is seen, the opposite effect was also observed in some tissues. Growth inhibition due to Lk6 overexpression in an eIF4E phosphorylation-dependent manner was also seen in another study (Reiling et al 2005). In this work, Reiling et al also had contradictory results to that of Arquier et al main results as their *Lk6* null animals were viable and fertile without obvious growth defects when grown under standard conditions. Although in the Reiling et al study, when *Lk6* null mutants were raised

under conditions of poor nutrient supply a reduction in size of flies was observed. This reduction in the amino acid supply also abolished the negative effects of Lk6 overexpression on growth, which suggests that the activity of Lk6 is also regulated in response to nutrient availability (Reiling et al 2005).

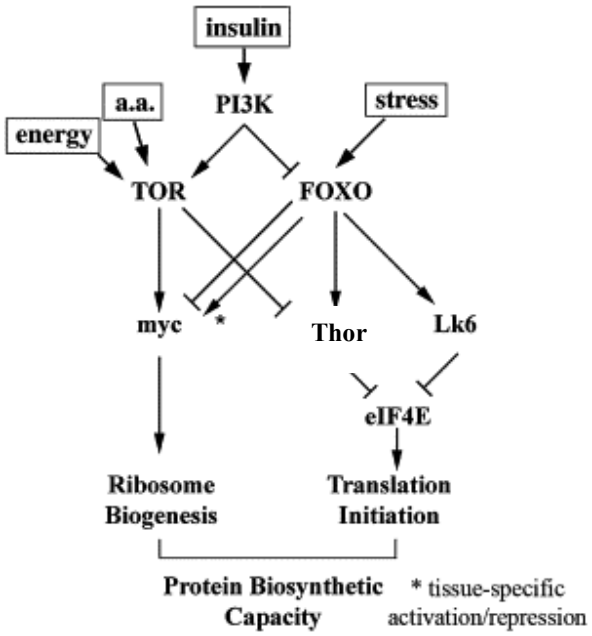


Figure 1.4. PI3K-dependent regulation of protein biosynthesis: Convergent regulation by TOR and FOXO in muscle. (adapted from Teleman et al 2008). Myc is transcriptionally repressed by FOXO and posttranscriptionally activated by TOR. Both inputs cause Myc activity to increase upon increased insulin signaling, leading to the expression of a set of E box-containing genes involved in ribosome assembly. Insulin signaling also regulates translation initiation via eIF4E. *Thor* and *Lk6* are direct transcriptional targets of FOXO that are induced under conditions of low insulin signaling. Both inhibit eIF4E activity either by direct binding and sequestration (4E-BP/Thor) or by phosphorylation (Lk6). 4E-BP/Thor binding to eIF4E is regulated by TOR (Teleman et al 2008).

1.2.3.7. eIF4E and the initiation of translation complex

As mentioned previously, eIF4E is a downstream target in the insulin signaling pathway of Thor and Lk6. Cellular eukaryotic messenger RNAs (except those in organelles) possess a cap structure at their 5′ terminus comprising, m^7GpppX (where X is any nucleotide) (Gingras et al 1999). This cap is one of the structural features of eukaryotic mRNAs involved in the modulation of ribosome binding to the mRNA. eIF4E is a translation initiation factor which is involved in the recognition of and binding to the cap (Hernández & Sierra 1995). eIF4E is part of a protein complex, eIF4F, made up of eIF4A (RNA helicase), eIF4G (a large scaffold protein), and eIF4E (Miron & Sonenberg 2001). The interaction of eIF4E with the cap structure, positions the eIF4F complex at the 5' end of the mRNA. The action of the complex is to unwind the inhibitory secondary structures present in the 5' untranslated region of the mRNA (Miron & Sonenberg 2001).

As mentioned above, in *Drosophila*, the activity of eIF4E is modulated through Thor and Lk6.

1.3. Previous research from the laboratory

As the work in this thesis carries on from research previously completed in our laboratory (Kimber 2005), a brief summary of what was has previously been found regarding Nitric Oxide signaling will be given below.

Previous work on NO in the lab has identified modulation of the insulin signalling pathway as providing a possible mechanism by which NO may act to inhibit growth.

Following microarray analysis on tissue culture cells that had been treated with a NO donor, S-nitroso-N-acetyl-penicillamine (SNAP), *Thor* was identified as one of seven transcripts up-regulated 4hrs after NO treatment, and it continued to be up-regulated over the next two time points (8 and 12hrs). As Thor is part of the insulin signalling pathway and is known to suppress growth under some conditions, it was therefore considered possible that this pathway may provide a relevant mechanism by which NO inhibits growth.

In order to analyse NO function *in vivo*, the mouse macrophage *NOS* gene (NOS2) was utilised to express NO under control of the UAS GAL4 system. When NOS2 was expressed in salivary glands there was a reduction in size and an increase in Thor-LacZ expression. However when NOS2 was expressed in a *dFOXO* loss-of-function mutant background, it resulted in the

suppression of the NOS2-induced phenotypes and a reduction in Thor-LacZ staining in the salivary glands. This showed that the phenotypic and molecular responses to NO are dependent on dFOXO. Thor signaling was also reduced when the endogenous dNOS expression was reduced using RNAi-NOS. Similarly, expression of Thor-LacZ was further reduced in a *dFOXO* mutant background. When RNAi-NOS was expressed throughout the larvae using a tubulin driver, it resulted in an overgrowth phenotype with third instar larvae that never pupated. These larvae simply wandered until they died.
Application of exogenous NO to either S2 tissue culture cells or 3rd instar larvae resulted in a drastic inhibition of protein synthesis.
All work above is from Kimber 2005 (Kimber 2005). A similar NO dependent inhibition of translation has been reported where NO treatment on 180-min-old embryos induced a reversible arrest of development, gene expression and turnover (Teodoro & O'Farrell 2003).

The results from Kimber 2005 did identify dFOXO as a possible target for the action of NO though further work was need to confirm these results and this is discussed below and in Chapter 3.

1.4. Aims of project

The aim of this research project was to confirm and identify the genetic and molecular components of NO signaling in *Drosophila melanogaster*. Particular emphasis was placed on how NOS can affect cell proliferation and/or growth. As previous work in the laboratory had identified dFOXO as a possible target for NO, the research was focused on confirming this result. As two known targets of dFOXO signaling include regulators of translation, and NO has been demonstrated to inhibit protein synthesis, the roles of these dFOXO targets was determined. The work also investigated the action of NO in the insulin signaling pathway and whether its inhibition of growth may result from action through the known sGC pathway. Finally as NO is a known inhibitor of growth, and in mammalian systems it has been shown to inhibit growth of tumor cells, this activity was investigated in *Drosophila* using two known and well defined oncogenes.
An introduction and discussion accompanies each results chapter.

Chapter 2: Material and Methods

2.1. General

2.1.1. Fly Husbandry

Drosophila stocks and crosses were raised on D+ food, in 8cm x 2.5cm plastic vials, stoppered with either cotton wool or rayon balls. Flies were raised at either 25^{o}C or 18^{o}C on a 12 hour light-dark cycle, unless otherwise stated. *Drosophila* were observed using CO_2, with a Nikon SM2645 dissecting microscope with a Microtec MFO-90 light source. Fly stocks are give in Appendix I.

D+ Glucose Food Media

Agar 40g
D+ Glucose anhydrous 551g (Fisher Scientific)
Maize meal 236g
Yeast 143g
Sucrose 185g
Nipagen 10%w/v 82mls
Propionic acid 25mls
Water 5500mls

Method

Yeast and anhydrous D+ Glucose were mixed into a paste using a small amount of water. The agar and maize were mixed with the rest of the water and brought to the boil to dissolve. The paste was then added and the mixture brought to the boil again. The mixture was allowed to cool before pouring into plastic vials or glass bottles.

2.1.2. Overexpression using the Gal4-UAS system

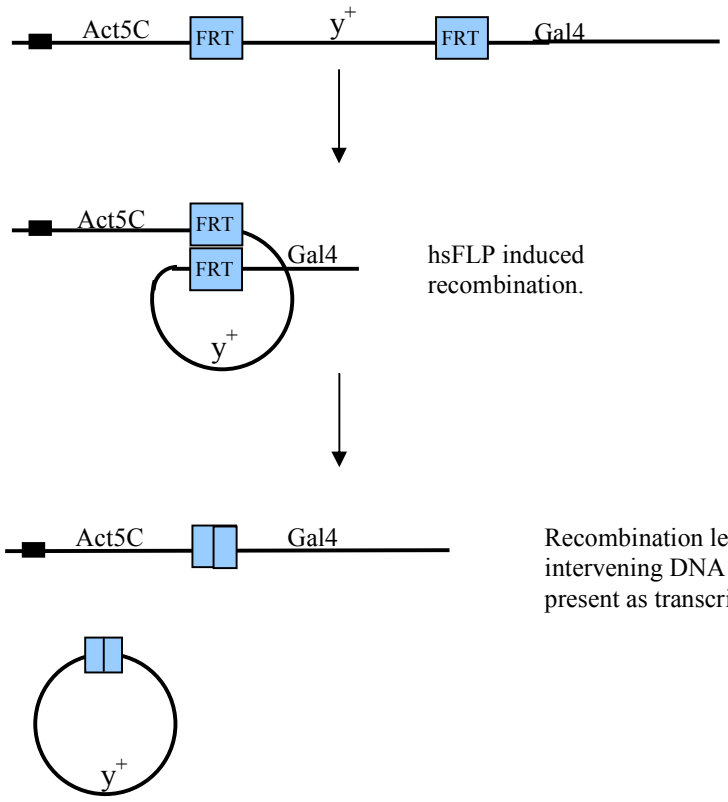
The Gal4-UAS system as described in Brand and Perimon (1993) was utilised for overexpression of specific genes. Flies carrying the gene to be expressed linked to an element containing multiple UAS sequences, were crossed to flies that carried the Gal4 coding sequence fused to a specific promoter. Offspring carrying both the UAS and Gal4 inserts were observed.

To drive expression in salivary glands Gal4 lines AB1 and c147 were exploited. Gal4 dependent *UAS-GFP* expression driven by either line was indistinguishable (data not shown). NOS2 and dFOXO expression by either Gal4 line either gave similar phenotypes. However, for genetic interaction experiments all transgenes were compared through expression driven by either AB1 or c147.

2.1.3. Generating Single Cell Clones

Single clones were generated using the FLP/FRT technique. FLP is a site-specific recombinase encoded by the *S. cerevisiae* 2μm plasmid. It efficiently catalyzes recombination between two copies of its specific 34 bp recognition site (called the Flp recognition target (FRT)). A heat shock FLP construct was used to remove a segment of DNA flanked by FRT sites, which lies in the middle of an ActinGal4 transcript preventing its expression. After recombination expression of the Gal4 transcript occurs so therefore the UAS will be expressed (Struhl & Basler 1993). Recombination was induced at a low level at 24-48 hours of development heat shocking for between 4 to 5 and half minutes, after the salivary gland cells had been determined (Demerec 1950). This regime produced single cells expressing GFP/mRFP as a marker or co-expressing the marker and *UAS-NOS2* (or any other genes/constructs). The single cells expressing NOS2 produced by this experimental design could then be used to assay the molecular and cellular consequences of high levels of NO production in otherwise unaffected salivary glands.

Figure 2.1. Schematic diagram of clone generation.



2.1.4. G418 selection

Materials

G-418 disulphate (Melford Laboratories Ltd.)

Method

Selection of recombinants was preformed using G418 a selection agent for the neomycin-resistance FRT sites. Flies were brooded for 24 hours then transferred. The eggs were left for approximately 24 hours to allow for hatching and development to 1st instar larvae. 150µl of G-418- disulphate was then added at 25mg/ml. Larvae were then left to develop to adults and recombinant flies were selected. Both positive and negative controls were preformed.

2.1.5. *Drosophila* Tissue Culture

Materials

$S2^{R+}$ *Drosophila* cells (Yanagawa et al 1998)

Schneider's *Drosophila* Medium *(*Gibco BRL, 21720)

10 % Fetal Bovine Serum (Sigma Aldrich, F-0643)

10ml Penicillin/Streptomycin solution per 1L (GIBCO BRL, 100ml, 10.000 units/ml Penicillin G Sodium and 10.000μg/ml Streptomycin Sulfate in 0.85% saline)

SNAP (Sigma Aldrich, N 3398)

DMSO (Sigma Aldrich)

Method

$S2^{R+}$ *Drosophila* cells were grown in Schneider's *Drosophila* Medium plus 10% FBS and 100 units per ml Penicillin, 100mg per ml Streptomycin. Cells were routinely cultured. For time course cells were grown to a cell density of $1x10^5$ cells/ml in 8-well Culture Slides (BD Falcon) containing 500μl/well of above supplemented Schneider's *Drosophila* Medium. Over the time course cells were either treated with 5μl DMSO as a control or 5μl SNAP. After given time the cells were Antibody stained as below (2.2.1) and examined on a LSM Zeiss inverted-LSM 510 META COMBI Confocal Microscope with Coherent Enterprise UV laser using 40x water lens.

Analysis of protein localisation was carried using imaging analysis software Volocity (www.improvision.com/products/Volocity/).

2.1. Immunohistochemistry

2.2.1. Antibody staining

Materials

1xPBS (10xPBS: 1.37M NaCl, 0.1M Na_2HPO_4, 0.01M NaH_2PO_4; pH7.4)

4% Paraformaldehyde in 1xPBS

100% Methanol

PBT (1xPBS, 0.1% Triton X-100, 0.2% BSA)

Normal Goat sera (Sigma)

Appropriate primary and secondary antibodies (Section 2.2.2.)

DAPI 1:20,000 in 1x PBS with 0.1% Triton X-100

Aquamount (Polyscience)

Method

Wandering third instar larvae were dissected by inversion and fixed in 4% paraformaldehyde for 20 minutes. Tissue was then washed 2x 5min in 1xPBS. If the tissue was to be stored it was washed twice in Methanol and stored at –20^{o}C in Methanol. The tissue was then washed in 1xPBS before permeabilization in PBT for 2 x 30 minutes. The tissue was then incubated in primary antibody overnight at 4^{o}C. Primary antibody was removed and stored, and the tissue was washed 4 x 20 minutes in PBT. The tissue was rinsed x 2 in PBT containing 2% goat sera. Then incubated with secondary antibody in PBT with 2% goat sera at room temperature for 2 hours. The tissue was then washed 4 x 15 minutes in PBT. DAPI staining of tissue was performed for 15 min. Tissue was then washed 2x 5minutes in 1xPBS before dissection. The required tissues were mounted on a slide in Aquamount and examined on a LSM Zeiss inverted-LSM 510 META COMBI Confocal Microscope with Coherent Enterprise UV laser using 40x water lens.

For tissue culture same protocol as above though staining was carried out in 8-well Culture Slides (BD Falcon).

2.2.2. Primary and Secondary Antibodies

dFOXO Antibody

Rabbit dFOXO antibody a kind gift from O.Puig was used at 1:1000 (Puig et al 2003).
Secondary antibody goat anti-rabbit Cy5 (Jackson ImmunoResearch Laboratories, Inc.) at 1:100.

β-Gal Antibody

Rabbit anti-βgal (Jackson ImmunoResearch Laboratories, Inc.) used at 1:5000.
Secondary antibody goat anti-rabbit Cy5 (Jackson ImmunoResearch Laboratories, Inc.) at 1:100.

Myc Antibody

Guinepig anti-dMyc a kind gift from Dr. Marco Milán used at 1:1000 (Herranz et al 2008).
Secondary antibody goat anti-Guinepig Alexa 555 (Molecular Probes, Invitrogen) at 1:200.

Thor Antibody

Rabbit anti-Thor a kind gift from Prof. Nahum Sonenberg used at 1:200 (Miron 2001).
Secondary antibody goat anti-rabbit Cy5 (Jackson ImmunoResearch Laboratories, Inc.) at 1:100.

2.3. 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 by inversion and fixed in 4% paraformaldehyde for 20 minutes. Tissue was then washed 2x 5min in 1xPBS. The tissue was then incubated in

diluted DAPI staining of tissue was performed for 15 min. Tissue was then washed 2x 5 minutes in 1xPBS before dissection. The required tissues were mounted on a slide in Aquamount and examined on a Zeiss AxioPhot Microscope at 40x unless other wise stated. Nuclear diameter was used as a measure of cell growth. Cell diameter and volume have large variation (not shown) due to the irregular cell shapes that occur in salivary glands and were therefore not used as a measure of size. These volume variabilities are probably also probably due to rapid expansion of cell volume independent of DNA replication during wandering larva stage due to accumulation of secretary products in preparation for glueing pupal case to vertical substrate.

In order to compare statistically the relative sizes of nuclei within each genotype, data was loaded into Minitab version 15 (MINITAB Inc) statistical software package. A histogram of residuals was used to show if the nuclei measurement data was normally distributed (graphs shown in Appendix II) and a one-way ANOVA, all pairway comparisons (Tukey) was carried out. ANOVA is a post hoc test which are used to identify which conditions are showing significant differences (Dytham 2003). Independence and equal variance across the data set were assumed (Dytham 2003). The raw data are recorded within the Appendix II. The P-values though are given for each graph. The P-value is the probability of the hypothesis being tested is true, the smaller the value the more confident we can be in the conclusions that are drawn for it (Dytham 2003). Using the Tukey 95.0% simultaneous confidence intervals generated for each comparison, a punet square was constructed to compare statistical significance across the data set which is shown in the Appendix II. Sampling variation from was kept to a minimum. Larvae to be compared were kept on the same batch of food plus the number of larvae in the vials was restricted to prevent variation in nutrients. The larvae were picked as they started to wander to reduce age variation.

Chapter 3: NO action is through dFOXO

3.1. Introduction

3.1.1. Regulation of growth by NO in *Drosophila melanogaster*

The identification of the signalling pathway through which NO acts to regulate growth is key to the understanding of the effect of NO on growth. Previous work our the lab has used Affymetrix gene arrays to identify any transcriptional changes that were induced in *Drosophila* S2 cells in response to exposure to NO, following treatment with the NO donor, S-nitroso-N-acetylpenicillamine (SNAP). These data demonstrated that many genes were up regulated in response to NO, and that many of these were also targets of the transcription factor dFOXO, part of the insulin signalling pathway (discussed in more detail in section 1.3.). Regulation of dFOXO activity itself occurs through Akt-dependent phosphorylation following exposure to insulin, resulting in dFOXO being retained in the cytoplasm (Puig et al 2003).

One of the known transcriptional targets of dFOXO found to be up regulated following NO treatment was *Thor* (*4E-BP*) (discussed in more detail in, section 1.2.3.5.). Thor is a member of the 4E-binding protein (4E-BP) family, which controls initiation of translation through the binding of eukaryotic initiation factor 4E (eIF4E) (Bernal & Kimbrell 2000). When levels of Akt activity are low, dFOXO is not phosphorylated, allowing it to enter the nucleus where it promotes transcription of *Thor* (and other genes) (Puig et al 2003). Thor in turn then binds eIF4E preventing initiation of translation and therefore growth is presumably inhibited.

It has also been demonstrated that *Thor*, is transcriptionally activated by NO *in vivo*. Expression of an active Nitric Oxide Synthase gene (NOS2) in the larval salivary glands resulted in increased expression of a Lac Z reporter construct of Thor. This increased expression of Thor in response to NO was shown to be completely suppressed by removal of dFOXO using a dFOXO null mutant (Kimber 2005). In the same study, the inhibition of growth of these cells by NO was also shown to be suppressed by the genetic removal of *dFOXO*. Therefore it was concluded that growth-inhibitory effects and activation of Thor by NO were dependent on dFOXO.

3.1.2. Analysis of NO expression in single cells of salivary glands

Although the above study by Kimber 2005 did identified a molecular target through which NO may be acting, the experiments were performed in whole salivary glands. Accordingly they did not take into account any physiological affects that the reduced growth may be having on the expression of genes, and could not distinguish between the direct consequences of NO signalling versus the effects of reduced growth of the cells. It was therefore decided to determine if NO signalled autonomously through dFOXO, and whether the apparent increase in dFOXO signalling could occur when growth was minimally affected. To achieve this, expression of NO would be induced in single cells within the salivary gland rather than in whole salivary gland as had been done by Kimber 2005. The expression in single cells would also allow any increase in expression of proteins out side of the target cell to be identified thereby showing if NO was have a cell autonomous or non-autonomous effect. This was done using the FLP/FRT technique. FLP is a site-specific recombinase encoded by the *S. cerevisiae* 2µm plasmid. It efficiently catalyzes recombination between two copies of its specific 34 bp recognition site (called the Flp recognition target (FRT)). A heat shock FLP construct was used to remove a segment of DNA flanked by FRT sites, which lies in the middle of an Actin 5C Gal4 transcript, thereby preventing its expression. Recombination results in expression of the Gal4 transcript leading to expression of the UAS (Struhl & Basler 1993). Recombination was induced at a low level at 24-48 hours after egg laying (AEL), after the salivary gland cells have been determined (Demerec 1950). This methodology produced single cells expressing GFP/mRFP as a marker, or co-expressing the marker and *UAS-NOS2* (or any other genes/constructs). The single cells expressing NOS2 produced by this experimental design could then be used to assay the molecular and cellular consequences of high levels of NO production in otherwise unaffected salivary glands.

The *UAS-NOS2* construct used was that previously used in the lab to drive NOS2 expression under the UAS/Gal4 system. The *NOS2* gene used, being the mouse macrophage Nitric Oxide Synthase gene (Lowenstein et al 1992), which unlike the *Drosophila NOS2* gene, is not regulated by calcium (Regulski & Tully 1995). Previously, this *NOS2* transgene, referred to as the mouse *NOS2* transgene (Kuzin et al 1996), has been utilised for experiments in *Drosophila*, under the control of a heat shock promoter. *NOS2* was cloned into pUAST (Brand & Perrimon 1993) and four transformants were obtained. These transformants of the UAS - mouse

macrophage *NOS2* gene, abbreviated here as *UAS-NOS2*, were mapped to their chromosome location by segregation (Kimber 2005). One transformant was obtained on the X chromosome, two on the second chromosome and one on the third chromosome. The one used for this study was *UAS-NOS2*, located on the X chromosome, as it gave the highest levels of expression.

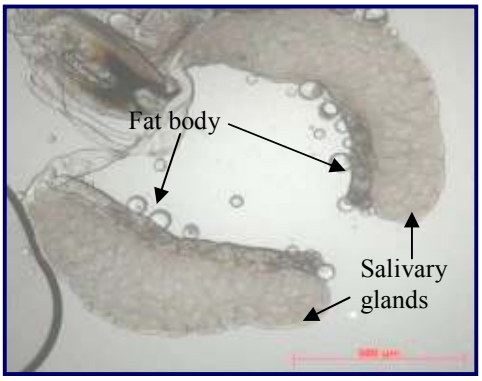
3.2. Results

3.2.1. Analysis of NOS2 and dFOXO expression in larval salivary glands

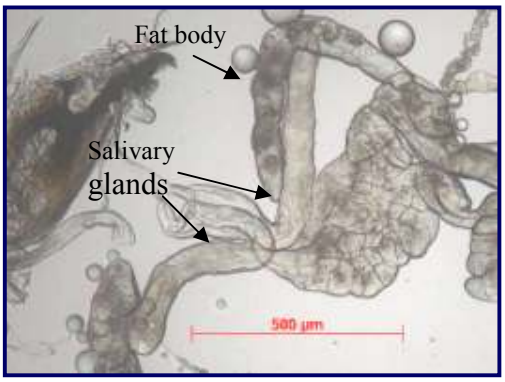
To confirm that NO acts as a negative regulator of growth, *UAS-NOS2* was expressed in the salivary glands using the GAL4 driver, c147-GAL4. Though some of this work had been completed by Kimber 2005 it was not quantified but rather judged by eye therefore to gain more detailed information on the effect of NO on cell proliferation and to also compare that with expression of dFOXO the experiment below was completed. The nuclei of wandering third instar larvae were measured and data statistically analysed as described in material and methods. The raw data are recorded within the appendix in section II. The salivary glands from third instar larvae expressing *UAS-NOS2* (NOS2) were overall smaller in size than that of the wild type control (Fig. 3.1Ai. and 3.1Aii.). The nuclei of the salivary glands expressing *UAS-NOS2* were also smaller than wild type (Fig. 6Bi. and 6Bii.) and upon measurement, it was found that the salivary gland nuclei of NOS2 expressing glands were statistically smaller than those from the wild type control larvae (Fig. 3.2.). As it was expected that NO may act through dFOXO to regulate growth, salivary glands from larvae expressing *UAS-dFOXO* using the c147-GAL4 driver were also examined. The salivary glands expressing *UAS-dFOXO* were also found to be smaller than those from the wild type control (Fig. 3.1Aiii.). The salivary gland nuclei were also shown to be statistically smaller than those from the wild type control larvae (Fig. 3.2.). Comparison of salivary gland nuclei expressing either *UAS-NOS2* or *UAS-dFOXO* showed that the nuclei from dFOXO were statistically smaller than those from NOS2 (Fig. 3.2.).

Figure 3.1. Salivary glands are reduced in size when NOS2 or dFOXO was over expressed.
A. Salivary glands **Ai.** Wild type (c147-GAL4/+). **Aii.** Increased expression of NOS2 (*UAS-NOS2*; c147-GAL4), salivary glands show reduced size. **Aiii.** Increased expression of dFOXO (c147-GAL4/*UAS-dFOXO*) salivary glands show reduced size.
B. Nuclei of salivary glands stained with DAPI. **Bi.** Wild type (c147-GAL4/+). **Bii.** Increased expression of NOS2 (*UAS-NOS2*; c147-GAL4), salivary glands nuclei show reduced size. **Biii.** Increased expression of dFOXO (c147-GAL4/*UAS-dFOXO*) salivary glands nuclei show reduced size.
Scale bars shown: A series, 500µm. and B series, 10µM.

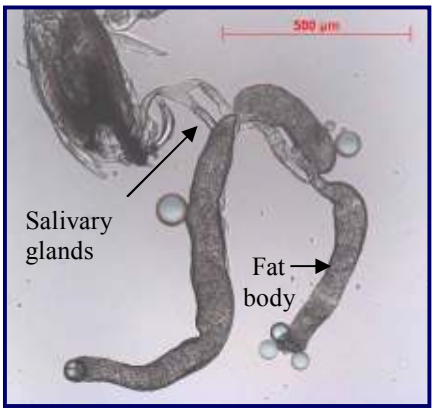
Ai. Wild Type.

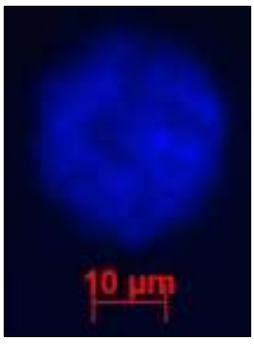


Aii. Increased NOS2 expression.

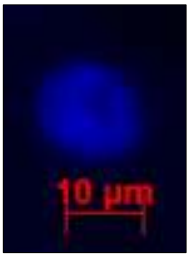


Aiii. Increased dFOXO expression.

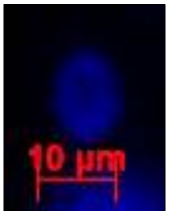




Bi. Wild Type



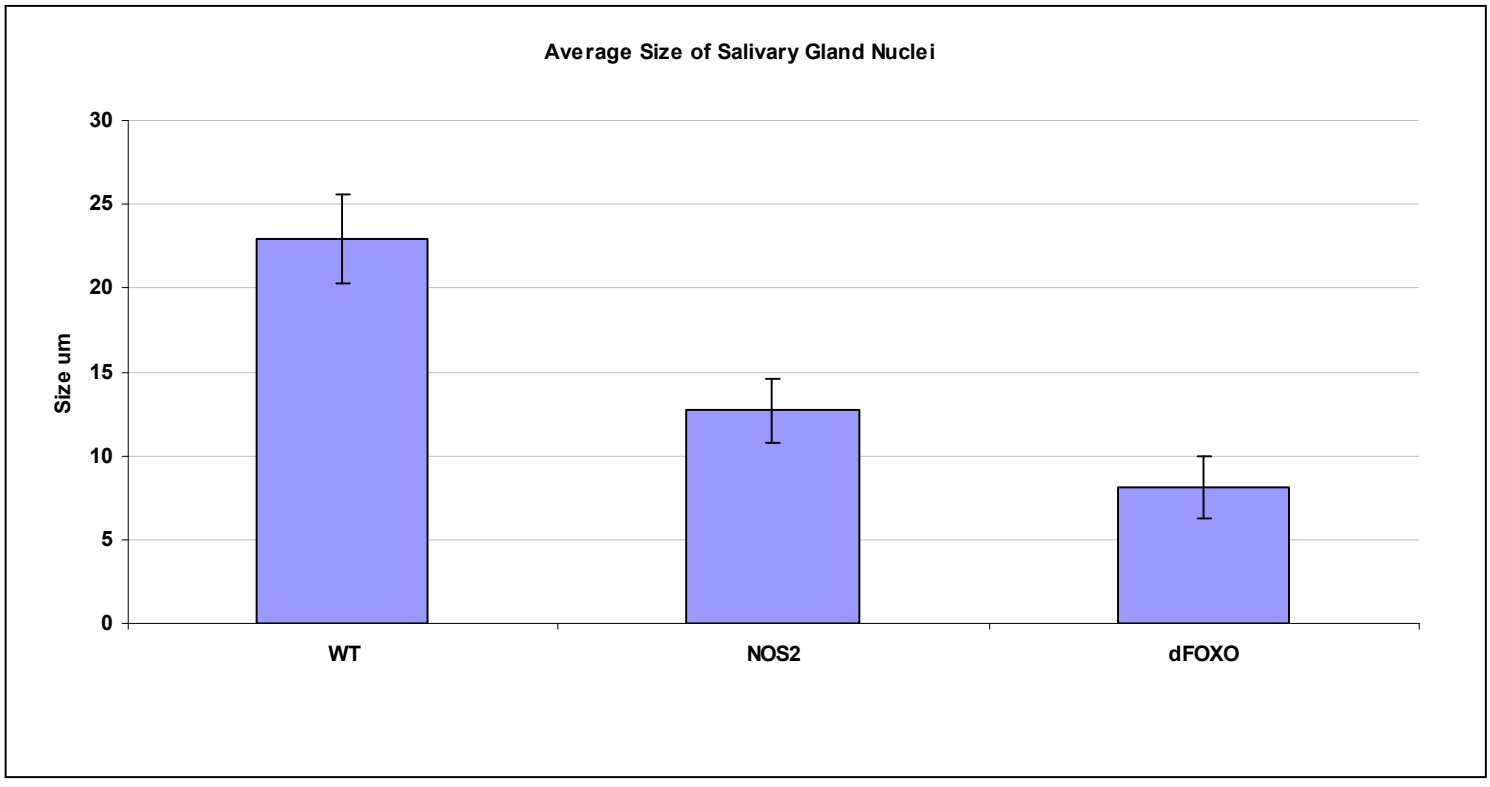
Bii. NOS2 expression



Biii. dFOXO expression

Figure 3.2. Average Size of Salivary Gland Nuclei was reduced following NOS2 or dFOXO expression.
c147-GAL4 was used to drive expression of *UAS-NOS2* or *UAS-dFOXO*. WT (c147-GAL4/+), NOS2 (*UAS-NOS2*; c147-GAL4) and dFOXO (c147-GAL4/*UAS-dFOXO*). Error bars represent the standard deviation. Both NOS2 and dFOXO nuclei are statistically smaller than those of WT (raw data is given in Appendix II.a.).

For WT, data was derived from 696 nuclei from 107 salivary glands. NOS2; 380 nuclei from 77 salivary glands. dFOXO; 68 nuclei from 8 salivary glands. P-Value = 0.001



3.2.2. Thor LacZ expression in NOS2 expressing clones

The reduced growth phenotype observed following expression of NO is thought to be a result of NO acting through the insulin pathway (Kimber 2005). This previous work showed that Thor, part of the insulin pathway, was up-regulated upon overexpression of NO. As previously mentioned, *Thor* is transcriptionally regulated by dFOXO, and accordingly the action of dFOXO can be monitored through the expression of Thor. The previous work demonstrated an increase of *Thor* by colourimetric monitoring the expression of a reporter of *Thor* expression P{w[+mC]=lacW}Thor[k07736] using β-galactosidase (X-Gal) staining. There was an increase in X-Gal staining when NOS2 was overexpressed in the salivary glands (Kimber 2005). This increase in Thor LacZ production coincided with a reduced size in the salivary glands. However, this increase in staining could have been a reflection of the reduced size of the cells and not in an increase in the expression of *Thor*. To verify the action of NO on expression of *Thor*, and thereby its action through dFOXO, clones were generated in the salivary glands that expressed *UAS-NOS2* in single, or small numbers of cells, in larvae carrying the Thor LacZ reporter. The expression of *Thor* was then identified through the expression of Thor LacZ reporter, using an anti beta-Gal antibody, and the UAS expression with *UAS-GFP*. NOS2 clones showed an increase in expression of Thor-LacZ reporter in a cell-autonomous (Fig. 3.3Ai.) or non-autonomous fashion (Fig. 3.3Aii.).

As well as the in the salivary gland, clones of NOS2 expressing cells were also generated in the wing discs of the developing larvae. These clones were much less prevalent, probably due to widespread induction of expression of NOS2 causing lethality. The 5 clones that were recovered showed varying results. A few did show an increase in Thor-LacZ expression (Fig. 3.4.), but due to lack of concordance, no real conclusion can be formed. Three clones did show an increase in Thor-LacZ expression and in one example it could be seen that the increased Thor-LacZ expression was observed with and surrounding the NOS expressing clone (Fig 3.4). This indicates that the diffusion of NOS can trigger increases in Thor-LacZ expression in cells neighboring those expressing NOS.

Figure 3.3. Thor LacZ expression is increased in NOS2 expressing clones in salivary glands.
Ai and Aii. Clones were generated using a 4min heat shock at 38°C between 24-48hr AEL, of animals with genotype: *UAS- NOS2*/hsFLP; Thor LacZ/Act5c>y^{+}>Gal4;*UAS-GFP*/+. Clones are therefore marked with GFP. **Ai.** NOS2 clone showing cell autonomous increase of Thor LacZ expression (arrow). **Aii.** NOS2 clone showing cell non-autonomous increase of Thor LacZ expression (white arrowheads show non-autonoumos and purple show clone cells a). Red: Thor LacZ, Blue: DAPI, Green: GFP Scale bar 50µm.

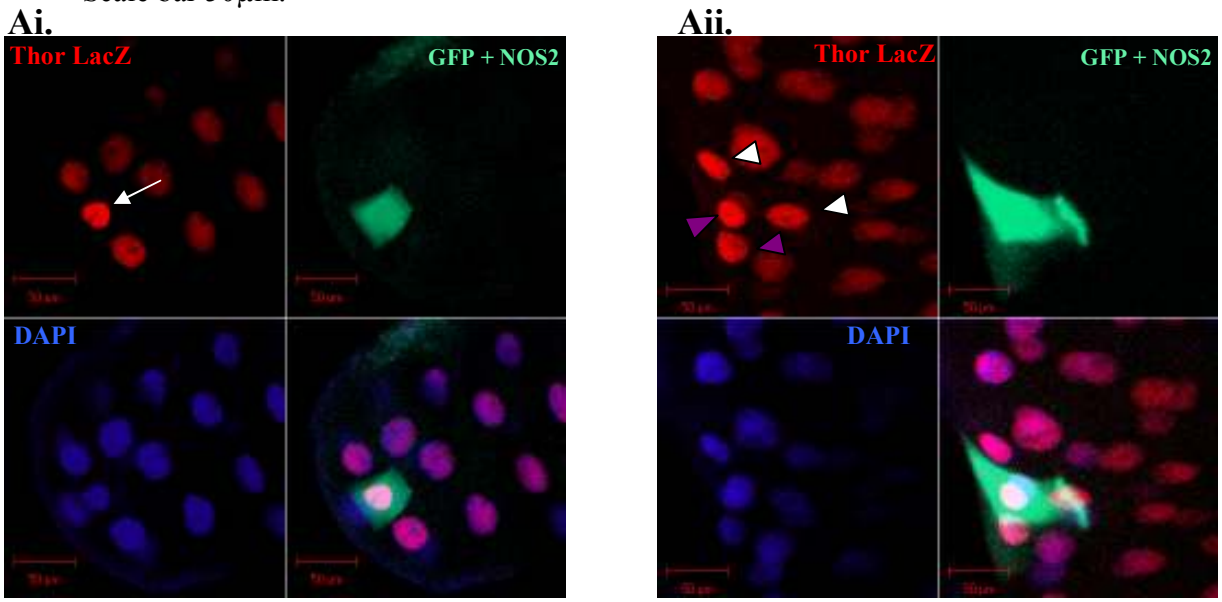
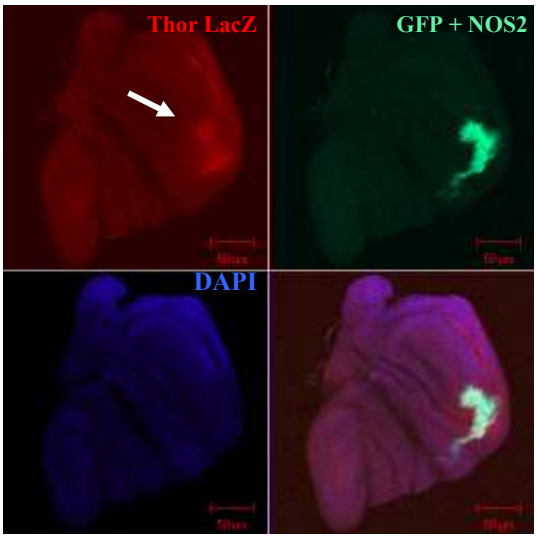


Figure 3.4. Thor LacZ expression is increased in NOS2 expressing clones in wing imaginal discs.
Clones were generated using a 4min heat shock at 38°C between 12-24hr AEL, of animals with genotype: *UAS- NOS2*/hsFLP; Thor LacZ/Act5c>y^{+}>Gal4;*UAS-GFP*/+. Clones are therefore labeled with GFP. Red: Thor LacZ, Blue: DAPI, Green: GFP. White arrow showing clone area. Scale bar 50µm.



