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Timing the Onset of Metamorphosis in Drosophila.
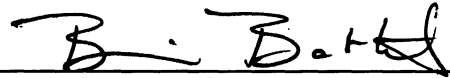
by

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A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

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HOUSTON, TEXAS
June , 2011

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Abstract

Timing the Onset of Metamorphosis in Drosophila

by

Magdalena A. Walkiewicz

Because Drosophila do not grow after initiation of metamorphosis, their final body size is determined by larval growth rate and duration of the larval growth phase. Drosophila metamorphosis is triggered by the steroid hormone ecdysone, which is produced in the prothoracic gland (PG). Ecdysone synthesis requires expression of the "Halloween" genes, which encode ecdysone biosynthetic enzymes. Growth rate is regulated by Insulin-like peptides, which are released from the insulin-producing cells (IPCs). Genetic ablation of the IPCs decreases growth rate and delays onset of metamorphosis, suggesting that ecdysone synthesis is induced by insulin signaling. Inhibiting PI3 Kinase (PI3K), the major effector of insulin signaling, in the PG similarly delays metamorphosis as a consequence of decreased ecdysone synthesis and decreased Halloween gene expression. In contrast, activating PI3K in the PG advances the onset of metamorphosis and increases Halloween gene expression. Here I report that increased insulin signaling, accomplished inhibiting the protein kinase A pathway in the IPCs increases insulin signaling and increases growth rate but also

advances the onset of metamorphosis by increasing expression of at least one Halloween gene. Ecdysone synthesis is promoted by a second peptide hormone, PTTH, which activates Halloween gene expression via the Torso receptor followed by Ras and Raf in the PG. Null mutations in the transcription factor *broad* (*br*) prevent *torso* transcription and thus prevent Halloween gene expression and metamorphosis. Here I identify Br as the mechanistic link between PI3K activity and Halloween gene expression. I found that PI3K activity is required for *br* expression by inhibiting the downstream kinase GSK-3. I provide evidence that three nuclear hormone receptors, βFTZ-F1, HR3 and E75, link GSK-3 activity with *br* expression: RNAi-mediated *βFTZ-F1* or *HR3* knockdown, or *E75A* overexpression, in the PG prevents *br* expression. I also found that ectopic Torso pathway activation, accomplished by expressing the constitutively active *Raf^{gof}*, restores Halloween gene transcription to larvae lacking *br* or *βFTZ-F1*, suggesting that these larvae fail to express Halloween genes because they fail to transcribe *torso*. These studies identify a potential molecular mechanism linking growth rate with competence to respond to the PTTH metamorphic signal and thus initiate metamorphosis.

Acknowledgments

I would like to express my endless gratitude to my advisor Dr. Michael Stern: thank you for all your wisdom, time, patience, and effort you have put into chiseling the scientist that I am today. It has been a wonderful process, which I enjoyed. I would also like to express many thanks to my committee: Dr. Bartel, Dr. Lane, Dr. Stewart, and Dr. McNew. for their guidance, support, time, and all the suggestions they have made over the years.

To my family, I say thank you for enduring on this adventure with me, Eric, Camil, and Sveva, thank you for your love, support, and patience. Thank you for keeping me grounded and always reminding me of what is important in life. To Kasia simply thank you. To my parents, thank you for never cutting my wings.

To all my lab member over the years, thank you for making the Stern Lab a fun place to be. And to my entire family especially Ania and Kuba, and all my friends, thank you for always believing in me, all your encouragements, and all the good times.

"The important thing in science is not so much to obtain new facts as to

discover new ways of thinking about them."

William Lawrence Bragg

Abbreviations

20E; 20-hydroxyecdysone (ecdysone)

AEL; after egg laying

AKH; Adipokinetic hormone

Akt; protein kinase B (PKB)

A.U.; arbitrary units

Br; Broad

Creb; cAMP response element binding protein

DAPI; 4',6-diamidino-2-phenylindole

Dib; Disembodied

DIC; Differential interference contrast microscopy

Dilp; Drosophila insulin like peptide

E75; Ecdysone 75

EcR; Ecdysone Receptor

GSK3; Glycogen Synthase Kinase 3

HR-3; Drosophila Hormone Receptor 3

IIS; Insulin/Insulin Growth Factor Signaling

InR; Insulin receptor

IPC; insulin producing cell

PDF; Pigment Dispersion Factor

PG; prothoracic gland

Phm; Phantom

PI3K; PI3 Kinase

PKA; Protein Kinase A

PTTH; prothoracicotropic hormone,

RG; ring gland

RTK; Receptor Tyrosine Kinase

SEM; standard error of mean

βFTZ-F1; β Fushi Tarazu-Transcription Factor 1

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Chapter 1: Introduction

1.1   Final body size determination

In many organisms, growth and reproduction are temporally separated: organisms grow during an early, sexually immature juvenile stage, and then at a certain time transition to non-growing, sexually mature adults. In humans, the very rapid childhood growth phase ends with the initiation of puberty, which will eventually result in a sexually mature adult. In holometabolous insects, a very rapid larval growth phase ends with the entry to metamorphosis, during which a sexually mature, non-growing organism is formed. In insects, the duration of the rapid growth phase is an important determinant in the final body size of the organism. For example, if an organism spends more time in the rapid growth phase growing at a normal rate it will produce a larger adult. Conversely, if an organism spends less time in the rapid growth phase and grows at a normal rate it will produce a smaller adult.

In both insects and humans, nutrition plays a critical role in determining not only growth rate, but also the duration of the rapid growth phase. In the Western world, the age of onset of puberty has decreased by one month per decade over the last 150 years. It is been suggested that this decrease is a consequence of much improved nutrition, which leads to increased growth rate. In contrast, Drosophila larvae raised on poor nutrients, the growth phase is extended and initiation of metamorphosis is delayed (Tu and Tatar, 2003). The

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developmental delay presumably allows for additional time for larval feeding, and at least partial restoration of normal body size.

These observations strongly suggest that there is a growth rate-dependent mechanism for the timing of the transition to adulthood. However, the observation that Drosophila larvae reared on poor nutrients pupariate at a smaller than normal size (Tu and Tatar, 2003) suggests that body size is not the sole regulator of the timing of onset of metamorphosis; apparently, other factors contribute to this initiation of metamorphosis as well.

1.2 The Drosophila life cycle

Drosophila, which is a holometabolous insect, develops through three larval instars. In the first 24 hours after egg laying (AEL), the embryo undergoes a very rapid development and hatches into a first instar larva (L1) (Edgar, 2006). After about 24 hours the first instar larva outgrows its exoskeleton and initiates molting to shed the old exoskeleton and to grow a new one (Edgar, 2006). After this process is completed the larva is now a second instar larva (L2) and in about 24 hours will again outgrow its exoskeleton and initiate another molt (Edgar, 2006). The newly molted third instar larva (L3) will feed until it enters wandering stage during which it leaves the food and finds a spot to initiate pupariation (Edgar, 2006). Several hours after wandering, metamorphosis is initiated (Edgar, 2006). During metamorphosis the organism remodels itself; it forms adult structures, which include the wings, halters, legs, antennae to produce a sexually

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mature adult.

In Drosophila, developmental transitions are triggered by the steroid hormone ecdysone. Titers of this hormone rise dramatically prior to each developmental transition (molts, pupariation and metamorphosis) (Edgar, 2006). This observation suggests that the timing of ecdysone synthesis is crucial in regulating the timing of developmental transitions of molting and ultimately metamorphosis.

1.3 Ecdysone synthesis in Drosophila

Ecdysone is synthesized in the prothoracic gland (PG), which is present between the larval brain lobes (King-Jones and Thummel, 2005). The PG is composed of 40 to 60 cells, which are arranged in two sheets. The PG is a part of a larger structure termed the ring gland (RG). The RG also includes the corpus allatum, where juvenile hormone is synthesized and the corpus cardiacam, where sugar metabolism is controlled (Kim and Rulifson, 2004; Rulifson et al., 2002). The RG is wrapped around the aorta (forming a ring) and located above the brain lobes (Siegmund and Korge, 2001). Detailed anatomical studies revealed that the RG is innervated by processes from several neurons (Siegmund and Korge, 2001).

Ecdysone is synthesized from cholesterol in a series of hydroxylation reactions (Gilbert, 2004). Following much effort, many of the genes encoding the ecdysone biosynthetic enzymes were identified. These genes are termed the

"Halloween genes", and encode the enzymes that carry out ecdysone biosynthesis ("Halloween enzymes").

1.3.1 The Halloween enzymes carry out ecdysone biosynthesis.

A set of mutants was initially identified with a common, mid-embryonic lethal terminal phenotype: a failure in cuticle formation. Embryos with this phenotype resembled ghosts; therefore this group of genes was named "Halloween genes" (Gilbert, 2004). These Halloween mutants also failed to involute the head, exhibited aberrant looping of the hindgut, and failed dorsal closure (Gilbert, 2004). To date the Halloween genes include a group of five genes: *spook (spo)* (Namiki et al., 2005; Ono et al., 2006), *phantom (phm)* (Warren et al., 2004), *disembodied (dib)* (Chavez et al., 2000) (Warren et al., 2002), *shadow (sad)* (Warren et al., 2002), *and shade (shd)* (Gilbert, 2004). Because of the inability of the Halloween mutants to form a differentiated first instar cuticle, a process that precedes the ecdysone-mediated molt, it was indicated that these genes maybe playing a role in ecdysone biosynthesis (Gilbert, 2004). Further characterization of these genes shed more light on their function in ecdysone biosynthesis (Gilbert, 2004).

