FOXO IS THE MEDIATOR LINKING TEMPORAL DIFFERENTIATION
AND THE INSULIN SIGNALING PATHWAY

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FOXO IS THE MEDIATOR LINKING TEMPORAL DIFFERENTIATION AND THE INSULIN SIGNALING PATHWAY

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The timing of differentiation is crucial for the correct development of an organism, because specific pathways can be used reiteratively to differentiate cells. Until recently, the molecular mechanism behind the temporal control of differentiation has remained elusive. Bateman and McNeill (2004) revealed a novel role for the insulin/insulin-like growth factor receptor (InR) pathway in regulating the timing of differentiation in neuronal photoreceptor cells in the *Drosophila* compound eye. The link between the InR pathway and temporal differentiation is significant, because of the implication that external factors, e.g. nutrition, are tightly coupled to the timing of differentiation. This proposal tests the hypothesis that FOXO, a crucial component of the InR pathway, mediates the regulation of developmental timing. The aims are the following: 1. Observe if dFOXO mutants affect temporal differentiation in the *Drosophila* eye. 2. Perform epistasis experiments to determine if dFOXO is downstream of other insulin signaling components. 3. Analyze the downstream targets of dFOXO that may play a role in neuronal differentiation.

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LIST OF ABBREVIATIONS

FOXO: Forkhead box, class O transcription factor

InR: insulin receptor

PI3K: phosphatidylinositol-3-kinase

PKB: protein kinase B

Chico: insulin receptor substrate

Dp110: phosphatidylinositol-3-kinase substrate

TOR: target of rapamycin

EGFR: epidermal growth factor receptor

RTK: receptor tyrosine receptor

PR: photoreceptor

MF: morphogenetic furrow

CTO: chodotonal organ

CNS: central nervous system

Flp/FRT: flippase/flp recognition target

GFP: green fluorescent protein

Tsc1: tuberous sclerosis complex 1

PTEN: phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase

Rheb: Ras homologue enriched in brain, small GTPase

S6K: p70 ribosomal S6 kinase

4E-BP: 4E binding protein, inhibitor of initiation factor eIF4E

dFOXOA3: constitutively active dFOXO

LY294002: phosphatidylinositol-3-kinase inhibitor

UAS: upstream activating sequence

FRE: FOXO responsive element

ChIP: chromatin immunoprecipitation

5'TOP: 5' oligopyrimidine tract (at transcription start site)

IGF-1: insulin-like growth factor 1

Introduction

The coordination of growth and differentiation is crucial for the correct development of a multicellular organism. Cell growth and differentiation pathways have been extensively studied in various organisms. These studies have identified a central role for insulin and insulin-like growth factors in growth and metabolism. The insulin-signaling pathway has an evolutionarily conserved general structure (Figure 1). In *Drosophila*, insulin-like receptor, dInR, mediates cell growth and body size (Tatar et al., 2001). In mammals, insulin-like growth factor 1 is involved in embryonic growth and lifespan (Holzenberger, M., 2003). In *C. elegans*, the insulin-signaling pathway controls metabolism, diapause (long-lived larval state), and longevity (Lee et al., 2003).

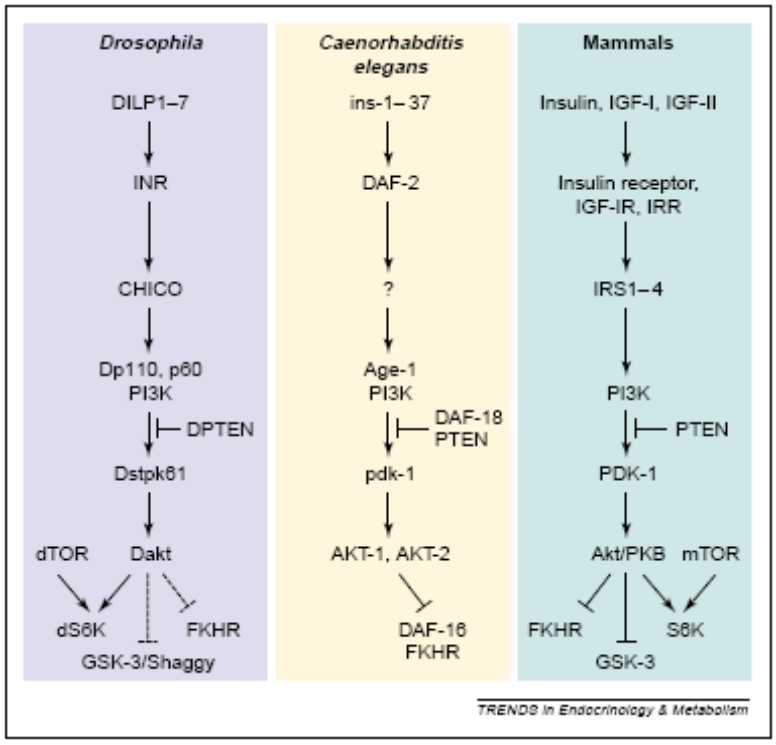


Figure 1. The insulin-signaling pathway has been well-characterized molecularly and shows evolutionary conservation across species (Garofalo, 2002).

The intracellular events triggered by insulin-like growth factors are also conserved across species. A key component of the insulin-signaling pathway is a forkhead transcription factor, FOXO (Forkhead box, class O). Without cellular stimulation, *FOXO* is localized in the nucleus where FOXO upregulates target genes (Birkenkamp, K.U. & Coffer, P.J., 2003). In the presence of insulin, the binding of the insulin peptide to the receptor tyrosine kinase leads to activation of the phosphatidylinositol-3-kinases (PI3K). PI3K activates protein kinase B (PKB) (also known as Akt) which translocates to the nucleus. PKB/Akt phosphorylates FOXO in the nucleus that allows for the binding of shuttling protein, 14-3-3, to FOXO. Binding of 14-3-3 to FOXO leads to its translocation to the cytoplasm and, consequently, its inactivation.