3.2.3. Analysis of Thor-YFP expression with NOS2 and dFOXO

Although LacZ is a commonly used reporter gene, it is only a reporter of a promoter activity and does not directly show native protein expression. Therefore we decided the use of a newly constructed Thor-YFP protein fusion (Cambridge Protein Tap Insertion Consortium, unpublished) would better facilitate the analysis of protein expression rather than just report promoter activity. As with the Thor LacZ experiments, both *UAS-NOS2* and *UAS-dFOXO* were expressed in the salivary glands using the c147 Gal4 driver in animals carrying the Thor-YFP fusion. Expression of either *UAS-NOS2* or *UAS-dFOXO* in whole salivary glands resulted in an increase in Thor-YFP expression when compared to wild type control (Fig. 3.5.). As with the case of Thor LacZ, this increase in expression of Thor-YFP may just have been a consequence of the reduced size or growth of the salivary glands. To resolve these issues, clones were made within the salivary gland that expressed either *UAS-NOS2* or *UAS-dFOXO*, and the expression of Thor-YFP examined. As for the Thor Lac Z clones, an increase in expression of Thor-YFP was observed in cells expressing either *UAS-NOS2* or *UAS-dFOXO*, compared to surrounding wild type tissue (Fig. 3.6.) The cells expressing Gal4 could be identified by the expression of *UAS-myr-mRFP* which expresses membrane-targeted monomeric RFP which also labels cell membranes (Chang 2003).

Figure 3.5. Thor-YFP expression in salivary glands over expressing either NOS2 or dFOXO. **Ai.** Wild type (c147-GAL4/Thor-YFP). **Aii.** Increased expression of NOS2 (*UAS-NOS2*; c147-GAL4/Thor-YFP) showing increase in Thor-YFP. **Aiii.** Increased expression of dFOXO (c147-GAL4/*UAS-dFOXO*;Thor-YFP) showing increase in Thor-YFP. Green; Thor-YFP. Scale bar 200µm. Red arrows indicate salivary gland and white arrows fat body.All images acquired and processed using identical settings.

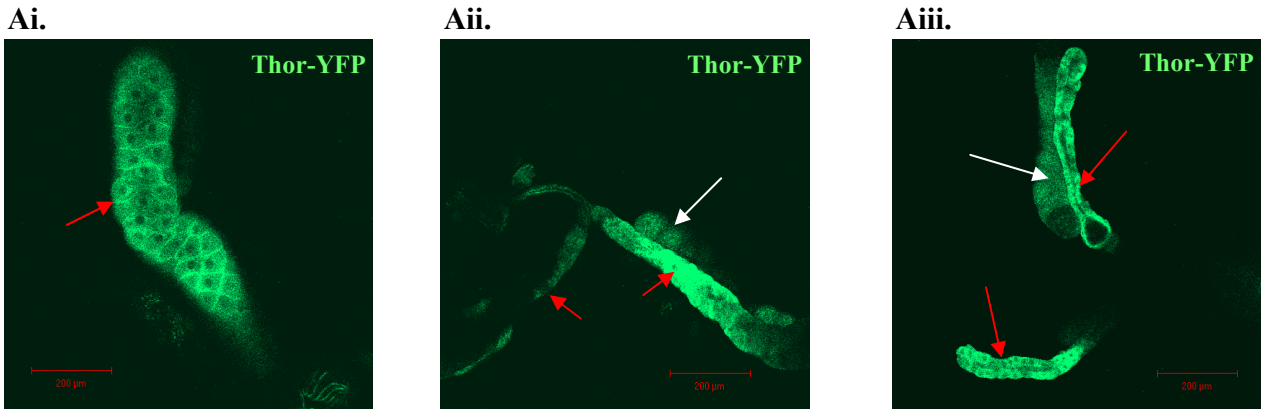
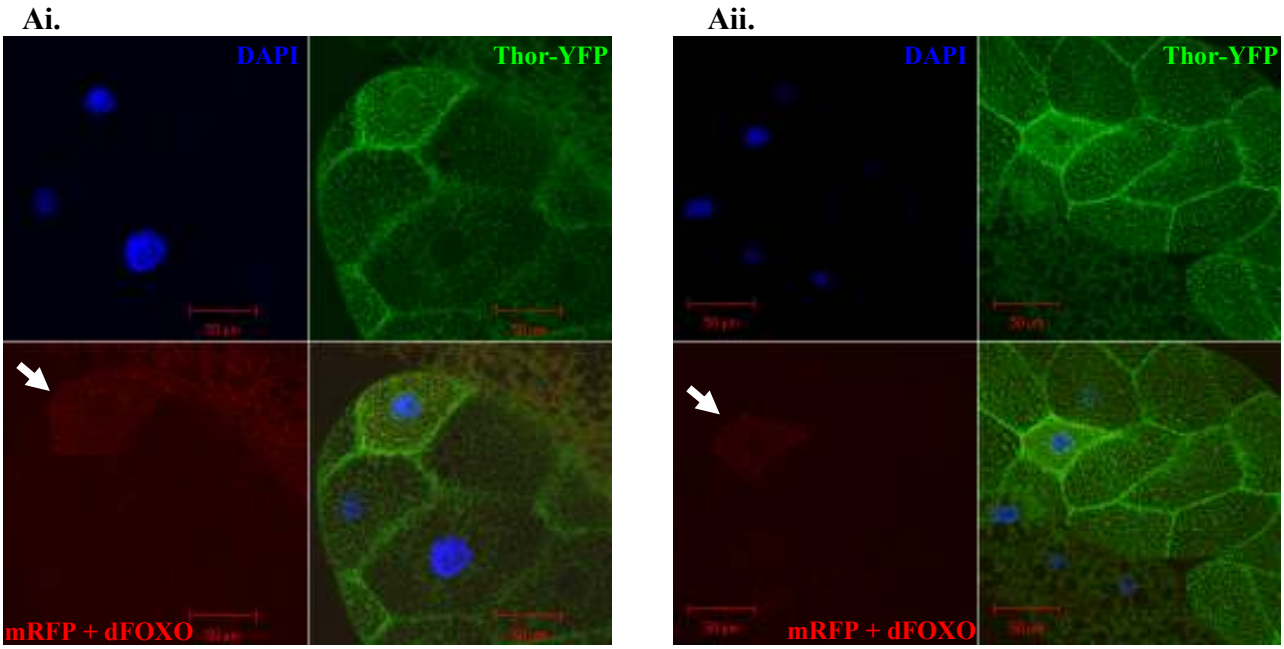


Figure 3.6. Thor-YFP expression is increased in single salivary gland cells expressing either NOS2 or dFOXO. Clones over expressing either NOS2 or dFOXO were generated using a 4min heat shock at 38°C between 24-48hr AEL. Clones are labeled with *UAS-myr-mRFP* (mRFP).
Ai. *UAS-NOS2* clone (UAS- NOS2/hsFLP; Thor-YFP/mRFP; Act5c>CD>Gal4/+).
Aii. *UAS-dFOXO* clone (hsFLP/+; *UAS-dFOXO*,Thor-YFP/ mRFP; Act5c>CD>Gal4/+).
Red: UAS-mRFP, Blue: DAPI, Green: Thor-YFP.White arrow showing clone cell.Scale bar 50µm



3.2.4. Analysis of Thor protein expression in response to NOS2 expression

To further investigate the increase in Thor expression in response to NO, an anti-Thor antibody was utilised. To first test this antibody for use on tissue, antibody staining was carried out on both larvae homozygous for a *Thor* null allele (*Thor*^{2}) and those expressing *UAS-NOS2* in clones. The homozygous *Thor*^{2} larvae showed the same level of staining as wild type larvae (Fig. 3.7.). The *UAS-NOS2* clones did show an increase in anti-Thor staining compared to that of surrounding wild type tissue but only when there was high expression of Gal 4 as indicated by very bright GFP expression in clones (Fig. 3.8.).

Figure 3.7. Antibody staining of Thor protein in wild type and *Thor* ^{2} salivary glands.
Ai Wild type third instar salivary gland (yw). **Aii** Thor null salivary gland (yw; *Thor* ^{2}).
Red: anti-Thor antibody, Blue: DAPI. The anti-Thor antibody Miron et al (2001)(Miron 2001).
Scale bar 100µm.

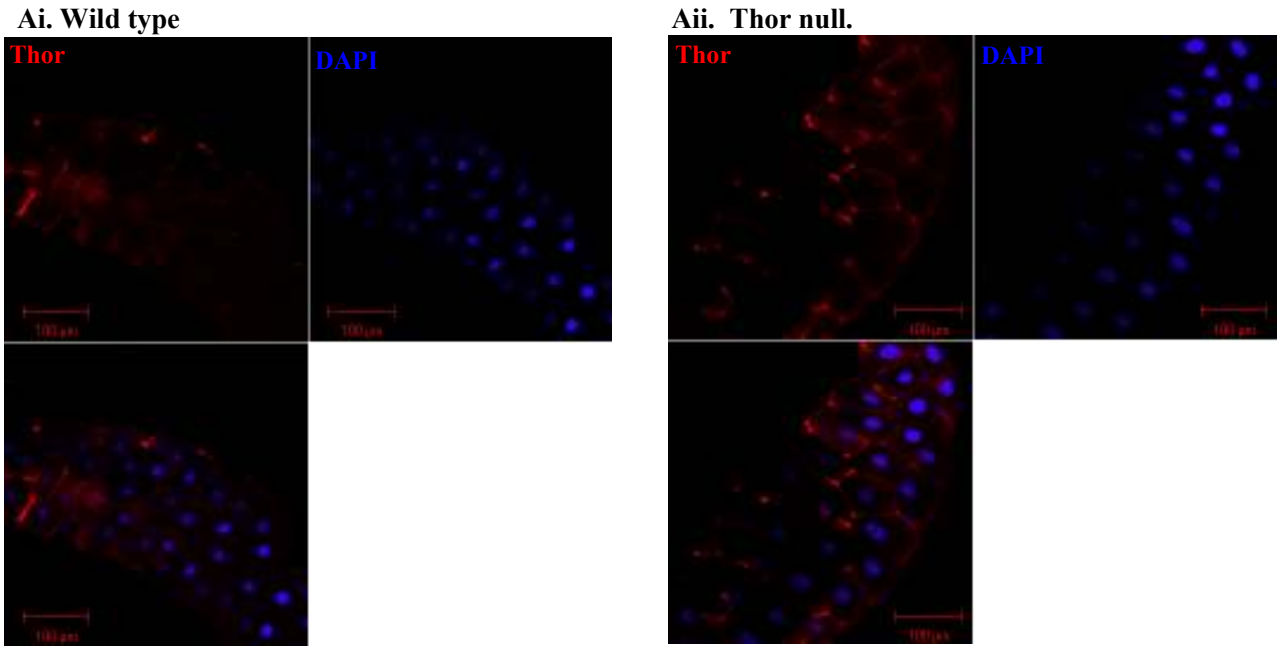
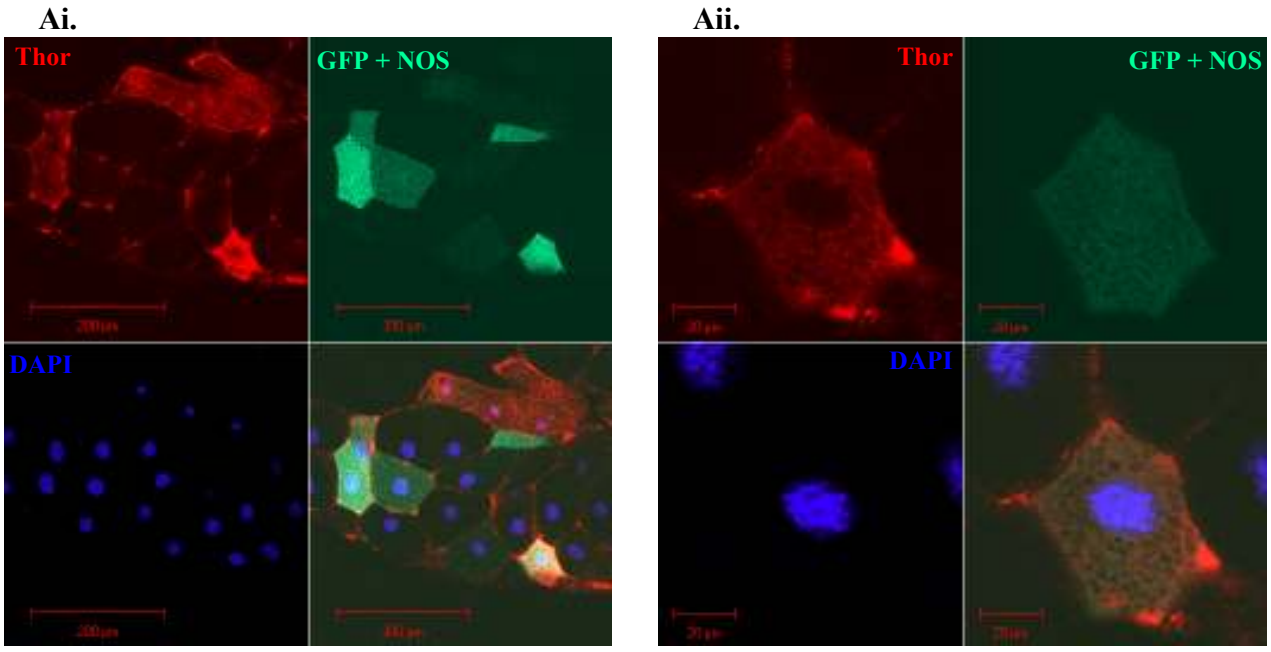


Figure 3.8. Antibody staining of Thor protein in NOS2 expressing clones in salivary glands.
Clones were generated using a 4min heat shock at 38°C between 24-48hr AEL. Clones are labeled with GFP.
Ai. *UAS-NOS2* clone (*UAS- NOS2*/hsFLP; Thor LacZ/Act5c>y^{+}>Gal4;*UAS-GFP*/+). Scale bar 200µm.
Aii. *UAS-NOS2* clone close up. Red: anti-Thor antibody, Blue: DAPI, Green: *UAS-GFP*. The anti-Thor antibody is as in Miron et al (2001)(Miron 2001). Scale bar 20µm



3.2.5. Analysis of dFOXO expression and localisation

NO has been shown to increase the expression of Thor in whole salivary glands, and I have demonstrated a similar increase in expression of Thor promoter activity, Thor-YFP and Thor protein expression in single cells expressing NOS2. Thor expression is known to be regulated by the transcription factor dFOXO (Puig et al 2003, Puig & Tjian 2005). It has also been shown that both the inhibition of growth and molecular responses to NO in *Drosophila* larvae are dependent on dFOXO (Kimber 2005). The phenotype of reduced size and increased Thor expression that is seen when NOS2 is expressed in the salivary glands is not seen when NOS2 is expressed in animals homozygous for a null allele of *dFOXO* (Kimber 2005).

To determine if the NO-induced effects on Thor expression in salivary glands were due to alterations in the expression of dFOXO, the levels and localization of dFOXO protein were determined. It has been shown that the sub-cellular localization, and therefore transcriptional activity, of dFOXO is altered by insulin (Puig et al 2003). Thus the localisation of dFOXO may change from cytoplasm to nucleus in response to an increase of NO. When dFOXO is located in the cytoplasm it is inactive, but when activated it moves in to the nucleus and initiates transcription of *Thor* (Puig et al 2003).

Expression and localisation of dFOXO was examined in whole salivary glands expressing *UAS-NOS2* using the c147 Gal4 driver. The data showed that there did not seem to be an identifiable change in the nuclear:cytoplasmic ratio of dFOXO distribution as compared to wild type cells (Fig. 3.9.). However, there is an apparent increase in the overall levels of dFOXO protein (Fig. 3.9.). Nevertheless, it should be noted that the results do show large and overlapping standard deviations.

dFOXO protein localisation was also examined in *UAS-NOS2* expressing clones within the salivary glands. A similar result is seen (Fig. 3.10.), with there being no obvious change in the nuclear:cytoplasmic ratio of dFOXO expression. However when the overall expression of dFOXO is compared to the surrounding wild type tissue there is again an overall increase of dFOXO expression within the clones (Fig. 3.10.).

Figure 3.9. Distribution of dFOXO expression in whole salivary glands.
dFOXO expression in the nucleus and the cytoplasm in wild type and *UAS-NOS2* expressing salivary glands. WT Nuclear dFOXO (+/+;c147/+), WT Cytoplasmic dFOXO (+/+;c147/+), NOS2 Nuclear dFOXO (NOS2/+;c147/+) and NOS2 Cytoplasmic dFOXO (NOS2/+;c147/+) Measurement of dFOXO expression was performed using Image J to calculate integrated density. Error bars indicate the standard deviation.

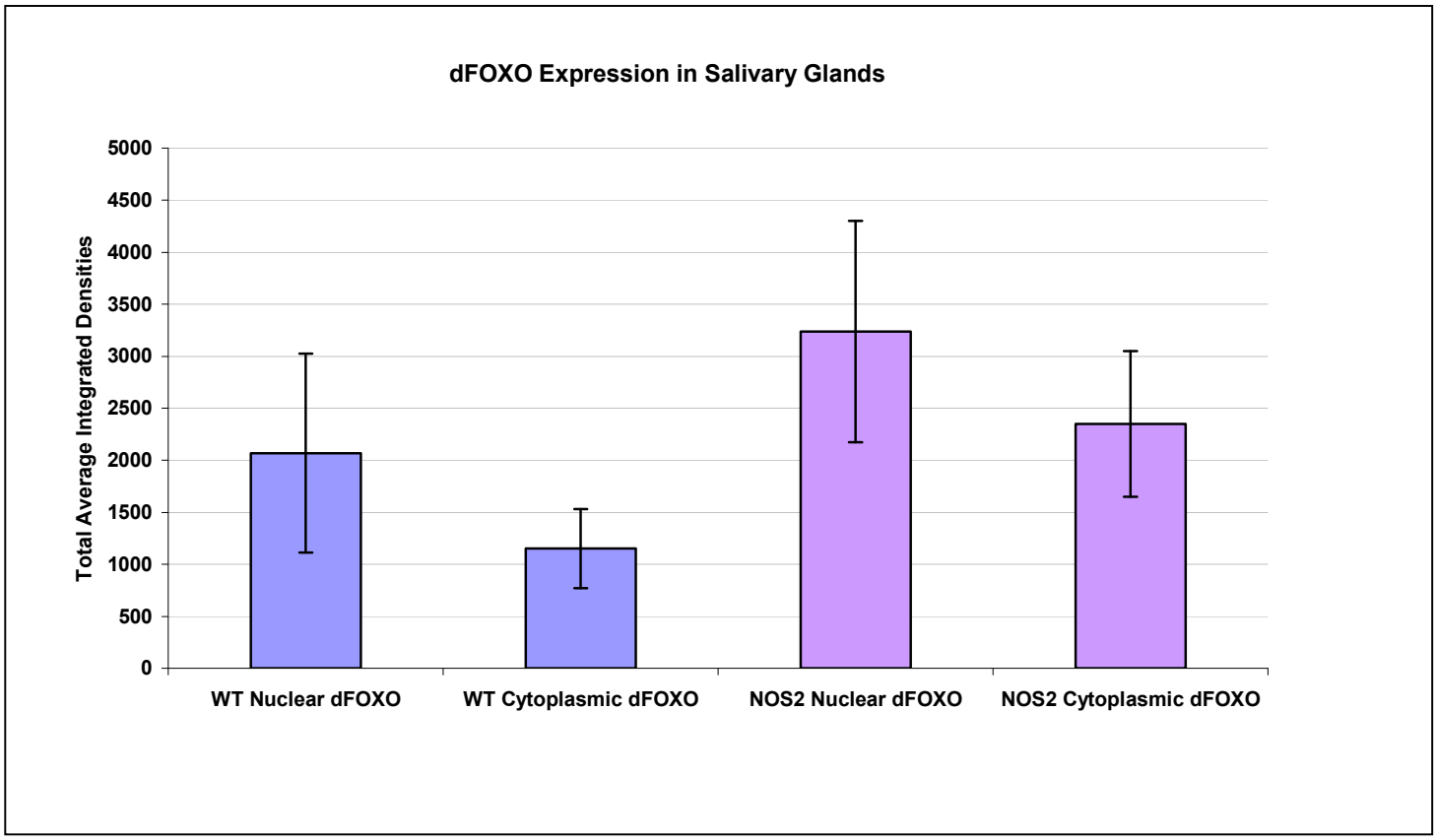
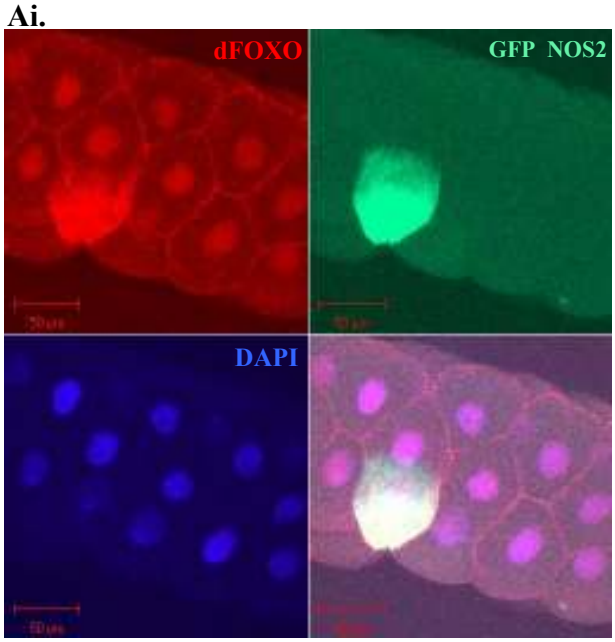
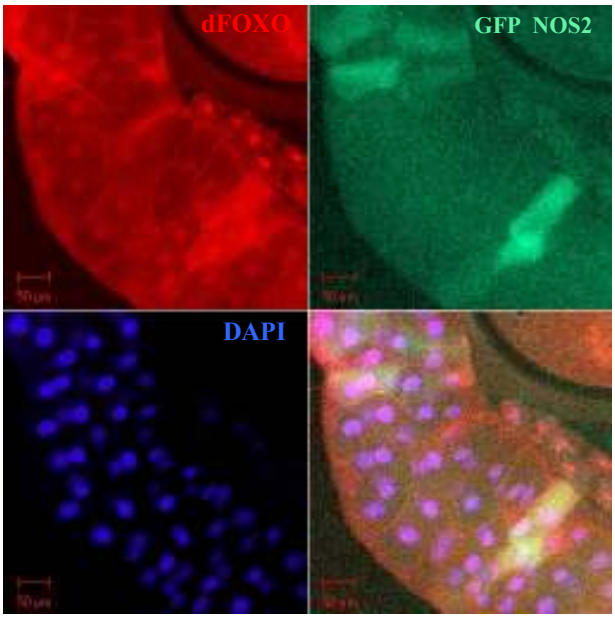


Figure 3.10. dFOXO protein expression and subcellular localization in NOS2 expressing cells in salivary glands. Clones of NOS2 expressing cells were generated using a 4 min heat shock at 38°C between 24 - 48hr AEL of animals with the genotype: *UAS- NOS2*/hsFLP; Act5c>y+>Gal4;*UAS-GFP*/+. **Ai** and **Aii** show increase in level but no obvious change in subcellular localisation of dFOXO protein in NOS2 expressing clones. Red: dFOXO expression using dFOXO primary antibody and cy5 secondary antibody, Blue: DAPI, Green: GFP. Scale bar 50µm

Ai.



Aii.



3.2.6. Analysis of dFOXO expression and localisation in cell culture

The previous work showing relocalisation of dFOXO protein was using cell culture (Puig et al 2003), therefore to examine if any similar effects could be observed in response to NO, S2 tissue culture cells were employed. The NO donor SNAP (S-nitroso-N-acetyl-penicillamine) was applied to S2 cells which were then stained with anti-dFOXO antibody. SNAP produces NO immediately upon contact with water, accordingly SNAP must be reconstituted prior to use in a non-aqueous solvent (DMSO), allowing the time of the start of NO production to be accurately recorded. The biological half-life of SNAP in aqueous media is predicted to be less than 30 minutes (Janssen et al 2000). The concentration of SNAP used was 200uM, the minimum concentration previously shown to induce cytostasis in S2 cells (Kimber 2005). Analysis of dFOXO expression was performed over a few different time-courses. Initial results suggested that there was an increase in dFOXO expression (Fig.3.11). The increase of dFOXO expression was apparent after 1hour, there seemed to be no effect at 30 min. The increase of dFOXO was seen through a six hour time course. Though there was an increase in dFOXO expression no consistent alterations to dFOXO localisation could be determined by eye, therefore analysis was done using the imaging analysis software Velocity (www.improvision.com/products/Volocity/) (Fig 3.12). The results though were highly variable, and I was not able to draw any definite conclusions from the data.

Figure 3.11. dFOXO protein expression and subcellular localization in S2 cells treated with SNAP. Two images of each treatment time with SNAP are given. Red: dFOXO expression using dFOXO primary antibody and cy5 secondary antibody, Blue: DAPI. All images acquired and processed using identical settings. Scale Bars 20μm.

A) Control cells with no SNAP treatment.

B) Cells after 30min SNAP treatment. dFOXO expression showed no increase compared to control.

C) Cells after 1hour SNAP treatment. dFOXO expression was increased compared to control.

D) Cells after 2 hours SNAP treatment. dFOXO expression was increased compared to control.

E) Cells after 3hours SNAP treatment. dFOXO expression was increased compared to control.

F) Cells after 4hours SNAP treatment. dFOXO expression was increased compared to control.

G) Cells after 5hours SNAP treatment. dFOXO expression was increased compared to control.

H) Cells after 6hours SNAP treatment. dFOXO expression was increased compared to control.

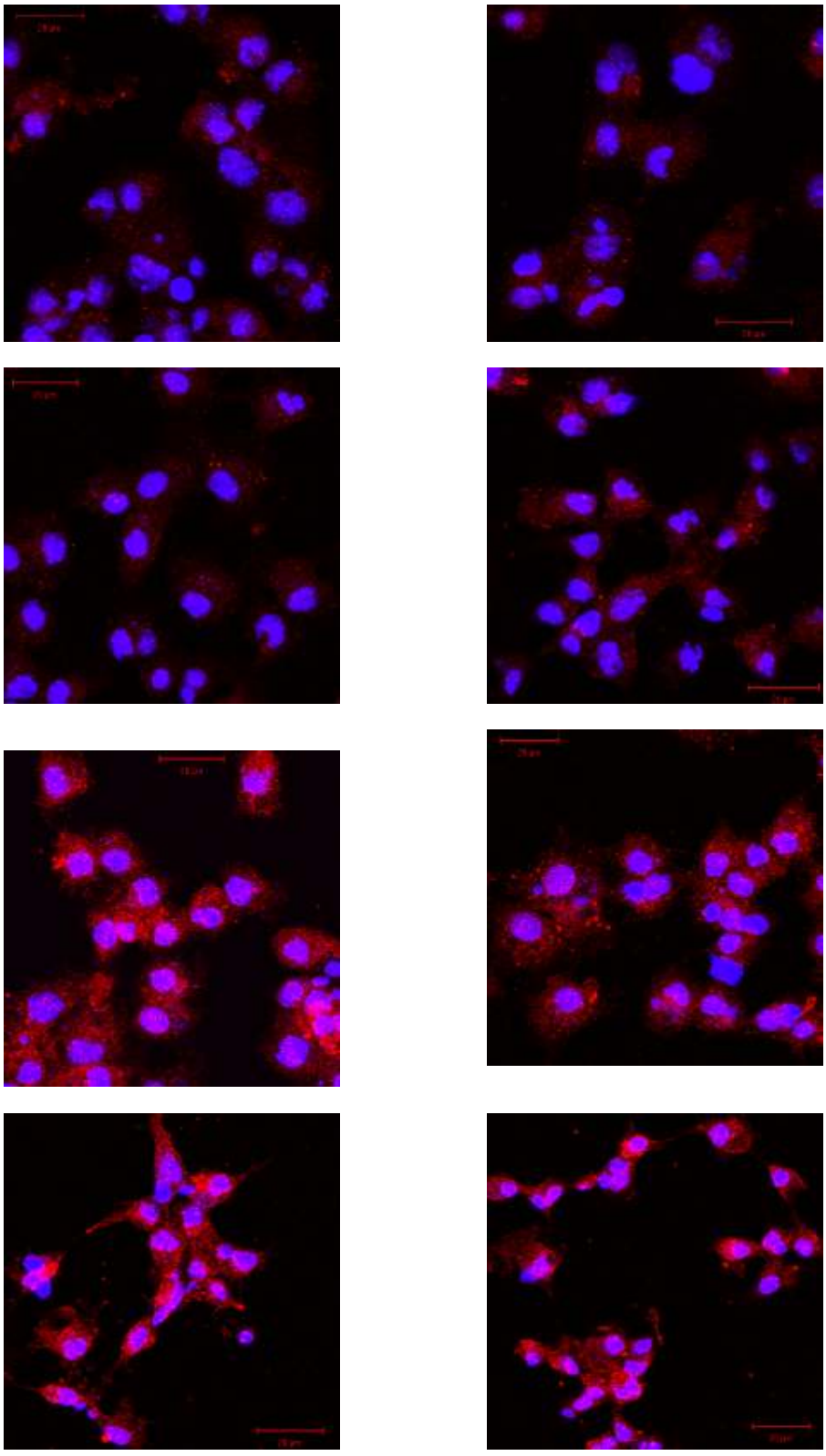
Figure 3.11.

A) No SNAP

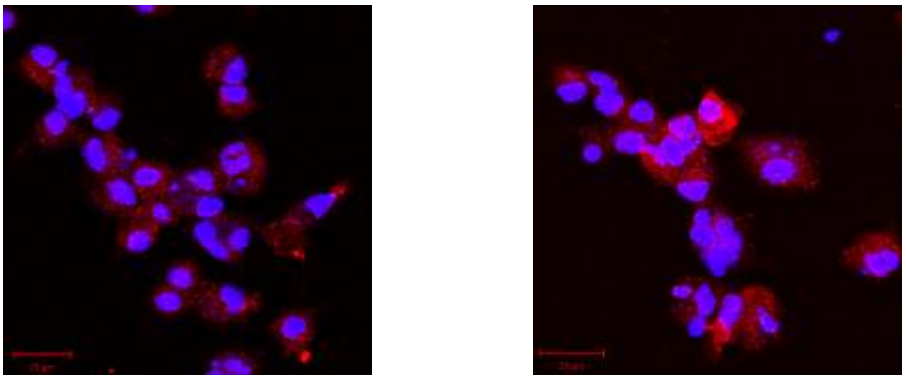
B) SNAP 30min

C) SNAP 1 hr

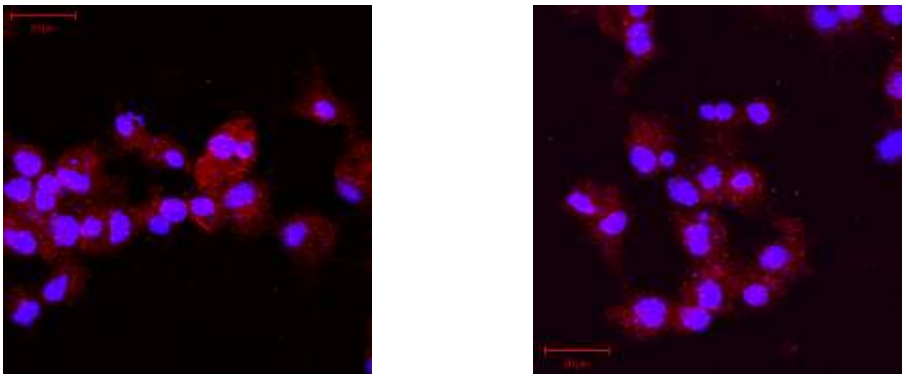
D) SNAP 2 hr



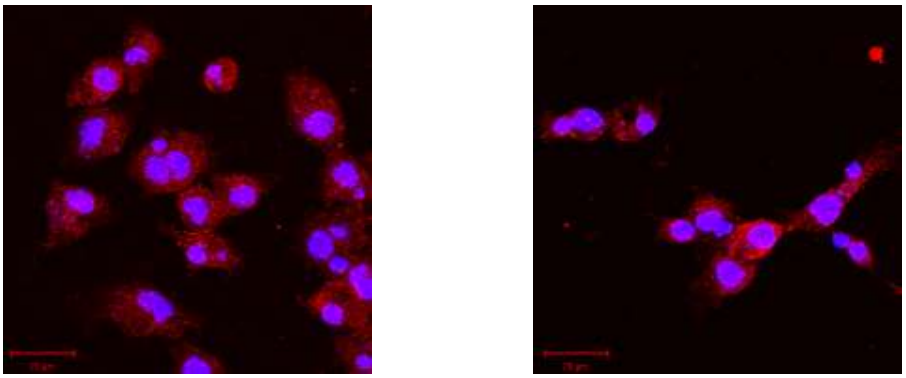
E) SNAP 3 hr



F) SNAP 4 hr



G) SNAP 5 hr



H) SNAP 6hr

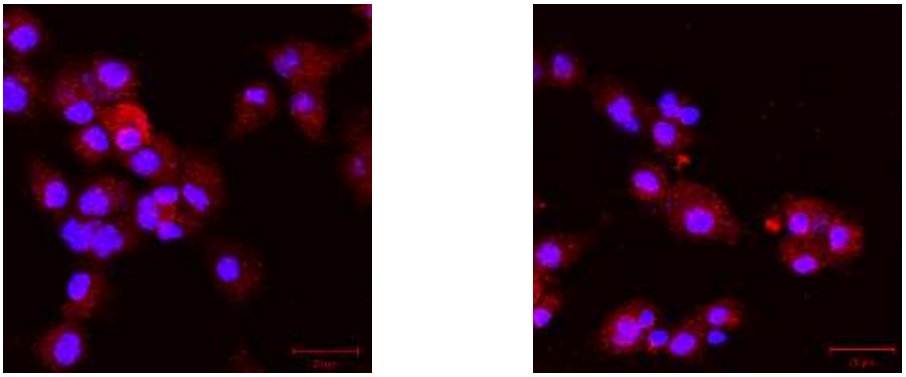
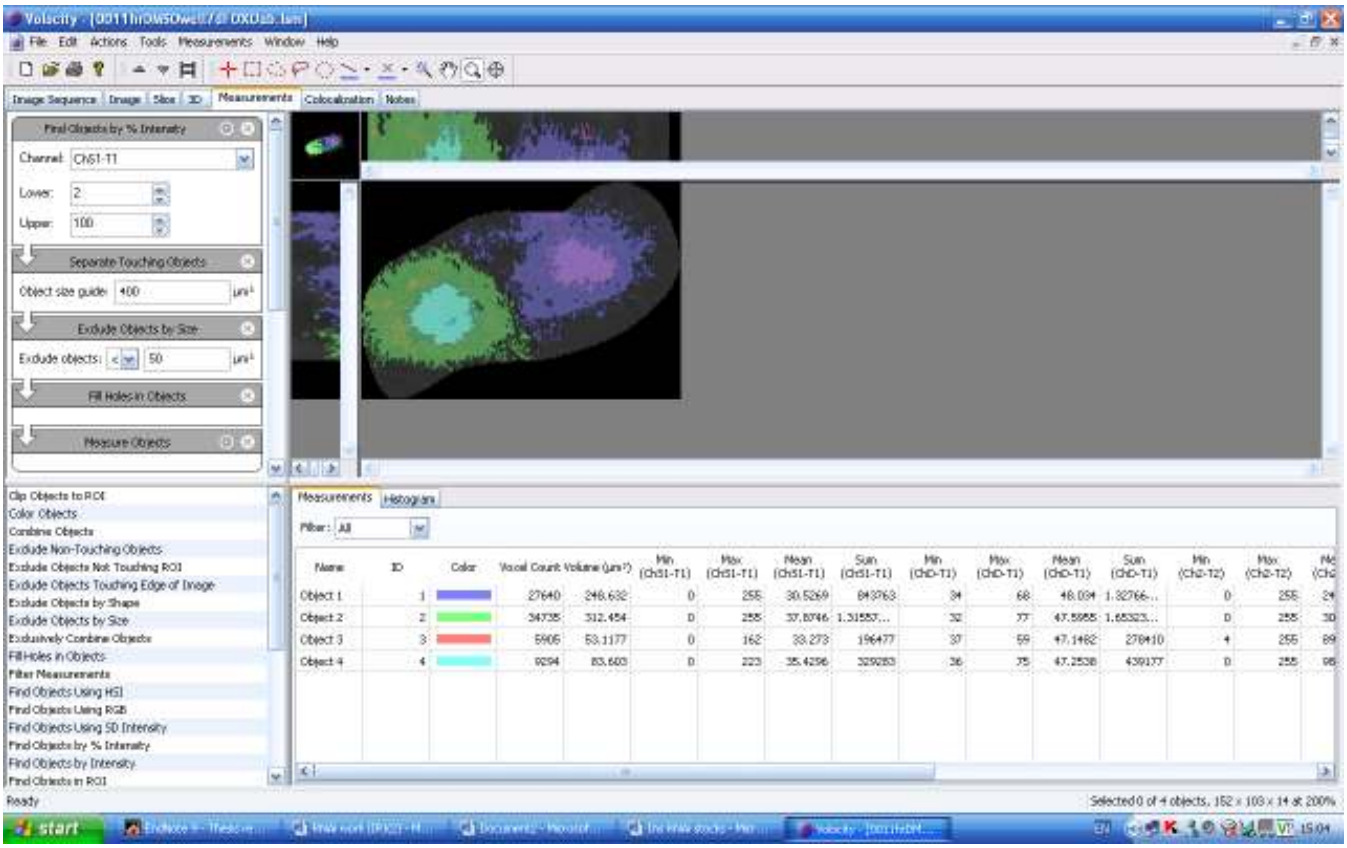


Figure 3.12. Screen capture showing the use of imaging analysis software Velocity. The software was utilised to identify any changed to dFOXO localisation in response to SNAP treatment on S2 cells.