1.3.1a. Disembodied (*dib*)

In situ hybridization experiments with *dib* transcripts showed expression in the PG, which is consistent with its possible function in ecdysone synthesis (Warren et al., 2002) (Chavez et al., 2000). Sequence analysis suggested that Dib belongs to the cytochrome p450 family and that it localizes to the mitochondria because a well conserved N-terminal amphipathic region (Warren et al., 2002). Colocalization studies of epitope-tagged *dib* and mitochondrial markers confirmed that Dib localizes to the mitochondria (Warren et al., 2002). Functional studies revealed that Dib is the C22-hydroxylase and it uses ketotriol as a substrate to produce 2-deoxyecdysone (Warren et al., 2002).

1.3.1b. Shadow (*sad*)

Similarly to *dib*, *sad* is also expressed in the PG (Warren et al., 2002). Sequence analysis of *sad* also suggested that it belongs to the cytochrome p450 family and it also contains a well conserved N-terminal amphipathic region placing Sad in the mitochondria (Warren et al., 2002).

Colocalization studies of epitope-tagged *sad* and mitochondrial markers confirmed that *sad* also localizes to the mitochondria (Warren et al., 2002). Functional studies placed Sad in the terminal step of ecdysone biosynthesis (Warren et al., 2002). Sad is the 2-hydroxylase, which converts 2-deoxyecdysone to ecdysone (Warren et al., 2002).

1.3.1c. Phantom (*phm*)

Like *dib* and *sad*, in situ experiments of *phm* revealed strong expression pattern in the epidermis, epidermal stripes in early embryos, and the in PG later in development (Warren et al., 2004). Sequence analysis of *sad* also suggested that it belongs to the cytochrome p450 family however, based on work in other insects it was predicted that Phm will localize to the microsome (Warren et al., 2004).

Colocalization studies of C-terminal HA-tagged *phm* and mSpitz-GFP, which labels the ER, confirmed that Phm is localized in the ER (Warren et al., 2004). Functional studies placed Phm a step ahead of Dib, where Phm is the 25-hydroxylase converting ketodiol to ketotriol (Warren et al., 2004).

1.3.1d. Spook (*spo*) and Spookier (*spok*)

Similarly to the other Halloween gene mutant animals, the *spo* mutants exhibit failure of dorsal closure, head involution and aberrant looping of the hindgut (Namiki et al., 2005). However, *in situ* experiments revealed that unlike *phm*, *dib*, and *sad*, *spo* is not expressed in the prothoracic gland PG but in the early embryo and developing adult ovary (Namiki et al., 2005).

Sequence analysis of the *spo* genes also classifies Spo as a cytochrome P450 and like Phm it contains a stretch of N-terminal hydrophobic resides, which
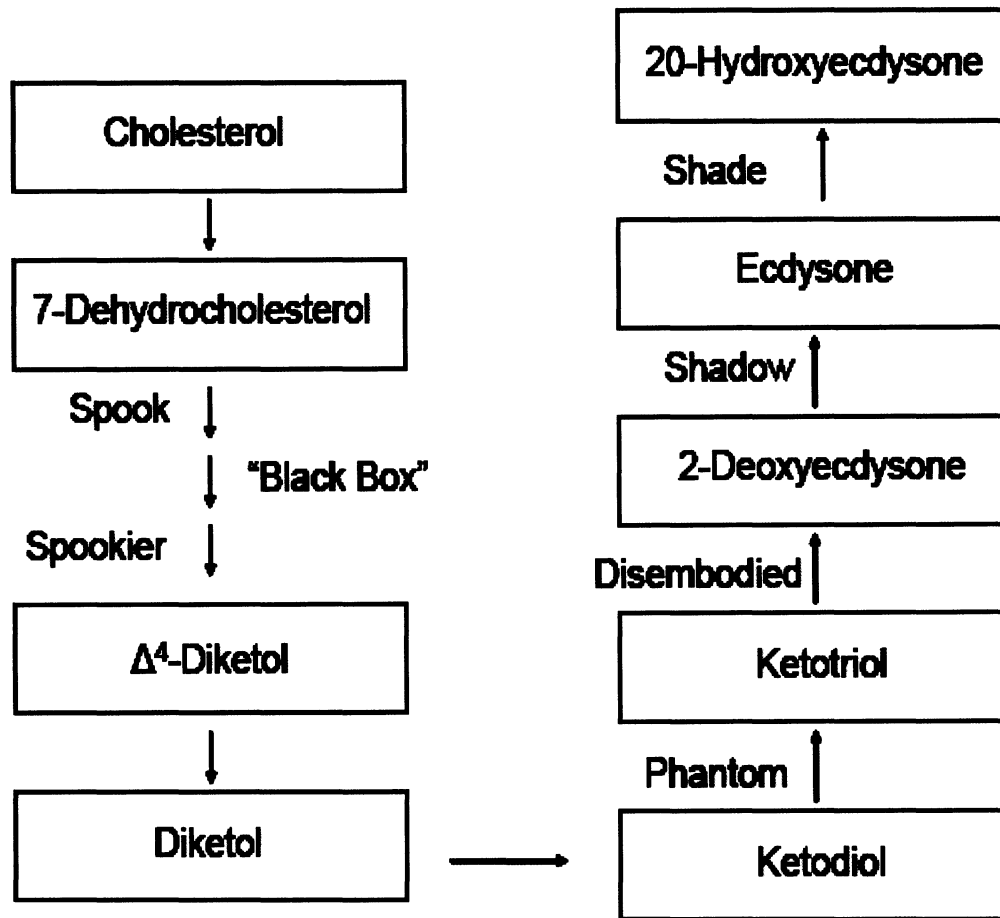
Figure 1.1 **Biosynthesis of 20E in Drosophila.** The Halloween enzymes including Spo, Spok, Phm, Dib, Sad, and Shd carry out ecdysone biosynthesis. Spo and Spok belong to the "Black Box", which includes additional unknown enzymes. The conversion of cholesterol to 7-Dehydrocholesteror is carried out by a 7,8-Dehydrogenase, which is encoded by an unknown gene. The gene encoding the proposed 5β[H]-reductase, which converts Δ^4-Diketol to Diketol is also unknown. Similarly, the conversion of Diketol to Ketodiol is also carried out by the proposed 3-Dehydroecdysteroid-3β-Reductase encoded by an unknown gene (Huang et al., 2008).

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allow targeting to the ER (Namiki et al., 2005). Colocalization studies confirmed that Spo localizes to the ER and not the mitochondria (Namiki et al., 2005).

The lack of *spo* expression in the PG led to the hypothesis that this enzyme might be encoded by a second gene, which was confirmed by identification of a second *spo*-like gene *spookier* (*spok*) (Ono et al., 2006). *In situ* analysis revealed *spok* expression in the embryonic and larval PG, similarly to *dib*, *phm*, and *sad* (Ono et al., 2006). Unlike *spo*, *spok* is missing a targeting sequence and no colocalization studies were performed to identify where in a cell it functions (Ono et al., 2006). Biochemical studies did not lead to identification of where in the ecdysone biosynthesis pathway *spok* functions (Ono et al., 2006).

1.3.1e. Shade (*shd*)

The final step in ecdysone biosynthesis is the conversion of ecdysone to 20-hydroxyecdysone (20E), which is the biologically active form of the hormone (Petryk et al., 2003). This final step is carried out by a 20-monooxygenase, which in Drosophila is encoded by *shd* (Petryk et al., 2003). In situ hybridization experiments revealed that *shd* is expressed in peripheral tissues and they include the fat body, the gut, and the Malphighian tubules (Petryk et al., 2003).

Surprisingly, *shd* transcripts were not detected in the brain, the ventral ganglion, or the RG (Petryk et al., 2003). However, this expression pattern was consistent with the expression patterns of 20-monooxygenase in other insects. Further analysis also revealed that *shd* mutants have normal levels of ecdysone

but reduced levels of 20-hydroxyecdysone (Petryk et al., 2003). Colocalization studies of Shd revealed it resides in the mitochondria; however Shd also has a hydrophobic signal that can target the protein to the ER (Petryk et al., 2003). This 'hybrid' nature of the protein can possibly allow for altered localization depending on tissue and developmental stage (Petryk et al., 2003).

Because developmental transitions in Drosophila are controlled by ecdysone, and the Halloween enzymes synthesize the hormone, it was hypothesized that transcription of the Halloween genes would be very tightly regulated to ensure ecdysone synthesis only at the appropriate time points in development. What regulates ecdysone synthesis in Drosophila?

1.3.2 Regulation of ecdysone synthesis in Drosophila

It is well established that ecdysone regulates the developmental transitions of molting and metamorphosis in Drosophila. If ecdysone synthesis is delayed, one would expect a delay in the onset of molting and metamorphosis. In contrast, if ecdysone synthesis is advanced, one would expect advancement in the onset of molting and metamorphosis. Therefore, I predicted that the factors that affect the timing of developmental transitions will affect the timing of ecdysone synthesis. I tested this hypothesis in chapters 3 and 4.

1.3.2a Nutrition regulates developmental timing in Drosophila

It was first reported in the 1960s that Drosophila third instar larvae removed from their normal food (containing yeast, sugar, cornmeal, and agar) to food lacking yeast exhibited delays in their development (Robertson 1960). It was later shown that larvae grown on food without yeast underwent metamorphosis about 2 days later then wild type (Tu and Tatar, 2003). More recently it was shown lowering or eliminating yeast from the larval diet extended larval development (Layalle et al., 2008). In addition to the delay in onset of metamorphosis, these larvae also have decreased body size.

These observations strongly suggest that nutrition plays a critical role in regulation of the timing of metamorphosis, and therefore in the timing of ecdysone synthesis. But what is the mechanism of this control? In both humans and Drosophila, insulin acts as a sensor of the nutritional state of an organism. Therefore, if insulin also acts in Drosophila as a sensor of the nutritional state, I predicted that genetic manipulation of insulin will also affect the time of onset of metamorphosis. I tested this hypothesis in chapter 3.