FOXO has a critical role in the growth aspect of the insulin signaling pathway. A study by Puig et al. (2003) demonstrated in *Drosophila* that dFOXO regulates the size of multicellular organisms by modulating cell number. Also, Junger et al. performed an epistasis analysis with dFOXO and other InR components in the heads of *Drosophila*. The results showed that $dFOXO^{-/-}$ significantly suppressed the growth-deficient phenotypes of loss-of-function DInR, chico, Dp110, and dPKB mutants (Junger et al., 2003). Furthermore, dFOXO also interacts with components of the TOR pathway. For example, dFOXO enhanced the bighead phenotype of $dTSC1^{-/-}$ flies. From this epistasis analysis, Junger et al. showed that dFOXO was downstream of PKB, and that dFOXO interacts with the TOR pathway, which closely interacts with the insulin pathway. These studies of the insulin-signaling pathway through PI3K/Akt and FOXO demonstrate their important roles in cell growth and proliferation.

Alongside growth pathways, differentiation pathways have also been extensively dissected. Differentiation has been well studied in the *Drosophila* eye, because of its repetitive and progressive nature. Differentiation begins during third larval instar stage, during which the morphogenetic furrow (MF) sweeps across the imaginal eye disc in the anterior direction (Voas et al., 2004). The Drosophila eye consists of about 800 ommatidia, which are stereotypical units consisting of eight photoreceptors (PRs) and accessory cells (Wernet et al., 2004). The eight photoreceptors are formed in a sequential and time-dependent manner. R8 is specified first, followed by R2/R5, R3/R4, R1/R6, and, lastly, R7. The specification of the different photoreceptors is dependent on the reiterative activation of the Notch and RTK (Receptor Tyrosine Kinase) pathways. However, the activation of these pathways is not sufficient to evoke photoreceptor differentiation. Constitutively active EGFR (epidermal growth factor receptor) causes overproduction of all photoreceptors types, except R8 that is independent of EGFR. But the response of an undifferentiated precursor cell to EGFR depended entirely upon the time of activation; for example, early EGFR stimulation led to photoreceptor neuron differentiation, whereas late EGFR stimulation led to pigment cell production (Freeman, 1996). Therefore, timing plays an important role in the differentiation of neural precursor cells in the *Drosophila* compound eye.

This concept is not specific to *Drosphila.* Vertebrate central nervous system (CNS) cell fates also appear to be controlled by a timing mechanism. For example, in the brain, later-born neurons migrate past earlier-born neurons prior to differentiation, resulting in cell fates dependent on 'birth order' (McConnell, 1989). These studies suggest the importance of timing on the differentiation of neuronal cells.

The observation that neuronal cell fate is dependent on 'birth order' has led to the question of whether a molecular 'clock' in involved in developmental events (Voas et al., 2004). There are many different types of molecular 'clocks'—one typically thinks of circadian rhythm in regard to 'clock'. However, this molecular 'clock' refers to the timing of birth of a neuron that determines its cell fate. Temporal differentiation in this paper will refer to time-dependent differentiation of neuronal cells. Determining the molecular nature of this 'clock' has remained an elusive challenge.

Recently, the insulin-signaling pathway has been shown to play a novel role in temporal neuronal differentiation. Bateman and McNeill (2004) demonstrated that the activation of the InR pathway resulted in precocious differentiation in the eye imaginal disc. In contrast, the lack of insulin signaling, induced by dInR loss-of-function mutant clones, led to delayed differentiation. These data suggested a tight coupling between growth/nutrition and differentiation in neuronal cells. Furthermore, increasing growth and cell size are not sufficient by themselves to induce differentiation. Bateman et al. showed that overexpression of cyclin D/CDK4 or myc did not induce precocious differentiation. Therefore, this effect of the InR pathway on temporal differentiation appears to be specific to the insulin pathway. Figure 2 is a model showing the link between the InR signaling pathway and neuronal differentiation as proposed by Bateman and McNeill.

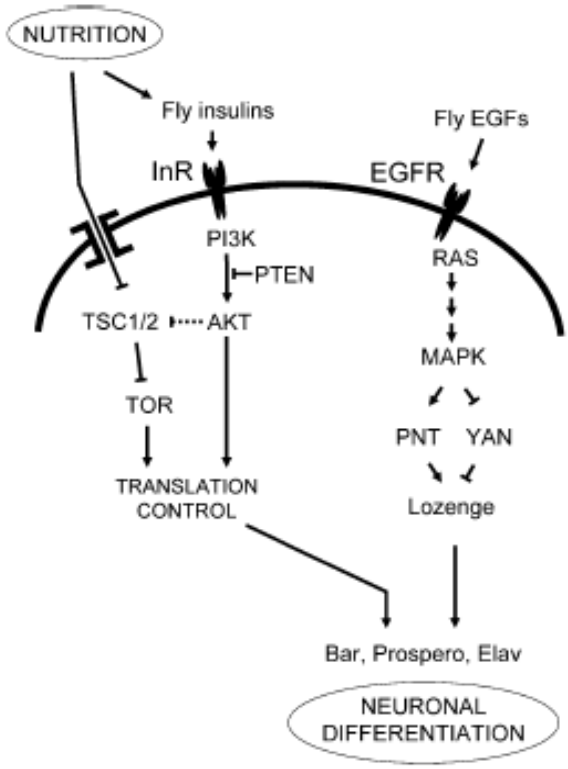


Figure 2. A model showing the link between the insulin signaling pathway and neuronal differentiation in accord with Bateman and McNeill's investigation (Leopold, 2004).

A question that remains from Bateman and McNeill's study is what mediates the actions of the insulin signaling pathway to control the timing of neuronal differentiation? In this paper, the 'mediator' will refer to the factor that serves as the link between the insulin signaling pathway and temporal differentiation. This study tests the hypothesis that FOXO is that mediator. The basis for hypothesizing FOXO to be the mediator is that FOXO plays a critical role in mediating growth in the insulin-signaling pathway. Further supporting FOXO's critical role in the insulin pathway, it has been proposed that FOXO acts as a 'nutritional sensor' (Tatar, personal communication). By serving the role of the mediator, FOXO can potentially coordinate differentiation with the number of cells in response to nutrients and external factors.