3.2.7. Analysis of Thor-YFP expression and localisation in salivary glands in tissue culture

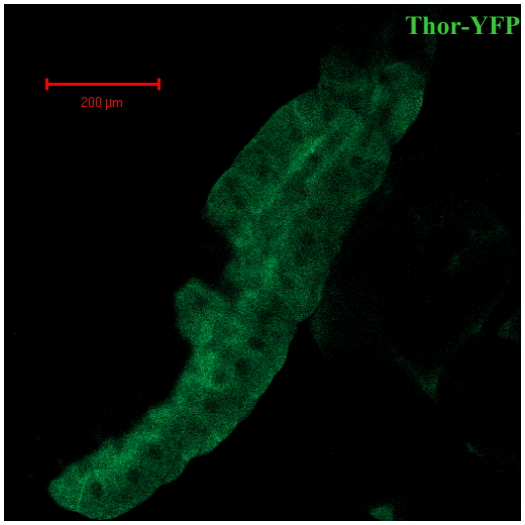
The work done by Kimber et al 2005 in cell culture was looking at expression of the gene rather than that of the protein its self, by utilizing the Thor-YFP construct the protein expression could be monitored. As seen above in 3.2.3 an increase in expression of Thor-YFP was observed in cells or whole salivary glands expressing *UAS-NOS2*. To identify the time frame in which this increase of Thor protein acts it was decided to use a time course of SNAP treated salivary glands. This was achieved by dissecting out the salivary glands of wandering third instar larvae and separating the pair. One of the pair was placed in tissue culture media plus and SNAP. The other gland was placed in tissue culture plus DMSO only. After specific incubation times images of both the SNAP treated (experimental) and DMSO treated (control) were taken. Most of the results that were incubated in time periods ranging from 30min to 7 hours showed increase in Thor-YFP expression when treated with SNAP (Fig. 3.13). Though through out the varing time periods the increase in expression was varible. The results that did not show increase were ones which had been slightly damaged during the dissection process or also glands that overall did not look normal. This result is in keeping with the results already shown in this chapter.

Figure 3.13. Thor-YFP expression is increased in single salivary gland cells incubated with SNAP. All images acquired and processed using identical settings.
Ai. 1 hour DMSO treated salivary gland 10x. **Aii.** 1 hour SNAP treated salivary gland 10x. With SNAP treatment Thor-YFP expression increased. Scale Bars 200µm

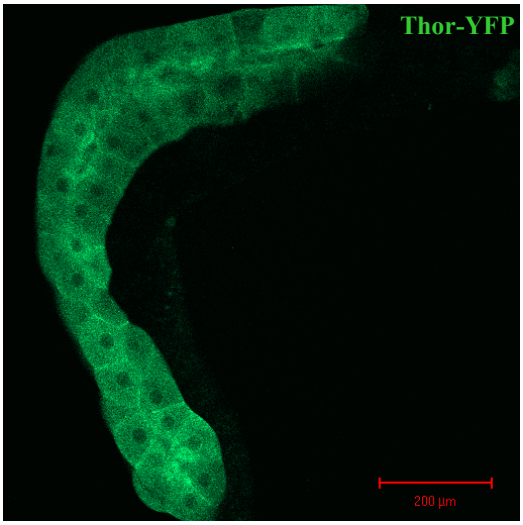
Bi. 4 hour DMSO treated salivary gland 63x. **Bii.** 1 hour SNAP treated salivary gland 63x. With SNAP treatment Thor-YFP expression increased. Scale Bars 200µm

Green: Thor-YFP

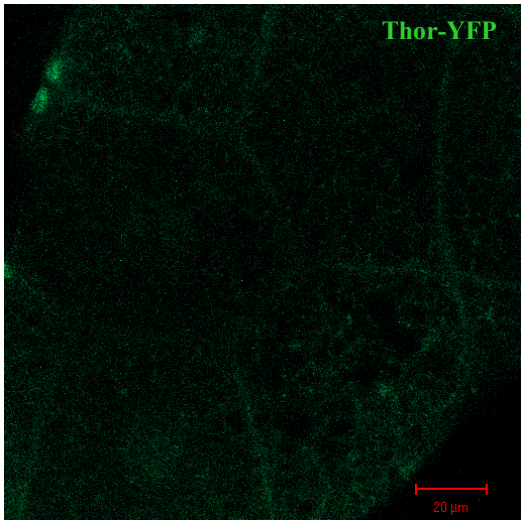
Ai. DMSO control



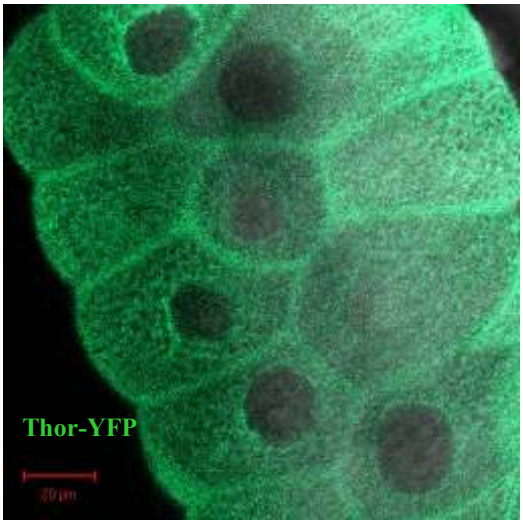
Aii. SNAP Treated



Bi. DMSO control



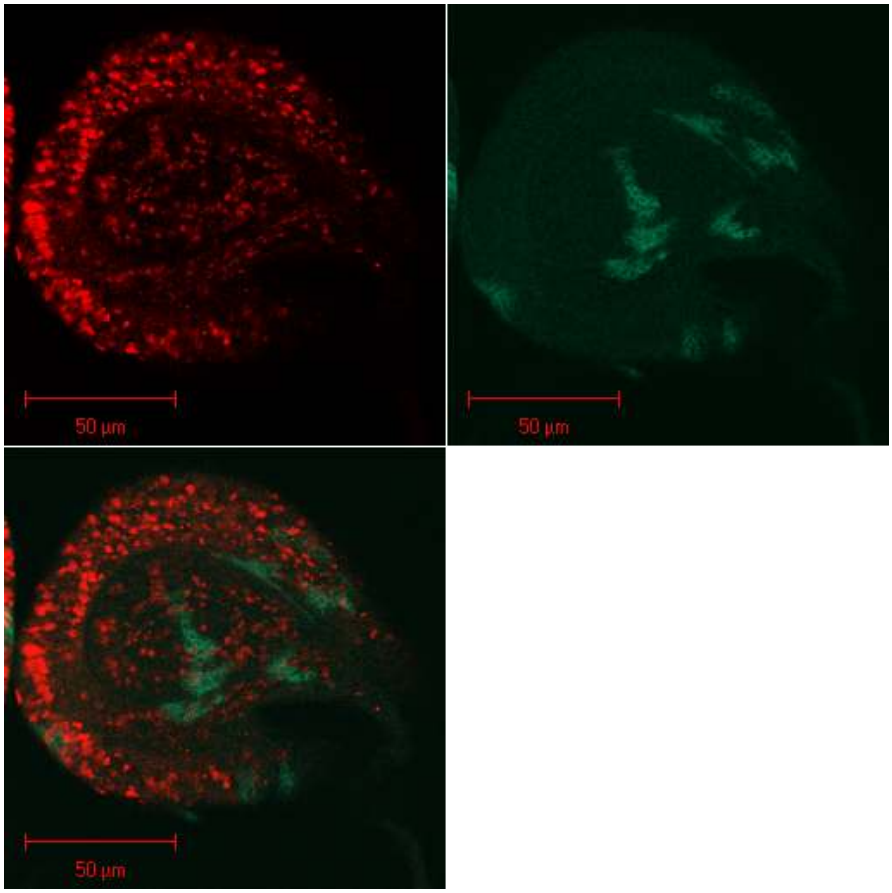
Bii. SNAP Treated



3.2.8. Analysis of DNA synthesis in response to removal of NOS

Kuzin et al 1996 investigated how NOS activity affected DNA synthesis. It was found that when NOS2 was expressed in the wing disc fewer went through S-phase as identified by there being less BrdU labeled cells. In contrast when cells were treated with L-nitroarginine methyl ester (L-NAME) a chemical which suppresses NOS activity, there were more BrdU labeled cells. Since this original paper a null mutant for NOS has been generated. This means that clones which are mutant for dNOS were able to be generated utilizing the dNOSc which is a null allele of the dNOS gene (Regulski et al 2004) therefore producing clones that are null rather than reduced NOS activity. Flies were generated to carry a FRT proximal to dNOSc. G418 selection was used as these flies inherited the transgenic neomycin resistance from the FRT site. Theses flies were then crossed to heat shock Flp stock to generate clones. BrdU (5-bromo2'-deoxy-uridine) staining was then done this was done because it would allow an estimation of the rate of cell division as it measures the number of cells which have proceeded through S-phase, which will be compared in dNOSc clones expressing tissue compared to wild type tissue. BrdU staining works as BrdU is a synthetic nucleotide which is an analogue of thymidine and will be incorporated into DNA in place of thymidine during DNA synthesis. Therefore during S-phase, in the presence of BrdU, cells will incorporate BrdU. Cells, which have incorporated BrdU into there DNA, can be detected using a monoclonal antibody against BrdU. The results showed that dNOSc clones were able to be generated. The generation of wing disc clones expressing NOS2 were as discussed previously were much less prevalent, probably due to widespread induction of expression of NOS2 causing lethality so no results were clear enough to analysis fully (Fig 3.14).

Figure 3.14. BrdU expression in NOS2 clones in wing imaginal discs.
Clones were generated using a 4min heat shock at 38°C between 12-24hr AEL, of animals with genotype: *UAS- NOS2*/hsFLP; Thor LacZ/Act5c>y^{+}>Gal4;*UAS-GFP*/+. Clones are therefore labeled with GFP. Red: BrdU, Green: GFP NOS2. Scale bar 50µm



3.3 Discussion

NO is a known regulator of growth. To verify a possible target of NO action, the expression of dFOXO was determined in both whole salivary glands and in single cell clones within the salivary glands. To first verify the effects of NO reduction on growth, *UAS-NOS2* was expressed in the salivary glands. NOS2 expression resulted in statistically smaller salivary glands and salivary gland nuclei. The same was seen in salivary glands expressing *UAS-dFOXO*, although the glands and nuclei were even smaller. These results show that both NO and dFOXO can inhibit growth, although dFOXO can achieve this to a higher level. This may be because NOS2 expression does not up regulate dFOXO expression/activity to the same level that is seen when *UAS-dFOXO* is driven by Gal4.

3.3.1 Thor expression increases in response to NOS2

As previously mentioned, it has been shown that Thor expression increases both *in vivo* and *in vitro* in response to NO treatments. In the *in vivo* study, NOS2 was expressed in whole salivary glands which meant the increase in Thor LacZ expression occurred in the background of a reduction in salivary gland size. Accordingly, the observed increase in staining could have been a reflection of this change in size, and not as a result of an increase in the expression of *Thor*. To verify the increase in Thor LacZ levels were in response to NOS2 expression, *UAS-NOS2* was expressed in small or single cell clones within the salivary gland. In this case, NOS2-expressing clones showed an increase in Thor LacZ as was expected. The clones also showed an interesting result in that the expression of NOS2 resulted in cell-autonomous as well as a cell non-autonomous action. This result coincides with the fact that NO is known to diffuse between cells (Haley 1998). The short-range of non-autonomous effects of NO observed are probably due to its half-life of approximately only a few seconds (Haley 1998), limiting its diffusion to those cells immediately neighboring the source of NO.
The increase in Thor expression in response to NOS2 was also observed with both Thor-YFP and using Thor antibody staining. These results verify those observed with the LacZ reporter. Thereby showing that NOS2 does act to increase the expression of Thor protein.

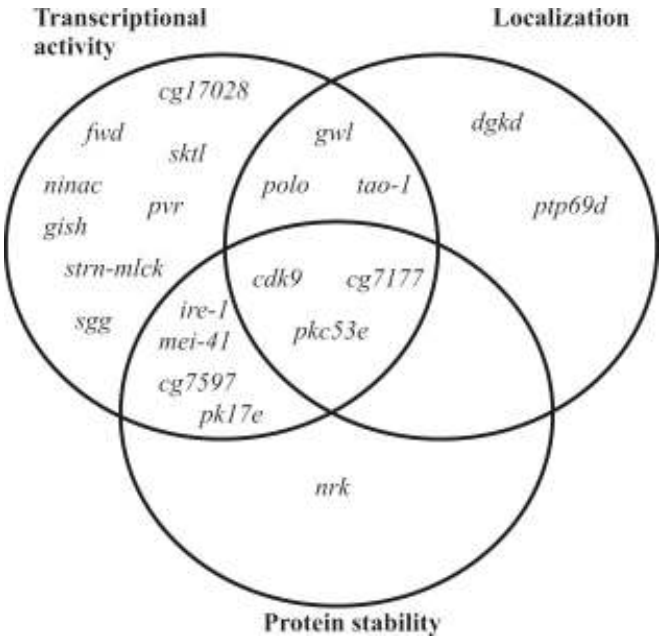
3.3.2. dFOXO expression increases in response to NOS2 activity

The increase in Thor expression that was found as described above is thought to be due to the action of the transcription factor dFOXO. It has been previously shown that reduced salivary gland size and increased Thor expression in response to NOS2 activity is dependent on dFOXO expression (Kimber 2005). Therefore dFOXO expression in response to NOS2 was examined using a dFOXO antibody. Firstly, expression of dFOXO was analysed in whole salivary glands in response to NOS2 expression. It was expected that there would be a change in sub-cellular localization from cytoplasm to nucleus, as that has been previously reported *in vitro* in *Drosophila* as a result of activity of the insulin pathway (Puig et al 2003). When NOS2 was expressed in the whole salivary gland an overall increase in dFOXO expression was seen in both cytoplasm and nucleus compared to wild type glands. The ratio of dFOXO in the nucleus to cytoplasm was similar for both wild type and NOS2 expressing salivary glands. However, dFOXO expression in response to NOS2 is difficult to determine in whole salivary gland due to the NOS2-induced reduction in size. Therefore clones of cells within the salivary glands expressing NOS2 were also examined. There was not an obvious change in nuclear to cytoplasmic ratio of expression, however, an increase in the amounts of dFOXO present in the clones expressing NOS2 was very noticeable. This increase in dFOXO expression in response to NOS2 expression may mean that NO acts through dFOXO, but by regulating dFOXO activity in different way than simply through modulation of sub-cellular localisation. This increase but no apparent sub-cellular localisation change of dFOXO was also observed in cell culture with S2 cells were treated with NO. The results also suggested that it took over 30 min for the NO to cause an increase in dFOXO expression.

Due to the lack of sub-cellar movement of dFOXO in response to NO it may mean that NO may be acting through different regulatory routes than those previously identified (such as through Akt-mediated phosphorylation). Although the increase in protein levels of dFOXO may be masking a subtle localisation change so Akt regulation cannot be completely ruled out. Other ways in which dFOXO is known to be regulated were mentioned briefly in Chapter 1 (section 1.2.3.4.). Sub-cellar localisation (through phosphorylation), transcriptional activity and protein stability may be ways in which NO is acting to regulate dFOXO and thereby growth. As an increase in dFOXO is observed with NOS2 expression it may be that dFOXO protein stability is altered by NO, thereby resulting in an increase in dFOXO protein. In a study of dFOXO regulators using *Drosophila* S2 cells, 21 protein were identified that modulated

dFOXO transcriptional activity, protein levels and/or sub-cellular localization (Fig. 3.15.) (Mattila et al 2008). Of these 21 proteins, 8 have been shown to regulate dFOXO through stability (Mattila et al 2008). Therefore these proteins may be of interest for future work to see if NO may act by affecting one or more of these proteins to regulate dFOXO and thereby growth. It may also be of interested to see if dFOXO mRNA is increased in response to NO. This would show that protein levels may be increasing, and increasing because of transcription of *dFOXO,* but this would not eliminate changes due to increased stability of the protein. This could be analysed in NOS2 clones using *in situ* hybridisation to *dFOXO* mRNA. It may also be possible that NO is directly effecting dFOXO through chemical modification such as S-nitrosylation (Asada et al 2009, Stamler 1994).

Figure 3.15. The identified dFOXO regulators grouped by their regulatory mode of action. Eighteen proteins regulate dFOXO dependent transcription, eight regulate its localization, and eight its stability. Three proteins have effects in all three readouts. Figure from Mattila et. al. (2008) (Mattila et al 2008).



3.3.3. In vitro versus in vivo

In this chapter both *in vitro* and *in vivo* techniques were used for looking at both Thor and dFOXO expression in response to both NOS expression and SNAP treatment to identify the action of NO anti-proliferation effect. Looking first at Thor expression, an increase in Thor expression was observed using Thor-LacZ , Thor-YFP and a Thor antibody in the salivary glands in response to NOS2 expression. The expression of Thor-YFP was as also looked at in response to *in vitro* expression of SNAP on cultured salivary glands, though results were variable in the amount of increase in Thor-YFP expression it did increase.
The expression of dFOXO was shown to increase in response to NOS2 through the use of dFOXO antibody though no sub-cellular localisation change was observed. The dFOXO antibody was also used in S2 cells to see if a change in dFOXO localisation could be observed in response to SNAP treatment. As for the NOS2 expression an increase in dFOXO was observed in response to SNAP treatment. A change in sub-cellular localisation though was not observed. Most of the previous work that had observed location change of dFOXO used a tagged version of dFOXO protein which would be one way forward. It would also be best to use a positive control such as insulin treatment to optimize the procedure to make sure the system was working. This was no done during this work as there was a limited supply of antibody meant work had to be limited

Together the results from this Chapter confirm NO as a growth regulator *in vivo* and that its action is through dFOXO. To investigate whether NO does act directly on dFOXO or via upstream components of the insulin signaling pathway or by other pathways, a genetic analysis examining the epistatic relationships between NO and known components of the signaling pathway was performed. The results of these experiments are detailed in the following chapters.

Chapter 4: Analysis of signal transduction required for NO dependent growth regulation

4.1. Introduction

Although NO has been shown to act through dFOXO, whether it acts directly on dFOXO or activates some component of the insulin signaling pathway, or a separate pathway is not known. To elucidate if NO acts directly on dFOXO, or if it acts further upstream in the insulin pathway or by another pathway, a genetic dissection of the pathway can be performed, assaying what components are necessary for the NO signal to be transduced. As the majority of the identified members of the insulin signaling pathway are required for viability in the whole organism or for tissue growth, a targeted knock down strategy to reduce the amounts of the proteins was undertaken. Data which analyses the signal transduction required for NO-dependent growth regulation will be presented in this chapter.

One way to reduce the expression of proteins is the use of RNA interference (RNAi), this allows for a targeted knockdown of protein expression. RNAi is a technique which results in specific gene-silencing, initiated by double-stranded RNA (Fire et al 1997). RNAi was first demonstrated in *Caenorhabditis elegans* showing that dsRNA could be used to silence specific genes in animals (Fire et al 1997). RNAi is an evolutionary conserved mechanism found in fungi, plants and animals. It has been determined that RNAi machinery is also conserved across species and that target mRNA degradation is directed by short (21–23-nucleotide) RNA fragments derived from long dsRNAs. This cleavage is performed by an RNase III-type enzyme, Dicer. So as well as dsRNA, small interfering (si)RNAs can be used directly as triggers to initiate gene silencing by binding to their target-mRNA. Once formed the small dsRNAs assemble into the RNA-induced silencing complex (RISC), which then targets the complementary mRNA.

In *Drosophila* one of the first examples of RNAi use was to demonstrate the actions of genes within the wingless pathway (Kennerdell & Carthew 1998). In this work dsRNA was injected directly into embryos, although since that study the UAS/Gal4 system has been utilised for delivery of the dsRNA. The Vienna *Drosophila* RNAi Center (VDRC) stock centre provides stocks enabling expression of RNAi directed towards 88% of the predicted protein-coding

genes in the *Drosophila* genome (Dietzl 2007). These RNAi constructs are linked to a UAS and so their expression is inducible and is able to be targeted with the use of Gal4 drivers. To analyze the signal transduction required for NO-dependent growth regulation, RNAi knockdown of specific genes was undertaken by expressing suitable constructs along with *UAS-NOS2* and observing any change to the growth phenotypes observed with *UAS-NOS2* alone.

Comparing the results of experiments when NOS2 was expressed alone, to the phenotypes produced by combinations of transgenes may show if NO is acting upstream or downstream of that component of a pathway. This technique is known as epistasis. Epistasis analysis works by examining the phenotype of a double mutant to identify if the products of the two mutant genes act in the same or independent pathway (Lawrence S. B. Goldstein 1994).

4.1.2. Is NO acting through soluble guanylyl cyclase to regulate growth?

As previously mentioned, a number of heme containing proteins are controlled by NO (Stamler 1994). Soluble guanylyl cyclase (sGC) is a heme-containing, heterodimeric NO receptor. The basal activity of sGC can be increased up to 200-fold by the binding of NO, but the resulting NO-heme complex has a half-life of only around 0.2s (Bruckdorfer 2005). sGC forms cyclic GMP from the nucleotide GTP. cGMP acts a secondary messenger, which can signal through cGMP-dependent protein kinases (PKGs). Active forms of PKG can phosphorylate many substrates including the IP_3 receptor and subunits of myosin light chain phosphatase (Krumenacker *et al* 2004).

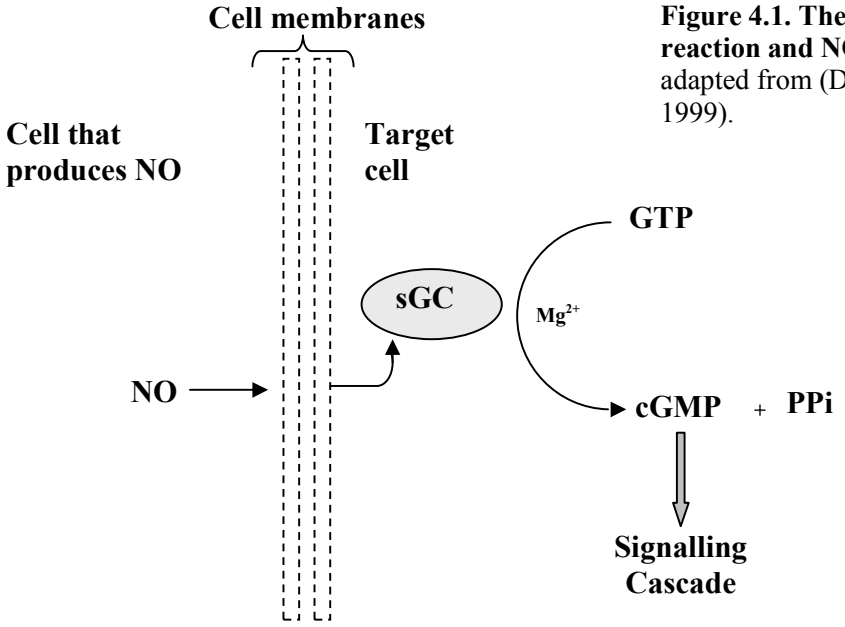


Figure 4.1. The guanylate cyclase reaction and NO signal transduction adapted from (Denningera & Marletta 1999).

The *Drosophila* genome contains 5 genes that code for sGC subunits – with different NO induced activities (Morton et al 2005). Two of these genes are Gycα-99B and Gycβ-100B, the α and β subunits that form a conventional, NO-sensitive heterodimeric sGC (Shah & Hyde 1995). Although several combinations of subunits exhibit sGC activity the majority of hetero- and homodimeric combinations are not greatly (only approximately 4 fold) stimulated by NO (Morton et al 2005). Only sGC complexes containing the Gycα-99B subunit are highly stimulated (30 fold) by NO (Morton et al 2005). Genetic removal of the Gycα-99B subunit has also been demonstrated to abolish any detectable increase in NO-induced increases in cGMP levels (Gibbs et al 2001). In *Drosophila,* NO has been shown to act through the interaction with sGC (as discussed in Chapter 1 section 1.1.4.). If NO signals to regulate growth via sGC, then the removal or reduction in the amount of NO-sensitive sGC should restrict the growth inhibition caused by NO signalling. To determine if this was so, a *UAS-RNAi-Gycα-99B* transgene obtained from VDRC was utilised. Co-expression of this *UAS-RNAi-Gycα-99B* with *UAS-NOS2* would determine whether NO regulation of growth was dependent on sGC expression, thereby indicating whether NO acted through sGC to regulate growth.

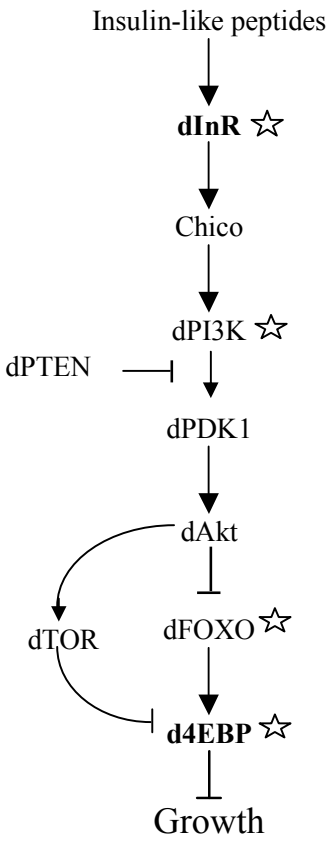
4.1.3. What components of the Insulin Signalling Pathway is NO affecting?

To determine what components of the insulin signaling pathway other than *dFOXO*, are required for NO-dependent growth inhibition, a similar RNAi-dependent knockdown approach was undertaken. The experiments performed were the co-expression of RNAi-NOS or NOS2 with components of the insulin pathway using epistasis analysis. For example, if NO activates dAkt, knockdown of any components of the pathway above dAkt would not suppress the phenotype associated with increased NO production. However, RNAi induced knockdown of components downstream (and including dAkt) should suppress the phenotypes in an analogous way to the genetic removal of *dFOXO*. As previously discussed in Chapter 1 section 1.3. an increased growth phenotype is produced by expression of UAS- RNAi-NOS with the tubulin-Gal4 driver. The expression of this construct under tubulin–Gal4 control results in an approximately 90% reduction in NOS protein levels (Kimber 2005). A selection of UAS-RNAi stocks from the VDRC stock centre were exploited to allow the knockdown of various specific components of the insulin signaling pathway (Fig. 4.2.).

As well as this genetic dissection of the proteins required for NO growth inhibition, a cellular analysis of the activation of the insulin signaling pathway was undertaken.

PI3K is upstream of dFOXO in the insulin signaling pathway (Fig. 4.2.) PI3K activity can be assayed *in vivo* by examining the localisation of a ß-tubulin promoter driven GFP-PH fusion (tGPH) (Britton et al 2002). tGPH works through the pleckstrin homology (PH) domain of the *Drosophila* homolog of the general receptor for phosphoinositides-1 (GRP1) being fused to GFP. This tagged receptor will bind specifically to phosphatidylinositol-3,4,5-P_3 (PIP_3), the secondary messenger generated by PI3K. PIP_3 generally resides in lipid membranes, particularly the plasma membrane. GRP1 is recruited to membranes when PI3K activity raises the cellular levels of PIP_3. Thus the fusion proteins containing the GRP1 PH domain will also be recruited to plasma membranes by binding PIP_3, and therefore serve as a reporter for PI3K activity (Britton et al 2002). tGPH has been shown to respond to increases or decreases in dPI3K signaling *in vivo* by increasing or decreasing its localization at the cell membrane (Britton et al 2002). Thus, this allows analysis of the effects of NO expression on dPI3K activity. Activated PI3K expression will be used as a positive control to show increased tGPH signal at cell membranes (Britton et al 2002, Wittwer et al 2005). If localisation of tGPH at the cell membrane decreases, this will suggest that the action of NO is not acting directly on dFOXO, but is instead acting further up the insulin pathway to reduce the activity of PI3K and thereby causing the increase in dFOXO. Conversely, if no change in the localisation of tGPH is observed, this will indicate that NO acts downstream of PI3K in this signaling cascade.

Figure 4.2. Insulin signalling pathway (adapted from (Puig et al 2003)) (☆ indicates components of the pathway tested with RNAi).



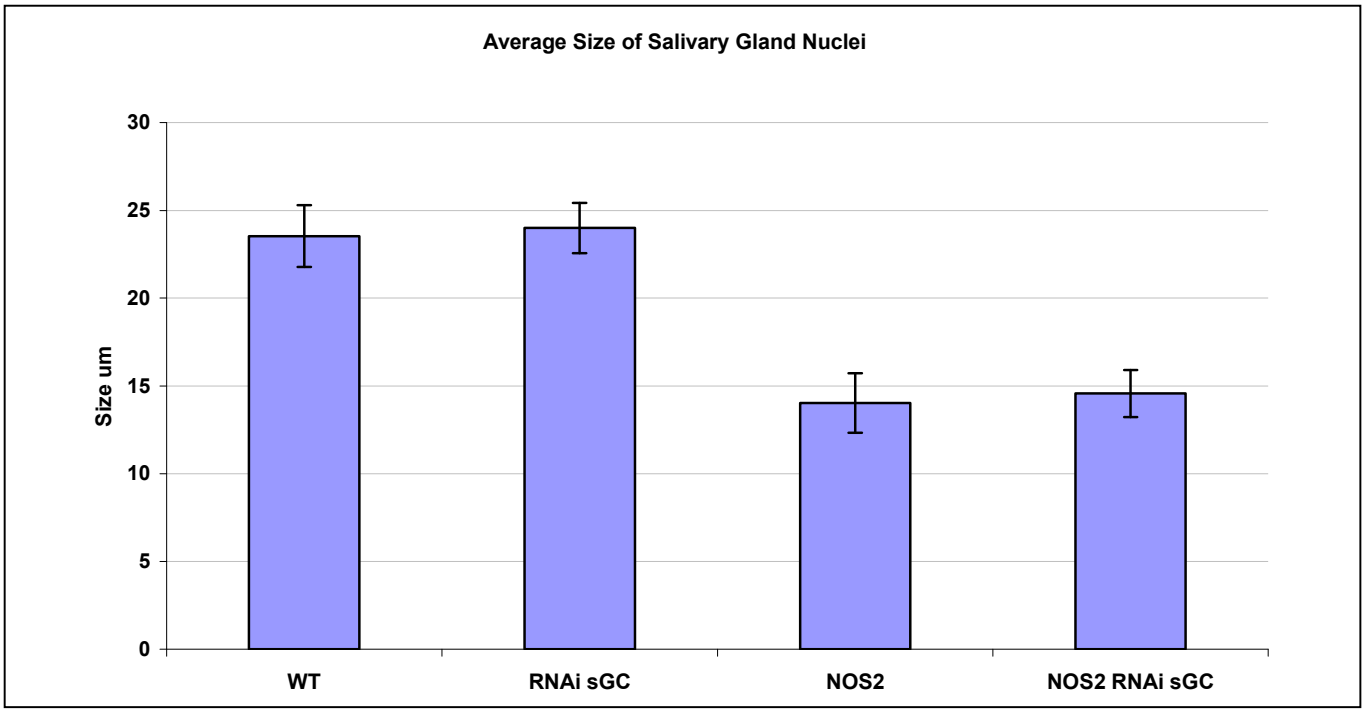
4.2. Results

4.2.1. Analysis of the requirement of sGC subunit- Gycα-99B for NO-dependent inhibition of growth

To determine if NO was acting through sGC to regulate growth, epistasis analysis was performed with targeted RNAi towards Gycα-99B which codes for the NO sensitive α subunit of soluble guanylyl cyclase (Shah & Hyde 1995). The c147-Gal4 driver was used to express *UAS-RNAi-Gycα-99B* and *UAS-NOS2* individually or together. Salivary gland nuclei of third instar larvae expressing *UAS-RNAi-Gycα-99B* were seen to be comparable in size to that of wild type larvae (Fig. 4.3.). Expression of *UAS-NOS2* showed the expected small nuclei when driven with c147-Gal4 (Fig. 4.3.). When *UAS-RNAi-Gycα-99B* and *UAS-NOS2* were co-expressed in the salivary gland, the salivary gland nuclei exhibited the reduced growth phenotype in a statistically similar way to that resulting from *UAS-NOS2* expression alone (Fig. 4.3.).

Figure 4.3. Average size of salivary gland nuclei expressing RNAi-Gycα-99B and NOS2.
c147 was used to drive expression UAS-RNAi- Gycα-99B, *UAS-NOS2* and both together. WT (+/+;c147-Gal4/+;+/+), RNAi-Gycα-99B (+/+;c147-Gal4/*UAS-RNAi-Gycα-99B*;+/+), NOS2 (*UAS-NOS2*/+;c147-Gal4/+;+/+) and NOS2 RNAi-Gycα-99B (*UAS-NOS2*/+;c147-Gal4/*UAS-RNAi-Gycα-99B*;+/+). Error bars represent the standard deviation (raw data is given in Appendix II.b.).

For WT, data was derived from 203 nuclei from 20 salivary glands. RNAi-Gycα-99B; 214 nuclei from 20 salivary glands. NOS2; 218 nuclei from 19 salivary glands. NOS2 RNAi-Gycα-99B; 265 nuclei from 21 salivary glands.



4.2.2. Analysis of interaction between RNAi-NOS expression and components of the insulin signalling pathway

The RNAi stocks from VDRC that were used in this part of the study were: *UAS-RNAi-dInR* and *UAS-RNAi-Pi3K* p110, both of which are components of the insulin pathway (Fig. 4.2.) and were expected to inhibit growth. Firstly, to verify the efficacy of the *UAS-RNAi-dInR* and *UAS-RNAi-Pi3K* transgenes, they were driven with the c147 salivary gland driver. As expected, the knockdown of positive components of the insulin pathway resulted in an overall reduction in size of salivary glands (Table 4.1). The lethality or growth phenotypes caused by the expression of these RNAi constructs when driven by tub-Gal4 was then determined (Table 4.1.). Expression via tubulin Gal4 driver of *UAS-RNAi-dInR* and *UAS-RNAi-Pi3K* p110 resulted in larval lethality before 3rd instar. When animals expressed either of these two RNAi constructs with RNAi NOS, again no 3rd instar larvae were recovered (Table 4.1.). Unfortunately as the RNAi-NOS dependent phenotype is only seen in 3rd instar larvae no conclusions about the interaction of NO and insulin signaling dependent growth could be drawn from these experiments.

Table 4.1. Size of salivary glands expressing RNAi-NOS and RNAi against insulin signalling pathway components.

UAS	Gal4 Driver	Genotype	3rd instar Salivary Gland size
UAS-RNAi-dInR	c147-Gal4	+/+; c147-Gal4/+; *UAS-RNAi-dInR*/+	very small
	tub-Gal4	+/+; +/+; *UAS-RNAi-dInR*/tub-Gal4	2nd instar lethal
UAS-RNAi-Pi3K p110	c147-Gal4	+/+; c147-Gal4/+; *UAS-RNAi-Pi3K* p110/+	small
	tub-Gal4	*+/+; +/+; UAS-RNAi-Pi3K p110/tub-Gal4*	2nd instar lethal
UAS-RNAi-dInR and *UAS-RNAi-NOS*	tub-Gal4	*UAS-RNAi-NOS/+; +/+; UAS-RNAi-dInR/tub-Gal4*	2nd instar lethal
UAS-RNAi-Pi3K p110 and *UAS-RNAi-NOS*	tub-Gal4	*UAS-RNAi-NOS /+; +/+; UAS-RNAi-Pi3K p110/tub-Gal4*	2nd instar lethal

4.2.3. Analysis of interactions between NOS2 expression and components of the insulin signalling pathway

An inhibition in growth phenotype was observed when *UAS-NOS2* was driven in the salivary gland by either c147-Gal4 or AB1-Gal4. The c147-Gal4 driven expression of *UAS-RNAi-Thor* transgene did not result in any observable change in salivary gland growth (Table 4.2.). Efficiency of the *UAS-RNAi-Thor* was assayed by examining expression of an endogenously expressed YFP tagged Thor protein. There appeared to be no reduction in expression of the YFP even with very high levels of RNAi-Thor expression driven by tubulin Gal4. Therefore no further work was undertaken with the *UAS-RNAi-Thor* stock. Expression of the *UAS-RNAi-dFOXO* with a salivary gland-specific Gal4, resulted in very small salivary glands that were smaller that those produced with either *UAS-NOS2* or the *UAS-dFOXO* with the same driver.