1.3.2b Insulin growth factors (Dilps) regulate developmental timing in Drosophila

The Drosophila genome has seven different *insulin-like peptides* (*dilp1-7*) (Brogiolo et al., 2001). Dilp2, Dilp3, and Dilp5 are produced by clusters of insulin-producing cells (IPCs), which are located bilaterally in the brain lobes (Brogiolo et al., 2001). The neuronal processes of the IPCs innervate the aorta, and Dilp2, Dilp3 and Dilp5 are released into the hemolymph (Kim and Rulifson, 2004; Rulifson et al., 2002). The circulating hemolymph delivers Dilp2, Dilp3, and Dilp5 to target tissues.

The *dilp* genes reveal dynamic expression pattern: *dilp2* is produced throughout development, *dilp5* is produced in second instar and persists throughout the rest of development, and *dilp3* is produced in the second half of the third instar and persists throughout the rest of development (Brogiolo et al., 2001). Furthermore, under starvation, expression of *dilp3* and *dilp5* is greatly reduced, suggesting that nutrient dependent growth is mediated by Dilp3 and Dilp5 whereas Dilp2 may be regulating growth independently of nutrient availability (Ikeya et al., 2002).

Genetic ablation of the IPCs, and therefore loss of Dilp2, Dilp3, and Dilp5, results in developmental delays (Rulifson et al., 2002). Wild type larvae take about 5 days to proceed from embryo to pupariation, but in larvae with ablated IPCs, this time is extended to 12 days. Furthermore, the prolonged development phenotype observed in larvae with ablated IPCs can be reduced to 6 days by overexpressing a *dilp2* transgene under control of the heat-shock promoter.

Therefore, the observation that limiting nutritional content of food and loss of insulin results in a delay of developmental transitions strongly suggests that

insulin acts as a signaling molecule in Drosophila in regulating developmental transitions and hence ecdysone synthesis. Based on this observation, manipulating the insulin signaling pathway is also predicted to affect developmental timing and hence ecdysone synthesis.

1.3.3c The insulin signaling pathway affects developmental timing

The seven different Dilp peptides bind and activate the Insulin receptor (InR). The Drosophila InR (DInR), similarly to mammalian INR, is composed of two alpha and two beta subunits; the beta subunit contains the cytoplasmic tyrosine kinase, which has been shown to be activated upon insulin binding. Upon activation, the DInR binds, phosphorylates, and activates Chico (the homolog of mammalian Insulin Receptor Substrate (IRS). Flies homozygous for either *InR* or *chico* exhibit developmental delays (Garofalo, 2002).

Upon activation, the InR and Chico activate the lipase kinase PI3K. Drosophila PI3K is composed of two subunits, Dp110 and Dp60. Mutations in the gene encoding Dp110 result in larvae that are unable to initiate pupariation and they live for up to 20 days in the third instar stage (Garofalo, 2002). In addition, it has been reported that mutations in the downstream effectors of PI3K also affect the timing of developmental transitions (Garofalo, 2002). Because loss of function mutations in the components of the insulin signaling pathway result in developmental delays, this observation raises the possibility that ecdysone synthesis is altered. However, these studies were performed with

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chromosomal mutations; therefore the identity of the affected tissue responsible for the developmental delay was not determined.

We hypothesize that if the insulin signaling pathway plays a role in regulating ecdysone synthesis, hence Halloween genes transcription, manipulation of the insulin signaling pathway directly in the PG is predicted to affect ecdysone synthesis and Halloween genes transcription.

1.3.2d The Insulin signaling pathway regulates ecdysone synthesis and Halloween gene transcription

We and others have shown that misexpression of PI3K within the PG alters the time of onset of metamorphosis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005): overexpression of the constitutively active *PI3K-CAAX* in the PG advances the onset of metamorphosis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). The transcript levels of *dib* and *phm*, which encode two of the ecdysone biosynthetic enzymes, in these larvae are increased (Colombani et al., 2005). In contrast, overexpression of dominant-negative *PI3K^{DN}* in the PG delays metamorphosis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). The transcript levels of *dib* and *phm* in these larvae are reduced (Colombani et al., 2005).

Final body size is also affected by these transgenes; *PI3K-CAAX* overexpression decreases body size, at least in part as a consequence of a truncated L3 stage. In contrast, *PI3K^{DN}* overexpression increases body size at

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least in part as a consequence of a prolonged L3 stage. The evidence above and work in other insects *Samia cynthia ricini* (Yoshida et al., 1998) and *Aedas aegypti* (Riehle and Brown, 1999) suggest that Insulin-like peptides have the ability to activate ecdysone synthesis. Furthermore, these results raise the possibility that insulin maybe sufficient to activate PI3K-mediated transcription of Halloween genes, and thus ecdysone synthesis in Drosophila. I tested this possibility directly in experiments described in chapter 3.

However, the observation that larvae reared on food with limited nutritional content pupariate at a smaller body size (Tu and Tatar, 2003) suggest that another mechanism, independent of the growth rate-dependent mechanism, exists to regulate the timing of the developmental transition of metamorphosis. What is the growth rate-independent mechanism?

1.3.3 The Prothoracicotropic hormone (PTTH) regulates ecdysone synthesis and hence the timing of developmental transitions in Drosophila.

1.3.3a PTTH overview

PTTH was first isolated from the heads of *Bombyx mori* (Kataoka et al., 1991). It was demonstrated that in *Bombyx* PTTH has the ability to induce ecdysone synthesis in the PG (Kataoka et al., 1991). Biochemical analysis revealed that active PTTH is a homodimer processed from a larger precursor protein (Kataoka et al., 1991). It was later shown that PTTH is produced in a pair

of neurosecretory cells located bilaterally in the brain lobes (Agui et al., 1979; Dai and Gilbert, 1991; Kawakami et al., 1990). The neuronal process of these cells innervate the corpus allatum, from which PTTH is released into the hemolymph (Gilbert et al., 2002). PTTH then binds to its receptor and stimulates ecdysone synthesis by activating the Ca^{2+}, cAMP, and the MAPK pathways in *Manduca sexta* (Rybczynski and Gilbert, 2003). Studies in *Manduca* and *Bombyx* prompted the quest to identify PTTH in Drosophila.

1.3.3b PTTH regulates ecdysone synthesis in Drosophila

First attempts to purify the Drosophila PTTH were not successful (McBrayer et al., 2007). Two peptides were identified but they showed little sequence homology to the PTTH molecule isolated from other insects (McBrayer et al., 2007). However, once PTTH sequences of additional insects were reported, a database search revealed a Drosophila gene with some sequence homology (McBrayer et al., 2007). Moreover, the length of the gene and a very highly conserved seven cysteine motif was identified in the Drosophila gene (McBrayer et al., 2007). In other insects, six of the cysteines form disulfide bridges, forming a cysteine knot type structure also present in other neuropeptides (for example the TGF-Beta, TGF, and PDGF) (McBrayer et al., 2007). The seventh cysteine links the two monomers to form a heterodimer, the biologically active molecule (McBrayer et al., 2007).

Detailed sequence analysis of the Drosophila *ptth* gene revealed additional hallmarks of a secreted peptide (McBrayer et al., 2007). First, a dibasic (KR) sequence is present just in front of a the first conserved cysteine serving most likely as a proteolytic cleavage site necessary for processing of the precursor protein (McBrayer et al., 2007). Second, a stretch of hydrophobic residues in the N-terminal end of PTTH functions either as a signal peptide or a type II transmembrane domain (McBrayer et al., 2007). This characteristic suggests the PTTH is secreted (McBrayer et al., 2007).

Similarly to other insects, PTTH is produced in two pairs of neurosecretory cells located bilaterally in the brain lobes (McBrayer et al., 2007). However, the neuronal processes of these cells to not innervate the corpus allatum, but rather they innervate the PG directly (McBrayer et al., 2007). The axon terminals form direct contact with the PG cells, suggesting that in Drosophila, PTTH is directly released from the PTTH neurons onto the PG (McBrayer et al., 2007).

Analysis of *ptth* expression pattern during in the third instar larvae revealed that *ptth* is not expressed uniformly but rather it exhibits an 8 hour cyclic pattern (McBrayer et al., 2007). About 12 hours prior to pupariation *ptth* levels sharply increase strongly suggesting that the Drosophila PTTH regulates developmental transitions (McBrayer et al., 2007).

To understand the role Drosophila PTTH plays in regulating developmental transitions genetic ablation studies were performed (McBrayer et al., 2007). The pro-apoptotic gene *grim* was overexpressed in the PTTH neurons, resulting in a loss of these neurons (McBrayer et al., 2007).

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Surprisingly, animals with ablated PTTH-neurons were still able to initiate developmental transitions and complete metamorphosis (McBrayer et al., 2007). However detailed analysis revealed that the timing of developmental transitions was altered (McBrayer et al., 2007). The duration of each larval stage was increased: the larvae spent approximately an additional 8 hrs in the first instar stage, the second instar stage was not increased further, and the larvae spent approximately an additional 5.5 days in the third instar stage (McBrayer et al., 2007). After pupariation was initiated, the time to adulthood was not increased any further (McBrayer et al., 2007). Even though the larvae were able to complete metamorphosis, the death rate of the ablated animals was increased compared to the wild type or mock-ablated controls (McBrayer et al., 2007).

1.3.3c PTTH regulates Halloween genes expression

It was also observed that the animals with ablated PTTH-neurons formed much larger puparia, and the adults exhibited increased body size (McBrayer et al., 2007). Similarly, third instar larvae in which the PTTH neurons were ablated were much larger that their wild type counterpart (McBrayer et al., 2007). This phenotype is often observed in larvae with low ecdysone titers (see above), raising the possibility that loss of PTTH decreases ecdysone synthesis hence Halloween gene expression (McBrayer et al., 2007).