All the proposed experiments will be conducted in the *Drosophila* compound eye. The *Drosophila* eye is a good model to use to study temporal differentiation, because cell fate specificity and timing are well characterized. Also, there are similarities in retina formation between *Drosophila* and mammals. For example, specialized groups of PRs are used to discriminate between colors (e.g. cones), and other PRs are specialized to distinguish shapes and motion (e.g. rods) (Wernet et al., 2004). However, there are drawbacks to studying temporal neuronal differentiation in the *Drosophila* eye. In vertebrates, the CNS neuronal precursor cells are linked to the cell cycle, whereas *Drosophila* neuronal photoreceptor cells are independent of the cell cycle (Voas et al., 2004). Nevertheless, the observation that neuronal differentiation depends on time in both vertebrates and invertebrates suggests that aspects of the *Drosophila* model will apply to vertebrate systems.

The aims in this study are:

1.) **Test if dFOXO mutants (null and constitutively active) affect temporal differentiation in the *Drosophila* eye.**
2.) **Determine whether or not dFOXO is epistatic to other components of the InR pathway.**
3.) **Test candidate downstream targets of dFOXO that may be involved in neuronal differentiation.**

Results

I. Effects of *dFOXO* mutants on temporal differentiation in the *Drosophila* eye

The first aim of this study is to test the effects of a null and constitutively active dFOXO mutant on temporal differentiation in the larval eye imaginal disc. These mutant clones will be expressed in the *Drosophila* eye using FRT-mediated recombination (Xu et al., 1993, Figure 3).

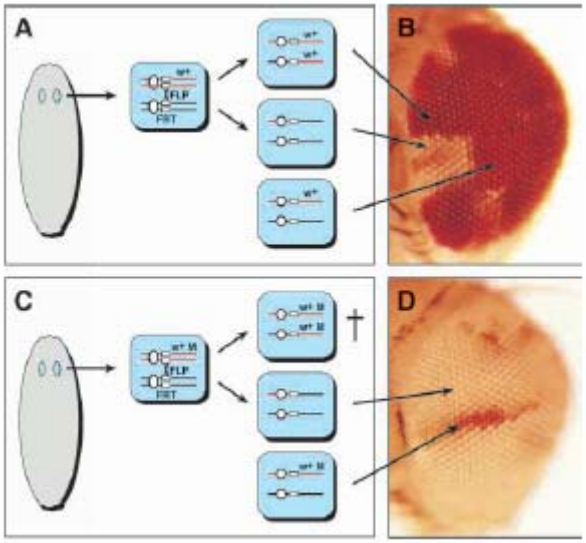


Figure 3. *Flp*-FRT mediated mitotic recombination in the *Drosophila* compound eye.
(A, B) The *white*$^{+}$(w^{+}) marker, present on the wild-type chromosome, results in dark red twin-spot wild-type clones, medium-red heterozygous clones, and white mutant clones.
(C, D) The *Minute* marker, which is homozygous lethal, kills twin-spot wild-type clones, allowing only heterozygous (red) and homozygous mutant clones (white) to survive.

The clones will be stained with specific antibodies to the transcription factors, Bar or Prospero, which are restricted to R1/R6 ommatidia or R7/non-neuronal cone cells respectively. The timing of appearance of the neuronal marker in the mutant clones will be compared to the GFP-marked wild-type clones to determine if the dFOXO mutant affects temporal differentiation. Two null dFOXO alleles and a constitutively active dFOXO mutant have already been constructed. Junger et al. (2003) isolated and

characterized two dFOXO null mutants, *dFOXO*^{21} and *dFOXO*^{25}, in which the W95 and W124 were mutated to stop codons respectively. The constitutively active *dFOXO* has its three Akt phosphorylation sites mutated to alanine. This constitutively active dFOXO mutant was shown to be active because it was constitutively localized in the nucleus and transcriptionally activated downstream genes (Puig et al., 2003). The expected result is precocious temporal differentiation in the *dFOXO*$^{-/-}$ mutant clones, indicating that the InR signaling pathway is activated (Figure 4A). Conversely, the mutant clones expressing the constitutively active *dFOXO* will have delayed temporal differentiation (Figure 4B).

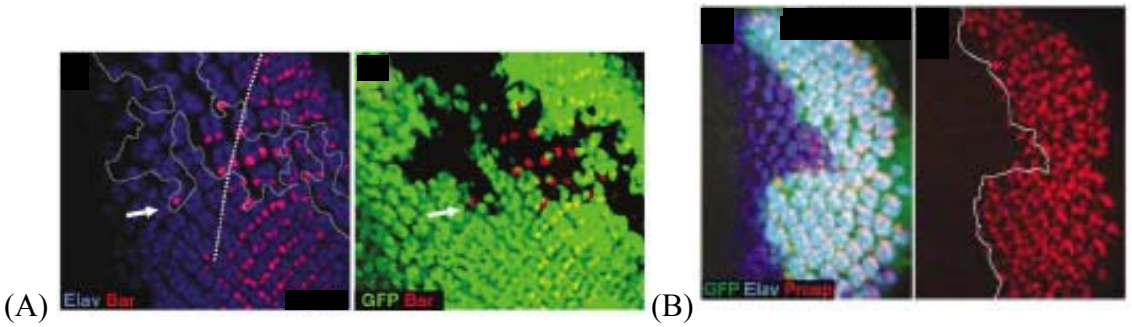


Figure 4. *dFOXO*$^{-/-}$ and constitutively active *dFOXO* accelerate and decelerate differentiation respectively.
(A) Loss-of-function dFOXO mutant clones, marked by a loss of GFP, show precocious staining of Bar (red) compared to wild-type clones. The arrow points to Bar staining in the mutant cells that is differentiating faster than wild-type.
(B) Constitutively active dFOXO mutant clones show delayed temporal differentiation.

There may be other results other than the expected results. For example, one of the dFOXO mutants may have accelerated/decelerated differentiation, whereas the other does not appear to have any effect on differentiation. In this case, other methods to make loss-of-function or gain-of-function dFOXO clones will be utilized. For example, *in vivo* RNAi can be used to make loss-of-function clones. For an alternate gain-of-function experiment, Junger et al. (2003) identified a dFOXO line with an EP transposable

element inserted in the second intron upstream of the open reading frame (EP35-147). By crossing this with an *ey*-Gal4 line, we can induce overexpression of the endogenous dFOXO in the eye. By testing other dFOXO loss-of-function or gain-of-function mutants, we can test if the lack of effect on temporal differentiation is actually due to inefficient expression of null/constitutively active dFOXO. However, if the phenotype of the mutant clone varies within the clone, e.g. a small patch of accelerated or decelerated photoreceptor differentiation, this may indicate that dFOXO is acting in a non-cell autonomous manner.