Table 4.2. Size of salivary glands expressing RNAi constructs targeted to Thor or dFOXO.

UAS	Gal4 Driver	Genotype	3rd instar Salivary Gland
UAS-RNAi-Thor	c147-Gal4	+/+;c147/+; UAS-RNAi-	wild type
UAS-RNAi-dFOXO	c147-Gal4	+/+;c147/ *UAS-RNAi-dFOXO*; +/+	very small

The inhibition of growth due to RNAi-dFOXO expression was not be expected, partly as null mutants of *dFOXO* are viable with no obvious growth phenotypes (Jünger et al 2003), and knockdown of dFOXO would decrease the expression of Thor and other negative regulators of growth. The reason for the observed reduction in growth may be due to the reported feed back loop of dFOXO signaling (Puig et al 2003, Puig & Tjian 2005). In situations where nutrients are limited, the insulin signaling pathway becomes inactivated, so dFOXO is not inhibited and is able to activate expression of genes. One of the genes targeted and up regulated by dFOXO when nutrients are limited, is *dInR*. Up regulating *dInR* expression allows cells to accumulate higher levels of dInR in the membrane, and thus they are more sensitive to any changes in DILP levels. Accordingly, cells with reduced levels of dFOXO may express lower levels of dInR compared to surrounding wild type tissues. These affected cells maybe unable to compete

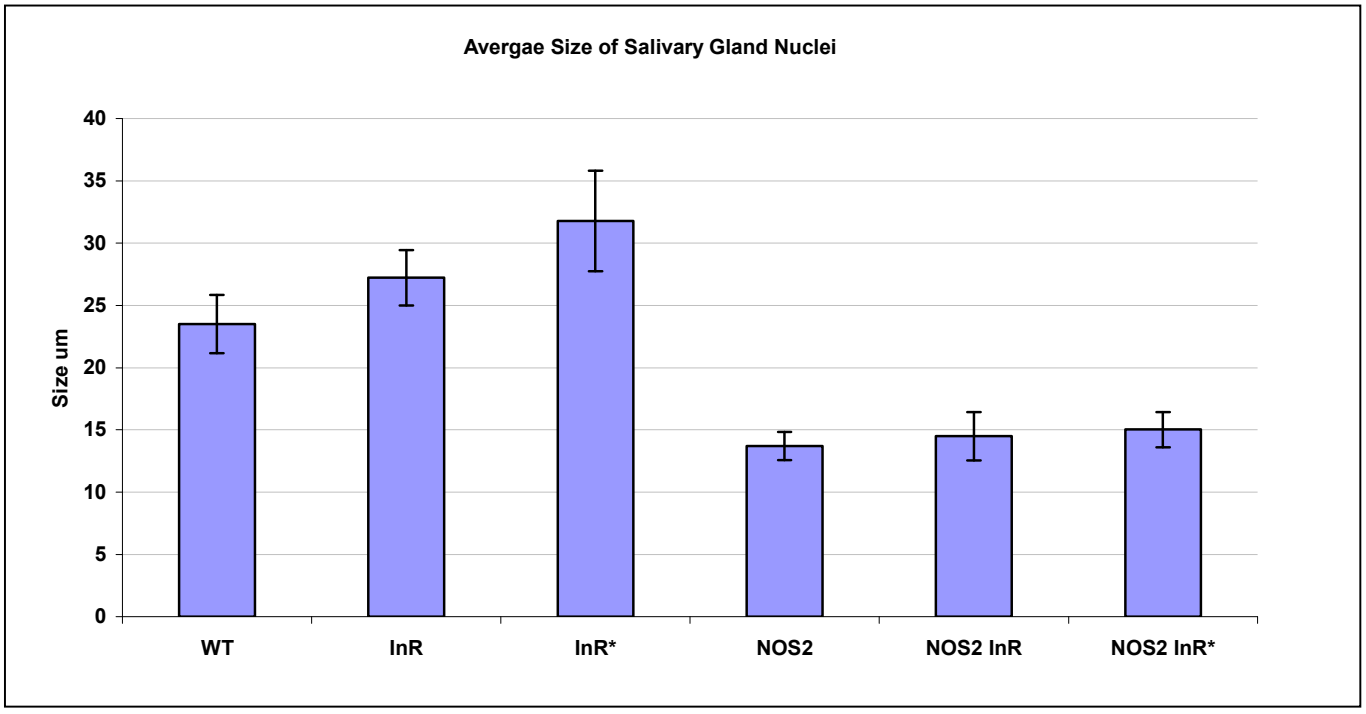
for DILPs and thus have reduced growth. It may also be possible that *UAS-RNAi-dFOXO* could be affecting expression of other genes, this is known as off-target RNAi and can occur where there is a degree of target sequence conservation in other genes (Saxena et al 2003). To further investigate the reduced growth phenotype caused by expression of *UAS-RNAi-dFOXO*, co-expression of wild type *UAS-dInR* (wt) and activated *UAS-dInR** was induced to determine if the small salivary gland phenotype could be rescued by dFOXO-independent expression of the dInR. Phenotypes caused by expression of wild type *UAS-dInR* (wt) or activated *UAS-dInR** and if their expression could modify the reduced growth phenotype associated with *UAS-NOS2* expression was also examined. It was determined if the reduced growth phenotype observed with NOS2 expression also occurred when RNAi-dFOXO was co-expressed, co-expression of *UAS-NOS2* and *UAS-RNAi-dFOXO* under c147-Gal4 control resulted in reduced salivary gland growth in 3rd instar larvae compared to wild types. The size of the nuclei of these salivary glands was not determined due to the very small size of the cells making measurements unreliable. However comparison by eye of both the salivary glands and the nuclei of these animals did not indicate any significant difference in size to those of animals expressing *UAS-RNAi-dFOXO* alone.

The co-expression of dInR with either *UAS-RNAi-dFOXO* or *UAS-NOS2* was performed using the salivary gland driver, AB1-Gal4. Animals co-expressing *UAS-RNAi-dFOXO* and either wild type *UAS-dInR* or activated *UAS-dInR**, exhibited small salivary glands that did not differ in size to those of *UAS-RNAi-dFOXO* expressing animals. As above, because of the resulting small gland size, it was not possible to take accurate measurements..

NOS2 expression with either wild type *UAS-dInR* or activated *UAS-dInR** (Fig. 4.4.) resulted in a decrease in growth compared to wild type *UAS-dInR* or activated *UAS-dInR** expression alone.

Figure 4.4. Average size of salivary gland nuclei expressing dInR and NOS2.
AB1-GAL4 was used to drive expression *UAS-dInR* wild type or *UAS-dInR** activated, NOS2 and both together. WT (+/+;+/+;AB1-GAL4/+), InR (*UAS-dInR* wt/+;+/+;AB1-GAL4/+), InR* (*UAS-dInR* activated/+;+/+;AB1-Gal4/+), NOS2 (*UAS-NOS2*/+;+/+;AB1-GAL4/+), NOS2 InR(*UAS-NOS2*/*UAS-dInR*wt;+/+; AB1-Gal4/+) and NOS2 InR* (*UAS-NOS2*/*UAS-dInR* activated;+/+; AB1-Gal4/+). Error bars indicate the standard deviation. (raw data is given in Appendix II.c.). P-Value= 0.001

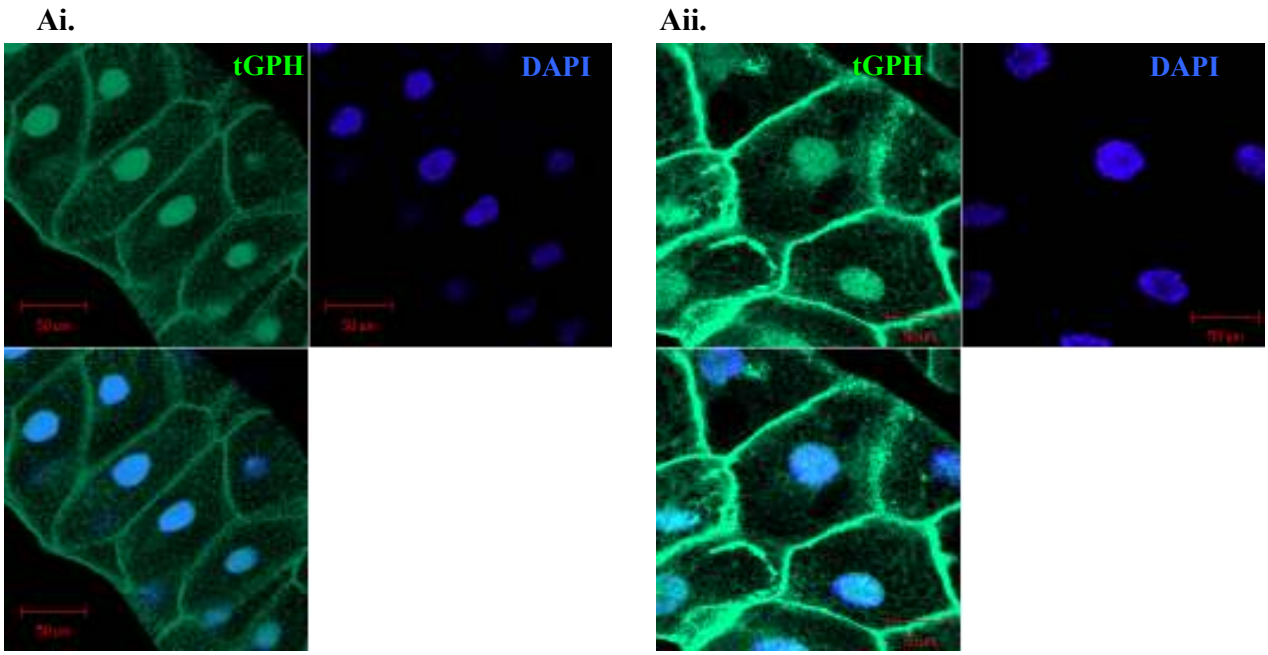
For WT, data was derived from 206 nuclei from 20 salivary glands. InR; 260 nuclei from 25 salivary glands. InR*; 269 nuclei from 22 salivary glands. NOS2; 315 nuclei from 26 salivary glands. NOS2 InR; 317 nuclei from 24 salivary glands. NOS2 InR*; 317 nuclei from 24 salivary glands.



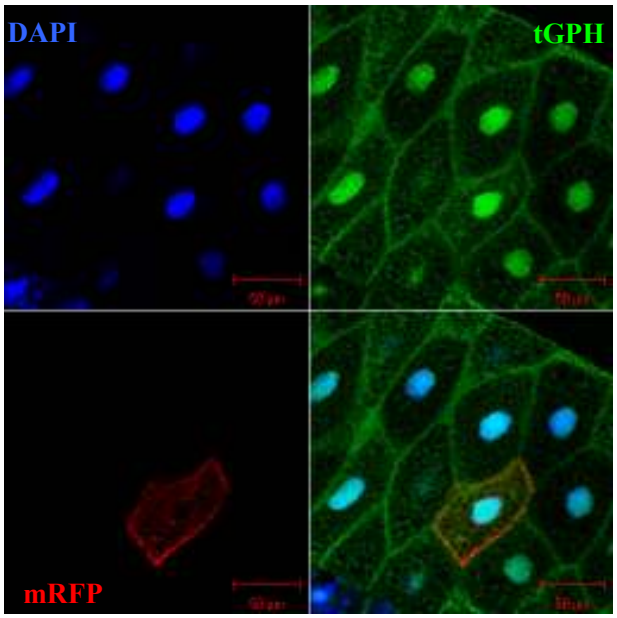
4.2.4. Localisation of tGPH reporter

To test if NO was affecting dPI3K activity, NOS2 was expressed in salivary glands using the salivary gland GAL4 driver c147 in larvae ubiquitously expressing the reporter of PI3 kinase activity, tGPH. *UAS-PI3K-CAAX* an activated, membrane-associated form of Pi3K92E, was used as a positive control (Leevers et al 1996). The expression of *UAS-PI3K-CAAX* resulted in an increase, compared to wild type, in tGPH localisation at the membranes of salivary gland cells (Fig. 4.5Aii.). There was also an increase in cell size which is expected with expression of *UAS-PI3K-CAAX* (Leevers et al 1996). Due to the small size of cells produced, the effects of *UAS-NOS2* expression in all cells of the salivary gland on the localization of tGPH were unclear. Therefore the FLP/FRT Gal4 technique was utilised to allow the localization of tGPH to be determined in single cells expressing NOS2. The tGPH localisation could be compared to that of surrounding wild type cells in the same salivary gland. The cells expressing Gal4 could be identified by the expression of *UAS-myr-mRFP* which expresses membrane-targeted monomeric RFP which also labels cell membranes (Chang 2003). Single salivary glands cells expressing only *UAS-myr-mRFP* (as a control) showed no change in tGPH localisation (Fig 4.6Ai. and Aii.). As expected, when *UAS-PI3K-CAAX* was expressed in single cells within the salivary gland an increase in tGPH localisation at the membranes was observed compared to surrounding wild type cells (Fig. 4.6Bi.). The PI3K-CAAX expressing cells were also larger in size compared to wild type cells. Expression of dFOXO in single cells resulted, in most cases, with increased localisation of tGPH to the membranes (Fig. 4.7A.i.and A.ii.). However this was not always the case, as no significant changes in localisation were observed in a few clones. NOS2 expression in single salivary gland cells showed variable results, with tGPH localisation increased at the membrane in some cells, and sometimes not in others (Fig. 4.8A.i., Aii. and A.iii.).

Figure 4.5. Localisation of tGPH in wild type and PI3K-CAAX expressing salivary glands.
Ai. Wild type (c147-GAL4/tGPH). **Aii.** Expression of PI3K-CAAX (c147-GAL4/tGPH; *UAS-PI3K-CAAX*). Blue; DAPI Green; tGPH. Scale bar 50µm. All images acquired and processed using identical settings



Ai.



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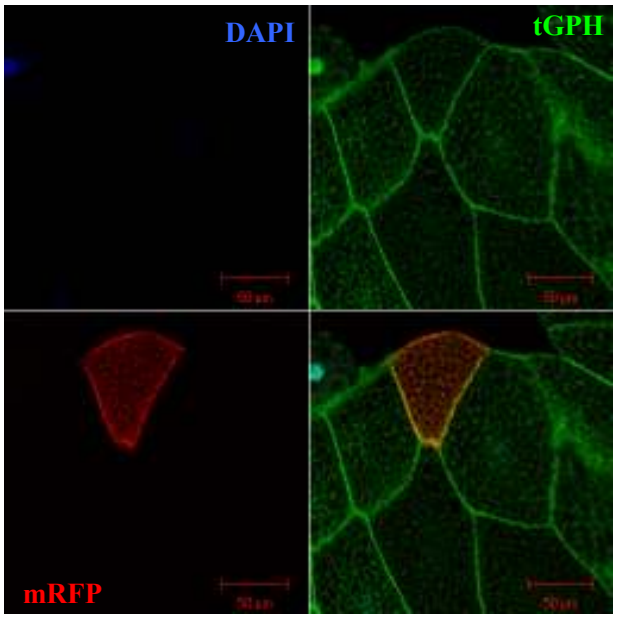


Bi.

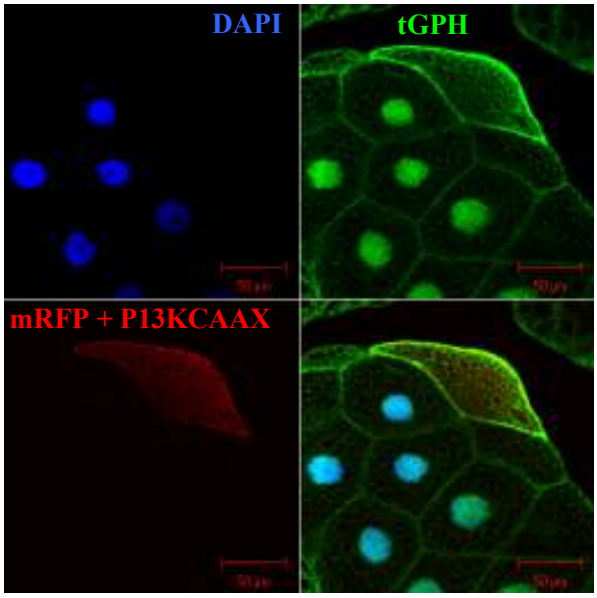
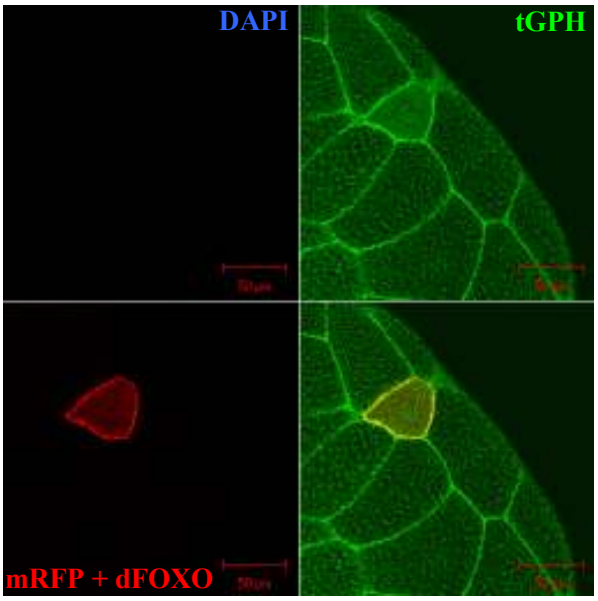


Figure 4.6. Localisation of tGPH in wild type and PI3K-CAAX expressing clones in salivary glands. Wild type and positive control clones for tGPH. Clones were generated using a 4 and half min heat shock at 38°C between 24-48hr AEL. Clones are labeled with mRFP. Scale bars 50µm.

Ai. and **Aii.** Wild type controls (hsFLP/+; *UAS-myr-mRFP*/+; tGPH/Act5C>CD>Gal4).
Blue; DAPI. Green; tGPH Red; myr-mRFP

Bi. PI3K CAAX positive control (hsFLP/+;*UAS-myr-mRFP*/tGPH; UAS-PI3K CAAX/ Act5C>CD>Gal4).
Blue; DAPI. Green; tGPH Red; myr-mRFP+ PI3K CAAX.

Ai.



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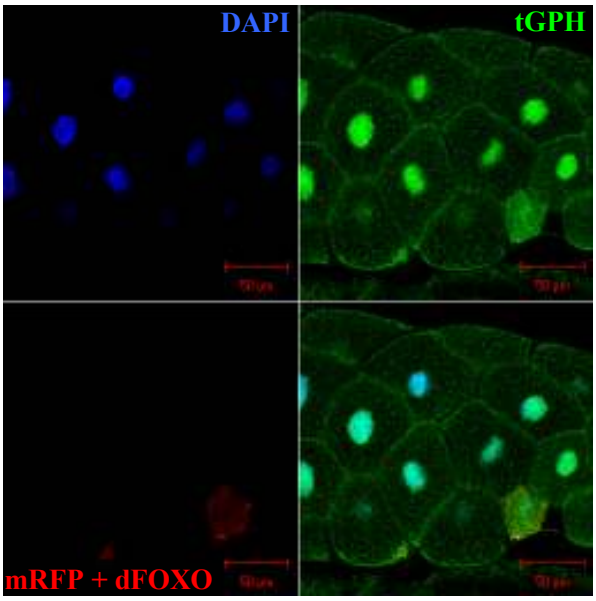


Aiii.

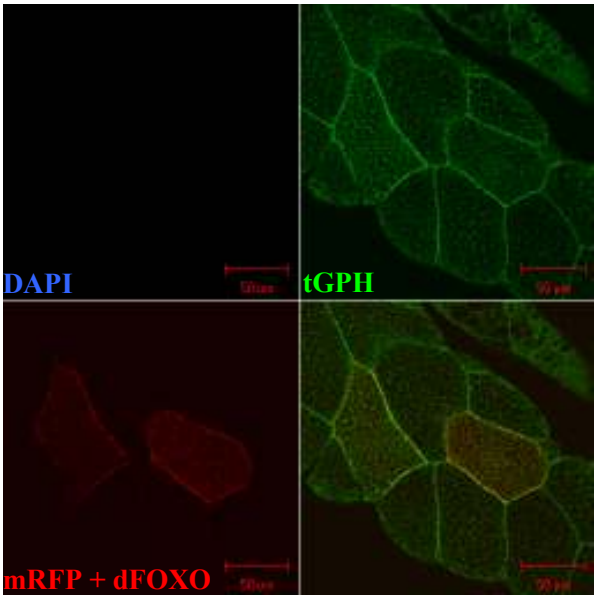
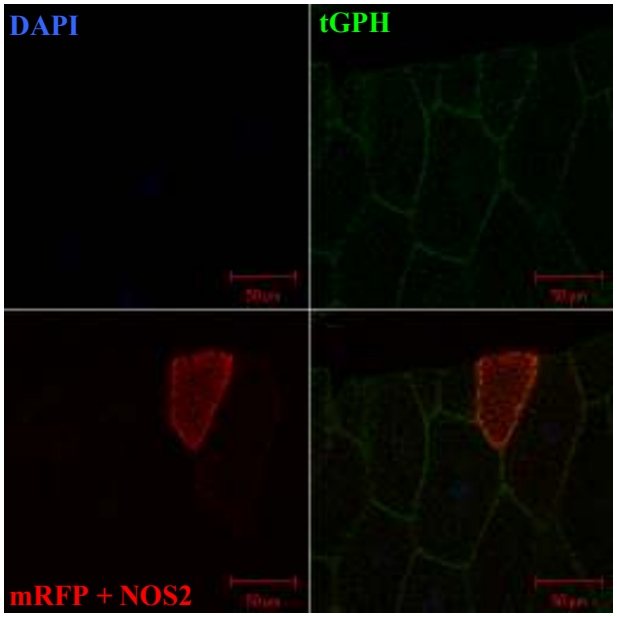
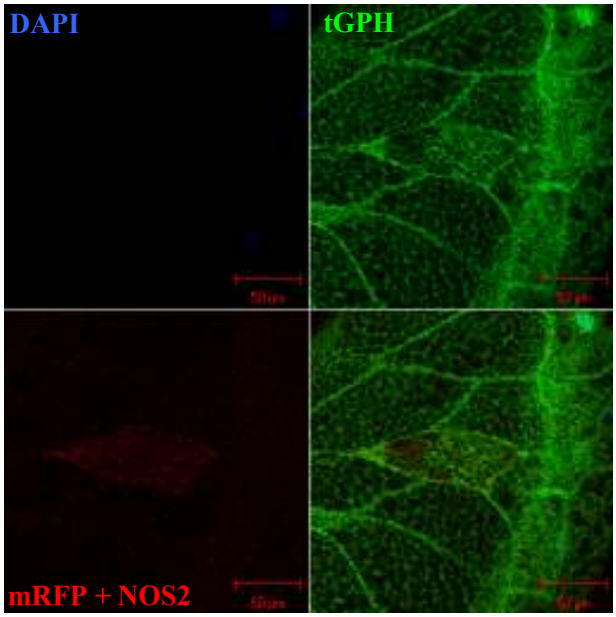


Figure 4.7. Localisation of tGPH in salivary gland cell clones over expressing dFOXO.
Clones were generated using a 4 and half min heat shock at 38°C between 24-48hr AEL. Clones are labeled with mRFP. Scale bars 50µm.
Ai., Aii. and **Aiii.** dFOXO (hsFLP/+; *UAS-dFOXO*/ UAS-myr-mRFP;tGPH/Act5C>CD>Gal4).
Blue; DAPI. Green; tGPH Red; myr-mRFP + dFOXO)

Ai.



Aii.



Aiii.

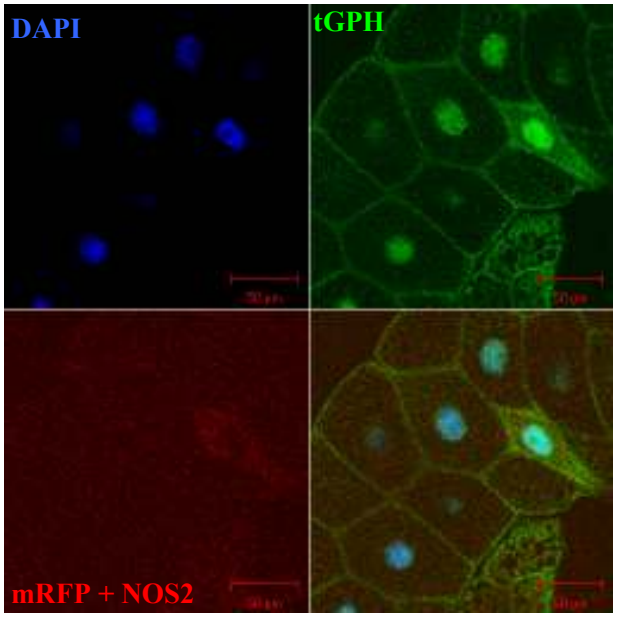


Figure 4.8. Localisation of tGPH in NOS2 expressing salivary gland cell clones.
Clones were generated using a 4 and half min heat shock at 38°C between 24-48hr AEL. Clones are labeled with mRFP.Scale bars 50µm.
Ai., Aii. and **Aiii.** NOS2 (hsFLP/*UAS-NOS2*; / UAS-myr-mRFP;tGPH/Act5C>CD>Gal4). Blue; DAPI. Green; tGPH Red; myr-mRFP + NOS2)

As well as the results shown for tGPH in the salivary glands in response to dFOXO, the fat body was also observed, results though were much more variable. In figures 4.9 it can be seen that in dFOXO expression clones in fat body does result in an increase in tGPH localisation at the membrane. This though didn't always occur in dFOXO clones and as it can be seen from figures 4.10 it did very in the intensity of expression at memebrane. The Movemnt of tGPH though was never observed in NOS2 clones in the fat body.

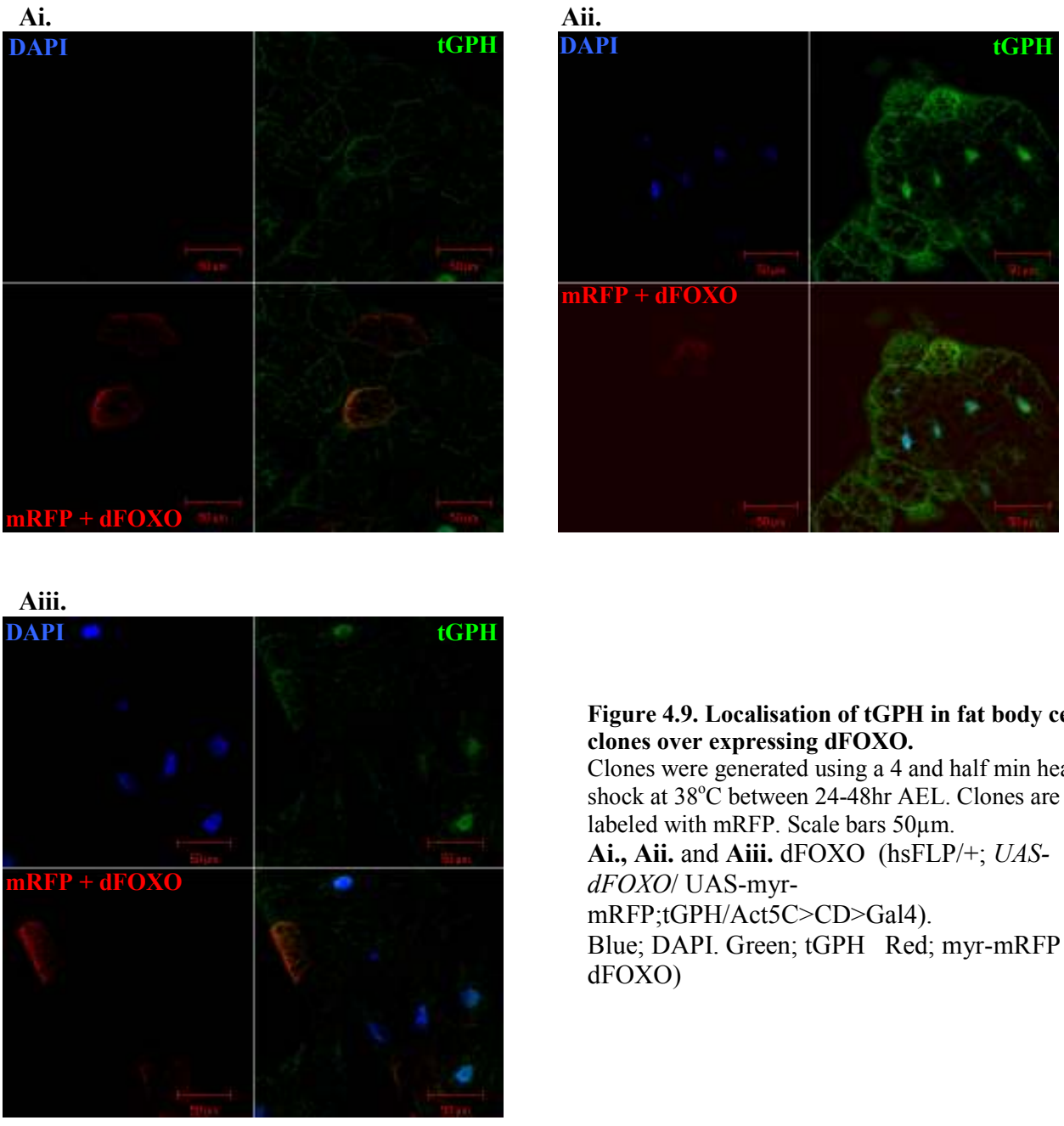


Figure 4.9. Localisation of tGPH in fat body cell clones over expressing dFOXO.
Clones were generated using a 4 and half min heat shock at 38°C between 24-48hr AEL. Clones are labeled with mRFP. Scale bars 50µm.
Ai., Aii. and **Aiii.** dFOXO (hsFLP/+; *UAS-dFOXO*/ UAS-myr-mRFP;tGPH/Act5C>CD>Gal4).
Blue; DAPI. Green; tGPH Red; myr-mRFP + dFOXO)

4.3. Discussion

The data presented in this Chapter have helped determine the role of sGC and components of the insulin signaling pathway in NO signaling. The expression of RNAi was utilised to genetically dissect the signaling pathway through the targeted knockdown of specific genes involved in the pathway. When combined with the study of other relevant mutations or overexpression of genes, this strategy allows the dissection of a pathway by showing which genes are genetically up or downstream of each other. The Gycα-99B subunit of sGC is a known target of NO. To assess if NO dependent growth regulation is acting through sGC, its expression was reduced via RNAi and the consequences of overexpression of NO was examined. As it was expected that NO may be acting within the insulin signaling pathway to regulate growth, a number of insulin pathway transgenes were selected to knockdown or increase signaling from parts of this pathway, while either increasing or reducing NO levels by expressing *UAS-NOS2* or *UAS-RNAi-NOS*.

As well as assaying the genetic requirements of NO signaling by knocking down parts of the insulin pathway, the cellular response to NO expression was determined by assaying the activity of PI3K by following tGPH localisation.

4.3.1. NO regulation of growth does not act through sGC subunit- Gycα-99B

Gycα-99B, an σ subunit of *Drosophila* sGC, is a known target for NO signaling (Morton et al 2005, Shah & Hyde 1995). To determine if NO regulates growth through sGC, *UAS-RNAi-Gycα-99B* was co-expressed with *UAS-NOS2* and the size of the salivary gland nuclei measured. Expression of RNAi-Gycα-99B in otherwise wild type animals had no observable effect on growth. The salivary gland nuclei in animals co-expressing *UAS-RNAi-Gycα-99B* and *UAS-NOS2* exhibited that same reduced size phenotype as that seen with expression of *UAS-NOS2* alone. This result indicates that NO-dependent regulation of growth does not act through the sGC subunit- Gycα-99B. It has to be noted though that the results from the use of *UAS-RNAi-Gycα-99B* can not be relyed upon until the RNAi its self is tested. This could be done using an anti-Gyc-99B or by anti-cGMP to clarify that the RNAi is actually having an effect. NO signaling has also been shown to act independently of sGC signaling in a number of ways including S-nitrosylation of target proteins (Asada et al 2009), through cytochrome oxidase (Cooper 2002) and through Metallothionein (St. Croix et al 2002), accordingly it is possible that NO is acting via one of these other pathways to regulate growth.

4.3.2. Interaction between RNAi-NOS expression and inhibition of insulin signaling

To try and determine the nature of the interaction between NOS signalling and insulin signaling to regulate growth, dNOS activity was reduced via the expression of RNAi-NOS, while the activity of the insulin signaling pathway was inhibited via the RNAi knockdown of activating components of the insulin signaling pathway. Co-expression of RNAi-NOS with either *UAS-RNAi-dInR* or *UAS-RNAi-Pi3K* p110 would demonstrate if NO was acting downstream of either dInR or Pi3K p110. Expression of either *UAS-RNAi-dInR* or *UAS-RNAi-Pi3K* p110 using c147 Gal4 resulted in reduced growth, but when expressed with tubulin Gal4 they were both 2nd instar lethal. The lethality with tubulin Gal4 is most likely due to their requirement throughout growth. The larvae co-expressing RNAi-NOS with either *UAS-RNAi-dInR* or *UAS-RNAi-Pi3K* p110 were also 2nd instar lethal. Therefore the knockdown of NOS was unable to suppress the 2nd instar lethality seen with both *UAS-RNAi-dInR* and *UAS-RNAi-Pi3K* p110 expression. It cannot be deduced from this result if this due to NO action not being downstream of dInR or Pi3k, or if it is the case that both signalling pathways are so vital for growth the animal cannot survive when they are both knocked down.

4.3.3. Interaction between NOS2 expression and inhibition and activation of insulin signaling

To determine if the growth regulation seen with NO expression is dependent on proteins within the insulin pathway, *UAS-NOS2* was co-expressed with transgenes to knockdown specific components of the pathway. dFOXO and Thor were chosen as they are proteins known to be negative regulators of growth, and have been shown to respond to NO expression. Expression of RNAi-Thor was shown not to reduce YFP tagged endogenous Thor expression, so no further work was carried out with this RNAi construct. There was an initially unexpected result when *UAS-RNAi-dFOXO* was driven in the salivary gland with c147-Gal4. In this instance, RNAi-dFOXO expression resulted in very small salivary glands which were smaller than those resulting from expression of either *UAS-NOS2* or *UAS-dFOXO*. This may be due to the reported feed-back loop where expression of dFOXO is required for full expression of dInR (Puig & Tjian 2005). To assess this, dFOXO-independent expression of dInR was induced in animals expressing RNAi-dFOXO. Expression of wild type *UAS-dInR* (wt) or activated *UAS-dInR** were unable to rescue the small salivary gland phenotype seen with expression of RNAi-

dFOXO. However, expression of wild type or activated *UAS-dInR* alone was able to increase growth in the salivary gland cells. An explanation for these data is that in the absence of dFOXO, insulin signaling cannot promote growth. Alternatively, the RNAi-dFOXO construct may target the degradation of other mRNA(s) required for growth.

The co-expression of *UAS-NOS2* with *UAS-RNAi-dFOXO* in the salivary gland resulted in very small glands that showed no significant difference in size to that of expression of *UAS-RNAi-dFOXO* alone. This finding does not agree with the results seen by Kimber (Kimber 2005) where the small salivary gland phenotype due to expression of NOS2 was suppressed in *dFOXO* null larvae. However, *dFOXO* null larvae do not show any growth phenotype unlike the expression of RNAi-dFOXO in salivary glands. To determine if the reduced growth phenotype caused by NOS2 expression, could be rescued by either wild type *UAS-dInR* or activated *UAS-dInR** co-expression of these constructs was undertaken. Expression of *UAS-NOS2* with either wild type or activated *UAS-dInR* resulted in decreased growth compared to expression of wild type or activated *UAS-dInR* alone. Thus NO-induced reduction of growth could not be rescued by the expression of either wild type or activated *UAS-dInR*. These data are consistent with NO acting genetically downstream or alongside of the insulin receptor.