Analysis of Halloween gene expression in larvae with ablated PTTH neurons revealed that transcript levels of ecdysone biosynthetic genes were

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reduced (McBrayer et al., 2007). Ecdysone feeding experiments rescued the prolonged development phenotype and the PTTH-ablated animals initiated metamorphosis at the same time as the wild type animals confirming that the defect reflected the inability to produce ecdysone and not the ability to respond to it (McBrayer et al., 2007). These observations suggest that PTTH is necessary to induce ecdysone synthesis at the appropriate time (McBrayer et al., 2007). How does PTTH activate ecdysone synthesis?

1.3.3d PTTH activates the Ras/Raf pathway in the Drosophila PG

We have shown that misexpression of the Ras/Raf pathway in the PG affects body size and the timing of onset of metamorphosis (Caldwell et al., 2005). Specifically, expressing the constitutively active Ras^{V12} or Raf^{GOF} in the PG decreases body size and advances the onset of metamorphosis, whereas inhibiting Ras or Raf by expressing the dominant-negative Ras^{N17} or Raf^{DN} in the PG increases body size and delays the onset of metamorphosis (Caldwell et al., 2005). We have also shown that these phenotypes were a consequence of altered ecdysone synthesis; overexpression of Ras^{V12} or Raf^{GOF} in the PG advances ecdysone synthesis whereas overexpression of dominant negative Raf^{DN} delays ecdysone synthesis (Caldwell et al., 2005). These observations strongly suggest that the MAPK pathway is involved in regulating ecdysone synthesis and presumably Halloween genes expression. Based on the fact that

the Ras and Raf are involved in ecdysone synthesis, we also predicted that the PTTH receptor would be a Receptor Tyrosine Kinase (RTK).

Several years later, Rewitz et al. (2009) investigated the identity of the PTTH receptor and made several observations ultimately identifying the RTK Torso as this receptor (Rewitz et al., 2009). First, Torso was a good candidate because it is expressed exclusively in the PG during larval development and its ligand Trunk has similar architecture to PTTH (Rewitz et al., 2009). This observation was consistent with the possibility that Torso can bind PTTH. Second, to test the prediction that *torso* encoded the PTTH receptor, Rewitz et al. (2009) examined the effects of RNAi-mediated *torso* knockdown in the PG and found that *torso* knockdown delayed pupariation about 5 days and increased body size to a level similar to larvae with ablated PTTH neurons (McBrayer et al., 2007; Rewitz et al., 2009). Third, similarly to the *ptth-* ablated animals, ecdysone feeding rescued the developmental delay conferred by *torso* knockdown (Rewitz et al., 2009). Taken together, these observations strongly suggested that Torso is the PTTH receptor.

Consistent with our previous data (Caldwell et al., 2005) Rewitz et al. (2009) also reported that RNAi-mediated knockdown of Ras, Raf, and Erk specifically in the PG also delayed metamorphosis and consequently increased body size (Rewitz et al., 2009). Finally, overexpression of Ras^{V12} in the PG not only rescues the delayed metamorphosis conferred by *torso* knockdown, but actually advances the onset of metamorphosis and decreases body size as we described previously (Rewitz et al., 2009).
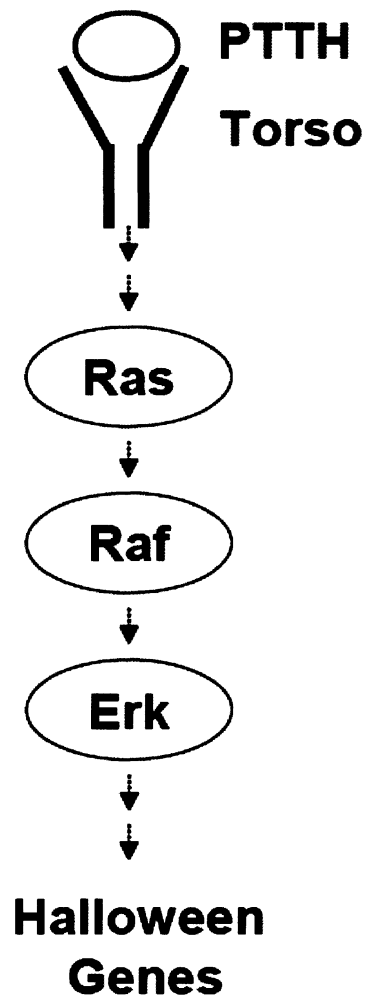
Figure1.2 **A model for PTTH signaling in Drosophila.** Upon PTTH release from the PTTH-producing neurons, PTTH binds and activates the RTK receptor Torso. This activation leads to Ras activation, which in turn activates Raf. Raf activates Erk, and Erk, via unknown transcription factor(s), activates Halloween gene transcription.

Therefore, PTTH, acting via Torso and the MAPK pathway, ensures the appropriate timing of ecdysone synthesis most likely via regulation of Halloween gene transcription. Because PTTH plays an important role in timing developmental transitions, how is PTTH synthesis regulated?

1.3.3e Retinoids regulate of *ptth* expression

The insulin growth factors, via PI3K, signal nutrient availability to the PG. What signals could PTTH communicate via the Ras and Raf pathway? Many reports have indicated that damage to imaginal discs, either induced by X-irradiation (Halme et al., 2010) or transgenes that inhibit translation (Stieper et al., 2008) or transgenes that induce apoptosis (Halme et al., 2010), delay the onset of metamorphosis, presumably to give the larva additional time to repair the damaged discs.

Recently, the signal used by discs to communicate damage was identified as the molecule retinoic acid: mutations in the retinoic acid biosynthetic pathway partially prevent the developmental delays conferred by disc damage (Halme et al., 2010). Furthermore, it was found that retinoic acid delays the onset of metamorphosis by inhibiting transcription of PTTH (Halme et al., 2010). These results suggest that PTTH communicates completion of a "healthy imaginal disc" checkpoint to enable ecdysone synthesis, and hence metamorphosis, to proceed.

1.3.3f The Pigment Dispersion Factor (PDF) regulates *ptth* expression

PDF is a small neuropeptide that responds to circadian rhythms in Drosophila (Williams and Sehgal, 2001). PDF is present in a set of lateral neurons (Williams and Sehgal, 2001). The mRNA levels of *pdf* do not cycle (Williams and Sehgal, 2001). PDF protein levels are constant in the cell body but cycle at the axon terminals raising the possibility of cyclic release (Williams and Sehgal, 2001). At the axon terminals PDF levels peak in the early part of the day and dip at night (Williams and Sehgal, 2001). More recently it was shown that PDF might be involved in transcriptional regulation of *ptth* (McBrayer et al., 2007). First, it was observed that the PDF-producing neurons form synapses with the dendritic arbors of the PTTH–neurons (McBrayer et al., 2007). Second, *ptth* levels were significantly increased in the *pdf* null mutant (McBrayer et al., 2007). Third, it was shown that the 8 hr periodicity observed in the wild type flies was gone in the *pdf* mutants (McBrayer et al., 2007). These observations suggest that PDF may be acting as a repressor of *ptth* transcription as well a controller of the timing of *ptth* transcription.

1.3.4 β Fushi Tarazu-Transcription Factor 1 (βFTZ-F1) regulates developmental timing and *dib* and *phm* transcription

The experiments described above demonstrated that altering PI3K or Ras/Raf in the PG, or ablating the PTTH neurons, affect transcription of

Halloween genes. These observations made it of interest to look for transcription factors that might participate in this regulation. One of such a candidate is βFTZ-F1. βFTZ-F1 belongs to the nuclear receptor superfamily (King-Jones and Thummel, 2005). Its ligand/ligands are unknown; therefore βFTZ-F1 is referred to as an orphan nuclear hormone receptor (King-Jones and Thummel, 2005). The *βftz-f1* gene gives rise to two isoforms the *αftz-f11* and *βftz-f1* (Lavorgna et al., 1993; Lavorgna et al., 1991). Both proteins share a common C-terminal region, which contains the nuclear hormone receptor-specific DNA-binging motif and the ligand/dimerization domain, the N-terminus, however, is distinct for these isoforms (Lavorgna et al., 1993; Lavorgna et al., 1991).

The *αftz-f1* and *βftz-f1* isoforms also exhibit distinct expression pattern: *αftz-f1* is maternally deposited to the egg and functions in the early embryo (Guichet et al., 1997; Yu et al., 1997). In contrast, *βftz-f1* is expressed in late embryos and during larval, prepupal, and pupal development (Lavorgna et al., 1993). Furthermore, antibody staining experiments revealed that βFTZ-F1 is present in the nuclei of most larval tissues, which included the salivary gland, fat body, trachea, epidermis, guts, Malpeghian tubules, and the RG (Yamada et al., 2000). Moreover, this expression pattern is temporally restricted to periods prior to a molt, or in prepupae (Yamada et al., 2000).

1.3.4a βFTZ-F1 serves as a competence factor

Competence has been defined as a cell's ability to undergo a developmental response triggered by a signal (Waddington, 1940). Competence can be achieved by preparing a cell to receive a signal, for example, by expressing the appropriate receptors and cofactors for the incoming signal (Broadus et al., 1999).