II. Epistasis experiments to determine if dFOXO is downstream of other insulin signaling components

Experiments will be performed to observe whether or not InR signaling mutants are dependent on dFOXO in temporal differentiation. These experiments will be critical in determining if dFOXO is the mediator between the insulin signaling pathway and temporal differentiation. Double mutants will be constructed by implementing the *ey-Flp*/FRT system to insert the mutant dFOXO (null or constitutively active) on the same FRT chromosome as the dInR signaling mutant. The InR signaling mutants are the following: *Tsc1*$^{-/-}$, *InR*$^{-/-}$, *PI3K*$^{-/-}$, *PTEN*$^{-/-}$, *Tor*$^{-/-}$, *Rheb*$^{-/-}$, *and S6 kinase*$^{-/-}$. The function of each of these components are listed in Table 1 and Figure 5 shows their role in the insulin signaling pathway.

Insulin signaling component	Function
InR	insulin receptor with conserved tyrosine kinase domain
PI3K	phosphatidylinositol 3-kinase
TSC1	tuberous sclerosis complex 1
PTEN	phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase
Tor	target of rapamycin
Rheb	Ras homologue enriched in brain; small GTPase downstream of TSC
S6 kinase	p70 ribosomal S6 kinase

Table 1. Insulin signaling components and their functions in the insulin pathway.

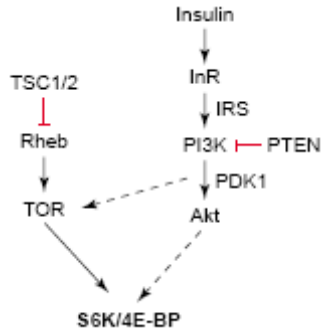
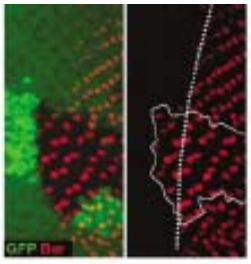
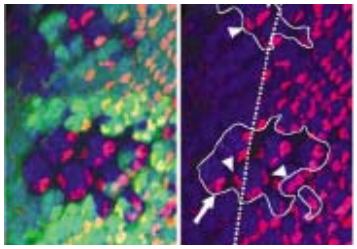
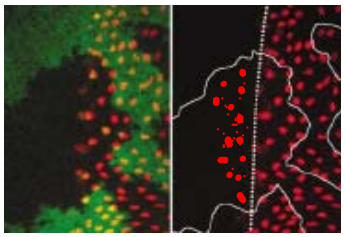


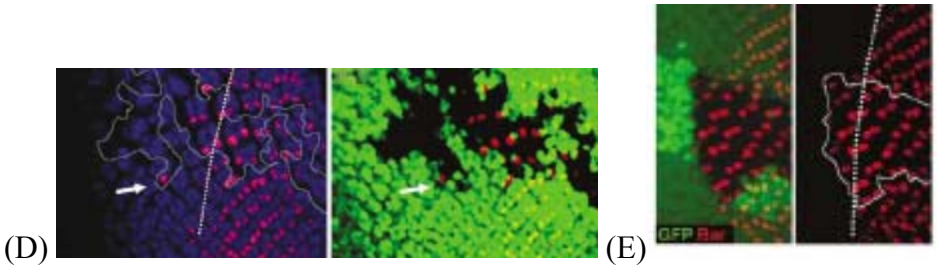
Figure 5. The components in the TOR/insulin signaling pathways. The TSC/TOR pathway converges with the canonical insulin signaling pathway to regulate growth (Pan et al., 2004).

These loss-of-function InR signaling mutants were chosen because Bateman and McNeill already studied their effects on temporal differentiation and showed that mutant clones activating the InR pathway (*Tsc1*$^{-/-}$, *PTEN*$^{-/-}$) had precocious differentiation, whereas the mutants inhibiting InR (*InR*$^{-/-}$, *Tor*$^{-/-}$, *Rheb*$^{-/-}$, *S6 kinase*$^{-/-}$) delayed

differentiation. Therefore, I will examine the effects of dFOXO mutants combined with these dInR mutants and compare their effects on temporal differentiation to that of single InR mutants. The double mutant clones will be expressed in the *Drosophila* compound eye by the FRT-mediated recombination. Imaginal discs will be stained by Bar or Prospero to assess timing as done in the prior experiment.

If dFOXO is the sole mediator between InR pathway and differentiation, dFOXO will be epistatic to the other InR components. In other words, the phenotype of the double mutants will match that of the dFOXO mutant, indicating that dFOXO is downstream of the InR mutant. The expected results are the following: *dFOXO*$^{-/-}$ will accelerate the delayed differentiation in loss-of-function *InR, PI3K, Rheb, Tor*, and *S6 kinase*, in all of which InR signaling is lacking, to precocious differentiation as shown with the *dFOXO*$^{-/-}$ clones (Figure 6A-E). *dFOXO*$^{-/-}$ will not change the *tsc1* and *pten* mutants significantly, since differentiation is already precocious (Figure 6F, 6G). Conversely, constitutively active dFOXO will delay differentiation in the *tsc1* and *pten* mutants (Figure 7A, 7B), but will not significantly affect the *InR, PI3K, Rheb, Tor, and S6 kinase* mutants since differentiation is already delayed.