4.3.4. tGPH localisation can be affected by NOS2 or dFOXO expression

To assess the cellular response of NO on the insulin pathway, the PI3K activity reporter, tGPH was utilised (Britton et al 2002). It was expected that NO would either have no effect on tGPH localisation, therefore indicating activity below dPI3K in the insulin pathway, or that the tGPH signal would decrease at the membrane suggesting that NO was acting above dPI3K. The initial experiments performed with tGPH were to use whole salivary glands using the Gal4 driver c147 to express *UAS-NOS2* or a positive control, the activated PI3K, *UAS-PI3K-CAAX*. The positive control worked as expected with increased growth of these cells and increased tGPH localisation at cell membranes. Thus tGPH localisation is capable of indicating increased PI3K activity due, in this case, to the expression of this constitutively active PI3K protein. However, when *UAS-NOS2* was expressed the results were less definitive. The salivary glands produced were much smaller than those of the controls making it hard to determine if there was any change in tGPH localisation. To circumvent this, the FLP/FRT Gal4 system was exploited to express the genes of interest in single cells of the salivary glands. As in the whole salivary glands, the expression of *UAS-PI3K-CAAX* in single cells showed an increase in size and tGPH

localisation at the membrane of those cells. The expression of *UAS-dFOXO* in single cells also resulted in increased tGPH signal at cell membranes. This result with *UAS-dFOXO* expression is consistent with the fact that changes in dFOXO activity are thought to feedback on the pathway by activating the expression of the insulin receptor (Puig et al 2003, Puig & Tjian 2005). However the result with *UAS-NOS2* expression was not as clear. The localisation of tGPH in the cells expressing NOS2 varied. This variability could be due to the increased levels of dInR due to increased dFOXO expression (Puig et al 2003, Puig & Tjian 2005) and temporal variability in this feedback. The feedback will allow the expression of NOS2 to affect tGPH localisation as a consequence of altering dFOXO activity. The extent of the feedback (dInR expression) will be dependent on how long the cells have been expressing NO and therefore there may be a lag in the activity of dFOXO dependent genes (e.g. dInR), their translation and the activation of the pathway.

The data presented in this chapter support the role of NO acting to regulate growth by inhibiting the insulin signaling pathway below or in parallel with the insulin receptor, dInR, but at or above the level of dFOXO activity. The work also confirms the known feedback mechanism involving dFOXO activity to regulate dInR expression (Puig et al 2003, Puig & Tjian 2005). Here I have demonstrated this feedback at the cellular level in the salivary gland through the localisation of tGPH reporter. Feedback at this cellular level has not been previously been demonstrated.
The molecular targets of dFOXO activation required for NO induced growth inhibition will be investigated in the following chapter.

Chapter 5: Genetic analysis of dFOXO transcriptional targets as possible growth inhibitors

5. Introduction

NO has been shown to act through the transcription factor dFOXO, and dFOXO itself has been shown to activate the transcription of many genes (Puig et al 2003, Teleman et al 2008). Some of these genes encode proteins that do, or can have, growth inhibitory effects. Data, intended to better define the roles of some of these potential effectors of the growth inhibitory phenotypes of NOS2 and dFOXO will be presented in this chapter.

NO can affect translation (Kimber 2005, Teodoro & O'Farrell 2003), and some of the dFOXO targets proteins are known regulators of translation. Accordingly we investigated whether it was NO modulation of these dFOXO target proteins that was responsible for the NO effect on translation. Given previous research in this area (as discussed in the introduction), the most likely candidates that may be required for both growth inhibition through the activity of dFOXO, and the reduced levels of translation induced by NO, are Thor and Lk6, both of which regulate the activity of the translation factor eIF4E. Genetic manipulation of these genes was undertaken to test the role of each of these genes in the regulation of growth by NO. The genetic manipulation undertaken and the rationale for selection of each gene is briefly explained below.

5.1. Thor

It has previously been shown that NO acts through dFOXO to regulate growth. One of the transcriptional targets that has been shown to have increased expression due to NO, via dFOXO, is Thor, indeed, Thor expression has been used as a reporter in studies as an indicator of dFOXO activity (Wittwer et al 2005).

It is thought that Thor may act to inhibit growth via its binding to the translation initiation factor eIF4E (Bernal & Kimbrell 2000). However, although Thor is considered to be a regulator of growth, this may not be the only way in which NO is acting, via dFOXO, to inhibit growth, as dFOXO has many transcriptional targets (Puig et al 2003, Teleman et al 2008). To test whether expression of Thor alone was sufficient for the growth phenotype observed with expression of either *UAS-NOS* or *UAS-dFOXO* (Chapter 3) a constitutively active form of

Thor, Thor^{LL} was expressed in the salivary gland. To determine if Thor is necessary for the action of NO, a null allele of Thor (*Thor*^{2}) (Bernal & Kimbrell 2000) was used. *Thor*^{2}, is a null mutant which has part of the promoter region, the translation site and a substantial part of the coding region deleted (Teleman et al 2005).

5.2. Lk6 and eIF4E

Another candidate effector protein for the growth inhibitory effects of NO is Lk6, the functional homolog of the mammalian Mnk kinases (Arquier et al 2005). Like Thor, it is an known transcriptional target of dFOXO (Teleman et al 2008) and has also been shown to regulate the activity of translation initiation factor eIF4E (Arquier et al 2005, Parra-Palau et al 2005, Reiling et al 2005). The regulation of eIF4E through Lk6 phosphorylation has been demonstrated (Arquier et al 2005, Reiling et al 2005), although the exact role of this phosphorylation event on eIF4E activity is unclear and will be discussed below. Lk6 has been shown in one study to be vital for normal growth and development (Arquier et al 2005). *Lk6* null mutants exhibited reduced viability, slower development and reduced adult size (Arquier et al 2005). These results were comparable to those seen in animals expressing a non-phosphorylatable form of eIF4E (Lachance et al 2002) where expression caused delayed development and flies were smaller in overall size. These data suggest that Lk6 acts to positively phosphorylate eIF4E which is then able to activate protein synthesis. In contrast however, results in mammalian studies have been contradictory, with it being shown that phosphorylation of eIF4E reduces its affinity for the capped mRNA (Scheper et al 2002). Moreover, it has also to be noted that even in the studies in which it was shown that phosphorylation of eIF4E through Lk6 acted to activate translation (Arquier et al 2005), Lk6 overexpression in the eye disc resulted in subtle growth impairments (Arquier et al 2005). Accordingly, even in studies where Lk6-mediated activation of translation is seen, the opposite effect was also observed in some tissues. Growth inhibition due to Lk6 overexpression in an eIF4E phosphorylation-dependent manner was also seen in another study (Reiling et al 2005). This work however, also produced contradictory results to those of Arquier et al, as Reiling et al found that their Lk6 nulls were viable, and fertile flies without obvious growth defects were produced when grown under standard conditions. However Reiling et al also found that when *Lk6* null mutants were raised under conditions of poor nutrient supply a reduction in size of flies was observed. The reduction in the amino acid supply also abolished the negative effects

of Lk6 overexpression on growth, which suggested that the activity of Lk6 was also regulated in response to nutrients.

Together these results show that Lk6 activity and its downstream effects are dependent on a variety of factors (nutrient availability and level of phosphorylation) and changing them will result in either to growth stimulation or growth inhibition (Reiling et al 2005) (Fig. 5.1.). Thus it may be that if dFOXO acts to upregulate Lk6 as a result of NO expression, it may then result in Lk6-mediated phosphorylation of eIF4E, which in turn would result in the inhibition of translation. To test this hypothesis both *Lk6* nulls and non-phosphorylatable form of eIF4E were utilised.

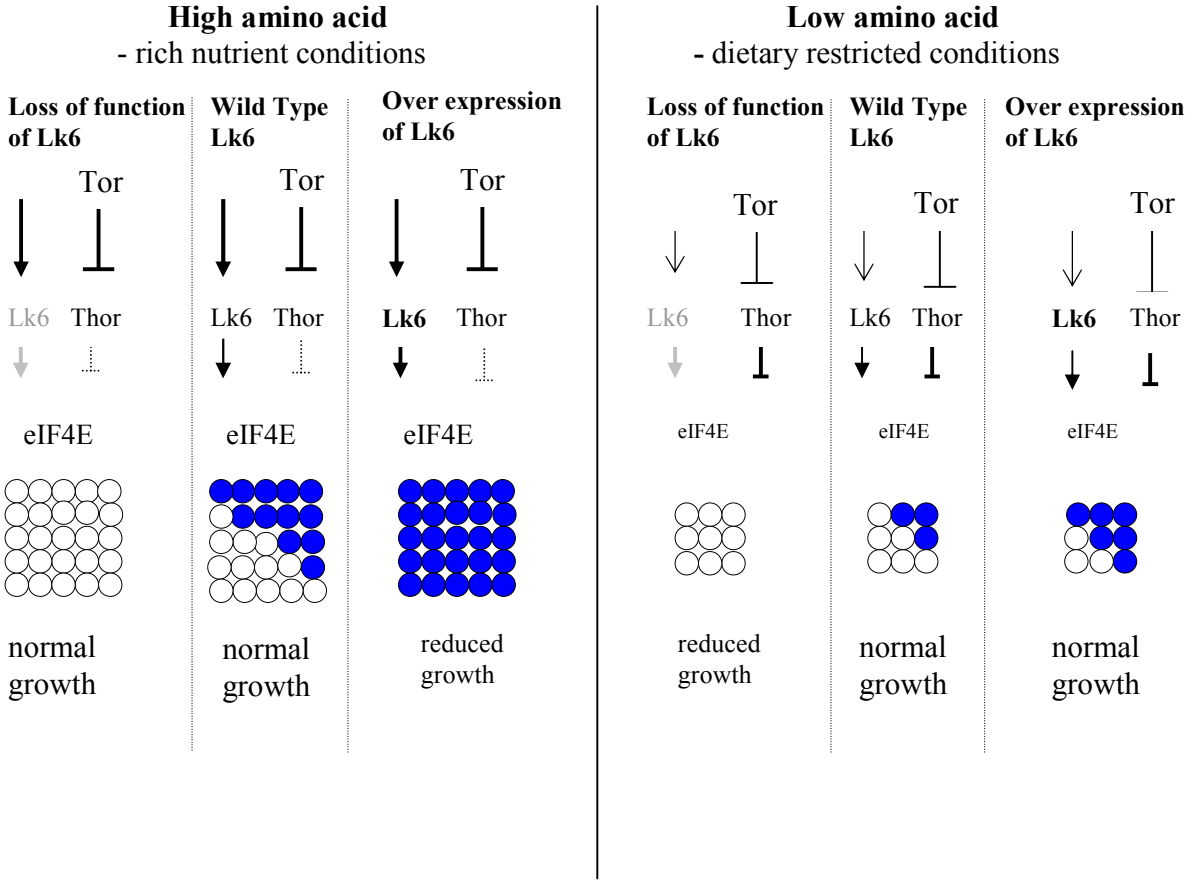


Figure 5.1. Effect of Diet and Lk6 Dosage on Growth.
In wild type, TOR activity is stimulated under rich nutrient conditions (high amino acids), resulting in the phosphorylation of Thor. This leads to an increase in the pool of free eIF4E (represented by circles), which assembles into functional translation initiation complexes. The translation efficiency is further modulated by phosphorylation of eIF4E by Lk6 (blue circles represents phosphorylated eIF4E). A high level of Lk6 results in complete phosphorylation of eIF4E and impairs growth because of premature eIF4E phosphorylation and/or a reduction of the affinity of phosphorylated eIF4E to capped mRNA. On the other hand, the loss of Lk6 is without impact on translation initiation under favorable conditions, because phosphorylation of eIF4E is not essential as long as the pool of free eIF4E is sufficiently large.

Under low amino acids conditions, however, the absence of Lk6 leads to impaired translation efficiency. Lk6 activity is presumably also regulated in response to amino acid availability. Figure adapted from Reiling et. al. (Reiling et al 2005).

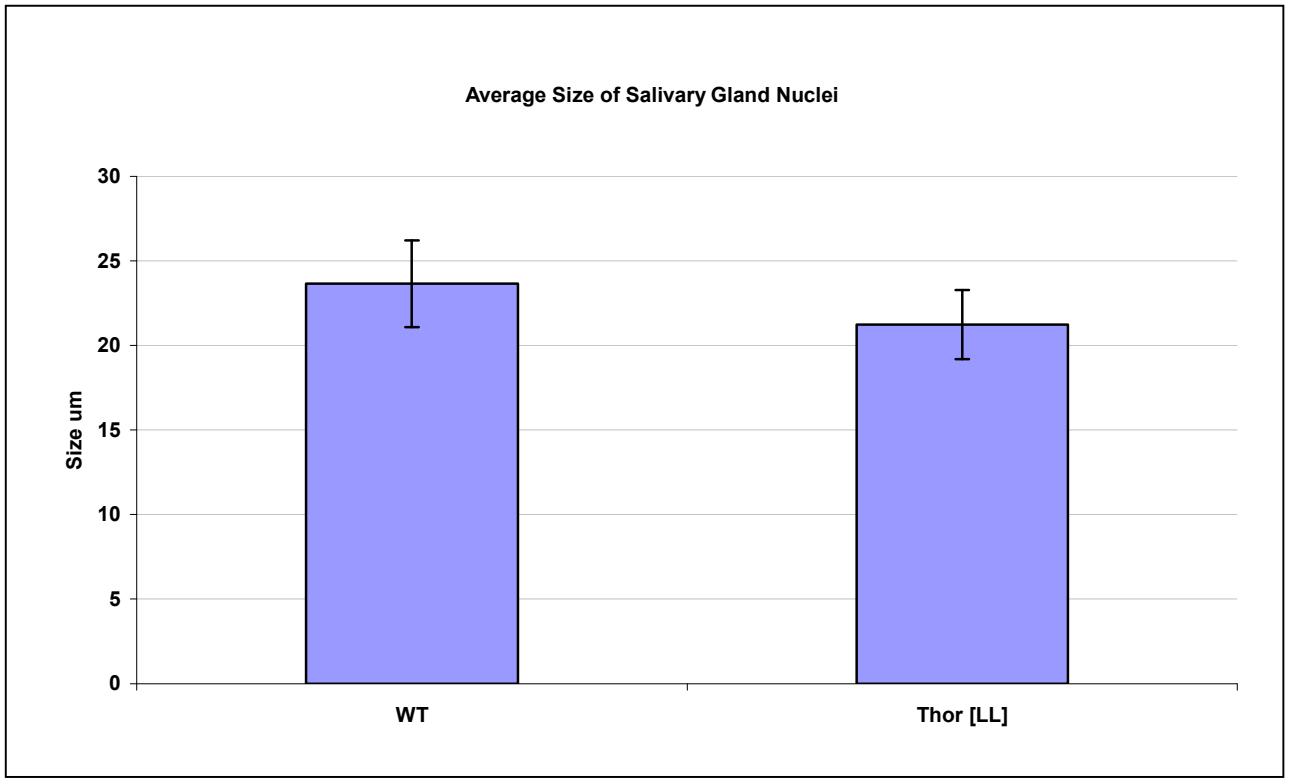
5.2. Results

5.2.1. Is Thor sufficient for NO induced growth inhibition?

Thor has been shown to respond to the action of NO, so further investigation into this action was undertaken. Expression of an active form of Thor (*UAS-Thor*^{LL}) and a Thor null mutant (*Thor*^{2}) were exploited. Thor^{LL} is an active form of Thor that binds deIF4E strongly (Miron 2001). This stronger binding of Thor^{LL} to deIF4E would result in deIF4E being unable to be recruited to the translation initiation complex and thereby inhibition of translation. Expression of *UAS-Thor*^{LL} within the wing-imaginal disc has been shown to decrease wing size. This reduction in size was not seen if wild type Thor was expressed (*UAS-Thor*) (Miron 2001). Expression of NO in the salivary glands using *UAS-NOS2* resulted in reduced salivary gland and nuclei size, and expression of *UAS-dFOXO* produced similar results (Chapter 3. Fig. 3.2.). It is thought that NO may be acting through dFOXO to increase transcription of Thor which thereby results in reduced growth. To see if expression of an active form of Thor would result in the same reduced growth in salivary glands, the salivary gland Gal4 driver c147 was utilised. The active form of *UAS-Thor* was chosen as previous work had demonstrated that wild type *UAS-Thor* did not cause any change in growth (Miron 2001). The expression of Thor^{LL} in the salivary glands did not show the same reduced nuclei size that was seen with *UAS-NOS2* or *UAS-dFOXO*. There was a small reduction when compared to the wild type (Fig. 5.2.) but this difference was not statistically significant (Appendix II. d.).

Figure 5.2. Average Size of Salivary Gland Nuclei expressing Thor^{LL}.
c147-GAL4 was used to drive expression *UAS-Thor*^{LL}. WT (c147-GAL4/+) and Thor^{LL} (c147-GAL4/*UAS-Thor*^{LL}). Error bars indicate the standard deviation. Wt; 209 nuclei of 19 salivary glands. Thor^{LL}; 193 nuclei of 21 salivary glands (raw data in Appendix II.d.). P-Value=0.001

For WT, data was derived from 209 nuclei from 19 salivary glands. Thor^{LL}; 198 nuclei from 21 salivary glands.

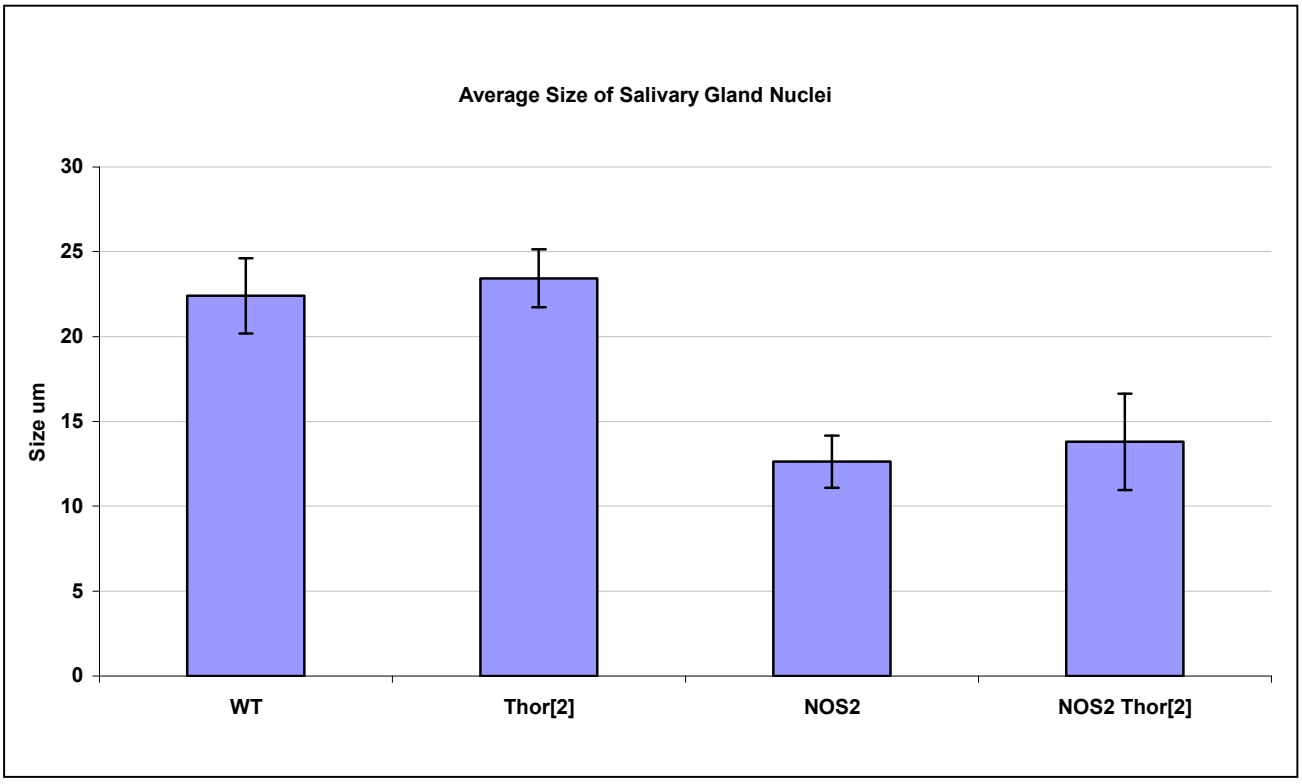


5.2.2. Is Thor necessary for NO induced growth inhibition?

dFOXO has many transcriptional targets, with Thor being just one of them. To identify if the reduced growth phenotype seen with NO is solely due to an increase in Thor levels, the *Thor* null mutant, $Thor^2$, was used. $Thor^2$ null mutant animals are homozygous viable and fertile (Bernal & Kimbrell 2000), and show no growth defects under normal conditions (Teleman et al 2005). However, $Thor^2$ null mutant larvae were found to have an impaired response to nutrient deprivation, thought to be due to an impaired ability to reduce energy burn rate (Teleman et al 2005). The salivary gland Gal4 driver AB1 was used to drive *UAS-NOS2* expression in the background of *Thor* null larvae. The nuclei of $Thor^2$ homozygous larvae were slightly bigger than wild type nuclei in size (Fig. 5.3.) but this effect was not statistically significant. The expression of *UAS-NOS2* in a *Thor* null background had little effect on the reduced growth phenotype seen with NO expression (Fig. 5.3.) with nuclei from larvae expressing *UAS-NOS2* in a wild type or a $Thor^2$ background being statistically similar (Appendix II. e.).

Figure 5.3. Average Size of Salivary Gland Nuclei in *Thor* null larvae.
AB1-GAL4 was used to drive expression NOS2 in salivary glands of Thor null larvae (Thor^2). WT (+/+; +/+; AB1-Gal4/+), *Thor*^{2} (+/+;*Thor*^{2}; AB1-Gal4/+), NOS2 (*UAS-NOS2*/+; +/+ ;AB1-GAL4/+) and NOS2 *Thor*^{2} (*UAS-NOS2*/+;*Thor*^{2} ;AB1-GAL4/+). Error bars indicate the standard deviation (raw data in Appendix II.e.). P-value= 0.001

For WT, data was derived from 216 nuclei from 21 salivary glands. *Thor*^{2}; 288 nuclei from 24 salivary glands. NOS2; 203 nuclei from 20 salivary glands. NOS2 *Thor*^{2}; 304 nuclei from 24 salivary glands.



5.2.3. Are Lk6 or the phosphorylation of eIF4E necessary for NO induced growth inhibition?

NO has been shown to inhibit growth through dFOXO. Thor is one of the transcriptional targets of dFOXO shown to be upregulated by NO through dFOXO, but as dFOXO is known to have many translational targets (Puig et al 2003) it may be that expression of Thor is not the target through which NO acts to regulate growth. Another candidate for NO action on growth through dFOXO is Lk6. Lk6 is of interest as it is a known transcriptional target of dFOXO and it also regulates the activity of the same translation factor, eIF4E. To investigate if NO may be acting via dFOXO on Lk6 to regulate growth, *Lk6* null mutations were utilised as well as a non-phosphorylatable form of eIF4E.

To generate *Lk6* null a loss-of-function animals, alleles of *Lk6* (*Lk6*^{15}and *Lk6*^{38}) were used, which were kind gift from Prof. Ernst Hafen (Reiling et al 2005). It has previously been shown that this mutant combination gave rise to viable and fertile flies without obvious growth defects (Reiling et al 2005). However, as with Thor , under conditions of limited nutrients Lk6 is required for normal growth (Reiling et al 2005). The salivary gland nuclei of third instar larvae of *Lk6* null animals were not different in size to that of wild type (Fig. 5.4.). To ascertain if NO was acting through Lk6, *UAS-NOS2* was expressed using the salivary gland Gal4 driver c147. The expression of *UAS-NOS2* in a *Lk6* null background did not show any rescue of the growth inhibition as was seen with *UAS-NOS2* alone (Fig. 5.4.).

A separate analysis to determine whether NO-reduced growth required Lk6 dependent phosphorlylation was also undertaken. A non-phosphorylatable form of eIF4E was utilised to show if phosphorylation of eIF4E was vital for the inhibition of growth by NO (presumably through Lk6 or other kinases). The *UAS-eIF4E*^{Ser251Ala} construct allows expression of a non-phosphorylatable form of eIF4E (Lachance et al 2002), and flies that express this construct in *eIF4E* mutant background are delayed in development and are smaller than control animals (Lachance et al 2002). As has already been discussed, the phosphorylation of eIF4E may act to

either activate or inhibit translation, but as it is expected that if dFOXO does act through Lk6 this would result in hyperphosphorylation of eIF4E and therefore inhibition of translation. An investigation whether expression of eIF4E^{S251A} can prevent the growth reduction seen with *UAS-NOS2* expression would determine if phosphorlylation (by Lk6 or other kinases) was necessary for the growth inhibition caused by NO. In this study, the salivary gland nuclei of third instar larvae expressing UAS-eIF4E^{S251A} did not appear different in size to wild type (Fig. 5.5.). To ascertain if NO was acting through Lk6, and therefore phosphorylation of eIF4E was inhibiting growth, *UAS-NOS2* was expressed using the salivary gland Gal4 driver c147 along with *UAS-eIF4E*^{S251A}. The expression of *UAS-NOS2* with *UAS-eIF4E*^{S251A} in the salivary gland did not show any rescue of the growth inhibition seen with *UAS-NOS2* alone, with the nuclei being slightly smaller in size to those produced as a result of *UAS-NOS2* alone, although this difference was not statistically significant (Fig. 5.5.).

Figure 5.4. Average Size of Salivary Gland Nuclei NOS2 expression in *Lk6* null larvae.
c147-GAL4 was used to drive expression *UAS-NOS2* in *Lk6* null larvae. WT (+/+;c147/+;+/+), *Lk6* null (+/+;c147/+;$Lk6^{15}/Lk6^{38}$), NOS2 (*UAS-NOS2*/ +;c147/+;+/+), NOS2 *Lk6* null (NOS2/+;c147/+;$Lk6^{15}/Lk6^{38}$). Error bars indicate the standard deviation (raw data is given in Appendix II. f.) P-Value= 0.001

For WT, data was derived from 247 nuclei from 20 salivary glands. *Lk6*; 245 nuclei from 24 salivary glands. NOS2; 186 nuclei from 15 salivary glands. NOS2 *Lk6*; 261 nuclei from 20 salivary glands.

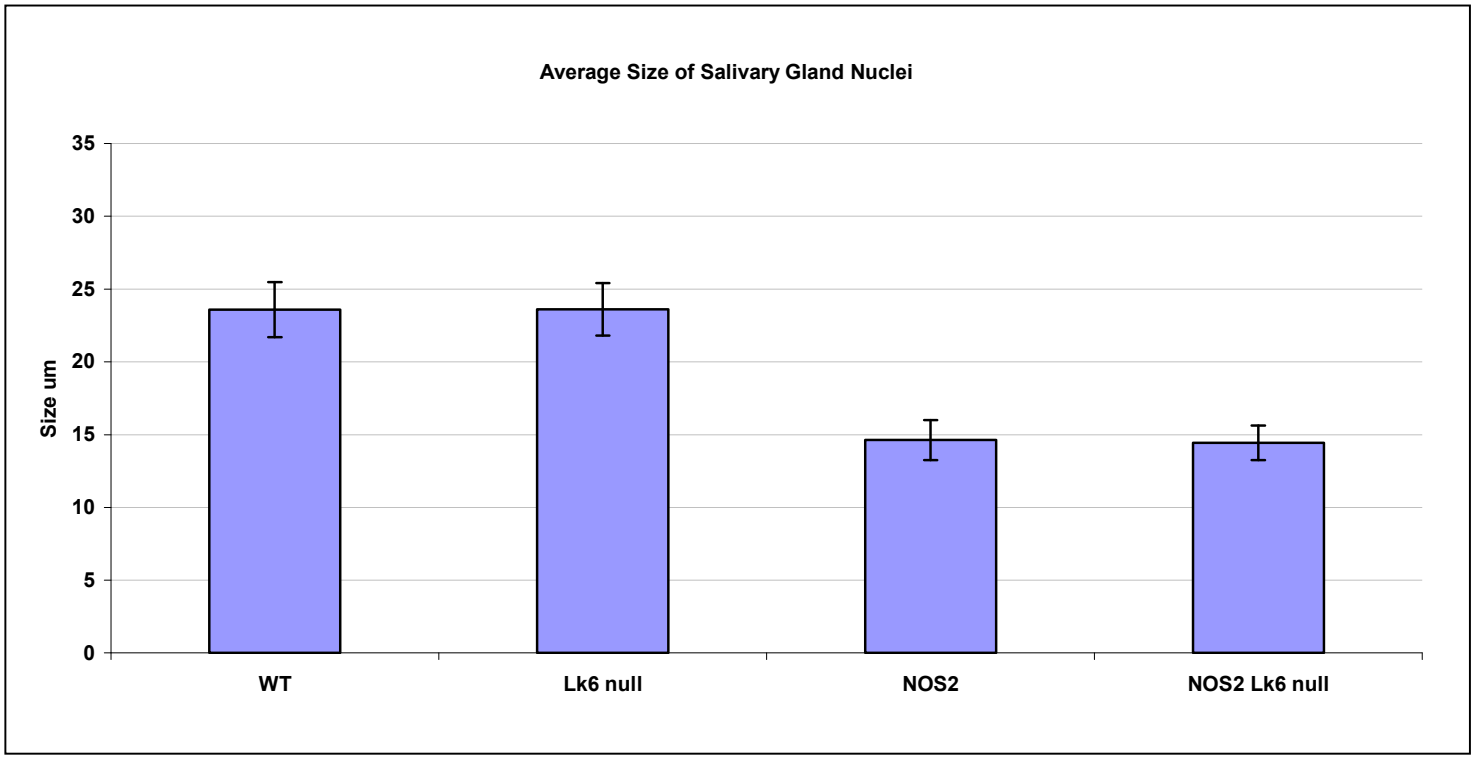
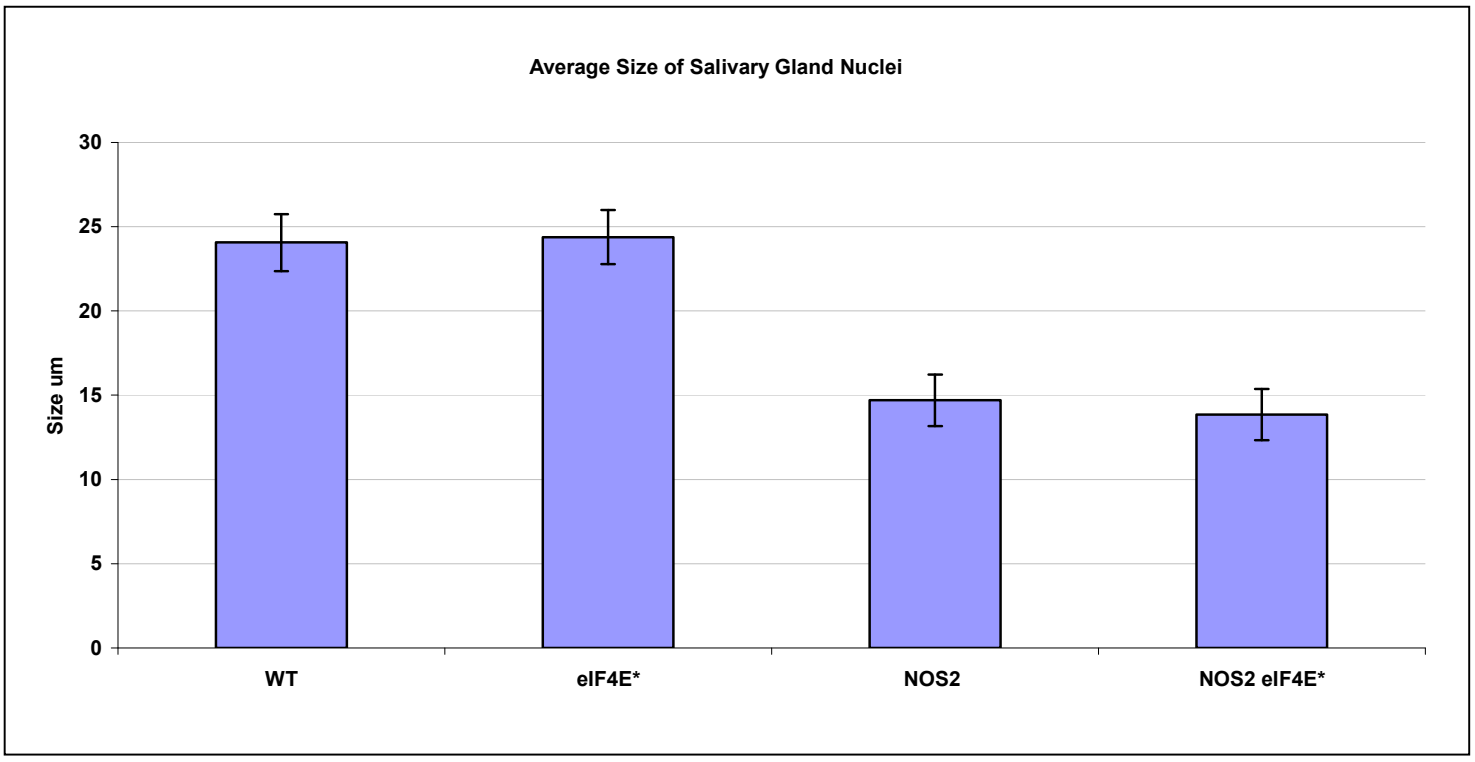


Figure 5.5. Average Size of Salivary Gland Nuclei expressing eIF4E^{S251A} and NOS2.
c147-GAL4 was used to drive expression *UAS- eIF4E*^{S251A}, NOS2 and both together. WT (c147-GAL4/+), eIF4E* (c147-GAL4/+; *UAS- eIF4E*^{S251A} /+), NOS2 (NOS2/+;c147-GAL4/+) and NOS2 eIF4E* (NOS2/+;c147-GAL4/+; *UAS- eIF4E*^{S251A} /+). Error bars indicate the standard deviation (raw data is given in Appendix II. g.). P-Value = 0.001

For WT, data was derived from 210 nuclei from 20 salivary glands. eIF4E*; 225 nuclei from 20 salivary glands. NOS2; 223 nuclei from 20 salivary glands. NOS2 eIF4E*; 224 nuclei from 20 salivary glands.

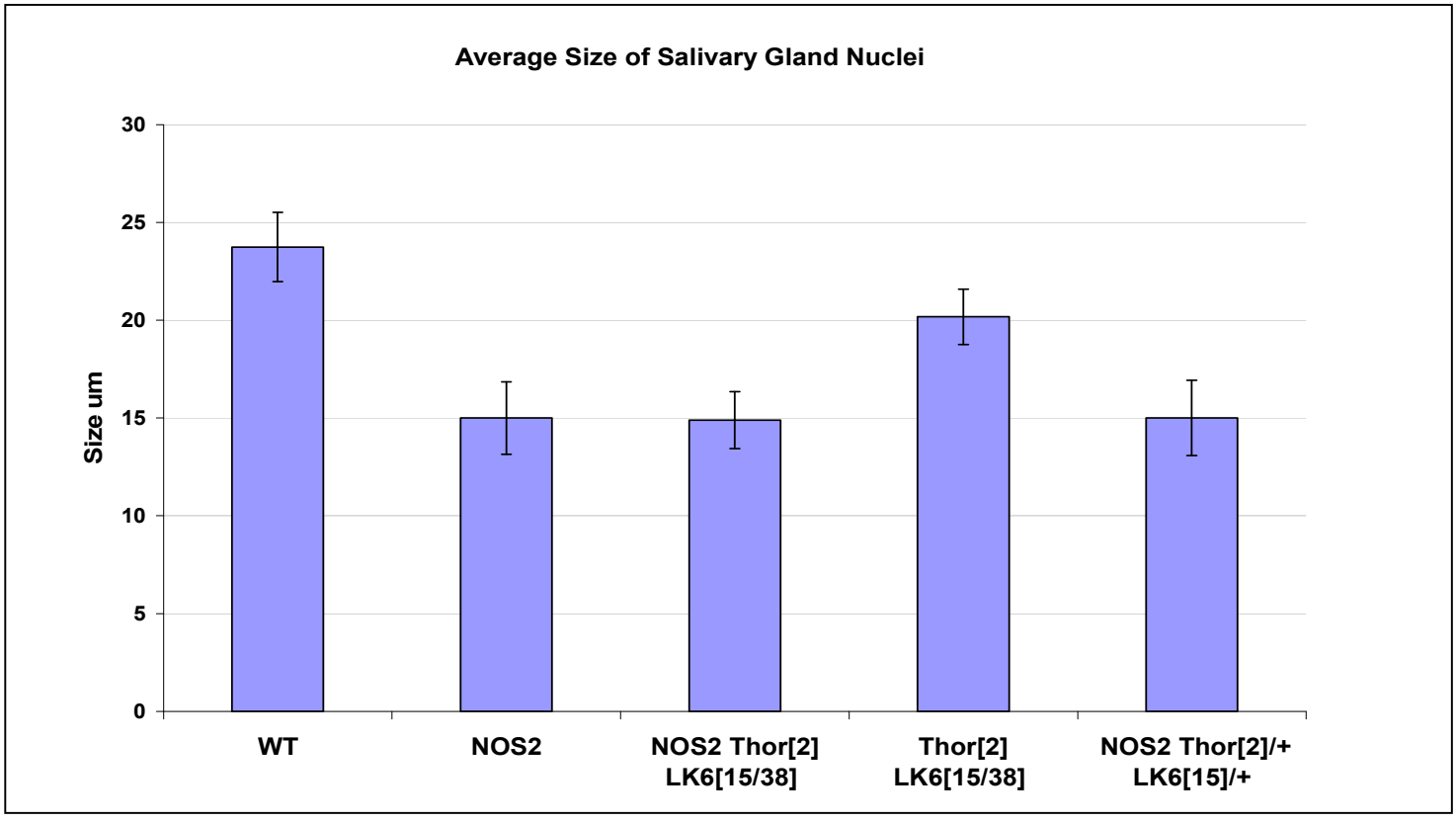


5.2.4. Are Thor and Lk6 required for NO induced growth inhibition?

Neither Thor nor Lk6 alone were shown to be individually required for the inhibition of growth seen with *UAS-NOS2* expression in the salivary gland. However it has been proposed (Reiling et al 2005) that Thor and Lk6 may be acting in partnership to control translation (Reiling et al 2005).Both these genes are targets of dFOXO (Teleman et al 2008). To test whether they are required for the signal transduction, *UAS-NOS2* was expressed in the salivary glands using a first chromosome Gal4 driver A9 in animals doubly mutant for $Thor^{2}$ and $Lk6^{15}/Lk6^{38}$. In these double null mutant animals, where both *Thor* and *Lk6* were null mutants, the average nuclei size was slightly smaller than wild type, although this difference was not statistically significant (Fig. 5.6.) (Appendix II. h.). When *UAS-NOS2* was driven in the salivary glands of double null mutants there was no change in the average size of nuclei when compared to *UAS-NOS2* expression alone (Fig. 5.6.).