Elegant studies suggested that βFTZ-F1 acts as a competence factor during Drosophila development. First, βFTZ-F1 is present prior to increases in ecdysone titers, either prior to molts, initiation of pupariation or during mid-prepupae stage (Lavorgna et al., 1993) and *βftz-f1* transcription is repressed by ecdysone, hence ensuring appropriate timing of *βftz-f1* expression (Woodard et al., 1994) fulfilling the requirements for providing competence (Richards, 1976). Second, βFTZ-F1 antibody staining of the salivary gland polytene chromosome showed that βFTZ-F1binds to some of the ecdysone-regulated puffs (Lavorgna et al., 1993). Third, ectopic *βftz-f1* overexpression has small effect on transcription of its target genes (Woodard et al., 1994). However, in the presence of ecdysone, βFTZ-F1 enhances transcription of the ecdysone-induced target genes (Woodard et al., 1994). Furthermore, a loss-of-function *βftz-f1* mutant is unable to induce the appropriate target genes even in the presence of ecdysone (Broadus et al., 1999). These four observations fulfill the criteria for a molecule to serve as a competence factor and strongly suggest the βFTZ-F1 is the

competence factor during Drosophila development (Broadus et al., 1999; Lavorgna et al., 1993; Richards, 1976; Woodard et al., 1994).

1.3.4b βFTZ-F1 is required for *dib* and *phm* expression

Because βFTZ-F1 is the Drosophila orthologue of mammalian steroidogenic factor (SF-1), which activates transcription of genes encoding cytochrome P450s, and because the Halloween genes encode cytochrome P-450 enzymes, Parvy et al. (2006) hypothesized that βFTZ-F1 might be the direct transcriptional activator for the Halloween genes. To test this possibility, Parvy et al. (2006) used mitotic recombination in early embryonic development to induce third instar larvae with PGs that were mosaic for *βftz-f1* mutant and wild type. Then they used antibodies against *dib* and *phm* to determine if the clones lacking *βftz-f1* also lacked *dib* and *phm* immunoreactivity (Parvy et al., 2005). They found that this was indeed the case.

This observation demonstrates that βFTZ-F1 is required cell autonomously for *dib* and *phm* expression. However, βFTZ-F1 was found in the PG only early in L3 (16-24 hours after the L2/L3 molt), and was not detectable late in L3 when Halloween genes expression is maximally induced. These observations argue against the possibility that βFTZ-F1 is a direct activator of Halloween gene expression. Rather, these results indicate that βFTZ-F1 most likely provides competence for the PG to respond to the incoming PTTH signal.

In chapter 4 of this dissertation I describe my experiments identifying the mechanism by which βFTZ-F1 promotes Halloween gene expression.

1.3.4c βFTZ-F1 regulates *broad* transcription

Br belongs to the ecdysone-induced "early genes", which are directly activated by ecdysteroids (King-Jones and Thummel, 2005). The *br* gene encodes four different isoforms, *br-z1, br-z2, br-z3,* and *br-z4*, which each belong to the Broad-Tramtrack-Bric-a-brac (BTB) family of zinc finger transcription factors. The alternatively spliced C-terminus encodes four zinc fingers, which most likely bind the DNA. The N-terminus is shared by all isoforms and it is required for protein-protein interactions.

Immunohistochemistry experiments revealed that Br is expressed in all tissues during metamorphosis (Zhou et al., 2004). Furthermore, immunohistochemistry studies within the PG revealed a dynamic expression pattern of each isoform (Zhou et al., 2004). Specifically, Br-Z2 and Br-Z3 appear in the prothoracic gland about 17 hours after the L2-L3 molt and are maintained until pupariation (Zhou et al., 2004). Because these isoforms appear well before the major peak of ecdysone that triggers metamorphosis, and because levels of these isoforms show little change throughout L3, Zhou et al. (2004) suggested the possibility that their expression in not controlled by ecdysone.

However, Br-Z1 and Br-Z4 exhibit a much more dynamic expression pattern: Br-Z4 levels increase slowly at mid-L3 and then very rapidly at

wandering, Br-Z1 is detected at wandering, and peaks at pupariation (Zhou et al., 2004). This dynamic expression pattern coincides with increasing ecdysteroid titer suggesting that *br-z1* and *br-z4* transcription are regulated by ecdysone (Zhou et al., 2004).

The hypothesis that *br-z2* and *br-z3* expression is not activated by ecdysone, taken together with the observation that βFTZ-F1 appears in the PG immediately prior to Br-z2 and Br-z3 raised the possibility that βFTZ-F1 might directly activated *br-z2* and *br-z3* expression in the PG. Previous reports support this possibility. In particular, it was previously reported that ectopic *βftz-f1* expression in a late third instar larvae increases *br* levels (Woodard et al., 1994). In addition, βFTZ-F1 directly binds at the *br* locus in salivary gland polytene chromosomes (Woodard et al., 1994). Finally, it was also observed that *bftz-f1* and *br* mutants exhibit similarities in certain phenotypes (Yamada et al., 2000). For example, both mutations in either gene arrest histolysis of the salivary gland (Yamada et al., 2000). The possibility that *βftz-f1* mutants exhibit this phenotype as a consequence of failure to express *br* is supported by the observation that ectopic *br* expression rescues this *βftz-f1* mutant phenotype (Yamada et al., 2000).

1.3.4d Br regulates transcription of the ecdysone biosynthesis genes

Animals caring a chromosomal mutation that result in loss of all *br* isoforms develop normally until L3. However, these flies are unable to initiate

metamorphosis, and rather exhibit prolonged wandering behavior (Kiss et al., 1980). This observation strongly suggests that Br is required for onset of metamorphosis, and is consistent with the possibility that Br is required for ecdysone synthesis (Kiss et al., 1980). More recently, it was shown that RNAi knockdown of *br* specifically in the PG mimics the chromosomal mutant; these larvae progress normally through development until late L3, but are unable to initiate metamorphosis (Xiang et al., 2010). The mechanism underlying this failure to pupariate was recently elucidated: both the *br* chromosomal null mutation or PG specific RNAi knockdown of *br* decrease transcript levels of all ecdysone biosynthetic genes tested (Xiang et al., 2010). In addition, the *InR* and *torso* transcript levels were also greatly reduced, raising the possibility that Br can directly and/or indirectly activate transcription of the Halloween genes and thereby activate ecdysone synthesis (Xiang et al., 2010).

Based on the observations that ectopic *βftz-f1* expression increases *br* transcription (Woodard et al., 1994), that ectopic *br* expression rescues *βftz-f1* mutant phenotype in the salivary gland (Yamada et al., 2000), that βFTZ-F1 is necessary for *dib* and *phm* transcription (Xiang et al., 2010), and it is only present in the prothoracic gland in the early and mid third instar (Parvy et al., 2005), we hypothesize that in the Drosophila prothoracic gland βFTZ-F1 serves as a competence factor to induce *br* transcription, which will activate either directly or indirectly Halloween genes transcription. This hypothesis also suggests that βFTZ-F1 does not directly activate *dib* and *phm* transcription and it raises a possibility that both *dib* and *phm* are activated directly or indirectly via *br*. This

hypothesis then suggests that the initial appearance of *βftz-f1* protein in the PG is an early trigger of Halloween genes expression and ecdysone synthesis. This hypothesis further suggests that the timing of expression of *βftz-f1* in the PG might be a key regulator of the timing of ecdysone synthesis. Next I describe previous experiments elucidating the regulation of *βftz-f1* transcription.

1.3.5 Regulation of *βftz-f1* expression

White et al. (1997) made three crucial observations that suggest that Drosophila Hormone Receptor 3 (HR3) activates *βftz-f1* expression. First, they showed the temporal patterns of HR3 and βFTZ-F1 in late third instar larvae and early prepupae, which support the following mechanism: HR3 appears first, followed by an overlap of both HR3 and βFTZ-F1, with a peak of βFTZ-F1 before loss of HR3 (White et al., 1997). Second, they showed that HR3 binds directly to the locus on the salivary gland polytene chromosome (White et al., 1997). Third, they demonstrated that heat-shock induced expression of a *HR3* transgene is sufficient to induce *βftz-f1* expression (White et al., 1997). Taken together, these three observations strongly suggest that HR3 activates *βftz-f1* expression by direct binding to the *βftz-f1* promoter.

White et al. (1997) also identified another nuclear hormone receptor, the ecdysone-inducible *E75B* gene, as a participant in *βftz-f1* transcriptional regulation. In particular, White et al. showed that the decreased βFTZ-F1 levels coincided with an increase of E75B levels, and that the ability of HR3 to activate
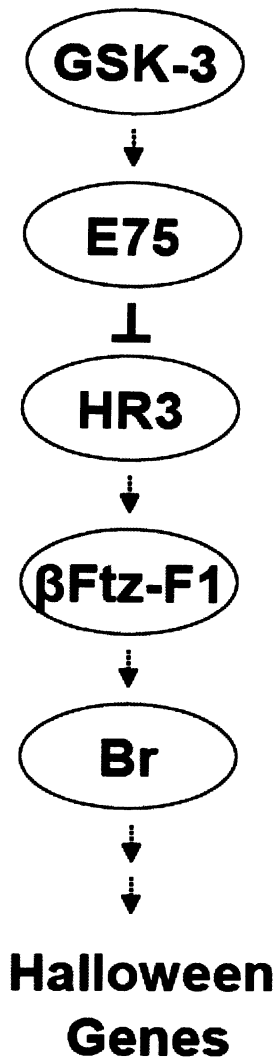
Figure 1.3 **A model for GSK-3mediated Halloween genes transcriptional activation in Drosophila.** GSK3 phosphorylates E75. This phosphorylation prevents E75 degradation. E75 binds to HR3 and prevents HR3-mediated activation of *βftz-f1* transcription. Since *βftz-f1* is not transcribed it can not activate *br* transcription, hence Halloween gene transcription.