(A)  (B)  (C) 



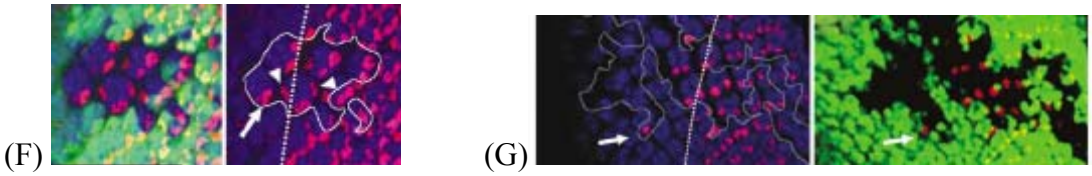


Figure 6. *dFOXO*$^{-/-}$ accelerates differentiation in loss of InR signaling mutants.
(A) *dFOXO*$^{-/-}$*/Dinr*$^{-/-}$ (F) *dFOXO*$^{-/-}$*/tsc1*$^{-/-}$
(B) *dFOXO*$^{-/-}$*/PI3K*$^{-/-}$ (G) *dFOXO*$^{-/-}$*/pten*$^{-/-}$
(C) *dFOXO*$^{-/-}$*/Rheb*$^{-/-}$
(D) *dFOXO*$^{-/-}$*/Tor*$^{-/-}$
(E) *dFOXO*$^{-/-}$*/S6K*$^{-/-}$

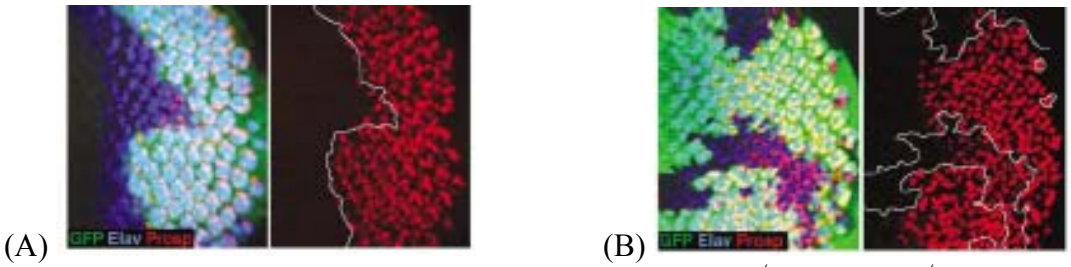


Figure 7. Constitutively active *dFOXO* delays differentiation in *tsc1*$^{-/-}$(A) and *pten*$^{-/-}$(B) mutant clones.

Alternate results would be that dFOXO mutants do not affect the phenotypes of one or several InR signaling mutants in temporal differentiation. For example, if *dFOXO*$^{-/-}$ does not affect the delayed differentiation phenotype of *tor*$^{-/-}$, then TOR would be epistatic, or downstream, to dFOXO. In this case where an InR signaling mutant is epistatic to dFOXO, it would be unlikely that FOXO is the mediator.

III. Analysis of effects of dFOXO target genes on temporal differentiation

The next step is to study dFOXO downstream target genes that could be involved in temporal differentiation. Microarray studies identified some genes upregulated by dFOXO by analyzing gene transcripts repressed upon insulin stimulation in *Drosophila* embryonic Kc167 cells (Junger et al., 2003). Junger et al. have identified several potential dFOXO target genes as shown in Table 2.

Gene product	Biological Process	FlyBase ID	Fold repressed
PEPCK (phosphoenolpyruvate carboxykinase)	Gluconeogenesis	FBgn0003067	4.6
Phosphorylase kinase gamma	Glycogen breakdown	FBgn0011754	2.4
CPTI (mitochondrial carnitine palmitoyltransferase)	Lipid catabolism	FBgn0027842	4.5
Long-chain-fatty-acid-CoA-ligase	Lipid catabolism	FBgn0027601	2.7
d4E-BP	Translation control	FBgn0022073	3.3
Cyclin-dependent kinase 8	Transcription control	FBgn0015618	3.3
Cyp4e2 (cytochrome P450)	Stress response	FBgn0014469	2.9
Cyp9c1 (cytochrome P450)	Stress response	FBgn0015040	4.1
DNA polymerase iota	DNA repair	FBgn0037554	3.4
Centaurin gamma	Cell-cycle control	FBgn0028509	3.2
CG3799	Cell-cycle control	FBgn0027593	4.2

Table 2. List of genes upregulated by FOXO (Junger et al., 2003).

Each putative upregulated dFOXO target gene listed in Table 2 will be ectopically expressed in *dFOXO*$^{-/-}$ mutant clones. This will be done by inserting a null (or hypomorphic) allele of the target gene on the same FRT chromosome as the constitutively active or null *dFOXO*. The null allele of the target gene can either be obtained, if null alleles already exist, or a null allele can be constructed, e.g. P-element excision. We would expect that the null (or hypomorph) dFOXO target gene would accelerate differentiation in a constitutively active dFOXO background. Since differentiation is precocious in *dFOXO*$^{-/-}$ clones, the co-expression of a target gene

upregulated by dFOXO will delay differentiation. Of the dFOXO target genes listed in Table 2, 4E-BP is the most well-known target. 4E-BP is a translational repressor and a downstream effector of the PI3K pathway in the regulation of cell proliferation and growth (Miron, et al., 2001). Therefore, if d4E-BP were involved in temporal differentiation, we would expect that the ectopic expression of d4E-BP to revert the precocious differentiation in *dFOXO*$^{-/-}$ clones to delayed differentiation, and the loss-of-function *d4E-BP* to accelerate differentiation in constitutively active dFOXO clones (Figure 8).

Figure 8. Ectopic expression of d4E-BP and loss-of-function *d4E-BP* in a *dFOXO*$^{-/-}$ or constitutively active *dFOXO* background respectively.
(A) *dFOXO*$^{-/-}$ clones show precocious differentiation (left), whereas d4E-BP co-expression delays differentiation (right).
(B) Differentiation is delayed in constitutively active dFOXO clones (left), whereas co-expression of loss-of-function *d4E-BP* accelerates differentiation (right).
(Modified from Bateman et al., 2004)

Puig et al. (2003) already demonstrated that FOXO directly binds to d4E-BP promoter and activates transcription of d4E-BP. However, if an uncharacterized dFOXO target gene is found to be involved in neuronal differentiation, experiments will be performed to determine if the target gene directly interacts with dFOXO. First, we want to determine if the expression of the putative target gene is upregulated in the presence of dFOXO and, second, if upregulation results from the binding of dFOXO to its promoter.

To show that dFOXO upregulates the target gene, we would perform RNase protection assays with mRNAs extracted from cells stably transfected with either dFOXO (wild-type) or dFOXOA3 (constitutively active) (Puig et al., 2003). dFOXO or

constitutively active *dFOXO* (dFOXOA3) are both V5-tagged and under the control of the metallothionein promoter. S2 cells stably transfected with dFOXO or dFOXOA3 are grown in the presence of insulin or CuSO4, or with both. Figure 9A shows the predicted results if dFOXO directly binds to the target gene. As seen in Figure 9A, dFOXOA3 activates the target gene in the presence or absence of insulin, whereas dFOXO only activates the target gene in the absence of insulin. Also, in the absence of CuSO4, dFOXO and dFOXOA3 cannot bind the target gene in the presence or absence or insulin. By putting dFOXO under the metallothionein promoter, we can show that insulin and $CuSO_4$ does not cause upregulation or downregulation of the target gene without dFOXO.