Figure 5.6. Average Size of Salivary Gland Nuclei NOS2 expression in *Thor* and *Lk6* null larvae. A9-GAL4 was used to drive expression *UAS-NOS2* in *Thor* and *Lk6* null larvae. WT (A9-Gal4/+;+/+;+/+), NOS2 (*UAS-NOS2*/ A9-Gal4;+/+;+/+), NOS2 $Thor^{2}$ $Lk6^{15/38}$ (*UAS-NOS2*/ A9-GAL4;$Thor^{2}$ / $Thor^{2}$; $Lk6^{15}/Lk6^{38}$), $Thor^{2}$ $Lk6^{15/38}$ (A9-GAL4/+; $Thor^{2}$ / $Thor^{2}$; $Lk6^{15}/Lk6^{38}$) and NOS2 $Thor^{2}$/+ $Lk6^{15}$/+ (*UAS-NOS2*/ A9-GAL4;$Thor^{2}$ / +; $Lk6^{15}$/+). Error bars indicate standard deviation (raw data in Appendix II. h.). P-Value=0.001.

For WT, data was derived from 211 nuclei from 20 salivary glands. NOS2; 223 nuclei from 20 salivary glands. NOS2 $Thor^{2}$ $Lk6^{15/38}$; 241 nuclei from 21 salivary glands. $Thor^{2}$ $Lk6^{15/38}$; 230 nuclei from 20 salivary glands. NOS2 $Thor^{2}$/+ $Lk6^{15}$/+; 246 nuclei from 20 salivary glands.



5.3. Discussion

5.3.1. Thor is not necessary or sufficient for NO induced growth inhibition.

To investigate how NO may inhibit growth through the action of the insulin pathway, components of the pathway that may act as negative regulators of growth were analysed more closely. NO has been shown to effect Thor expression. To test whether Thor was sufficient for the growth inhibition induced by NO, an high affinity active form of Thor (*UAS-Thor*^{LL}) was expressed in salivary glands. To determine whether Thor was necessary for the phenotype, a *Thor* null (*Thor*^{2}) was utilised. As dFOXO is known to upregulate the transcription of *Thor* (Puig et al 2003), it was hypothesised that expression of an active form of Thor may inhibit the size of the salivary gland nuclei that were observed with either *UAS-NOS2* or *UAS-dFOXO*. This was not the case with targeted expression of Thor^{LL} to the salivary glands. This resulted in salivary gland nuclei that were not significantly different in size from that of wild type. This may be due to increased Thor expression not being the only way in which NO, acting through dFOXO, is inhibiting growth of these cells. It may also be that although Thor^{LL} binds to deIF4E more strongly, this is not having the same growth inhibitory effect that is caused by the large increase in normal Thor expression that occurs when NO is expressed. Nevrtheless, it should be noted that Thor^{LL} has been shown in wing dics to reduce growth in wing discs (Miron 2001).

To further investigate if the action of NO is specifically through Thor via dFOXO, NO was expressed in *Thor* null mutant animals to test if the reduced growth phenotype was Thor dependent. The reduced growth phenotype was still observed following NO expression in *Thor* null homozygous larvae. However, this is in contrast to the results of a similar experiment previously observed in our laboratory showing that dFOXO nulls abolish the growth inhibitory effects of NO (Kimber 2005). Thus, although the action of NO has been shown to increase Thor expression through dFOXO, this increase does not appear necessary for NO to regulate growth. Taken together these data imply that there may be other transcriptional targets of dFOXO whose modulation is required to reduce growth.

5.3.2. Lk6 or eIF4E phosphorylation are not required for NO-induced growth inhibition

Lk6 is another transcriptional target of dFOXO (Teleman et al 2008) that has been previously shown to be a regulator of the translation factor eIF4E (Arquier et al 2005, Parra-Palau et al 2005, Reiling et al 2005) and thus may be responsible for dFOXO regulated growth. The Lk6-

dependent regulation of eIF4E is thought to be both inhibitory and activating (Arquier et al 2005, Reiling et al 2005). It was hypothesised that if NO acted through Lk6 (via dFOXO) it would be due to the increase in *Lk6* expression resulting in hyperphosphorylation of eIF4E reducing its affinity for capped mRNA. However, it was found that neither complete loss of Lk6, or expression of a non-phosphorylatable form of eIF4E ($eIF4E^{S251A}$),was able to suppress the reduced growth phenotype observed with NO expression in the salivary gland. Thus neither Lk6 nor other kinases which may phosphorylate eIF4E are responsible for the reduced growth of the salivary gland caused by NO.

5.3.3. Thor and Lk6 are not required for NO induced growth inhibition

It is has been suggested that translational control through the dFOXO branch of the insulin pathway acts directly via parallel, independent targets (Lk6 and Thor) to regulate protein translation (Teleman et al 2008). Therefore although *Thor* null and *Lk6* nulls alone were unable to suppress the reduced growth seen following expression of NO, it may be that removal of both simultaneously would prevent NO-dependent growth inhibition.
However, it was found that despite removing both *Thor* and *Lk6* at the same time, NO was still able to inhibit growth. Therefore NO growth inhibition through dFOXO does not require either Thor, Lk6, or a combination of the two. Thus, although I have demonstrated some evidence for the proposed parallel independent functions in growth regulation by Thor and Lk6 (the slightly reduced growth of *Thor2*; *Lk6* doubly mutants animals), there is no evidence that either of these regulators of translation (either separately, or redundantly in parallel) are required for the inhibition of growth caused by NO. Therefore, although dFOXO is necessary (and sufficient) for growth inhibition of the salivary glands, the biologically relevant targets of this transcription factor have yet to be identified.

Chapter 6: Oncogenes and NO

6.1. Introduction

Nitric Oxide is known to have an antiproliferative activity (Kuzin et al 1996, Peunova & Enikolopov 1995, Peunova et al 2001), and FOXO, a target of NO, has been linked to cell-cycle arrest and apoptosis in mammalian studies (Zanella et al 2008). These observations suggest that NO may have a function as a tumor suppressor, having anti-oncogenic effects. To test this, two known oncogenes, Ras and Myc, were analysed with respect to their response to NO. Although both Ras and Myc were discussed in Chapter 1, they are briefly discussed again below.

6.1.2. Ras

Ras is a guanine nucleotide binding protein, a GTPase, and is controlled by the GDP/GTP cycle. Ras is inactive when bound to GDP, but becomes active when bound to GTP. Ras in the active GTP-bound form acts in signal transduction pathways to transmit extracellular signals from receptor tyrosine kinases to downstream serine/threonine kinase targets. (Vojtek & Der 1998).

The microarray data produced from NO donor treated *Drosophila* S2 cells versus untreated S2 cells (Kimber 2005), was compared to that from microarray analysis of hemocytes expressing activated Ras (Ras^{V12}) versus hemocytes expressing wild type Ras (Asha et al 2003). There is an inverse correlation in the up and down regulation of transcripts identified from these data sets. In the hemocyte data, 1286 genes were found to be up regulated in association with the expression of activated Ras^{V12}. When compared to the data for S2 cells derived 12 hours after NO, 83 genes of those 1286 were found to be downregulated. Analysis of these 83 common genes revealed that those most upregulated by expression of Ras^{V12} are in turn the most downregulated genes following NO treatment (Kimber 2005). These results give an indication that the proliferation control produced by NO may work in an opposite way to that of the overproliferation induced by Ras. This was tested in this study, by co-expression of Ras^{V12} with NO to see if NOS2 could suppress the overgrowth seen with Ras^{V12} alone.

6.1.3. Myc

Myc is part of a network of transcription factors which act to regulate a wide variety of processes including growth and proliferation, differentiation, apoptosis and oncogenesis (Pierce et al 2004). This transcription network, known as the Max transcription factor network, it is made up of a group transcription factors which share two common features. They contain a basic-helix-loop-helix-leucine zipper (bHLHZ) motif which mediates in protein-protein binding and DNA binding (Grandori et al 2000). They also form individual heterodimers with Max, which is also a bHLHZ protein (Grandori et al 2000). *Drosophila* has one ortholog to the mammalian Myc transcription factor dMyc.

To test if NO was able to suppress the overgrowth induced by Myc, NOS2 and Myc were also co-expressed in the same manner as NOS2 and Ras^{V12}.

Myc is also an interesting potential target for NO signaling as *myc* has been identified as a direct and also indirect target of dFOXO, with *myc* mRNA levels being controlled by dFOXO in a tissue specific manner. dFOXO can inhibit or increase *dMyc* expression (Teleman et al 2008). FOXO has been shown to suppress Myc driven proliferation (Bouchard et al 2007) and Myc dependent gene expression in cell culture (Delpuech et al 2007). The FOXO-dependent nature of Myc induced transformation has demonstrated cooperativity between Myc and Ras (Bouchard et al., 2004).

To investigate if NO can effect expression of Myc, possibly through dFOXO, Myc protein levels were also examined in salivary gland clones expressing NOS2 compared to the surrounding wild type salivary gland tissue.

6.2. Results

6.2.1. Interaction between Ras and NOS-controlled cell proliferation

To examine whether Ras-induced over proliferation could be suppressed by NOS2, an activated form of Ras, Ras^{V12}, was expressed in the larval salivary gland using the salivary gland GAL4 driver c147, both alone and in combination with *UAS-NOS2*.

The late third instar larvae which were expressing just the *UAS- Ras^{V12}*, had salivary glands which showed a marked increase in overall size (Fig. 6.1.). The nuclei in the salivary glands expressing the *UAS- Ras^{V12}* were also much larger than the control nuclei in wild type controls (Fig. 6.1. and 6.2.). In contrast, when both *UAS- Ras^{V12}* and *UAS-NOS2* were co-expressed in the salivary gland, the salivary glands showed a marked decrease in size compared to wild type, looking closer in size to NOS2 expressing salivary glands (Fig. 6.1. and 6.2.). Co-expression of *UAS- Ras^{V12}* and *UAS-NOS2* resulted in a decrease in the size of the nuclei when compared to those expressing Ras^{V12} alone. There was a range of nuclear sizes but all were smaller than nuclei expressing Ras^{V12} alone (Fig. 6.1. and 6.2).

Figure 6.1. Salivary glands and salivary gland nuclei expressing Ras^{V12}.
c147-Gal4 was used to drive the expression of Ras^{V12} alone (A,C), and in the presence of *UAS-NOS2* (D). (A) c147-GAL4/UAS-Ras^{V12} salivary gland pair and c147-GAL4/+ salivary gland. Scale bar 200µm (B) c147-GAL4/UAS-Ras^{V12} salivary gland nucleus. (C) c147-GAL4/+ salivary gland nucleus. (D) NOS2/+;c147-GAL4/+ salivary gland nucleus. (E) NOS2/+;c147-GAL4/UAS-Ras^{V12} salivary gland nucleus. Scale bars 20µm.

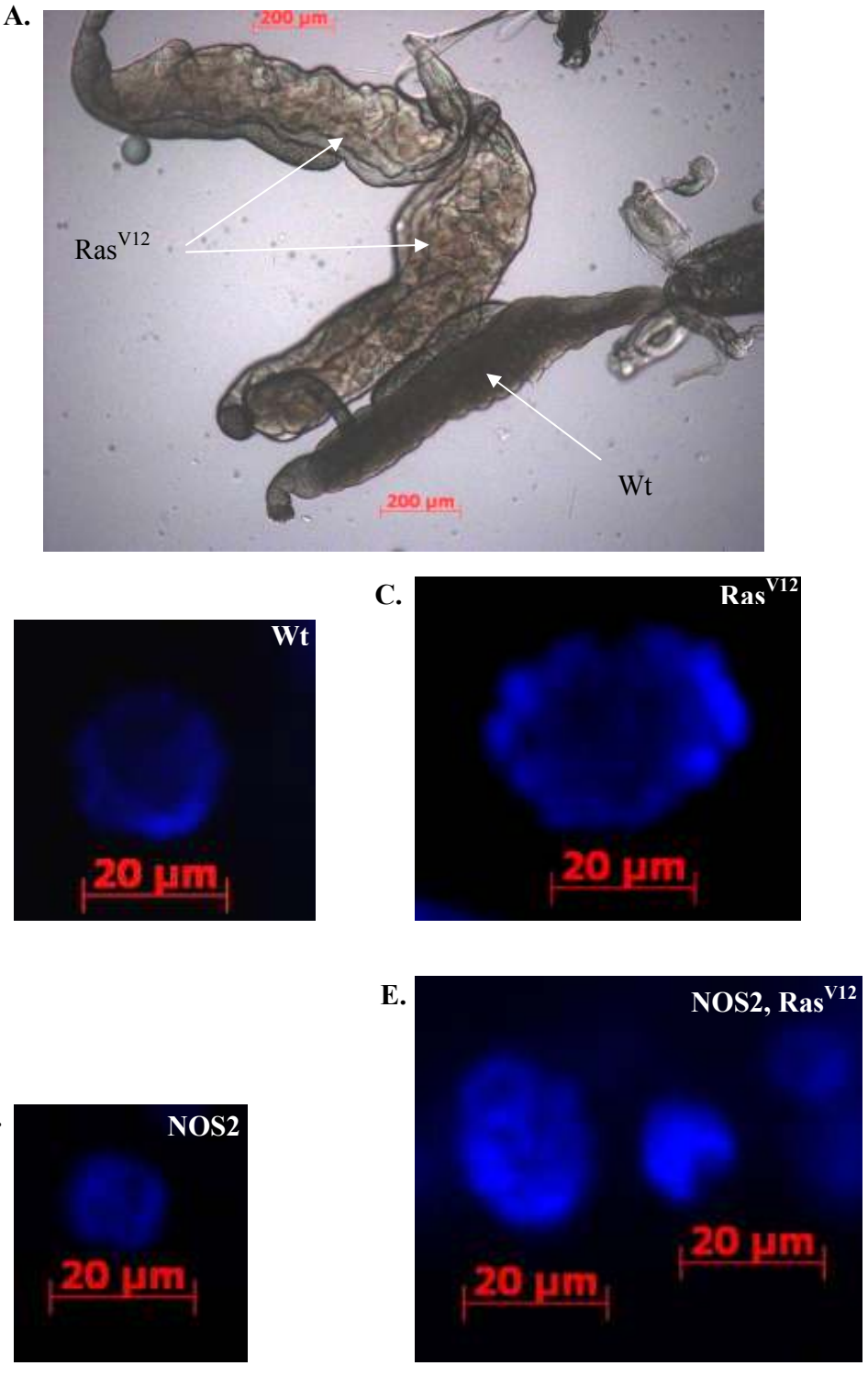
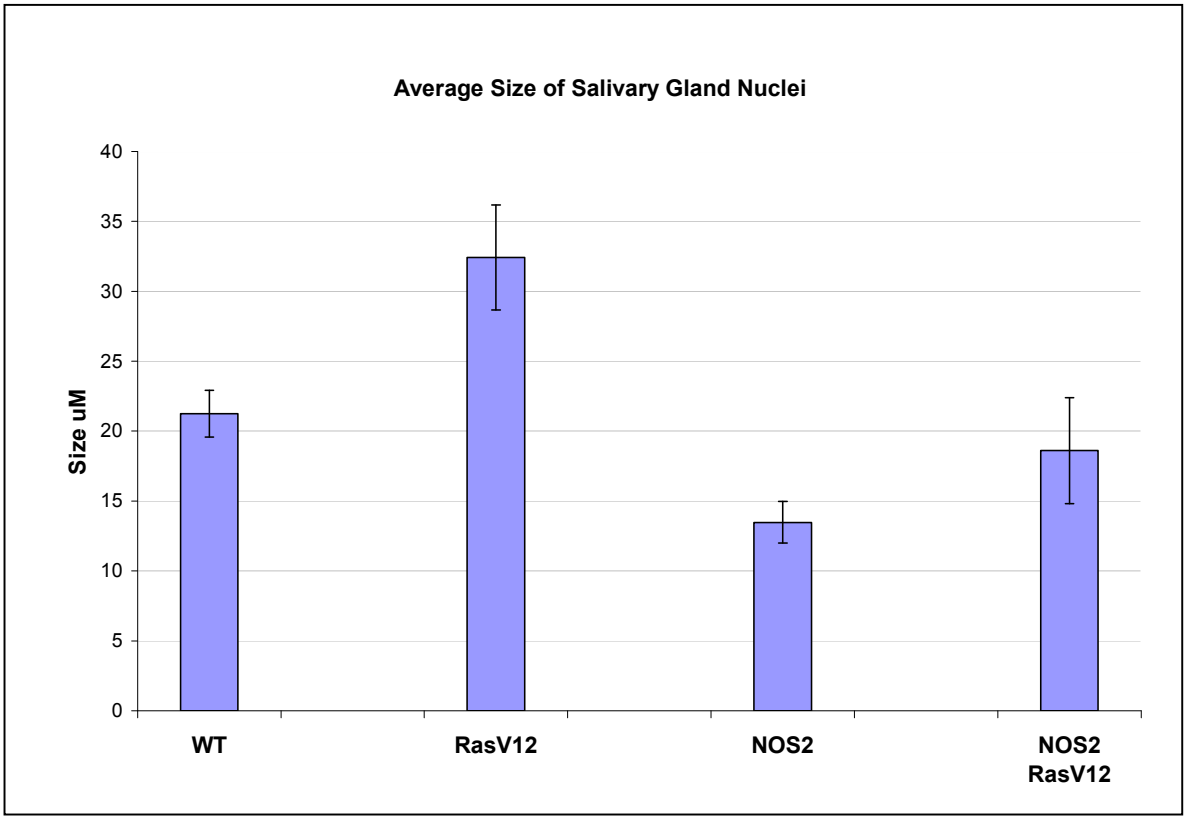


Figure 6.2. Average size of salivary gland nuclei expressing Ras^{V12} and NOS2.
c147-GAL4 was used to drive expression UAS-Ras^{V12}, NOS2 and both together. WT (c147-GAL4/+), RasV12 (c147-GAL4/UAS-Ras^{V12}), NOS2 (NOS2/+;c147-GAL4/+) and NOS2 Ras^{V12} (NOS2/+;c147-GAL4/UAS-Ras^{V12}). Error bars indicate the standard deviation (raw data is presented in Appendix II.i.). P-Value=0.001

For WT, data was derived from 223 nuclei from 20 salivary glands. Ras^{V12}; 236 nuclei from 20 salivary glands. NOS2; 282 nuclei from 21 salivary glands. NOS2 Ras^{V12}; 246 nuclei from 22 salivary glands.



6.2.2. Interaction between dMyc and NOS controlled proliferation

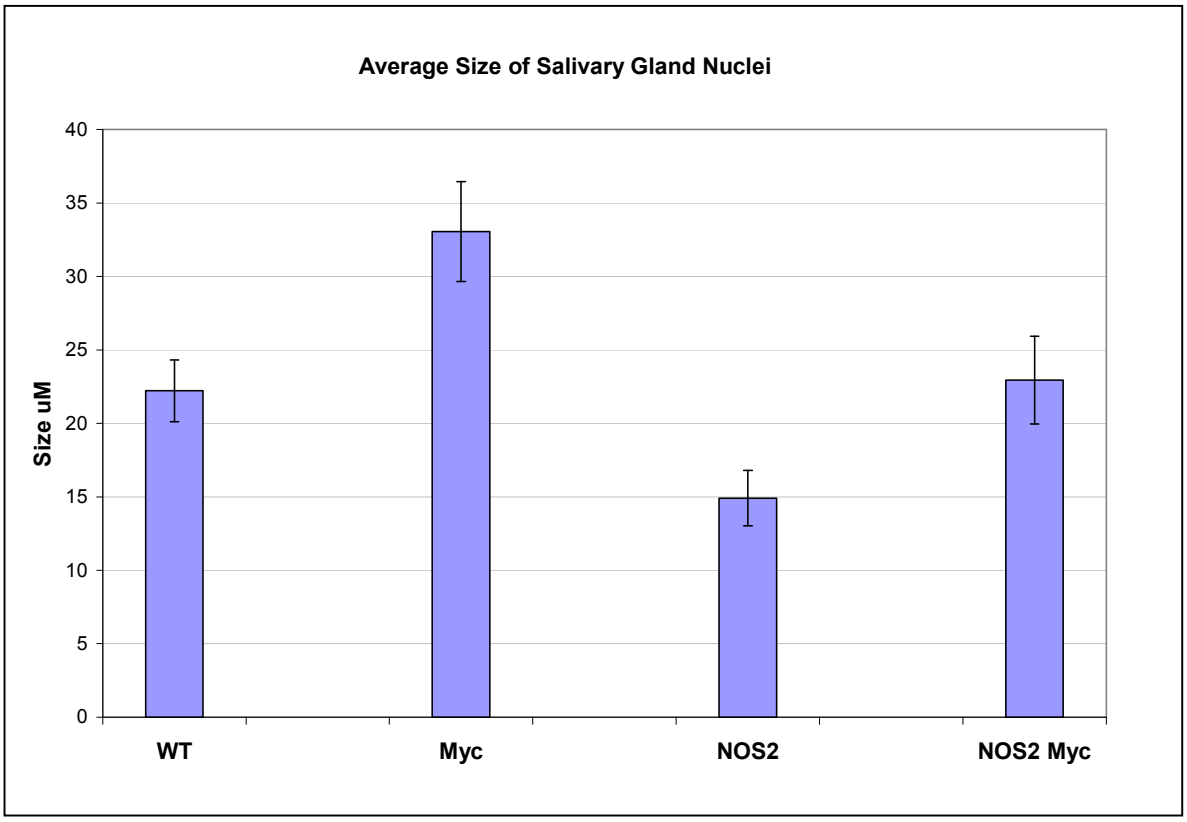
To examine whether dMyc-induced growth could be suppressed by NOS2 expression, dMyc was overexpressed in the larval salivary gland using the salivary gland GAL4 driver c147, both alone and in combination with *UAS-NOS2*.

Late third instar larvae which were expressing just *UAS-dMyc* had salivary glands which showed a marked increase in overall size (Fig. 6.3.). The nuclei in the salivary glands expressing *UAS-dMyc* were much larger than nuclei in wild type controls (Fig. 6.3.). When both *UAS-dMyc* and *UAS-NOS2* were co-expressed in the salivary gland, the salivary glands showed a marked decrease in size when compared to glands in which just *UAS-dMyc* was overexpressed (Fig. 6.3.). The size of the nuclei co-expressing dMyc and NOS2 was also decreased when compared to those expressing dMyc alone, although co-expression resulted in a range of nuclei sizes, all were smaller than nuclei expressing dMyc alone, which was very similar to that of wild type nuclei (Fig. 6.3.).

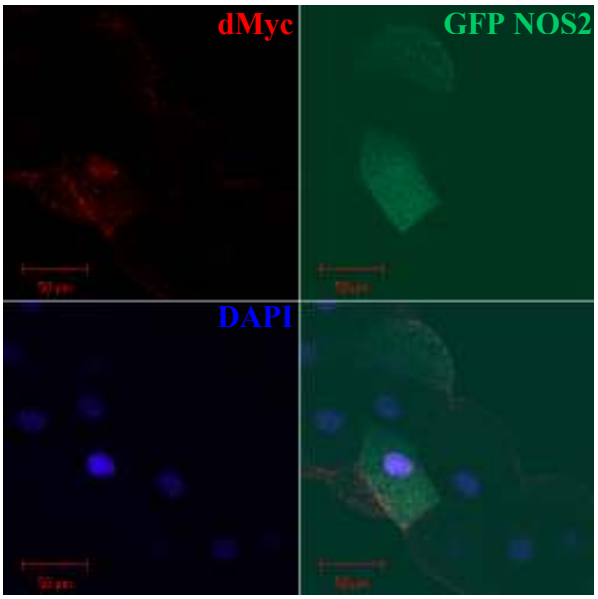
To examine if NO is able to not only suppress the overgrowth phenotype seen with *UAS-dMyc* expression, but also to regulate expression of *dMyc*, salivary glands expressing NOS in single cell clones were stained with a dMyc antibody to examine for any change in dMyc expression. The antibody was shown to be specific as tested in wing discs. *UAS-dMyc* expression was driven using a Gal4, which drove expression in a specific pattern in the wing discs (Dpp pattern). When dMyc antibody stained the discs showed uniform weak expression as well as increased staining in the specific pattern of Dpp expression. The level of Myc expression was analysed in salivary glands containing small clones of cells expressing NOS2. In 17 clones studied in salivary glands, 10 showed a slight increase in dMyc expression (Fig. 6.4.), 5 showed no increase and in the remaining 2, the staining was not clear enough to make a judgement. The clones which did show an increase demonstrated both cell-autonomous and non-autonomous up regulation.

Figure 6.3. Average size of salivary gland nuclei expressing dMyc and NOS2.
c147-GAL4 was used to drive expression UAS-Myc, NOS2 and both together. WT (c147-GAL4/+), Myc (c147-GAL4/+; *UAS-dMyc*/+), NOS2 (NOS2/+;c147-GAL4/+) and NOS2 Myc (NOS2/+;c147-GAL4/+;*UAS-dMyc*/+). Error bars indicate the standard deviation. (raw data is given in Appendix II.j.) P-Value= 0.001

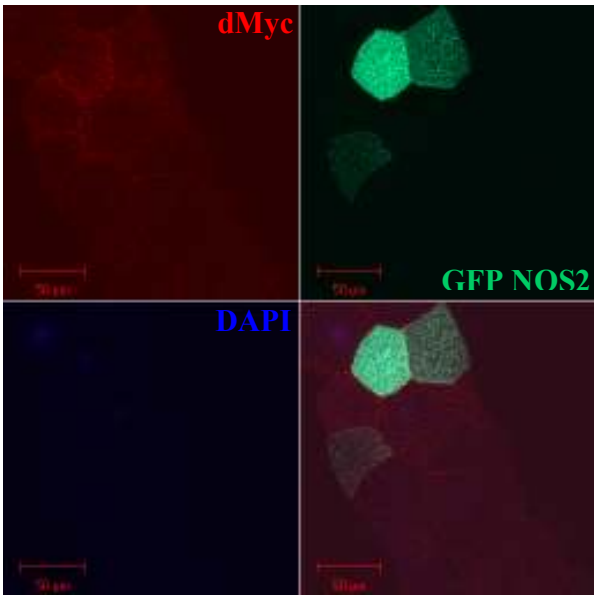
For WT, data was derived from 286 nuclei from 26 salivary glands. Myc; 319 nuclei from 26 salivary glands. NOS2; 223 nuclei from 20 salivary glands. NOS2 Myc; 376 nuclei from 26 salivary glands.



Ai.



Aii.



Aiii

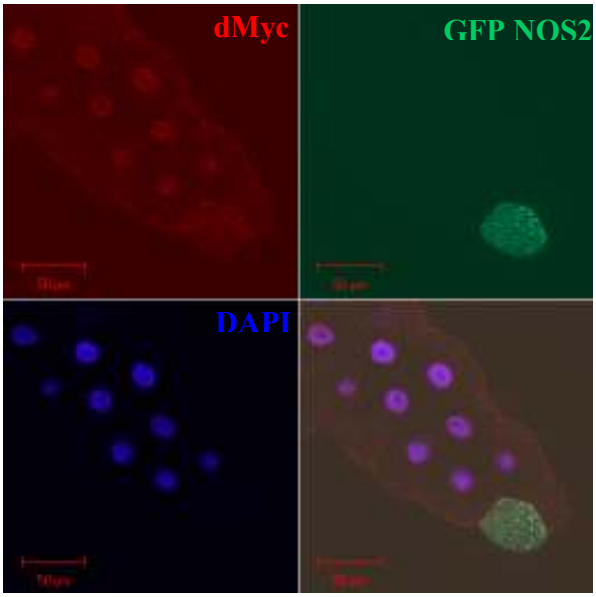


Figure 6.4. dMyc expression in NOS2 expressing clones in salivary glands.
Clones were generated using a 4 and half min heat shock at 38°C between 24-48hr AEL. Clones are marked with GFP. Scale bars 50µm.
Ai., Aii. and Aiii. NOS2; UAS- NOS2/hsFLP; +/Act5c>y^{+}>Gal4;*UAS-GFP*/+. Red; dMyc Green; GFP + NOS2 Blue; DAPI.

6.3. Discussion

6.3.1. Ras

NO has been shown to have antiproliferative activity (Kuzin et al 1996, Peunova & Enikolopov 1995, Peunova et al 2001and this thesis). To determine if Ras-induced overproliferation could be suppressed by NO, an activated Ras^{V12} protein was co-expressed with NOS2. Expression of activated Ras^{V12} in salivary glands results in increased of both the overall salivary gland size and the nuclear size. This increase in size is consistent with results seen from overexpression of Ras^{V12} in other *Drosophila* tissues as well as the results seen by Berry & Baehrecke 2007 where expression of Ras^{V12} in the salivary glands resulted in increased growth. It should be noted that in Berry & Baehrecke 2007 growth was measured by cell area measurements on *Drosophila* 13.5 hours after puparium formation, whereas I was examining nuclear size of late third instar larvae although the results are similar. When Ras^{V12} was co-expressed with NOS2, NOS2 was able to rescue the overproliferation effects of increased nuclear size induced by Ras^{V12} alone. The average nuclear size of salivary glands expressing both NOS2 and Ras^{V12} was smaller than in wild type salivary glands, but larger than in glands expressing NOS2 alone. Thus NOS2 is capable of suppressing the Ras^{V12} overgrowth phenotype, but in turn Ras^{V12} can suppress, to some degree, the small salivary gland phenotype of NOS2. This supports the molecular data showing they have opposing effects on the regulation of many genes (Kimber 2005).

These data also show that NOS2 expression is capable of suppressing Ras^{V12} induced overproliferation supporting the idea of NOS as a tumour suppressor, having anti-oncogenic effects. As dFOXO is a proposed target for NO, that has been linked to cell-cycle arrest and apoptosis in mammalian studies (Zanella et al 2008), it suggests that NO may be acting through dFOXO to exert its anti-oncogenic effects. To further investigate this it would be necessary to repeat the experiment, but in animals homozygous for *dFOXO* null mutations. Activated Ras^{V12} is known to act through PI3K, a component of the insulin pathway, to increase growth, and NO is thought to act in this pathway to suppress growth. It is interesting to note that NOS2 can suppress Ras^{V12} induced overproliferation.

6.3.2. Myc

As with Ras^{V12}, NOS2 was able to suppress the overgrowth phenotype resulting from dMyc expression. This overgrowth phenotype was similar to previous studies where dMyc is overexpressed in *Drosophila*, resulting in an increase in the size of cells, with both mitotic and endoreplicating cells (including those of the salivary gland) being larger than normal (Grewal et al 2005, Neufeld et al 1998, Pierce et al 2004). NOS2 co-expression with dMyc results in salivary gland nuclei which were statistically similar to that of wild type salivary glands.
I was unsure what levels of dMyc expression would be observed in NOS2 expressing clones, but was surprised when in more than half, an obvious increase was apparent, given that dFOXO expression can inhibit *dMyc* expression in *Drosophila* muscle (Demontis & Perrimon 2009) and inhibition of N-Myc expression by NO has been reported neuroblastoma cells (Ciani et al 2004).

However it should be noted that in some clones no change in dMyc expression was observed and the background in the staining was relatively high, accordingly in future verification studies this would need to be optimised. dMyc has been identified as a direct and also indirect target for the transcription factor dFOXO, with *dMyc* mRNA levels being controlled by dFOXO in a tissue-specific manner. dFOXO can inhibit or increase *dMyc* expression (Teleman et al 2008).
In vertebrates FOXO has been shown to suppress Myc-driven proliferation (Bouchard et al 2007) and Myc-dependent gene expression in cell culture (Delpuech et al 2007), and in *Drosophila,* dFOXO has been shown to inhibit dMyc function (Demontis & Perrimon 2009). Thus, although increased Myc expression is usually associated with increased growth and proliferation, the activity of dMyc in these NOS2 expressing cells may be suppressed by the increased dFOXO activity in these same cells. Alternatively, the increase in dMyc expression may reveal a control mechanism whereby once dFOXO expression reaches specific levels in the cell, this activates dMyc expression, therefore preventing over inhibition of proliferation.

Overall the data presented in this Chapter demonstrates that NO can suppress the increased growth of salivary gland cells expressing ectopic Myc or activated Ras, thereby supporting the possible use of NO-donating compounds in the treatment of misregulated growth.

Chapter 7: Discussion

7.1. Introduction

The work presented in this thesis has confirmed the role Nitric Oxide in the inhibition of growth in *Drosophila* and demonstrated a potential molecular mechanism for its action. NO was also shown to be able to not only regulate normal growth in *Drosophila* but also to inhibit misregulated overgrowth. The mechanism as to how NO functions in regulating growth elucidated through the data presented in this thesis, as well as possible lines worthy of further investigation, are discussed below.

7.2. Expression of NOS in *Drosophila*

Nitric Oxide levels were controlled via expression of NOS2, a constitutively active mouse macrophage nitric oxide synthase gene, under the control of GAL4 (section, 3.2.1.). The ectopic expression of NOS2 in the salivary glands was achieved by using either c147 or AB1 Gal4 as the driver. Expression of NOS2 in the salivary glands resulted in a reduced growth phenotype (section 3.2.1.). This reduced growth phenotype was similar to that following overexpression of dFOXO (section 3.2.1.). The transcription factor dFOXO, which is part of the insulin signaling pathway, has previously been identified as a possible target for NO signaling (Kimber 2005), and was also shown to be necessary for NO action in inhibiting growth (Kimber 2005). The data presented in this thesis confirm these initial findings and further demonstrate that NO can upregulate the protein levels of dFOXO. However it is not clear how NO has this effect. It is speculated that it may be post transcriptionally as no changes in *dFOXO* mRNA levels were observed in NO treated tissue culture cells (Kimber 2005).

7.2.1. Thor expression in NOS2 expressing larvae

To confirm the role of dFOXO as a target for NO signaling in *Drosophila,* the expression of one known dFOXO transcriptional target, Thor, was studied. This allowed the activity of dFOXO to be monitored, albeit indirectly. The expression of Thor, and thereby dFOXO activity, was examined in three ways: through the use of a LacZ promoter fusion, a YFP protein fusion and via antibody staining of the endogenous Thor protein. In the previous *in vivo* study of Kimber et al. NOS2 was expressed in whole salivary glands which resulted in the increase in Thor LacZ expression, coinciding with a reduction in the size of the salivary glands

(Kimber 2005). However, the observed increase in LacZ staining may have been a reflection of this change in size and not in an increase in the expression of *Thor*.

To verify the apparent increase of Thor LacZ reporter levels in response to NOS2 expression, NOS2 was expressed in small or single cell clones within the salivary gland. NOS2 expressing clones showed an increase in Thor LacZ levels confirming the direct effect of NO on *Thor* expression. Interestingly, the clones also demonstrated that the expression of NOS resulted in cell non-autonomous as well as cell autonomous effects. These non-autonomous effects are consistent with the fact that NO is known to diffuse between cells (Haley 1998). The short-range of the non-autonomous effects of NO are probably due to its half-life of approximately only a few seconds (Haley 1998) limiting its diffusion to those cells immediately neighboring the source of NO.

As the Thor LacZ is only a reporter of the *Thor* promoter activity and does not directly reflect protein levels, a Thor-YFP protein fusion was also utilised to observe the behavior of Thor protein itself to increased levels of NO. This construct comprises an insertion of a YFP protein trap construct into the coding region of the *Thor* locus. Thus it reflects the expression of the endogenous protein rather the just activity of the promoter. Experiments using the Thor-YFP fusion also indicated an increase in expression in response to expression of NOS2 in whole salivary glands, and in single salivary gland cells.

To validate expression of Thor-YFP as an indicator of increased dFOXO activity, *UAS-dFOXO* was also expressed in whole salivary glands and in single salivary gland cells. The results from this, was as expected, showing an increase in Thor-YFP expression. The final verification was achieved using antibody raised against Thor. Although this antibody was later identified not to be specific for only Thor protein, this was probably due to being from an early bleed as the original purified antibody had run out. The antibody though did show that there was an increase in Thor protein expression in response to NOS2. Taken together these data strongly suggests that NO can increase Thor expression through what is thought to be increased dFOXO activity.