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βftz-f1 requires a decrease in E75B levels. In addition, it was shown that E75B binds to the same sites on the polytene chromosome as HR3 and in some instances HR3 is required for E75B binding (White et al., 1997). But the most direct evidence that E75B is involved in regulating *βftz-f1* expression as an inhibitor came from the observation that larvae overexpressing both *HR3* and *E75B* do not express *βftz-f1* (White et al., 1997). However, in the absence of E75B, *βftz-f1* is expressed (White et al., 1997). This interaction between E75B and HR3 is direct: in vitro binding experiments confirmed that HR3 and E75B interact directly (White et al., 1997). These observations strongly suggest that E75B represses *HR3*, and this repression inhibits HR3 mediated *βftz-f1* activation. However, when E75B is not present, HR3 activates *βftz-f1* expression. These results suggest that the appearance of *βftz-f1* in the PG occurs following the inhibition or inactivation of E75B protein. If so, then the timing of ecdysone synthesis will be affected by the timing of E75B inactivation.

Next I describe previous experiments in which mechanisms regulating E75 levels were elucidated. The mammalian orthologue of E75, REV-ERBA, is phosphorylated by (GSK-3) (Wang et al., 2006). This phosphorylation event provides protection from proteolysis (Wang et al., 2006), thus stabilizing REV-ERBA.GSK3, in turn, is a well-established target of PI3K: activated PI3K elicits the Akt-dependent phosphorylation of GSK3 on position serine 9 (Cross et al., 1995). This phosphorylation event inhibits GSK3 activity (Cross et al., 1995).

1.3.6 Proposed molecular mechanism for critical size

Critical size is defined as a larval body size at which "the physiological processes that end in metamorphosis are irreversibly initiated and no longer delayed by undernourishment" (Stieper et al., 2008). Based on the observations above we hypothesize that when larvae reach a certain body size (critical size), insulin-dependent PI3K activation in the PG reaches threshold, active Akt then phosphorylates and inhibits GSK3, leading to the dephosphorylation and destabilization of E75B, which then permits HR3-mediated *βftz-f1* expression. Destabilization of E75B maybe the key event that signals that the larvae is now ready to initiate metamorphosis. However, when the larvae have not yet reached the appropriate size, GSK3 phosphorylates and protects from proteolysis E75B. E75B then prevents the HR3-dependent activation of *βftz-f1* transcription. Lack of *βftz-f1* leads to lack of Br, and hence lack of Torso: thus, the PG in this state is not competent to respond to the PTTH ecdysonergic signal. I hypothesize that this mechanism explicitly links attainment of a critical body size with induction of ecdysone synthesis.

Figure 1.4 **A model for PI3K-mediated Halloween genes transcriptional activation in Drosophila.** Insulin binds and activates the InR, which activates PI3K. PI3K activation leads to Akt phosphorylation and activation. Akt then phosphorylates and inhibits GSK3. This inhibition prevents E75 phosphorylation resulting in E75 degradation. Once E75 is degraded it can not bind and inhibit HR3. HR3 can now activate *βftz-f1* transcription. βFTZ-F1 activates *br* transcription and once Br is present in the PG it will activate Halloween gene transcription.

1.4 Ecdysone signaling in Drosophila

Once ecdysone is made in the prothoracic gland it is released into the hemolymph and converted into the 20-hydroxyecdysone (20E) in the peripheral tissues (Gilbert et al., 2002). 20E binding is mediated by the Ecdysone Receptor (EcR) and Ultraspiracle (Usp), which form a heterodimer (Koelle et al., 1991; Yao et al., 1993). Both EcR and Usp belong to the canonical nuclear hormone receptor family; they both have the common structural elements, including the DNA binding domain, the ligand binding domain, and a flexible linker region (King-Jones and Thummel, 2005).

The highly-conserved DNA binding domain contains two zinc fingers, which navigate DNA binding (King-Jones and Thummel, 2005). The less-conserved ligand binding domain contains a hydrophobic pocket that binds the ligand and also binds other receptors to form dimers. In addition, the N-terminus contains an activation domain that acts independently of ligand binding, whereas the C-terminal activation domain depends on ligand binding (King-Jones and Thummel, 2005). The EcR is an ortholog of either vertebrate farnesoid X receptor or liver X receptor (King-Jones and Thummel, 2005). Usp is an ortholog of the vertebrate retinoid X receptor (King-Jones and Thummel, 2005).

Both *EcR* and *usp* are encoded by single genes. However *EcR* expresses three different isoforms: *EcR-A*, *EcR-B1*, and *EcR-B2* (Talbot et al., 1993) whereas *usp* expresses only one (Oro et al., 1990). The three EcR isoforms share a common C-terminal region, which includes the DNA binding domain and

a ligand binding domain (Talbot et al., 1993). The N-terminal region varies between the different isoforms. The EcR-A protein is predicted to be 91.2 kDa, EcR-B1 to be 93.9kDa and EcR-B2 to be 73.4 kDa (Talbot et al., 1993).

Biochemical analysis revealed that the EcR-B isoforms are ligand-dependent transcriptional activators, whereas the EcR-A is a poor transcriptional activator (Hu et al., 2003; Mouillet et al., 2001). Usp lacks transcriptional activation capability on its own, but genetic studies showed that Usp by itself can function as a repressor, as is the case with the vertebrate retinoid X receptors (Zhang and Dufau, 2004).

All three EcR isoforms are expressed during postembryonic development; however, they have different tissue specificity. In general, at metamorphosis all the larval tissues exhibit strong nuclear staining of EcR-B1 and weak staining of EcR-A, with one exception being the prothoracic gland, which exhibits strong EcR-A staining. This observation raises the possibility that EcR maybe involved in ecdysone synthesis and it has been shown that in *Manduca sexta,* indeed, ecdysone acts on the prothoracic gland to inhibit its own synthesis (Gilbert et al., 2002; Sakurai and Williams, 1989).

Twenty E directly induces a set of primary response genes including the *br*, *E74*, and *E75* (Fletcher et al., 1995; Fletcher and Thummel, 1995; King-Jones and Thummel, 2005). These gene products have two roles: they repress their own transcription and activate a large set of secondary response late genes. The E74 gene encodes two transcripts: *E74A* and *E74B* (Fletcher et al., 1995; Fletcher and Thummel, 1995). Transcript levels of both *E74A* and *E74B* are

used as an indirect readout of ecdysone synthesis (Caldwell et al., 2005; Colombani et al., 2005).

Chapter 2: Methods and Materials

2.1 Fly stocks and crosses maintenance.

All fly stocks (Table 1) were maintained on the standard cornmeal/agar Drosophila media. In some cases the Drosophila larvae were reared on media with very high yeast content (35g/L) and low carbohydrate content as specified in chapter 3. For long term storage flies were maintained at 18°C, for daily use stock were kept at room temperature, and experiments were carried out either at room temperature or at 25°C or 30°C.

2.2 Developmental staging

Depending on fecundity of each genotype anywhere from five to ten mating pairs were placed in a vial for two days. The flies were then transferred to a new vial every 12 hours for 5 to 7 days. These developmentally staged larvae were used for different experiments, which are listed below. The vials were kept either at room temperature or at 25°C.

2.3 Larval collections

Developmentally staged larvae were removed from the vials, washed with water, dried with a paper towel, and collected. These larvae were then used for variety of different experiments, which are described below.

2.4 mRNA extractions

Developmentally staged larvae were placed in RNase-free tubes and transferred to the -80°C freezer. Once the larvae were frozen, they were removed from the freezer, placed on dried ice and mashed with a RNase-free pestle. Then, 1 ml of Trizol reagent (Invitrogen) was added to the sample and incubated at room temperature for 5 min. Next 0.2 ml of chloroform was added; samples were mixed, and incubated at room temperature for 3 minutes. The samples were then transferred into a microcentrifuge and spun for 20 minutes at 14 000 rpm at 4°C. The samples were then removed from the centrifuge and the top aqueous layer was collected and placed in a new RNase-free tube. Then, 0.5 ml of RNase-free 2-propanol was added to each tube, the samples were mixed and incubated at room temperature for 10 minutes. Samples were transferred to the centrifuge and spun at 12 000 g for 10 minutes at 4°C. The solution was removed from the tubes without disturbing the pellet. The samples were washed with 70% RNase-free ethanol and spun again at 8 000 g for 5 minutes at 4°C. The ethanol solution was removed and samples were dried. Once dried, the samples were resuspended in nuclease free water and incubated

at 55°C for 10 minutes. The RNA concentration was then measured using a Ultraspectophotometer 2100 pro (Biochrom, Ltd.).

2.5 Reverse transcription

Using the Invitrogen Super Script III reverse transcription kit, mRNA was transcribed into cDNA. Two µg of RNA, 1 µl of 50 µM oligo dT, 1 µl of 10 mM dNTPs, and water to the total volume of 10 µl was placed in a PCR tube. The tubes were incubated at 65°C for 5 minutes and then placed immediately on ice for two minutes. A mix containing 4 µl of 25 mM MgCl₂, 2 µl of 0.1M DTT, 2 µl of 10 Xbuffer, 1 µl of 200U/ µl Super Script III reverse transcriptase, and 1 µl of 40U/ µl RNase out was added to each sample and the samples were incubated for 50 min at 50°C followed by 5 minutes of 85°C, and 5 minutes on ice. One µl of 2U/ µl RNase H was added to the samples and the samples were incubated at 37°C for 20 minutes. The samples were then placed on ice, 80 µl of water was added and the samples were used for the quantitative-real time PCR (q-PCR).

2.6 Quantitative PCR (Q-PCR)

Q-PCR reactions were set up either in 50 µl or 25 µl total volume (half reactions). For the 50 µl reactions 5 µl of cDNA, 0.9 µl of each oligo (Table 2), 1.25 µl of probe (Table 3), 25 µl of master mix (Applied Biosystems), and 16.95 µl of water was added. For the half reactions all ingredients were cut in half. The

reactions were run using the Applied Biosystems 7000 and the Applied Biosystems 7500. All the biological samples were run in triplicates and either one or two biological samples were measured. The sequences of the oligos, probes and primer and probes sets are listed in Tables 2.2-2.4.