An additional experiment will test if endogenous dFOXO can upregulate the target gene. We treat S2 cells with either LY294002 (PI3K inhibitor) or insulin and perform RNase protection assay. Figure 9B shows the predicted results that the target gene is upregulated in the presence of LY294002 but not insulin.

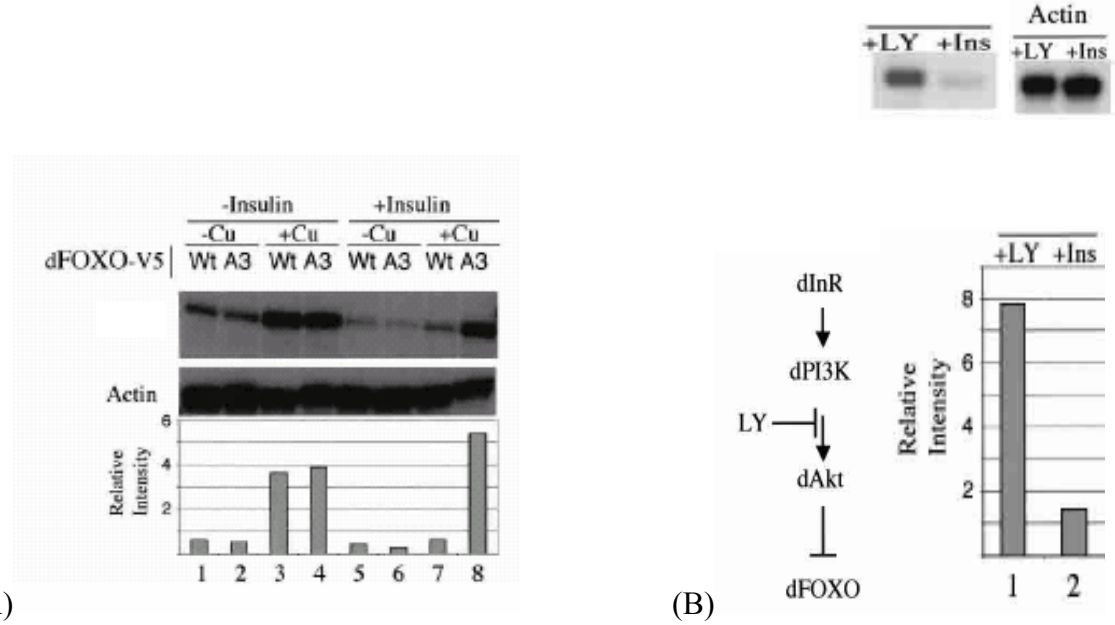


Figure 9. dFOXO upregulates its target gene in the absence of insulin.

(A) dFOXOA3 (constitutively active) activates the target gene in the presence or absence of insulin. However, dFOXO can only activate in the absence of insulin.
(B) The target gene is upregulated in the presence of PI3K inhibitor, LY294002, but inhibited in the presence of insulin.
(Modified from Puig et al., 2003)

If we determine that dFOXO upregulates the target gene, experiments showing direct binding of dFOXO to the target gene promoter will be conducted. Different lengths of the promoter of the target gene will be fused to luciferase reporter and co-transfected into S2 cells with dFOXOA3 (Figure 10). Different lengths of the target gene promoter would be tested in order to locate the FREs (FOXO responsive elements) in the promoter. In this case, the FREs appear to be spread out in the promoter, since the 194 bp construct still activates luciferase activity significantly better than the negative control.

The positive control is a construct containing four tandem FOXO4-binding sites upstream of alcohol dehydrogenase distal core promoter driving luciferase (pGL4xFRE) (Puig et al., 2003). FOXO4 has 85% identity to the forkhead DNA-binding domain in dFOXO, so dFOXO binds to this construct. The negative control would be a construct with upstream activating sequences (UAS), a promoter sequence to which dFOXO is known not to bind.

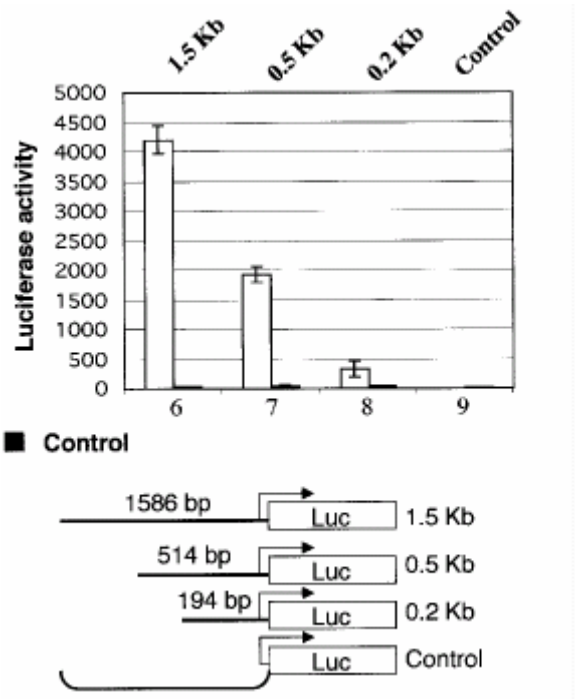


Figure 10. The constructs are composed of varying lengths of the promoter of the target gene fused to the luciferase reporter. Binding of dFOXO to the target promoter activates luciferase. In this case, FREs (FOXO responsive elements) appear to be apread out along the 1.5 kb region of the target promoter. The control is an empty vector in place of the target promoter. (Modified from Puig et al., 2003)

A gel shift experiment would also be performed to show that dFOXO binds the target gene promoter. The target gene promoter would be P^{32}-labeled and purified recombinant dFOXO would be added. Figure 11 shows the predicted results if dFOXO binds the target promoter. Increasing levels of dFOXO binds the promoter (lanes 1-4), whereas the control (multiple cloning site of pBluescript SK II, Puig et al., 2003) shows no binding. Furthermore, addition of unlabeled promoter competes for dFOXO binding with the labeled promoter (lanes 9-12), whereas unlabeled control promoter does not compete (lanes 13-16). This provides strong support that dFOXO directly binds the target gene promoter.