7.2.2. dFOXO expression in response to NOS2 expression

To confirm that the NOS2-induced increase in Thor expression was affected through an increase in dFOXO activity, the expression of dFOXO was analysed in response to NOS2 expression. dFOXO activity has been previously been shown to be regulated by localisation (Jünger et al 2003, Puig et al 2003). The phosphorylation of dFOXO by Akt causes it to be

retained in the cytoplasm, and thereby unable to act as a transcription factor (Jünger et al 2003, Puig et al 2003). Therefore to study dFOXO activity, the localisation of dFOXO protein was examined in salivary glands using an antibody specific to dFOXO. A change in localisation of dFOXO in response to NOS2 expression in whole salivary glands was not observed, although an overall increase in dFOXO expression was detected. To test that this increase in expression was not a consequence of reduced cell size, dFOXO expression was analysed in single cells expressing NOS2. These single cells also showed an increase in dFOXO protein levels in both the cytoplasm and nuclei. Accordingly, it is suggested that NO acts on dFOXO by increasing its expression. The regulation of dFOXO expression has been suggested to play a role in its activity, and I have shown that increased expression of *dFOXO* can mimic the effects of NO. In a study of dFOXO regulators using *Drosophila* S2 cells, 21 proteins were identified that modulated dFOXO transcriptional activity, protein levels and/or sub-cellular localization (Mattila et al 2008). Of these 21 proteins, 8 were shown to regulate dFOXO stability (Mattila et al 2008). Therefore, these proteins may be of interest for future work to examine if NO may act by affecting one or more of these proteins to regulate dFOXO stability and thereby growth. It may also be worthwhile to determine if *dFOXO* mRNA levels are increased in response to NO. This would show that protein levels may be increasing due to increased transcription of dFOXO, although of course this would not exclude changes due also to other factors such as changes in the stability of the protein and splice variants, as well as other post transcriptional modifications. This analysis could be performed in NOS2 expressing clones using *in situ* hybridisation to *dFOXO* mRNA or real-time PCR could be utilised.

7.2.3. NOS acts in the insulin signalling pathway

It was shown that NO was acting within the insulin signalling pathway through dFOXO, but not whether this was a direct action. Therefore the effects of NO on components of the insulin signaling pathway, above dFOXO, were also investigated.

We first examined whether knocking out parts of the pathway (which result in reduced growth) had any effect on NOS signaling. This part of the study was attempted using RNAi to knockdown dNOS expression, along with dInR or PI3K p110. However, no results were obtained because of the lethality that resulted.

To verify the previous data demonstrating that NOS2-induced growth inhibition was dFOXO-dependent (Kimber 2005), a new RNAi-dFOXO transgene was utilised. Expression of this RNAi-dFOXO transgene induced a reduced growth phenotype in the salivary glands,

producing glands that were smaller than those resulting from expression of either *UAS-NOS2* or *UAS-dFOXO* alone. However, it is possible that this reduced growth may be due to the known feedback loop, whereby dFOXO acts to regulate expression of *dInR* (Puig & Tjian 2005). In this case, it could mean that although dFOXO is known to act in this feedback loop where it is thought to function to regulate sensitivity to insulin, acting as an insulin sensor to activate insulin signaling, thereby allowing a fast response to the hormone after each meal (Puig & Tjian 2005). It may be the case that dFOXO is needed for further expression of *dInR* during development as well, so when dFOXO is not there to modulate *dInR* expression, reduced growth is observed as is seen with RNAi-dFOXO.

There is a caveat to this theory, as *dFOXO* null mutants are viable and do not show reduced growth (Jünger et al 2003). To address this, dFOXO-independent expression of *dInR* was examined by expressing *UAS-dInR*, and co-expressing it with *UAS-RNAi-dFOXO*. Expression of wild type *UAS-dInR* (wt) or activated *UAS-dInR** was unable to rescue the small salivary gland phenotype seen with *UAS-RNAi-dFOXO*. However, expression of wild type or activated *UAS-dInR* alone was able to increase growth of salivary gland cells. An explanation for these observations is that in the absence of dFOXO, insulin signaling cannot promote growth. Alternatively, the RNAi-dFOXO construct may target the degradation of other mRNA(s) required for growth, so called 'off-targets' (Saxena et al 2003). It may be worth determining if RNAi-dFOXO expression does reduce dFOXO protein levels however this would not exclude the possibility of off-targets causing the observed effects. It would be also interesting to examine dInR proteins levels in animals expressing RNAi-dFOXO, or in *dFOXO* nulls to determine if there is reduced expression of dInR. The discrepancy between salivary gland growth in *dFOXO* null animals compared to those expressing RNAi-dFOXO may be due to competition for insulin. For example, cells expressing RNAi-dFOXO are likely to have reduced expression of dInR compared to wild type cells and thus will be at a growth disadvantage, whereas in *dFOXO* null animals, all cells will be expressing equally low levels of dInR.

7.2.4. NO acts downstream of dInR

To determine if the reduced growth phenotype caused by NOS2 expression could be rescued by either wild type *UAS-dInR* or activated *UAS-dInR*, epistasis analysis was undertaken. Co-expression of *UAS-NOS2* with either wild type or activated *UAS-dInR* resulted in decreased growth compared to that of wild type or with activated *UAS-dInR* expression alone. Thus NO-

induced reduction of growth was unable to be rescued by the expression of either wild type or activated *UAS-dInR*. These data are consistent with NO acting genetically downstream of, or in parallel to, the insulin receptor.

To further examine where NO may be acting in the insulin signalling pathway, a reporter of PI3K activity was utilised. This reporter, known as tGPH, responds to PIP_3 levels, a secondary messenger in the pathway, which increases as a result of active PI3K phosphorylation of phosphatidylinositol-4,5-P_2 (PIP_2).. tGPH is recruited to plasma membranes by binding PIP_3, and therefore serves as a reporter for PI3K activity (Britton et al 2002).

As NOS signalling was shown to act through dFOXO, and dFOXO is known to be part of a feedback loop, the localization of tGPH in single cells of the salivary glands expressing dFOXO or NOS2 was determined. Single cells expressing dFOXO showed increased localisation of tGPH to the cell membrane when compared to wild type cells. This result is consistent with the fact that changes in dFOXO activity are thought to feedback on the pathway by activating the expression of the insulin receptor (Puig et al 2003, Puig & Tjian 2005), but this is the first demonstration of this feedback loop in salivary gland cells.

However, the data obtained with NOS2 expression were not as clear. The localisation of the tGPH in cells expressing NOS2 showed some variation. Mostly, as for dFOXO, tGPH was observed at higher levels at the cell membranes. However, in a minority of cases there was no alteration in tGPH localisation. This variability could be due to the feedback were dFOXO acts to increase expression of the insulin receptor although the extent of this feedback will be dependent on how long the cells have been expressing NO. There may also be a lag in the activity of dFOXO dependent genes, their translation and the activation of the pathway. Thus, although clones are induced at 24 –28 hours AEL, there may be slight differences in the level and time of NO expression in these cells which results in the perceived variability in expression of dFOXO and thereby its feedback loop. Indeed, when levels of dFOXO were determined directly in single cells expressing NOS2, there were noticeable differences between individual cells. This explanation could be tested by directly correlating levels of dFOXO expression and tGPH localisation simultaneously in clones of cells expressing NOS2 using the FLP/FRT technique.

7.2.5. NO inhibition of growth does not require soluble Guanyl Cyclase (sGC)

The activity of sGC is known to be regulated directly by NO in *Drosophila* (Gibbs et al 2001). To determine if NO was regulating growth through sGC, the effects of NO were determined in animals with reduced sGC expression. sGC was knocked down via expression of RNAi-Gycα-99B. There was no obvious change in the growth inhibition induced by NOS2 when expression of this sGC was reduced. Indeed, there were no observed changes in growth of cells expressing RNAi-Gycα-99B, indicating that cGMP levels are not controlling growth. These data indicate that NO dependent regulation of growth does not act through sGC. This is not the first instance where NO signaling has been shown to act independently of sGC signalling. Other examples of sGC-independent NO signaling have also been identified including by S-nitrosylation (Asada et al 2009), through cytochrome oxidase activity (Cooper 2002) and through Metallothionein (St. Croix et al 2002), so it may be that NO is acting via one of these other pathways to regulate growth.

However, it has to be noted that the *Drosophila* genome contains 5 genes that code for soluble guanylyl cyclase subunits. Each subunit is differently activated by NO (Morton et al 2005). The only subunit targeted by RNAi was Gycα-99B (the α subunit). This subunit along with the β subunit, Gycβ-100B, form a conventional, NO-sensitive heterodimeric soluble guanylyl cyclase (Dietzl 2007, Shah & Hyde 1995). Only sGC complexes containing the Gycα-99B subunit are highly stimulated (approx. 30 fold) by NO (Morton et al 2005), and the genetic removal of the Gycα-99B subunit has also been demonstrated to abolish detectable increase in NO induced increases in cGMP levels (Gibbs et al 2001). Thus although Gycα-99B is the only likely sGC that can be responding to NO it is possible that other sGCs may function independently of Gycα-99B and these can be stimulated to increase cGMP levels by NO. To further investigate the possible role(s) of sGC, if any, in NO inhibition of growth, the levels of cGMP could be directly observed using anti-cGMP antibodies. However, any change observed in cGMP levels would still not conclusively demonstrate whether cGMP was required for NO dependent growth inhibition. To definitively test the role of sGC and cGMP the simultaneous knock down of all sGC genes would be required.

7.2.6. dFOXO transcriptional targets: roles in NO signalling

dFOXO was shown to be vital for NO signaling and thus an analysis of the roles of some of its transcriptional targets was undertaken. As mentioned above, an increase in Thor expression was identified in response to NO.

To identify if expression of Thor alone was acting to regulate growth due to NO via dFOXO, expression of an active form of Thor ($Thor^{LL}$) was utilised to see if Thor expression would be able to induce the reduced growth seen with either NOS2 or dFOXO expression. In this study, the expression of $Thor^{LL}$ resulted in a small reduction in growth, but this difference was not statistically significant. This may be due to increased Thor expression not being the only way in which NO, acting through dFOXO, is inhibiting growth of these cells. It may also be that although $Thor^{LL}$ binds to deIF4E more strongly, this is not having the same growth-inhibitory effect that is caused by the large increase in Thor expression that occurs when NO is expressed. Therefore further investigation was required.

To test if the increase in Thor was required for NO inhibition of growth, a null mutation of *Thor* was utilised. When NOS2 was expressed in this *Thor* null background, a reduction of growth was still observed. Thus Thor is not required for NO induced inhibition of growth.

Another transcriptional target of dFOXO examined was Lk6. Like Thor, Lk6 is also known to regulate the activity of the translation initiation factor, eIF4E. Lk6 is also a target of dFOXO transcriptional regulation. The activity of Lk6 in response to NO expression was studied via two routes: firstly through the expression of NO in an *Lk6* mutant null background, and secondly by regulation of eIF4E. Expression of NO in *Lk6* null animals did not alter the ability of NO to inhibit growth. These data indicate that neither Lk6 nor Thor are required individually for NO controlled growth. However it has been proposed that Thor and Lk6 may act in partnership to regulate translation (Reiling et al 2005). Thus they may have redundant roles in growth regulation. To test any redundant roles, double mutant animals were constructed. NOS2 expression in these double mutants still resulted in growth inhibition. Thus there is no evidence that either of these regulators of translation (separately or in parallel) are required for the inhibition of growth caused by NO. Although no role for Lk6 was revealed, it is still possible that other kinase(s) may regulate the activity of its substrate, eIF4E, in response to NO. The role of phosphorylation of eIF4E in NO dependent growth control was tested using a non-phosphorylatable form of eIF4E ($eIF4E^{Ser251Ala}$). Expression of $eIF4E^{Ser251Ala}$ in parallel with NOS2 was however unable to suppresses the NO-inhibition of the growth phenotype.

Thus there is no evidence that NO-induced growth inhibition is dependent on Thor, Lk6 or their effector, eIF4E. Accordingly, although dFOXO is necessary (and sufficient) for growth inhibition of the salivary glands, the biologically relevant targets of this transcription factor have yet to be identified and this may be of interest for further study.

7.2.7. NO acts as an inhibitor of misregulated growth

Nitric Oxide is known to have an antiproliferative activity (Kuzin et al 1996, Peunova & Enikolopov 1995, Peunova et al 2001) and FOXO, a target for NO, has been linked to cell-cycle arrest and apoptosis in mammalian studies (Zanella et al 2008), this suggests that NO may function as a tumor suppressor, with anti-oncogenic effects.

To test this in *Drosophila,* the interaction of NO signaling with two known and well-defined oncogenes, Ras and dMyc, was analysed.

Both Ras and dMyc induced overgrowth were shown to be inhibited by co-expression of NOS2.

The expression levels of dMyc in response to NOS2 expression were examined as FOXO has been shown to suppress Myc driven proliferation (Bouchard et al 2007), and Myc dependent gene expression in cell culture (Delpuech et al 2007). In *Drosophila* Telman *et. al.* 2008 have also shown that dFOXO does not only act indirectly to inhibit *dMyc* expression but it may also act directly to maintain constant levels of dMyc in the adipose tissue upon fasting. In my studies dMyc protein levels often increased in response to NOS2 expression. However in some instances dMyc levels appeared unaltered. The increased dMyc levels in these growth-inhibited cells is somewhat counterintuitive as increased dMyc is normally associated with increased growth. However, FOXO can inhibit the function of Myc (Bouchard et al 2007, Delpuech et al 2007), and thus the effects of increased dMyc expression (usually associated with increased growth and proliferation) may be being suppressed by the increased dFOXO activity in these cells.

Alternatively the observed increase in dMyc expression may indicate a control mechanism, whereby once dFOXO expression (induced by NO) reaches a specific level in the cell, dMyc expression is activated thereby preventing the over-inhibition of growth. This hypothesis could be tested by examining dMyc expression as well as dFOXO expression levels in clones overexpressing dFOXO. Slight over expression of dFOXO might be predicted to result in a decrease in dMyc expression, whereas high levels of dFOXO could result in an increase of dMyc.

7.3. Further work

As well as the specific lines of enquiry that have been discussed above, several other aspects of interest that may be worth investigating are noted below.

dFOXO has been shown to act as a key effector of NO signalling in this thesis as well as in previous work (Kimber 2005), therefore it would be revealing to investigate how NO acts on dFOXO by examining a known regulator of dFOXO activity, dAkt. Although the data presented in this thesis indicates that levels dFOXO are regulated by NO, changes due to alterations to the localisation of dFOXO cannot be eliminated. Accordingly it may prove useful to determine whether dAkt activity is altered in response to NO. It is also relevant to point out a study in which NO was shown to inhibit proliferation of hematopoietic cells (Wang et al 2007). This work provides evidence that NO-induced G0/G1 arrest is mediated through the regulation of cell cycle related proteins, which may be depend on Akt deactivation by NO (Wang et al 2007).

There is also a mammalian cell line which has been utilised to analysis FOXO nuclear-cytoplasmic shuttling in response to chemical stimuli. This cell line expresses a GFP-FOXO3a fusion protein and has previously been used to test the ability of compounds to alter FOXO3a localisation (Zanella et al 2008). It would be interesting to determine if NO could alter localisation of this mammalian FOXO by using a NO donor such as SNAP. This would allow the analysis of mammalian FOXO in response to NO treatment and in turn it may demonstrate that NO can effect FOXO localisation. This work in tissue culture would back up work that could be continued with the tagged version of dFOXO in cell culture in response to SNAP treatment.

The use of Thor-YFP in this study has also identified a method for being able to identify proteins which maybe part of the NO signalling pathway using cultured salivary glands and SNAP treatment. The Cambridge Protein Trap YFP Insertion stocks can be utilised to identify NO responsive proteins. The YFP-protein fusions are expressed at endogenous levels so that both localization and protein complexes are likely to reflect the normal cellular physiological condition. It must be noted that though SNAP treatment on cultured salivary gland did result in the expected increase in Thor-YFP expression, the results were variable. So though this method may prove to be a way to quickly screen and identify proteins that may respond to NO and

there by be part of NO signalling pathway, care must be taken to then do further work to verify those findings.

A method that could be used to identify genes required for NO induced cytostasis is that of RNAi screens in cell culture. Cells could be cultured in the presence of SNAP at a concentration that inhibits cell division (200µm (Kimber thesis 2005)) and it could be determined if any single RNAi suppressed this growth inhibition. The localisation of dFOXO in these cells could also be determined.

The model proposed in this thesis of NO signalling to FOXO to inhibit growth eliminates sGC (upstream of FOXO) and Thor and Lk6 as molecules required for this pathway. However, the RNAi screens referred to above may reveal critical molecules required for this signalling.

A specific protein who's role in NO signalling that has not been investigated in this work was that of AKT. AKT has been identified in previous studies to firstly have a role in the insulin pathway acting to inhibit dFOXO (ref). Secondly it has been also identified as a target for NO whereby NO acts to inhibited AKT by preventing its phosphorylation. To identify if a similar mechanism is occurring in *Drosophila* the amount of phosphorylated Akt could be determined after NO treatment by using antibodies that recognise the phosphorylated form of the protein (Torroglosa et al 2007). Additionally, activated Akt could be co-expressed with NOS2 to test whether activated Akt could rescue the NOS induced phenotypes.

Appendices

I. Fly stocks used

Table 1. Bloomington stocks, http://flystocks.bio.indiana.edu/

Stock #	Geneotype	Comments
1824	y[1] w[*]; P{w[+mW.hs]=GawB}AB1	GAL4 in salivary gland, basal expression of P{GawB}
4780	y[1] w[*]; P{w[+mC]=GAL4-Act5C(FRT.CD2).P}S	Ubiquitous expression of GAL4 in FLP-generated clones
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.
7012	y[1] w[*] P{ry[+t7.2]=hsFLP}1; P{w[+mC]=UAS-Tor.WT}III	Wild type Tor expressed under the control of UAS
7118	w[1118]; P{w[+mC]=*UAS-myr-mRFP*}1	P{*UAS-myr-mRFP*} expresses membrane-targeted monomeric RFP
7119	w[1118]; P{w[+mC]=*UAS-myr-mRFP*}2/TM6B, Tb[1]	P{*UAS-myr-mRFP*} expresses membrane-targeted monomeric RFP
8163	w[118]; P{w[+mC]=tGPH}2; Sb[1]/TM3, Ser[1]	Expresses a fusion protein composed of GFP and the pleckstrin homology domain.
8262	y[1] w[1118]; P{w[+mC]=UAS-InR.Exel}2	Expresses wild type InR under the control of UAS
8263	y[1] w[1118]; P{w[+mC]=UAS-InR.A1325D}2	Expresses a constitutively active InR under the control of UAS
8294	P{w[+mC]=UAS-Pi3K92E.CAAX}1, y[1] w[1118]	Expresses a constitutively active Pi3K92E with a farnesylation signal (CAAX) appended to the C terminus.
8651	w[1118]; P{w[+mC]=UAS-p35.H}BH1; P{w[+mC]=*UAS-Thor*.LL}s	Expresses a mutant Thor protein that cannot bind eIF-4E effectively under the control of UAS
8708	w[1118]; P{w[+mC]=UAS-Lk6.A}1/TM6B, Tb[1]	Wild type Lk6 expressed under the control of UAS
8709	w[1118]; P{w[+mC]=UAS-Lk6.T424D}6	Constitutively-active Lk6 expressed under the control of UAS
8711	w[1118]; P{w[+mC]=UAS-eIF-4E.S251A}8	Non-phosphorylatable eIF-4E expressed under the control of UAS
8761	P{w[+m*]=GAL4}A9, w[*]	Expresses GAL4 in the wing and haltere discs. Also weak salivary gland expression observed.
9559	y[1] w[*]; Thor[2]	Thor loss of function mutants
9575	y[1] w[*]; P{w[+mC]=UAS-foxo.P}2	Expresses wild type dfoxo under UAS control

Table 2. Vienna *Drosophila* RNAi Center (VDRC), http://stockcenter.vdrc.at/control/main

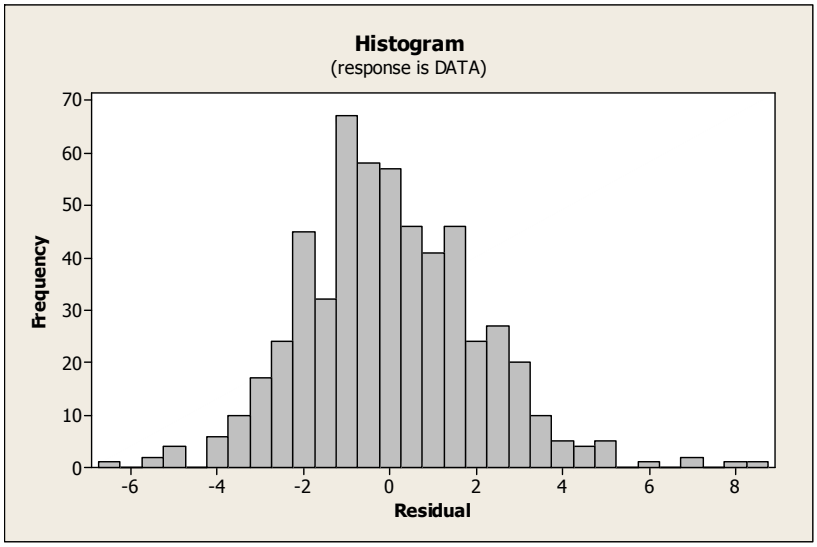
Stock #	Genotype	Chromosome	CG number of Gene effected
992	UAS-RNAi-dInR	3	CG18402
30556	*UAS-RNAi-dFOXO*	2	CG3143
38985	*UAS-RNAi-Thor*	3	CG8846
38985	UASRNAi-dPI3K p110	3	CG4141
43711	*UAS-RNAi-Gycα-99B*	2	CG1912

Table 3. Fly stocks from other sources.

Genotype	Chromosome	Comments and Reference
Lk6[15]	3	Lk6 null mutant. A kind gift from Prof. Ernst Hafen (Reiling et al 2005)
Lk6[38]	3	Lk6 null mutant. A kind gift from Prof. Ernst Hafen (Reiling et al 2005)

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

II. a. Wild type, NOS2 and dFOXO expression



Genes **1** **WT**
2 **NOS2**
3 **dFOXO**

One-way ANOVA: DATA versus GENES

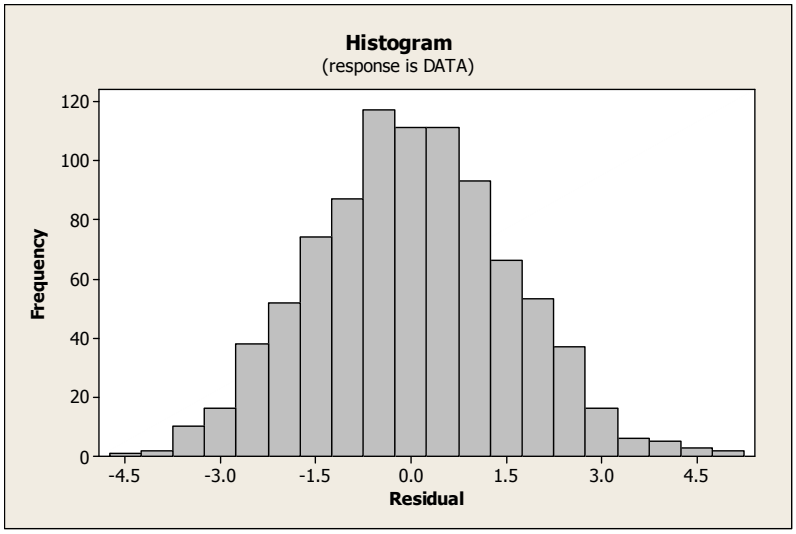
```
Source   DF        SS       MS        F      P
GENES     2  11668.31  5834.16  1381.55  0.001
Error   553   2335.26     4.22
Total   555  14003.57

S = 2.055   R-Sq = 83.32%   R-Sq(adj) = 83.26%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level    N    Mean  StDev   -+---------+---------+---------+--------
1      108  22.964  2.664                                        (*)
2      380  12.711  1.888              (*
3       68   8.098  1.835   (*)
                           -+---------+---------+---------+--------
                          8.0      12.0      16.0      20.0

Pooled StDev = 2.055

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of GENES

Individual confidence level = 98.04%
```

```
GENES = 1 subtracted from:

GENES    Lower   Center    Upper  ------+---------+---------+---------+---
2      -10.778  -10.253   -9.729          (*)
3      -15.611  -14.866  -14.122  (*)
                                 ------+---------+---------+---------+---
                                   -12.0      -6.0       0.0       6.0



GENES = 2 subtracted from:

GENES   Lower  Center   Upper  ------+---------+---------+---------+---
3      -5.246  -4.613  -3.980                  (*)
                               ------+---------+---------+---------+---
                                 -12.0      -6.0       0.0       6.0
```

Genes	1 WT	2 NOS2	3 *UAS-dFOXO*
1 WT	-	-	-
2 NOS2	SD- smaller	-	-
3 *UAS-dFOXO*	SD- smaller	SD- smaller	-

Columns are compared to rows. SD- statistically different

II. b. Wild type, NOS2 and dFOXO expression



Genes **1** **WT**
2 **RNAi-Gycα-99B**
3 **NOS2**
4 **NOS2 RNAi-Gycα-99B**

One-way ANOVA: DATA versus GENES

```
Source   DF        SS       MS        F      P
GENES     3  20042.25  6680.75  2781.42  0.001
Error   896   2152.12     2.40
Total   899  22194.37

S = 1.550   R-Sq = 90.30%   R-Sq(adj) = 90.27%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level    N    Mean  StDev  ----+---------+---------+---------+-----
1      203  23.539  1.749                                    *)
2      214  24.000  1.443                                     (*)
3      218  14.029  1.690  (*
4      265  14.569  1.335    (*
                          ----+---------+---------+---------+-----
                           15.0      18.0      21.0      24.0

Pooled StDev = 1.550



Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of GENES

Individual confidence level = 98.96%


GENES = 1 subtracted from:
```

```
GENES   Lower  Center   Upper  -------+---------+---------+---------+--
2       0.071   0.461   0.851                   (*
3      -9.898  -9.510  -9.122   *)
4      -9.341  -8.970  -8.599   (*)
                               -------+---------+---------+---------+--
                                   -6.0       0.0       6.0      12.0
```

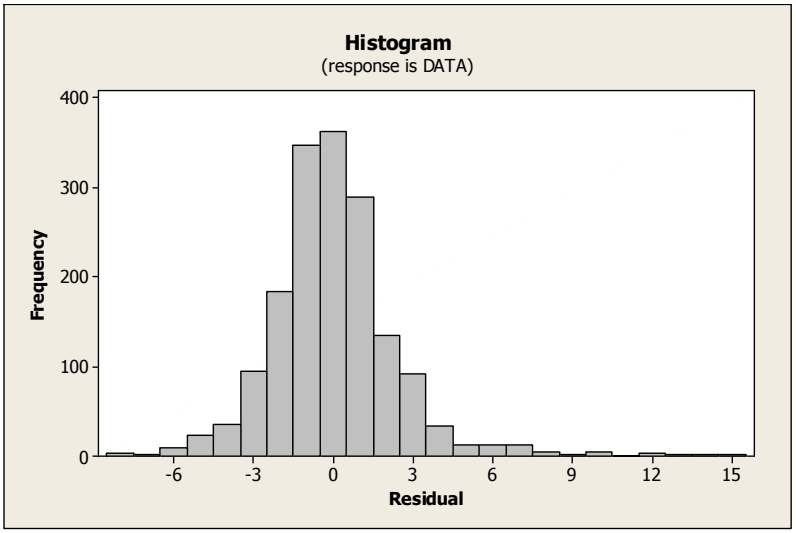
```
GENES = 2 subtracted from:

GENES    Lower  Center   Upper  -------+---------+---------+---------+--
3      -10.353  -9.971  -9.588  *)
4       -9.796  -9.431  -9.065   *)
                               -------+---------+---------+---------+--
                                   -6.0       0.0       6.0      12.0
```

```
GENES = 3 subtracted from:

GENES  Lower  Center  Upper  -------+---------+---------+---------+--
4      0.176   0.540  0.903                   (*)
                             -------+---------+---------+---------+--
                                 -6.0       0.0       6.0      12.0
```

Genes	1	2	3	4
1 WT	-			
2 RNAi-Gycα-99B	ND	-		
3 NOS2	SD-smaller	SD -smaller	-	
4 NOS2 RNAi-Gycα-99B	SD- smaller	SD- smaller	ND	-

Columns are compared to rows. SD- statistically different. ND- statistically not different.

II. c. Wild type, NOS2 and dInR expression



Genes

	1	**WT**
	2	**dInR**
	3	**dInR***
	4	**NOS2**
	5	**NOS2 dInR**
	6	**NOS2 dInR***

One-way ANOVA: DATA versus GENES

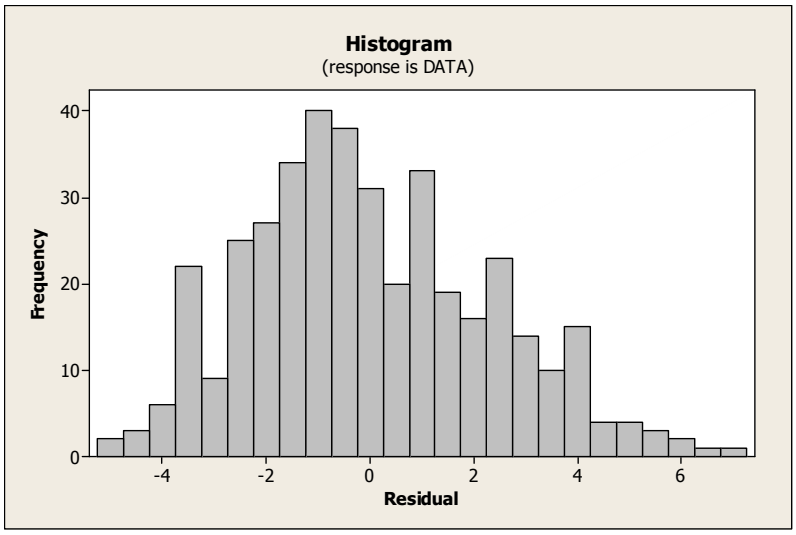
```
Source    DF        SS        MS        F      P
GENES      5  82469.37  16493.87  3053.76  0.001
Error   1659   8960.55      5.40
Total   1664  91429.92

S = 2.324   R-Sq = 90.20%   R-Sq(adj) = 90.17%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level    N    Mean  StDev  ---+---------+---------+---------+------
1      209  23.497  2.344                    (*)
2      260  27.218  2.233                            *)
3      269  31.763  4.028                                     (*
4      315  13.706  1.129  *)
5      317  14.504  1.938   (*)
6      295  15.025  1.416     *)
                          ---+---------+---------+---------+------
                          15.0      20.0      25.0      30.0

Pooled StDev = 2.324


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of GENES
```

```
Individual confidence level = 99.56%


GENES = 1 subtracted from:

GENES    Lower  Center   Upper  ---------+---------+---------+---------+
2        3.106   3.721   4.336                      (*
3        7.655   8.266   8.876                           *)
4      -10.381  -9.790  -9.200           *)
5       -9.583  -8.993  -8.403           (*)
6       -9.070  -8.471  -7.873            (*
                                ---------+---------+---------+---------+
                                       -10         0        10        20


GENES = 2 subtracted from:

GENES    Lower   Center    Upper  ---------+---------+---------+---------+
3        3.969    4.545    5.121                     (*
4      -14.066  -13.511  -12.956       *)
5      -13.268  -12.714  -12.160        *)
6      -12.755  -12.192  -11.629        (*
                                ---------+---------+---------+---------+
                                       -10         0        10        20


GENES = 3 subtracted from:

GENES    Lower   Center    Upper  ---------+---------+---------+---------+
4      -18.606  -18.056  -17.506  (*
5      -17.808  -17.259  -16.710   (*
6      -17.295  -16.737  -16.179    *)
                                ---------+---------+---------+---------+
                                       -10         0        10        20


GENES = 4 subtracted from:

GENES  Lower  Center  Upper  ---------+---------+---------+---------+
5      0.271   0.798  1.324                    (*
6      0.783   1.319  1.856                     *)
                           ---------+---------+---------+---------+
                                  -10         0        10        20


GENES = 5 subtracted from:

GENES   Lower  Center  Upper  ---------+---------+---------+---------+
6      -0.014   0.522  1.057                     (*
                           ---------+---------+---------+---------+
                                  -10         0        10        20
```

Genes	1	2	3	4	5	6
1 Wt						
2 dInR	SD-greater					
3 dInR*	SD-greater	SD-greater				
4 NOS2	SD-smaller	SD-smaller	SD-smaller			
5 NOS2 dInR	SD-smaller	SD-smaller	SD-smaller	ND		
6 NOS2 dInR*	SD-smaller	SD-smaller	SD-smaller	ND	ND	

Columns are compared to rows. SD- statistically different. ND- statistically not different.

II. d. Wild type and Thor^{LL} expression



Genes 1 WT
2 Thor L.L.

One-way ANOVA: DATA versus GENES

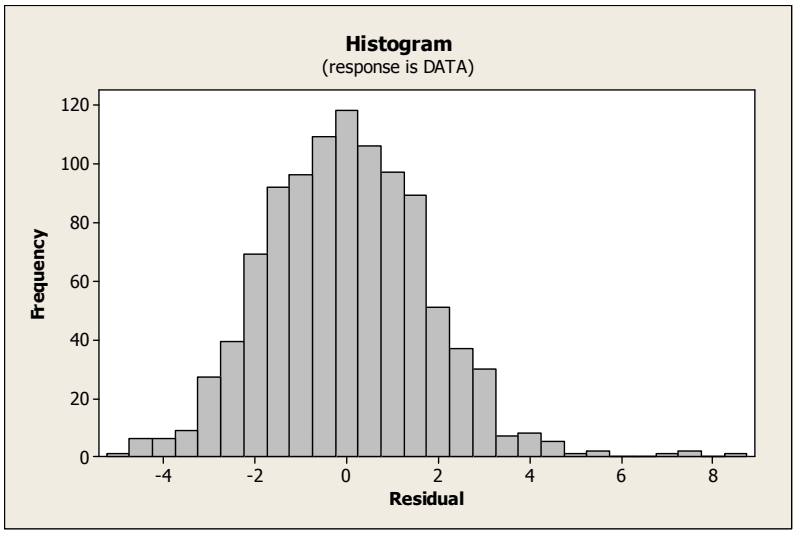
```
Source   DF       SS      MS      F      P
GENES     1   354.79  354.79  65.62  0.001
Error   400  2162.74    5.41
Total   401  2517.53

S = 2.325   R-Sq = 14.09%   R-Sq(adj) = 13.88%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level    N    Mean  StDev  ----+---------+---------+---------+-----
1      209  23.653  2.556                            (----*---)
2      193  21.772  2.046  (----*----)
                          ----+---------+---------+---------+-----
                           21.70     22.40     23.10     23.80

Pooled StDev = 2.325


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of GENES

Individual confidence level = 95.00%


GENES = 1 subtracted from:

GENES   Lower  Center   Upper  ---------+---------+---------+---------+
2      -2.337  -1.880  -1.424  (----*-----)
                               ---------+---------+---------+---------+
```

-1.60 -0.80 -0.00 0.80

Genes	1	2
1 Wt	-	
2 Thor L.L	SD- slightly smaller	-

Columns are compared to rows. SD- statistically different. ND- statistically not different.

II. e. Wild type, NOS2 and Thor null larvae



Genes **1** **WT**
2 **Thor[2]**
3 **NOS2**
4 **NOS2 Thor[2]**

One-way ANOVA: DATA versus GENES

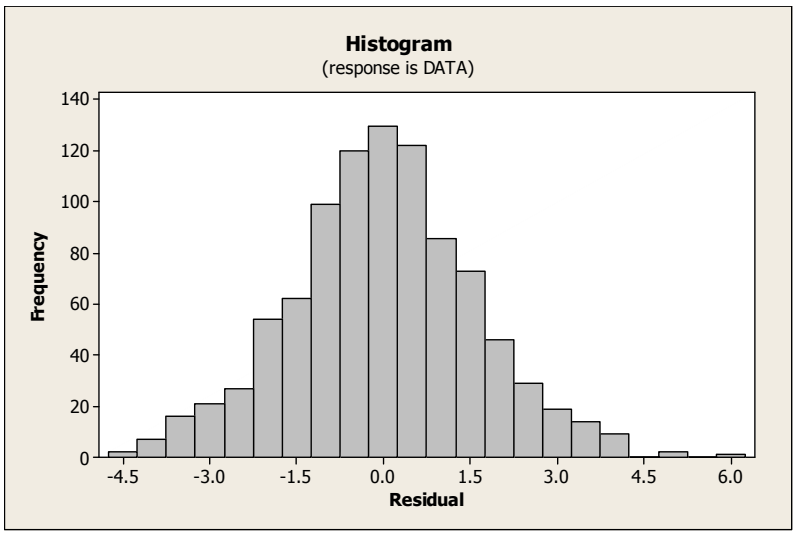
```
Source    DF        SS       MS        F      P
GENES      3  24838.42  8279.47  2714.92  0.001
Error   1005   3064.87     3.05
Total   1008  27903.29

S = 1.746   R-Sq = 89.02%   R-Sq(adj) = 88.98%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level    N    Mean  StDev  ---------+---------+---------+---------+
1      216  22.399  2.228                                    (*
2      288  23.431  1.706                                       (*)
3      203  12.627  1.533  (*)
4      302  13.432  1.512     (*
                           ---------+---------+---------+---------+
                                 15.0      18.0      21.0      24.0

Pooled StDev = 1.746


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of GENES

Individual confidence level = 98.96%
```

```
GENES = 1 subtracted from:

GENES    Lower  Center   Upper  ---------+---------+---------+---------+
2         0.629   1.032   1.436                     (*
3       -10.211  -9.773  -9.334    (*
4        -9.367  -8.968  -8.568     (*)
                                 ---------+---------+---------+---------+
                                       -6.0       0.0       6.0      12.0


GENES = 2 subtracted from:

GENES    Lower   Center    Upper  ---------+---------+---------+---------+
3       -11.216  -10.805  -10.394  (*)
4       -10.369  -10.000   -9.631    *)
                                  ---------+---------+---------+---------+
                                        -6.0       0.0       6.0      12.0


GENES = 3 subtracted from:

GENES  Lower  Center  Upper  ---------+---------+---------+---------+
4      0.398   0.805  1.212                    *)
                            ---------+---------+---------+---------+
                                  -6.0       0.0       6.0      12.0
```

Genes	1	2	3	4
1 Wt	-			
2 Thor 2	ND	-		
3 NOS2	SD smaller	SD smaller	-	
4 NOS2 Thor2	SD smaller	SD smaller	ND	-

Columns are compared to rows. SD- statistically different. ND- statistically not different.