2.7 Larval dissections

Larvae at appropriate developmental stage were selected and placed on a dissection plate containing 1XPBS under a dissecting microscope. Using magnetic insect pins, larvae were pinned ventral side down. Larvae were then opened and all tissues other then the epidermis, brain lobes, ventral ganglion and the ring gland were discarded. These larvae were then used for assessing the size of the ring gland or immunohistochemistry.

2.8 Immunohistochemitry

Dissected larvae were fixed in 4% formaldehyde and 1XPBS-TritonX (PBS-T) solution for 15 minutes in a safety hood. After the 15 minutes the fixing solution was removed, disposed of, and 1XPBS-T solution was applied for 10 minutes to wash the residual formaldehyde. This process was repeated 3 times. After the washes, the fixed larvae were placed in 0.5 mL tubes containing the appropriate primary antibody dilution in 1XPBS-T solution. The larvae were incubated in the primary antibody overnight at 4°C. After this process was

completed the primary antibody solution was removed and three ten minutes washes with 1XPBS-T followed. Once the washes were completed the appropriate secondary antibody was added. The larvae were incubated with the secondary antibody for 2 hours at room temperature. The secondary antibody was then removed and 3 washes in 1XPBS were done to remove the nonspecifically bound antibody. The larvae were then placed back on the dissection plate under the dissecting microscope and secondary dissections were performed: either the ring gland alone or the ring gland and brains lobes were dissected out onto a slide containing mounting medium (Vectashield).

2.9 Ring gland, prothoracic gland, and nuclear size.

Dissected larvae were fixed in 4% formaldehyde and 1XPBS-T solution for 15 minutes in a safety hood. After the 15 minutes the fixing solution was removed, disposed of, and 1XPBS-T solution was applied for 10 minutes to wash the residual formaldehyde. This process was repeated 3 times. After the washes secondary dissections were performed and the ring glands or the ring glands with the brain lobes were placed on a slide containing mounting media with DAPI (UltraCruz, Santa Cruz Biotechnology, Inc.), which stains chromatin and allows visualization of the nuclei under fluorescent light.

2.10 Microscopy

Immunochistochemistry and ring gland size experiments were visualized using Zeiss Axioplan 2 with Metamorph. Images were taken with either the 20X or the 40X objective. Zeiss confocal laser-scaning microscope was used for some of the publication quality images.

2.11 Developmental timing measurements

Developmentally staged larvae were grown at 25°C. Once the animals started to pupariate, the number of new pupae form was recorded in 12 hour intervals. When pupariation was completed the percentage of pupae at any given time was calculated and graphed. For publication purposes ten biological samples for each genotype were scored.

2.12 Adult weight measurements

Four days after eclosion adults were collected, etherized, and weighted on a laboratory scale. Females and males were weighted separately. At least ten females and ten males of each biological sample were measured and at least 5 biological samples of each genotype were measured.

2.13 Growth rate measurements

Developmentally staged larvae were grown at 25°C until the appropriate developmental stage. The larvae were then removed from the vial, washed, dried, and weighed on a laboratory scale. Unless the measured sample did not contain 10 larvae, all the weight measurements were performed with N=10. At least 5 biological samples of each genotype were measured.

2.14 Pupal length measurements

Pupae from not crowded vials were selected and measured using a microscope. The pupae were then placed in individual vials and once they eclosed the sex of the animals was determined by visual inspection. For the pupal length measurement at least 10 pupae of each genotype and sex were measured.

2.15 Western blot analysis

Developmentally stagged larvae were collected, frozen and incubated on ice for 15 minutes in lysis buffer (120 nM NaCl, 50 mM Tris pH=7.2, 2 mM EDTA, 15 mM $Na_4O_7P_2$, 20 mM NaF, 1.5 µM pepstatin A, 2.3 µM leupeptin and 100 µM PMSF). Samples were centrifuged at 18,000 g for 30 minutes at 4°C. Equal

amounts of protein lysates were aliquoted, 2× sample buffer was added, and samples were boiled for 5 min. Samples were then resolved on 8% SDS-PAGE gel and transferred to nitrocellulose (Walkiewicz and Stern, 2009). The blots were blocked in 5% non-fat milk and incubated with rabbit anti-*Drosophila* pAkt antibody at 1:1000 (Cell Signaling Technologies) and rabbit anti-Akt antibody at 4°C overnight (Walkiewicz and Stern, 2009). Anti-vinculin at 1:500 was used as a loading control (Santa Cruz Biotechnology, Inc.). HRP-conjugated secondary antibodies were used and chemiluminescence was detected on film, and digitized (Walkiewicz and Stern, 2009).

2. 16 The GAL4-UAS system

The GAL4-UAS system, also knows as the "fly geneticist's swiss army knife" (Duffy, 2002) allows for targeted gene manipulation in Drosophila. GAL4, a yeast transcription factor, encodes an 881 amino acid protein that binds a specific sequence termed the Upstream Activating Sequence (UAS) (Duffy, 2002). It was then shown that GAL4 can stimulate transcription of a reporter gene that is under the control of the UAS in Drosophila and that GAL4 overexpression in Drosophila has no phenotypic consequences (Fischer et al., 1988). These two observation led to the development of the GAL4/UAS system for targeted gene expression in Drosophila (Brand and Perrimon, 1993). In this system two fly lines are crossed together: one line carries the GAL4 element inserted in either the promoter region or a gene region (driver line) and one line

that carries the UAS flanked by either a reporter gene (LacZ, GFP, or RFP), or a gene of interest (responder line) (Brand and Perrimon, 1993). The experiments are then performed on the progeny produced by the cross between the Gal4 and the UAS lines. GAL4 is temperature sensitive, which allows for different expression levels (Duffy, 2002). With the development of RNA interference (RNAi), the GAL4-UAS system also allows for a tissue specific knockdown of a protein of interest (Duffy, 2002). All the Gal4 drivers and UAS-transgenes are listed in Table 2.1.

Table 2.1. Gal4 drivers and UAS transgenes used in experiments described below.

Gal 4 drivers:	Chromosome	Expression pattern
amnesiac^{c651}(amn ^{c651})	X	Protoracic gland, neurons
amn ^{c651}Gal4Gal80^{TS}	X	Protoracic gland, neurons
armadillo (arm)	II	Ubiquitous
daughterless (da)	III	Ubiquitous
DDC	III	Seratonergic and dopanergic neurons
dilp2	II	Insulin producing cells (IPC)
elav	X	Nervous system
phantom (phm)	II, III	Prothoracic gland
phantom>dicer (phm>dicer)	III	Prothoracic gland
UAS transgenes:		**Note: obtained from**
UAS- βFTZ-F1	(III)	Constructed by John Merrian Sent by Craig Woodard
UAS- βFTZ-F1^{RNAi}	II	VDRC
UAS-BR-Z3	III	Lyn Riddiford
UAS-BR-Z4	III	Lyn Riddiford
UAS-Cdk4, UAS-CycD	II	Bruce Edgar
UAS-Creb2^{DN}	II	John Kiger
UAS-dilp2	II	Gyunghee Lee
UAS-E75A	II	Henry Krause

UAS-E75^{RNAi}	III	VDRC
UAS-Gsk-3^{S9A}	II	Bloomington Stock Center
UAS-Hr-3	III	Henry Krause
UAS-Hr-3^{RNAi}	II	VDRC
UAS-LacZ	III	Bloomington Stock Center
UAS-myc	II	Bloomington Stock Center
UAS-p35	III	Andreas Bergmann
UAS-PI3K-CAAX	X	Sally Leevers
UAS-PI3K^{DN}	II	Sally Leevers
*UAS-PKAR**	II	Dan Kalderon
UAS-PTEN	III	Sally Leevers
UAS-Raf^{DN}	III	Bloomington Stock Center
UAS-Raf^{GOF}	III	Bloomington Stock Center

Table 2.2. Names and sequences of primers used in the Q-PCR experiments.

Primer Name	Primer Sequence
E74A-F	5'GTTGCCGGAACATTATGGATATA3'
E74A-R	5'GCCCTATGTCGGCTTGCT3'
E74B-F	5'ATCGGCGGCCTACAAGAAG3'
E74B-R	5'TCGATTGCTTGACAATAGGAATTTC3'
Dib-F	5'GCCCAAGCTCACCAGATTGA3'
Dib-R	5'TGCAGACGAGCTCCAAAGGT3'
RPL13A-F	5'TCCGTGCGGTTCGTAAAAAT3'
RPL13A-R	5'TGGCCGCGACCATCA3'

Table 2.3. Names and sequences of probes used in the Q-PCR experiments.

Probe Name	Probe Sequence
E74A-Probe	5′FAM-CTTGAGATGAGGCCGCA-MGB3′
E74B-Probe	5′FAM-TTGATGAAGCGATATTACAC-MGB3′
Dib-Probe	5′FAM-TTTGGAATTAACCTGTTTGCT-MGB3′
RPL13A-Probe	5′VIC-TGGTTTGAACAGGACC-MGB3′

Table 2.4. Names and catalog numbers of primer and probes sets used in the qPCR experiments.