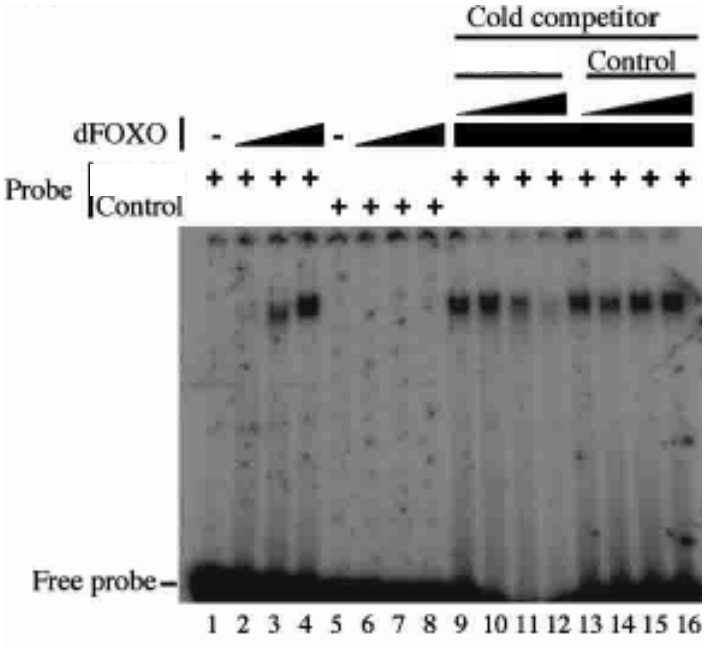


Figure 11. Gel shift assay. With increasing dFOXO concentration, we see binding to the promoter of the target gene (lanes 1-4). With the control probe, there is no apparent binding to dFOXO. When unlabeled promoter of the target gene is added, dFOXO binding to the labeled probe is decreased due to competition (lanes 9-12). The addition of an unlabeled control probe has no effect on dFOXO binding to the target promoter (lanes 13-16). (Modified from Puig et al., 2003)

Lastly, to show dFOXO binding to the target promoter *in vivo*, ChIP assay (Chromatin Immunoprecipitation) will be performed in S2 cells transfected with either dFOXO or dFOXOA3 in the presence of insulin. As in a prior experiment, both dFOXO constructs are epitope-tagged with V5 and under the control of the metallothionein promoter. After activation with $CuSO_4$, cellular proteins are cross-linked by treating cells with formaldehyde and subjected to immunoprecipitation with α-dFOXO and α-V5, both specific for dFOXO. After reversal of crosslinking by the addition of RNase A and NaCl and heating, DNA would be detected by PCR with primers encompassing regions corresponding to the FREs. The expected result is shown in Figure 12. dFOXOA3 shows strong binding to the promoter (lanes 1 and 3), whereas wild-type dFOXO shows

less binding to the promoter due to the presence of insulin (lanes 6 and 8). The negative controls are the pre-immune serum and an unrelated antibody (α-Myc) which shows no binding (lanes 2, 4, 7, 9). An additional negative control is to probe for a promoter that does not bind to dFOXO, e.g. U6 snRNA (not shown).

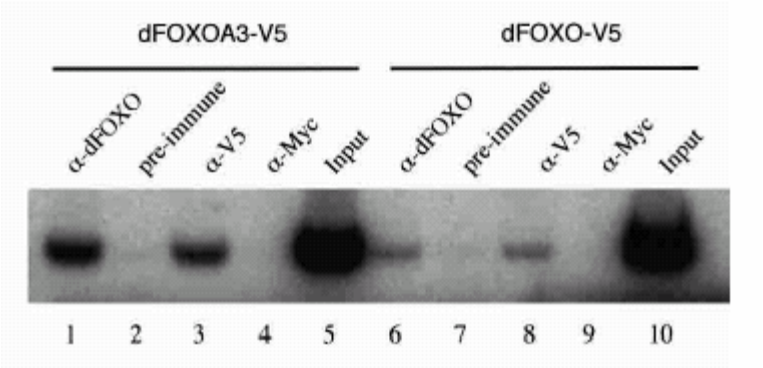


Figure 12. ChIP assay shows strong binding of dFOXOA3 to the target promoter *in vivo*. Binding is weaker for wild-type dFOXO in the presence of insulin. (Modified from Puig et al., 2003)

Table 2 lists some of the downstream targets of dFOXO, but it is neither conclusive nor comprehensive. Xuan et al. (2005) utilized comparative genomics to identify FOXO target genes by finding orthologues of FOXO target genes and by identifying genes with conserved FOXO binding motifs in gene promoter. Table 3 lists FOXO target genes identified by comparative genomics.

Human gene	Definition and function
Known FOXO target genes	
INSR	Insulin receptor
PDK4	Pyruvate dehydrogenase kinase, isoenzyme 4
Potential FOXO target genes with known function	
TXNIP	Thioredoxin interacting protein, GO: biological process unknown
TLP19	Endoplasmic reticulum thioredoxin superfamily member, 18 kDa, GO: electron transport
FEN1	Flap structure-specific endonuclease 1, GO: DNA replication/repair
BTG1	B-cell translocation protein 1, GO: cell proliferation
PLXNC1	Plexin C1, GO: cell adhesion/development
MLH3	Mismatch repair gene MLH3, GO: meiotic recombination/mismatch repair
IGF1R	Insulin receptor signaling pathway
SLC12A6	Solute carrier family 12 (potassium/chloride transporters), member 6
TFAP4	Transcription factor AP-4 (activating enhancer binding protein 4)
SSB3	SPRY domain-containing SOCS box protein SSB-3, GO: intracellular signaling cascade
PER1	Period (*Drosophila*) homolog 1, GO: regulation of transcription, DNA-dependent
DHX8	DEAH (Asp-Glu-Ala-His) box polypeptide 8, GO: RNA splicing
FLJ10597	Function unknown, GO: protein ubiquitination.
STK11	Serine/threonine protein kinase 11, related to growth suppression (Tiainen et al., 2002)
TFDP2	Transcription factor Dp-2 (E2F dimerization partner 2), GO: regulation of cell cycle
ELOVL6	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2)
LCP2	Lymphocyte cytosolic protein 2, GO: regulation of blood and lymphatic vascular separation
CITED2	Cbp/p300-interacting transactivator, GO: regulation of transcription from Pol II promoter
AP4M1	Adaptor-related protein complex 4, mu 1 subunit, GO: intracellular protein transport
ASBABP2	Pregnancy-associated plasma protein A, pappalysin 1. (Chen et al., 2003)
Potential FOXO target genes with function unknown	
FLJ12168	TBC1 domain family, member 17, unknown function
FLJ12221	Zinc finger, SWIM domain containing 4, unknown function
CNNM3	Cyclin M3,ancient conserved domain protein 3, unknown function
FLJ23142	Secernin 3, unknown function
KIAA1155	Unknown function
DJ465N24.2.1	NPD014 protein, function unknown
C5orf6	Unknown function
FLJ13611	Hypothetical protein
FLJ23209	PDZK7 protein, hypothetical protein, function unknown
LOC220213	OTUD1: OTU-like cysteine protease, function unknown