II. f. Wild type, NOS2 and Lk6 null larvae



Genes **1** **WT**
2 **LK6[15/38]**
3 **NOS2**
4 **NOS2 LK6[15/38]**

One-way ANOVA: DATA versus GENES

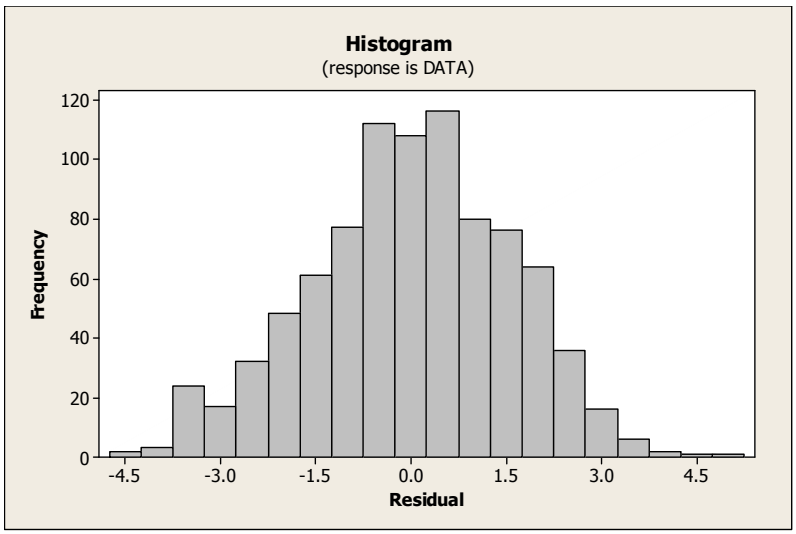
```
Source   DF        SS       MS        F      P
GENES     3  19331.37  6443.79  2537.68  0.001
Error   935   2374.20     2.54
Total   938  21705.56

S = 1.594   R-Sq = 89.06%   R-Sq(adj) = 89.03%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level    N    Mean  StDev  ---+---------+---------+---------+------
1      247  23.582  1.884                                     *)
2      245  23.607  1.791                                     *)
3      186  14.625  1.382   *)
4      261  14.428  1.185  (*
                          ---+---------+---------+---------+------
                          15.0      17.5      20.0      22.5

Pooled StDev = 1.594


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of GENES

Individual confidence level = 98.96%
```

```
GENES = 1 subtracted from:

GENES   Lower  Center   Upper  ---------+---------+---------+---------+
2      -0.344   0.025   0.394                    (*)
3      -9.354  -8.957  -8.560  (*)
4      -9.516  -9.153  -8.790  (*
                               ---------+---------+---------+---------+
                                     -5.0       0.0       5.0      10.0


GENES = 2 subtracted from:

GENES   Lower  Center   Upper  ---------+---------+---------+---------+
3      -9.380  -8.982  -8.584  (*)
4      -9.542  -9.178  -8.814  (*
                               ---------+---------+---------+---------+
                                     -5.0       0.0       5.0      10.0


GENES = 3 subtracted from:

GENES   Lower  Center  Upper  ---------+---------+---------+---------+
4      -0.589  -0.196  0.196                   (*
                              ---------+---------+---------+---------+
                                    -5.0       0.0       5.0      10.0
```

Genes	1	2	3	4
1 WT	-			
2 LK6 null	ND	-		
3 NOS2	SD- smaller	SD- smaller	-	
4 NOS2 LK6 null	SD- smaller	SD- smaller	ND	-

Columns are compared to rows. SD- statistically different. ND- statistically not different.

II. g. Wild type, NOS2 and eIF4E larvae



Genes **1** **WT**
5 **eIF4E***
6 **NOS2**
7 **NOS2 eIF4E***

One-way ANOVA: DATA versus GENES

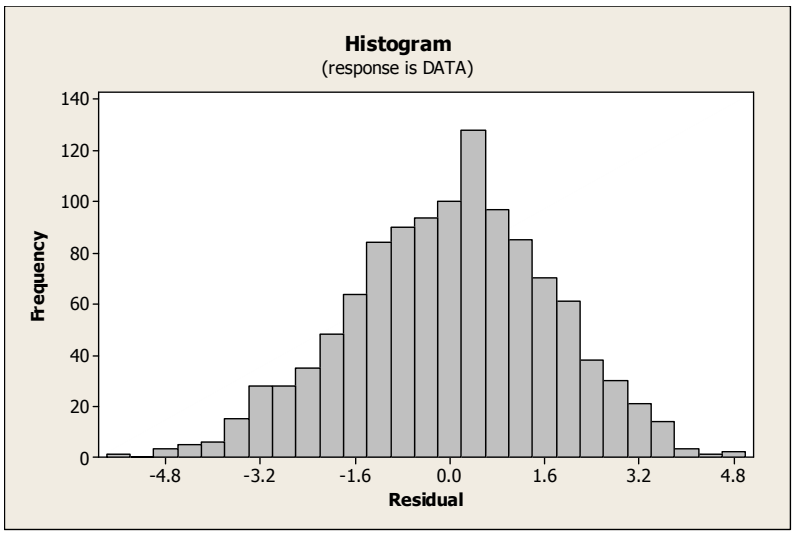
```
Source   DF        SS       MS        F      P
GENES     3  21899.50  7299.83  2914.70  0.001
Error   878   2198.94     2.50
Total   881  24098.45

S = 1.583   R-Sq = 90.88%   R-Sq(adj) = 90.84%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level    N    Mean  StDev  -----+---------+---------+---------+----
1      210  24.055  1.689                                    (*)
2      225  24.372  1.608                                      *)
3      223  14.700  1.517     (*)
4      224  13.848  1.516  (*)
                           -----+---------+---------+---------+----
                             15.0      18.0      21.0      24.0

Pooled StDev = 1.583


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of GENES
```

```
Individual confidence level = 98.96%


GENES = 1 subtracted from:

GENES    Lower   Center   Upper  --------+---------+---------+---------+-
2       -0.073    0.317   0.707                    (*
3       -9.746   -9.355  -8.964    *)
4      -10.597  -10.207  -9.817  (*)
                                --------+---------+---------+---------+-
                                     -6.0       0.0       6.0      12.0


GENES = 2 subtracted from:

GENES    Lower   Center    Upper  --------+---------+---------+---------+-
3      -10.056   -9.672   -9.288   (*)
4      -10.907  -10.524  -10.140  *)
                                 --------+---------+---------+---------+-
                                      -6.0       0.0       6.0      12.0


GENES = 3 subtracted from:

GENES   Lower  Center   Upper  --------+---------+---------+---------+-
4      -1.236  -0.852  -0.468                  (*
                              --------+---------+---------+---------+-
                                   -6.0       0.0       6.0      12.0
```

Genes	1	2	3	4
1 WT	-			
2 eIF4E*	ND	-		
3 NOS2	SD-smaller	SD- smaller	-	
4 NOS2 eIF4E*	SD- smaller	SD- smaller	ND	-

Columns are compared to rows. SD- statistically different. ND- statistically not different.

II. h. Wild type, NOS2, Thor null and Lk6 null larvae



Genes

1	**Wt**
2	**NOS2**
3	**NOS2 Thor[2] LK6[15/38]**
4	**Thor[2] LK6[15/38]**
5	**NOS2 Thor[2]/+ LK6[15]/+**

One-way ANOVA: DATA versus GENES

```
Source    DF        SS       MS        F      P
GENES      4  14412.50  3603.12  1252.85  0.001
Error   1146   3295.84     2.88
Total   1150  17708.34

S = 1.696   R-Sq = 81.39%   R-Sq(adj) = 81.32%


                          Individual 95% CIs For Mean Based on Pooled StDev
Level    N    Mean  StDev    -+---------+---------+---------+--------
1      211  23.745  1.778                                      (*)
2      223  14.999  1.853    (*)
3      241  14.895  1.459    (*
4      230  20.173  1.410                        (*)
5      246  15.008  1.921    (*)
                            -+---------+---------+---------+--------
                          15.0      17.5      20.0      22.5

Pooled StDev = 1.696


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of GENES
```

Individual confidence level = 99.36%

GENES = 1 subtracted from:

```
GENES   Lower  Center   Upper  ---+---------+---------+---------+------
2      -9.190  -8.745  -8.300  (*)
3      -9.286  -8.850  -8.413  (*)
4      -4.013  -3.571  -3.130              (*)
5      -9.170  -8.736  -8.302  (*)
                              ---+---------+---------+---------+------
                              -8.0      -4.0       0.0       4.0
```

GENES = 2 subtracted from:

```
GENES   Lower  Center  Upper  ---+---------+---------+---------+------
3      -0.535  -0.104  0.326                      (*)
4       4.739   5.174  5.609                                  (*)
5      -0.419   0.009  0.437                      (*)
                            ---+---------+---------+---------+------
                            -8.0      -4.0       0.0       4.0
```

GENES = 3 subtracted from:

```
GENES   Lower  Center  Upper  ---+---------+---------+---------+------
4       4.851   5.278  5.705                                  (*)
5      -0.306   0.113  0.533                      (*)
                            ---+---------+---------+---------+------
                            -8.0      -4.0       0.0       4.0
```

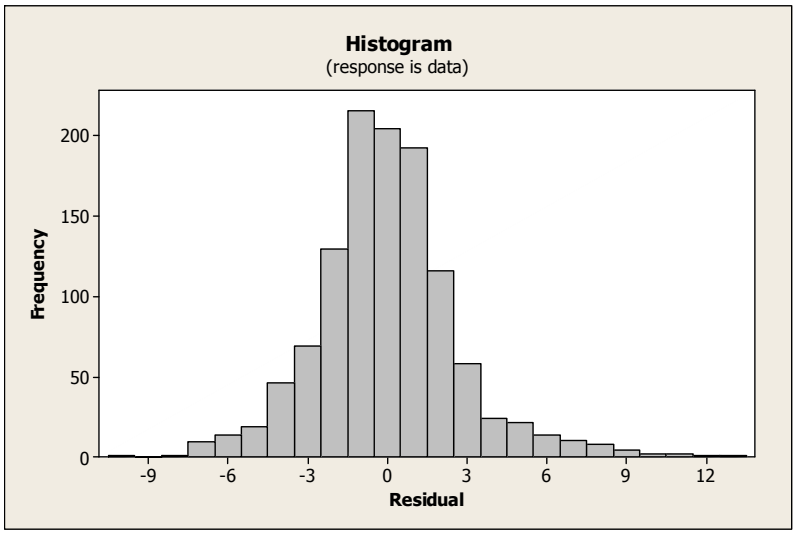
GENES = 4 subtracted from:

```
GENES   Lower  Center   Upper  ---+---------+---------+---------+------
5      -5.589  -5.165  -4.740           (*)
                              ---+---------+---------+---------+------
                              -8.0      -4.0       0.0       4.0
```

Genes	1	2	3	4	5
1 WT	-				
2 NOS2	SD- smaller	-			
3 NOS2 THOR2 LK6null	SD- Smaller	ND	-		
4 THOR 2 LK6 null	SD- smaller	SD- greater	SD- greater	-	
5 NOS2 Thor[2]/+ LK6[15]/+	SD- Smaller	ND	ND	SD- smaller	-

Columns are compared to rows. SD- statistically different. ND- statistically not different.

II. i. Wild type, NOS2, and Ras^{V12} expression



Gene **1** **WT**
2 **RASV12**
3 **NOS2**
4 **NOS2 RASV12**

One-way ANOVA: data versus genes

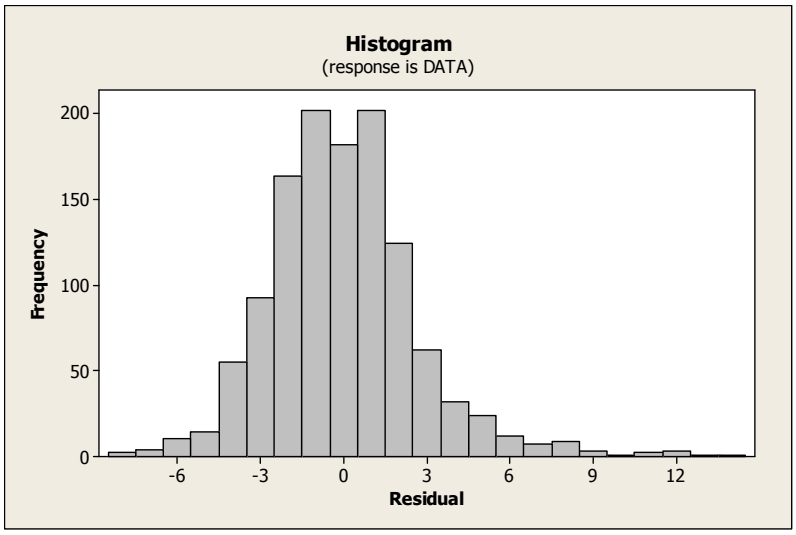
```
Source    DF        SS        MS        F      P
genes      3  48131.13  16043.71  2228.14  0.001
Error   1156   8323.79      7.20
Total   1159  56454.91

S = 2.683   R-Sq = 85.26%   R-Sq(adj) = 85.22%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level    N    Mean  StDev  ----+---------+---------+---------+-----
1      396  20.951  1.512                (*
2      236  32.416  3.749                                      (*)
3      282  13.476  1.479  (*)
4      246  18.607  3.782            *)
                          ----+---------+---------+---------+-----
                           15.0      20.0      25.0      30.0

Pooled StDev = 2.683
```

```
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of genes

Individual confidence level = 98.96%


genes = 1 subtracted from:

genes   Lower  Center   Upper    +---------+---------+---------+---------
2      10.899  11.465  12.032                                    *)
3      -8.012  -7.475  -6.938                (*
4      -2.903  -2.344  -1.785                     (*
                                +---------+---------+---------+---------
                              -20       -10         0        10


genes = 2 subtracted from:

genes    Lower   Center    Upper    +---------+---------+---------+---------
3      -19.548  -18.940  -18.333    (*)
4      -14.437  -13.809  -13.182          *)
                                   +---------+---------+---------+---------
                                 -20       -10         0        10


genes = 3 subtracted from:

genes  Lower  Center  Upper    +---------+---------+---------+---------
4      4.530   5.131  5.732                            *)
                              +---------+---------+---------+---------
                            -20       -10         0        10
```

Genes	1	2	3	4
1 WT	-			
2 Ras V12	SD- greater	-		
3 NOS2	SD- smaller	SD- smaller	-	
4 NOS2 Ras V12	SD smaller	SD- smaller	SD- greater	-

Columns are compared to rows. SD- statistically different. ND- statistically not different.

II. j. Wild type, NOS2 and dMyc expression



Genes **1** **Wt**
2 **Myc**
3 **NOS2**
4 **NOS2 Myc**

One-way ANOVA: DATA versus GENES

```
Source    DF        SS        MS        F      P
GENES      3  44832.26  14944.09  2096.24  0.001
Error   1200   8554.81      7.13
Total   1203  53387.07

S = 2.670   R-Sq = 83.98%   R-Sq(adj) = 83.94%


                           Individual 95% CIs For Mean Based on Pooled StDev
Level    N    Mean  StDev    -+---------+---------+---------+--------
1      286  23.927  1.936                      (*
2      319  33.046  3.391                                          *)
3      223  14.900  1.483    (*)
4      376  22.940  2.985                   (*
                              -+---------+---------+---------+--------
                           15.0      20.0      25.0      30.0

Pooled StDev = 2.670


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of GENES

Individual confidence level = 98.96%
```

```
GENES = 1 subtracted from:

GENES   Lower  Center   Upper  ---------+---------+---------+---------+
2       8.560   9.118   9.677                              *)
3      -9.639  -9.027  -8.414           (*)
4      -1.525  -0.988  -0.450                    (*)
                                ---------+---------+---------+---------+
                                       -10         0        10        20


GENES = 2 subtracted from:

GENES    Lower   Center    Upper  ---------+---------+---------+---------+
3      -18.743  -18.145  -17.547  (*
4      -10.628  -10.106   -9.584          (*
                                 ---------+---------+---------+---------+
                                        -10         0        10        20


GENES = 3 subtracted from:

GENES  Lower  Center  Upper  ---------+---------+---------+---------+
4      7.460   8.039  8.618                           (*)
                             ---------+---------+---------+---------+
                                    -10         0        10        20
```

Genes	1	2	3	4
1 WT	-			
2 MYC	SD- greater	-		
3 NOS2	SD- smaller	SD-smaller	-	
4 NOS2 Myc	ND	SD-smaller	SD- greater	-

Columns are compared to rows. SD- statistically different. ND- statistically not different.

Bibliography

Arquier N, Bourouis M, Colombani J, Léopold P. 2005. Drosophila Lk6 Kinase Controls Phosphorylation of Eukaryotic Translation Initiation Factor 4E and Promotes Normal Growth and Development. *Current Biology* 15: 19-23

Asada K, Kurokawa J, Furukawa T. 2009. Redox- and Calmodulin-dependent S-Nitrosylation of the KCNQ1 Channel *J. Biol. Chem.* 27: 6014-20.

Asha H, Nagy I, Kovacs G, Stetson D, Ando I, Dearolf CR. 2003. Analysis of Ras-Induced Overproliferation in Drosophila Hemocytes. *Genetics* 163: 203-15

Bernal A, Kimbrell DA. 2000. Drosophila Thor participates in host immune defense and connects a translational regulator with innate immunity. *PNAS* 97: 6019-24

Berry DL, Baehrecke EH. 2007. Growth Arrest and Autophagy Are Required for Salivary Gland Cell Degradation in Drosophila. *Cell* 131: 1137-48

Bogdan C. 2001. Nitric oxide and the immune response. *Nature Immunology* 2: 907 - 16

Böhni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, et al. 1999. Autonomous Control of Cell and Organ Size by CHICO, a Drosophila Homolog of Vertebrate IRS1-4. *Cell* 97: 865-75

Bouchard C, Lee S, Paulus-Hock V, Loddenkemper C, Eilers M, Schmitt CA. 2007. FoxO transcription factors suppress Myc-driven lymphomagenesis via direct activation of Arf. *Genes Dev.* 21: 2775–87

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118: 401-15

Bredt DS, Snyder SH. 1994. Nitric Oxide: A Physiologic Messenger Molecule. *Annual Review of Biochemistry* 63: 175-95

Britton JS, Edgar BA. 1998. Environmental control of the cell cycle in Drosophila: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. *Development* 125: 2149-58

Britton JS, Lockwood WK, Li L, Cohen SM, Edgar BA. 2002. Drosophila's Insulin/PI3-Kinase Pathway Coordinates Cellular Metabolism with Nutritional Conditions. *Developmental Cell* 2: 239-49

Brogiolo W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E. 2001. An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. *Current Biology* 11: 213-21

Bruckdorfer R. 2005. The basics about nitric oxide. *Mol Aspects Med.* 26: 3-31

Casas-Tintoa S, IIc MTM, Andreua P, Puig O. 2007. Characterization of the Drosophila insulin receptor promoter. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* 1769: 236-43

Champlin D, Truman J. 2000. Ecdysteroid coordinates optic lobe neurogenesis via a nitric oxide signaling pathway. *Development* 127: 3543-51

Chang HC. 2003. Chang constructs and insertions. . ed. Pct FlyBase

Ciani E, Severi S, Contestabile A, Bartesaghi R, Contestabile A. 2004. Nitric oxide negatively regulates proliferation and promotes neuronal differentiation through N-Myc downregulation. *J Cell Sci* 117: 4727-37

Contestabile A, Ciani E. 2004. Role of nitric oxide in the regulation of neuronal proliferation, survival and differentiation. *Neurochemistry International*

Nitric Oxide and Cyclic GMP Signal Transduction in Brain 45: 903-14

Cooper CE. 2002. Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? . *Trends in Biochemical Sciences* 27: 33-9

Delpuech O, Griffiths B, East P, Essafi A, Lam EWF, et al. 2007. Induction of Mxi1-SR{alpha} by FOXO3a Contributes to Repression of Myc-Dependent Gene Expression. *Mol. Cell. Biol.* 27: 4917-30

Demerec M. 1950. *Biology of Drosophila*: Cold Spring Harbor Laboratory Press. 632 pp.

Demontis F, Perrimon N. 2009. Integration of Insulin receptor/Foxo signaling and dMyc activity during muscle growth regulates body size in Drosophila. *Development* 136: 983-93

Denningera JW, Marletta MA. 1999. Guanylate cyclase and the NO/cGMP signaling pathway. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1411: 334-50

Dietzl G, Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., Couto, A., Marra, V., Keleman, K., Dickson, B.J. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* 448: 151-6

Dong J, Pan D. 2004. Tsc2 is not a critical target of Akt during normal Drosophila development. *Genes & Development* 18: 2479-84

Dytham C. 2003. *Choosing and Using Statistics. A Biologist's Guide.* Oxford, UK: Blackwell Science

Edgar BA, Orr-Weaver TL. 2001. Endoreplication Cell Cycles: More for Less. *Cell* 105: 297-306

Fernandez R, Tabarini D, Azpiazu N, Frasch M, Schlessinger J. 1995. The Drosophila insulin receptor homolog: A gene essential for embryonic development encodes two receptor isoforms with different signaling potential. *EMBO J* 14: 3373–84

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1997. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391: 806-11

Galloni M, Edgar BA. 1999. Cell-autonomous and non-autonomous growth-defective mutants of Drosophila melanogaster. *Development* 126: 2365-75

Gansauge S, Nussler AK, Beger HG, Gansauge F. 1998. Nitric oxide-induced apoptosis in human pancreatic carcinoma cell lines is associated with a G1-arrest and an increase of the cyclin-dependent kinase inhibitor p21WAF1/CIP1. *Cell Growth Differ* 9: 611-7

Gao X, Neufeld TP, Pan D. 2000. Drosophila PTEN Regulates Cell Growth and Proliferation through PI3K-Dependent and -Independent Pathways. *Developmental Biology* 221: 404-18

Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, et al. 2002. Tsc tumour suppressor proteins antagonize amino-acid−TOR signalling. *Nature Cell Biology* 4: 699 - 704

Garofalo RS. 2002. Genetic analysis of insulin signaling in Drosophila. *Trends in Endocrinology and Metabolism* 13: 156-62

Gibbs SM, Becker A, Hardy RW, Truman JW. 2001. Soluble Guanylate Cyclase Is Required during Development for Visual System Function in Drosophila. *J. Neurosci.* 21: 7705-14

Gingras A-C, Raught B, Sonenberg N. 1999. eIF4 INITIATION FACTORS: Effectors of mRNA Recruitment to Ribosomes and Regulators of Translation. *Annual Review of Biochemistry* 68: 913-63

Goberdhan DCI, Paricio N, Goodman EC, Mlodzik M, Wilson C. 1999. Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway
Genes Dev. 13: 3244-58
Grandori C, Cowley SM, James LP, Eisenman RN. 2000. The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annual Review of Cell and Developmental Biology* 16: 653-99
Grewal SS, Li L, Orian A, Eisenman RN, Edgar BA. 2005. Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. *Nature Cell Biology* 7: 295-302
Haley JE. 1998. Gases as neurotransmitters. *Essays Biochem* 33: 71-91
Halfar K, Rommel C, Stocker H, Hafen E. 2001. Ras controls growth, survival and differentiation in the Drosophila eye by different thresholds of MAP kinase activity. *Development* 128: 1687-96
Harvey KF, Mattila J, Sofer A, Bennett FC, Ramsey MR, et al. 2008. FOXO-regulated transcription restricts overgrowth of Tsc mutant organs
J. Cell Biol. 180: 691-6
Hernández G, Sierra JM. 1995. Translation initiation factor eIF-4E from Drosophila: cDNA sequence and expression of the gene. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* 1261: 427-31
Herranz H, Pérez L, Martín FA, Milán M. 2008. A Wingless and Notch double-repression mechanism regulates G1–S transition in the Drosophila wing. *The EMBO Journal* 27: 1633-45
Huang H, Potter C, Tao W, Li D, Brogiolo W, et al. 1999. PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. *Development* 126: 5365-72
Ikeya T, Galic M, Belawat P, Nairz K, Hafen E. 2002. Nutrient-Dependent Expression of Insulin-like Peptides from Neuroendocrine Cells in the CNS Contributes to Growth Regulation in Drosophila. *Current Biology* 12: 1293-300
Jacobs FMJ, van der Heide LP, Wijchers PJEC, Burbach JPH, Hoekman MFM, Smidt MP. 2003. FoxO6, a Novel Member of the FoxO Class of Transcription Factors with Distinct Shuttling Dynamics. *J. Biol. Chem.* 278: 35959-67
Janssen LJ, Premji M, Lu-Chao H, Cox G, Keshavjee S. 2000. NO+ but not NO radical relaxes airway smooth muscle via cGMP-independent release of internal Ca2+. *Am J Physiol Lung Cell Mol Physiol* 278: L899-905
Jarry A, Charrier L, Bou-Hanna C, Devilder M-C, Crussaire V, et al. 2004. Position in Cell Cycle Controls the Sensitivity of Colon Cancer Cells to Nitric Oxide-Dependent Programmed Cell Death. *Cancer Res* 64: 4227-34
Jünger M, Rintelen F, Stocker H, Wasserman J, Vegh M, et al. 2003. The Drosophila Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *Journal of Biology* 2: 20
Kennerdell JR, Carthew RW. 1998. Use of dsRNA-Mediated Genetic Interference to Demonstrate that frizzled and frizzled 2 Act in the Wingless Pathway. *Cell* 95: 1017-26
Kimber S. 2005. *The regulation of Growth by Nitric Oxide Signalling in Drosophila Melanogaster*. DPhil thesis. University of Sussex, Brighton. 276 pp.
Krumenacker JS, Hanafy KA, Murad F. 2004. Regulation of nitric oxide and soluble guanylyl cyclase. *Brain Research Bulletin*
Neuroprotection and the Eye 62: 505-15

Kuzin B, Regulski M, Stasiv Y, Scheinker V, Tully T, Enikolopov G. 2000. Nitric oxide interacts with the retinoblastoma pathway to control eye development in Drosophila. *Current Biology* 10: 459-62

Kuzin B, Roberts I, Peunova N, Enikolopov G. 1996. Nitric Oxide Regulates Cell Proliferation during Drosophila Development. *Cell* 87: 639-49

Lachance PED, Miron M, Raught B, Sonenberg N, Lasko P. 2002. Phosphorylation of Eukaryotic Translation Initiation Factor 4E Is Critical for Growth

Mol. Cell. Biol. 22: 1656-63

Lawrence S. B. Goldstein EAF. 1994. *Drosophila melanogaster: Practical Uses in Cell and Molecular Biology* Academic Press. 67-9 pp.

Leevers SJ, Weinkove D, MacDougall LK, Hafen Ernst, D. WM. 1996. The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. *EMBO J* 15

Lowenstein C, Glatt C, Bredt D, Snyder S. 1992. Cloned and Expressed Macrophage Nitric Oxide Synthase Contrasts with the Brain Enzyme. *PNAS* 89: 6711-5

Mattila J, Bremer A, Ahonen L, Kostiainen R, Puig O. 2009. Drosophila FoxO regulates organism size and stress resistance through an adenylate cyclase. *Mol. Cell. Biol.* : MCB.00302-09

Mattila J, Kallijärvi J, Puig O. 2008. RNAi screening for kinases and phosphatases identifies FoxO regulators. *Proceedings of the National Academy of Sciences* 105: 14873-8

Meffert MK, Premack BA, Schulman H. 1994. Nitric oxide stimulates Ca2+-independent synaptic vesicle release. *Neuron* 12: 1235-44

Miron M, J. Verdu, P. E. Lachance, M. J. Birnbaum, P. F. Lasko, and Sonenberg, N. 2001. The translational inhibitor 4E-BP is an effector of PI (3) K/Akt signalling and cell growth in Drosophila. *Nature Cell Biology* 3: 596-601

Miron M, Lasko P, Sonenberg N. 2003. Signaling from Akt to FRAP/TOR Targets both 4E-BP and S6K in Drosophila melanogaster. *Mol. Cell. Biol.* 23: 9117-26

Miron M, Sonenberg N. 2001. Regulation of Translation via TOR Signaling: Insights from Drosophila melanogaster. *J. Nutr.* 131: 2988S-93

Morton DB, Langlais KK, Stewart JA, Vermehren A. 2005. Comparison of the properties of the five soluble guanylyl cyclase subunits in Drosophila melanogaster. *J Insect Sci.* 5

Murillo-Carretero M, Ruano MJ, Matarredona ER, Villalobo A, Estrada C. 2002. Antiproliferative effect of nitric oxide on epidermal growth factor-responsive human neuroblastoma cells. *Journal of Neurochemistry* 83: 119-31

Nathan CF, Hibbs JB. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Current Opinion in Immunology* 3: 65-70

Nelson DW, Padgett RW. 2003. Insulin worms its way into the spotlight. *Genes & Development* 17: 813-8

Neufeld T. 2003. Shrinkage control: regulation of insulin-mediated growth by FOXO transcription factors. *Journal of Biology* 2: 18

Neufeld TP, Cruz AFAdl, Johnston LA, Edgar BA. 1998. Coordination of Growth and Cell Division in the Drosophila Wing. *Cell* 93: 1183-93

Palmer RMJ, Ferrige AG, Moncada S. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524 - 6

Parra-Palau JL, Scheper GC, Harper DE, Proud CG. 2005. The Drosophila protein kinase LK6 is regulated by ERK and phosphorylates the eukaryotic initiation factor eIF4E in vivo. *Biochem. J.* 385: 695-702

Pervin S, Singh R, Chaudhuri G. 2001. Nitric oxide-induced cytostasis and cell cycle arrest of a human breast cancer cell line (MDA-MB-231): Potential role of cyclin. *PNAS* 98: 3583-8

Peunova N, Enikolopov G. 1995. Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells. 375: 68-73

Peunova N, Scheinker V, Cline H, Enikolopov G. 2001. Nitric Oxide Is an Essential Negative Regulator of Cell Proliferation in Xenopus Brain. *J. Neurosci.* 21: 8809-18

Pierce SB, Yost C, Britton JS, Loo LWM, Flynn EM, et al. 2004. dMyc is required for larval growth and endoreplication in Drosophila. *Development* 131: 2317-27

Polysciences I. Warrington, PA

Potter CJ, Pedraza LG, Xu T. 2002. Akt regulates growth by directly phosphorylating Tsc2. *Nature Cell Biology* 4: 658 - 65

Prast H, Philippu A. 2001. Nitric oxide as modulator of neuronal function. *Progress in Neurobiology* 64: 51-68

Prober DA, Edgar BA. 2000. Ras1 Promotes Cellular Growth in the Drosophila Wing. *Cell* 100: 435-46

Puig O, Marr MT, Ruhf ML, Tjian R. 2003. Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes Dev.* 17: 2006-20

Puig O, Tjian R. 2005. Transcriptional feedback control of insulin receptor by dFOXO/FOXO1 *Genes Dev.* 19: 2435-46

Regulski M, Stasiv Y, Tully T, Enikolopov G. 2004. Essential function of nitric oxide synthase in Drosophila. *Current Biology* 14: R881-R2

Regulski M, Tully T. 1995. Molecular and Biochemical Characterization of dNOS: A Drosophila Ca2+/ Calmodulin-Dependent Nitric Oxide Synthase. *PNAS* 92: 9072-6

Reiling JH, Doepfner KT, Hafen E, Stockera H. 2005. Diet-Dependent Effects of the Drosophila Mnk1/Mnk2 Homolog Lk6 on Growth via eIF4E. *Current Biology* 15: 24-30

Rulifson EJ, Kim SK, Nusse R. 2002. Ablation of Insulin-Producing Neurons in Flies: Growth and Diabetic Phenotypes. *Science* 296: 1118-20

Saxena S, Jonsson ZO, Dutta A. 2003. Small RNAs with Imperfect Match to Endogenous mRNA Repress Translation: Implications for off-target activity of small inhibitory RNA in mammalian cells *J. Biol. Chem.* 278: 44312-9

Scheper GC, van Kollenburg B, Hu J, Luo Y, Goss DJ, Proud CG. 2002. Phosphorylation of Eukaryotic Initiation Factor 4E Markedly Reduces Its Affinity for Capped mRNA. *J. Biol. Chem.* 277: 3303-9

Schmidt HHHW, Walter U. 1994. NO at work. *Cell* 78: 919-25

Secombe J, Pierce SB, Eisenman RN. 2004. Myc: A Weapon of Mass Destruction. *Cell* 117: 153-6

Shah S, Hyde DR. 1995. Two Drosophila Genes That Encode the α and β Subunits of the Brain Soluble Guanylyl Cyclase *Journal of Biological Chemistry* 270: 15368-76

St. Croix CM, Wasserloos KJ, Dineley KE, Reynolds IJ, Levitan ES, Pitt BR. 2002. Nitric oxide-induced changes in intracellular zinc homeostasis are mediated by metallothionein/thionein. *Am J Physiol Lung Cell Mol Physiol* 282: L185-92

Stamler JS. 1994. Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* 78: 931-6

Struhl G, Basler K. 1993. Organizing activity of wingless protein in Drosophila. *Cell* 72: 527-40

Stuehr DJ. 1997. Structure-function Saspects in the Nitric Oxide Synthases. *Annual Review of Pharmacology and Toxicology* 37: 339-59

Torroglosa A, Murillo-Carretero M, Romero-Grimaldi C, Matarredona ER, Campos-Caro A, Estrada C. 2007. Nitric oxide decreases subventricular zone stem cell proliferation by inhibition of epidermal growth factor receptor and phosphoinositide-3-kinase/Akt pathway. *Stem Cells*. 25(1):88-97.

Teleman AA, Chen Y-W, Cohen SM. 2005. 4E-BP functions as a metabolic brake used under stress conditions but not during normal growth. *Genes & Development* 19: 1844-8

Teleman AA, Hietakangas V, Sayadian AC, Cohen SM. 2008. Nutritional Control of Protein Biosynthetic Capacity by Insulin via Myc in Drosophila. *Cell Metabolism* 7: 21-32

Teodoro RO, O'Farrell PH. 2003. Nitric oxide-induced suspended animation promotes survival during hypoxia. *The EMBO Journal* 22: 580-7

Tettweiler G, Miron M, Jenkins M, Sonenberg N, Lasko PF. 2005. Starvation and oxidative stress resistance in Drosophila are mediated through the eIF4E-binding protein, d4E-BP. *Genes Dev.* 19: 1840–3

Verdu J, Buratovich MA, Wilder EL, Birnbaum MJ. 1999. Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. *Nature Cell Biology* 1: 500 - 6

Villalobo A. 2006. Nitric oxide and cell proliferation. *FEBS J* 273: 2329 - 44

Vojtek AB, Der CJ. 1998. Increasing Complexity of the Ras Signaling Pathway. *J. Biol. Chem.* 273: 19925-8

Wang X, Sun H, Li C. 2007. Nitric oxide induces promyelocytic cell growth arrest and apoptosis through deactivation of Akt pathway. *Leukemia Research* 31: 653-60

Wanga X, Sunb H, Lib C. 2007. Nitric oxide induces promyelocytic cell growth arrest and apoptosis through deactivation of Akt pathway. *Leukemia Research* 31: 653-60

Waskiewicz AJ, Flynn A, Proud CG, Cooper JA. 1997. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *The EMBO Journal* 16: 1909-20

Weinkove D, Leevers SJ. 2000. The genetic control of organ growth: insights from Drosophila. *Current Opinion in Genetics & Development* 10: 75-80

Wittwer F, Jaquenoud M, Brogiolo W, Zarske M, Wüstemann P, et al. 2005. Susi, a Negative Regulator of Drosophila PI3-Kinase. *Developmental Cell* 8: 817-27

Wullschleger S, Loewith R, Hall MN. 2006. TOR Signaling in Growth and Metabolism. 124: 471-84

Yanagawa S-i, Lee J-S, Ishimoto A. 1998. Identification and Characterization of a Novel Line of Drosophila Schneider S2 Cells That Respond to Wingless Signaling. *J. Biol. Chem.* 273: 32353-9

Zanella F, Rosado A, García B, Carnero A, Link W. 2008. Chemical Genetic Analysis of FOXO Nuclear-Cytoplasmic Shuttling by Using Image-Based Cell Screening. *ChemBioChem* 9: 2229-37

Zhang H, Stallock JP, Ng JC, Reinhard C, Neufeld TP. 2000. Regulation of cellular growth by the Drosophila target of rapamycin dTOR. *Genes & Development* 14: 2712-24