Table 3. Mammalian orthologues of FOXO target genes containing conserved dFOXO binding motifs as identified by comparative genomics (Xuan, et al., 2005).

Therefore, if the dFOXO target genes listed in Table 2 do not have any effect on temporal differentiation, there are many other FOXO target genes that can be tested. However, as stated previously, *in vitro* and *in vivo* experiments are needed to show that FOXO binds directly to and activates the target gene.

Bateman and McNeill hypothesize that a 5'TOP proneural factor is the mediator between the insulin signaling pathway and neuronal differentiation. 5'TOPs are 5' oligopyrimidine tracts at the transcriptional start site in ribosomal proteins and protein synthesis elongation factors (Bateman et al., 2004). The reasoning behind Bateman and

McNeill's hypothesis is that regulation of growth by the insulin signaling pathway is mediated through translational control (Figure 13, Bateman et al., 2004).

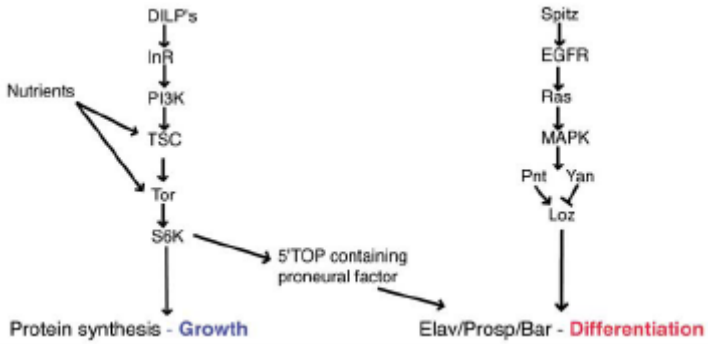


Figure 13. Bateman and McNeill's model in which a 5'TOP proneural factor mediates signaling between the insulin signaling pathway and neuronal differentiation.

The insulin signaling pathway mediates translational control through Tor and S6K. S6K also selectively promotes translation of 5'TOP mRNAs (Goberdhan, 2003). So a possible mechanism, according to Bateman and McNeill's theory, is that insulin activation leads to upregulated translation of 5'TOP mRNA encoding a proneural factor through S6K action. A proneural factor can also initiate neuronal differentiation (Isshiki et al., 2003). Therefore, by the activation of the insulin signaling pathway, translation of a proneural factor can be upregulated and initiate differentiation.

There are several problems with Bateman and McNeill's hypothesis. Translational control is not the only means of regulating growth; the insulin signaling pathway regulates growth by both transcriptional and translational control (Goberdhan, et al., 2003). A study by Stolovich et al. (2002) showed that complete inhibition of mammalian TOR and S6K only had a mild repressive effect on the translation of TOP mRNAs. Stolovich et al. concluded that translation of TOP mRNAs was mainly regulated through the PI3K pathway, not the TOR pathway. Furthermore, Bateman and

McNeill constructed a *S6K*$^{-/-}$ mutant clone and observed a very weak delay in phenotype (Bateman et al., 2004). This observation does not support S6K upregulating the translation of a 5'TOP proneural factor. Another potential problem with having a 5'TOP proneural factor as mediator is that it may upregulate the translation of several genes in addition to those involved in neuronal differentiation.

The experiments proposed in this study only focus on the neuronal development of the photoreceptors in the *Drosophila* eye. Earlier in the introduction, I discussed the similarity of retinal development and time-dependent neuronal development in vertebrates and invertebrates. But what is the relevance to mammals and organismal systems as a whole? Although Bateman and McNeill's research has not yet been applied to the central nervous system, there is reason to believe that the insulin signaling pathway also plays a role in the central nervous system. A study by Garofalo and Rosen (1988) measured dInR mRNA levels in various development stages in *Drosophila*. They discovered that dInR mRNA levels were markedly higher in the developing nervous system during embryogenesis. From this observation, Garofalo and Rosen hypothesized that the InR may be involved in active neurite outgrowth. Furthermore, this elevated dInR mRNA level persisted through larval and adult stages. The cortex of the brain and ganglion cells were among the most prominently labeled tissues (Garofalo and Rosen, 1988). Other studies have also studied the effect of the insulin pathway in the central nervous system. For example, Feldman et al. (1997) found that insulin-like growth factor I (IGF-1) promoted neurite outgrowth and enhanced growth cone mobility. These studies suggest that the insulin signaling pathway may affect the neurons in the central nervous system in addition to the peripheral nervous system.

Significance

The novel role of the insulin pathway in regulating developmental timing is an important finding, because of the implication that growth and nutrients is linked to the timing of differentiation. Loss of regulated control over temporal differentiation can lead to mispatterning and deformities, such as those observed in the *Drosophila* eye. Therefore, tightly regulated temporal control of differentiation is essential for the correct formation of an organism. By proposing that FOXO mediates signaling between the insulin pathway and temporal neuronal differentiation, I am suggesting a model in which tight regulation and coordination between growth and differentiation can be achieved by FOXO's role as mediator.

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