Primer & Probe Sets	Applied Biosystems Assay ID
Thor	Dm01842928_g1
Dilp2	Dm01822534_g1
Dilp3	Dm01801937_g1
Dilp5	Dm01798339_g1
Dilp6	Dm01829746_g1
Phantom (phm)	Dm 01844264_G1
RpII140	Dm02134593_g1
Rpl32	Dm02151827_g1

Chapter 3: Increase Insulin/Insulin Growth Factor Signaling Advances the Onset of Metamorphosis in Drosophila[1]

3.1 Inhibiting the PKA pathway in the insulin producing cells increases insulin signaling

To evaluate the effects of altered IIS on growth rate and the timing of metamorphosis, we began by altering activity of genes of the protein kinase A (PKA) pathway within the insulin producing cells (IPCs). We had two reasons for hypothesizing that altered PKA pathway activity within the IPCs might alter IIS.

First, PKA and its downstream transcription factor Creb activate transcription of the mammalian insulin gene (Eggers et al., 1998; Jhala et al., 2003) and insulin-receptor substrate 2 (Jhala et al., 2003). Second, a dwarf body size phenotype, indicative of altered IIS, is observed in Drosophila Creb and PKA mutants (Belvin et al., 1999; Li et al., 1995; Skoulakis et al., 1993). My observation that immunoreactivity to Creb is enriched in the IPCs (Figure 1A) supports the possibility of a role for PKA and Creb in IIS.

To test this possibility, I inhibited PKA signaling specifically in the IPCs by use of the Gal4/UAS system (Brand and Perrimon, 1993). In particular, I used the *dilp2-Gal4* driver, which expresses specifically in the IPCs (Ikeya et al., 2002; Rulifson et al., 2002), to induce expression of the dominant-negative *PKAR** *(Li et al., 1995)*, which encodes a PKA regulatory subunit that fails to bind cAMP and

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thus constitutively inhibits PKA activity, and $Creb2^{DN}$ transgenes (Eresh et al., 1997), which encodes the b-zip dimerization domain and blocks the ability of wildtype Creb2 to activate transcription.

I used three distinct assays to demonstrate that IPC-specific PKA pathway inhibition during larval development increases IIS. First, I measured larval weight gain, which is increased by IIS, by weighing developmentally-staged larvae at specific times after egg laying (AEL). I found that *dilp2>PKAR** and *dilp2>Creb2^{DN}* larvae grew faster than wildtype controls (Figure 1B). For example, by 108 hours AEL, *dilp2>PKAR** and *dilp2>Creb2^{DN}* were about 40% heavier than *dilp2>YFP*.

Second, I used quantitative RT-PCR (Q-PCR) on developmentally-staged larvae to measure transcript levels of the *Thor* gene, which encodes initiation factor 4E binding protein and is repressed transcriptionally by IIS (Puig et al., 2003). I found that *Thor* transcript levels were decreased in *dilp2>PKAR** and *dilp2>Creb2^{DN}* compared to *dilp2>YFP* 108 and 120 hours AEL (Figure 1C). Third, I used Western blot analysis levels on developmentally staged larvae to measure amount of the phosphorylated form of the kinase Akt (p-Akt), which is increased by IIS-mediated PI3 Kinase (PI3K) activation (Stokoe et al., 1997). I found that p-Akt levels were substantially increased in *dilp2>PKAR** and *dilp2>Creb2DN* larvae compared to *dilp2>YFP* 108 and 120 hours AEL (Figure 1D and not shown). Thus we conclude that blocking PKA pathway activity in the IPCs increases IIS.

A dilp2>YFP anti-Creb Merge

B

C

D

E

F

G
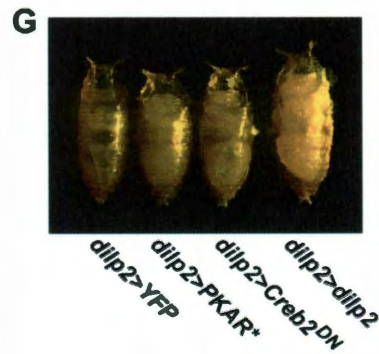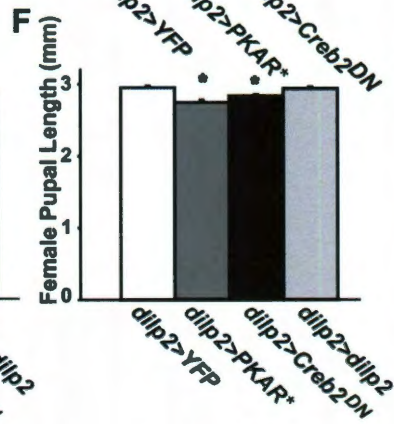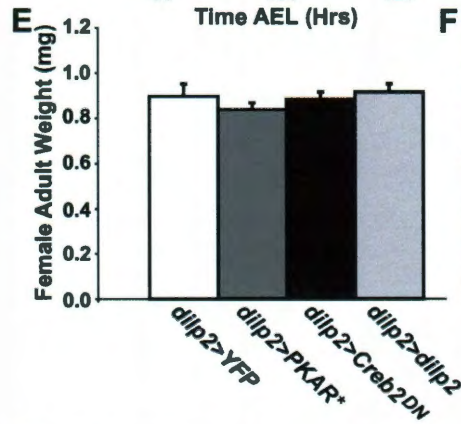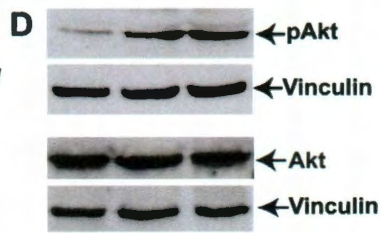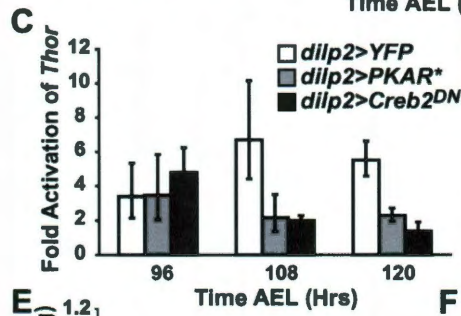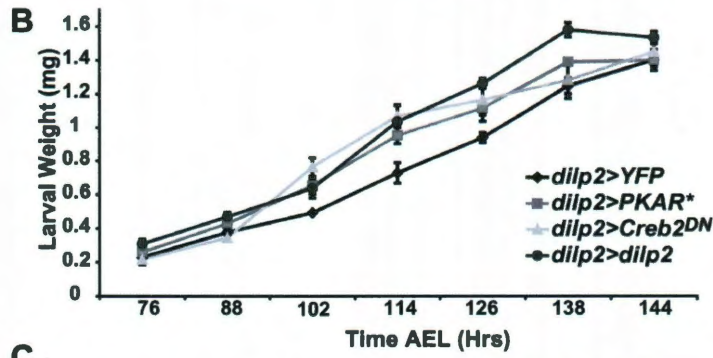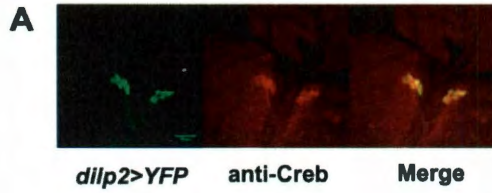
Figure 3.1 **Inhibition of the PKA pathway in the IPCs increases IIS.** (A) Brain lobes and ring gland from *dilp2>YFP* wandering third instar larva, showing enrichment of anti-Creb immunoreactivity in the IPCs. Red: anti-Creb, green: YFP. The scale bar is 35 µm. (B) Mean larval weight (y-axis) of the indicated genotypes (x-axis) from 76 to 144 hours after egg laying (AEL). Error bars represent SEM. At least 3 independent biological samples of each genotype, each containing at least 3 larvae, were measured. (C) Mean *Thor* transcript levels (y-axis) were determined from two biological samples collected from each genotype (x-axis) and measured in triplicate at the indicated time AEL. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method using the *Thor* and *RPII140* expression assay (Applied Biosystems). Error bars represent SEM. (D) Western blots from protein extracts obtained from developmentally staged larvae (108 hours AEL) of the genotypes indicated below the gels, using the antibody indicated to the right of each gel. The top two panels and bottom two panels represent blots made from separate gels. (E) Female adult weight (y-axis) of the indicated genotypes (x-axis). At least six biological samples each containing at least five flies were measured. Means+/−SEM are indicated. (F) Length (y-axis) of >10 female pupae of the indicated genotypes (x-axis). Means+/−SEM are indicated. (G) Photographs of female pupae of the indicated genotypes.

Despite the more rapid growth in *dilp2>PKAR** and *dilp2> Creb2^{DN}* larvae, final pupal and adult size was altered only modestly. The *dilp2>PKAR** and *dilp2>Creb2^{DN}* genotype did not significantly affect either female or male adult weight (Figure 1E and data not shown). However, the *dilp2>PKAR** and *dilp2> Creb2^{DN}* female and male pupae were about 7% shorter than wildtype (Figure 1F and data not shown), and the resulting decreased length to weight ratio in many cases gave *dilp2>PKAR** and *dilp2>Creb2^{DN}* pupae a "chubby" appearance (Figure 1G).

3.2 Increased IIS advances the onset of metamorphosis by precocious activation of ecdysone synthesis

Why did the increased growth rate in *dilp2>PKAR** and *dilp2>Creb2^{DN}* larvae fail to increase pupal or adult size? It was previously shown that activation of PI3K specifically within the prothoracic gland (PG) caused a precocious metamorphosis (Caldwell et al., 2005; Mirth et al., 2005) as a consequence of increased transcription of at least two ecdysone biosynthetic genes, *disembodied* (*dib*) and *phantom* (*phm*) (Colombani et al., 2005) and hence precocious ecdysone synthesis. By attenuating the duration of the larval stages, this precocious metamorphosis decreased the amount of time available for larval growth and was at least partly responsible for the resulting dwarf phenotype.

Given that PI3K is the major intracellular effector of IIS, we asked if increasing IIS might similarly cause a precocious metamorphosis via increased

transcription of *dib* and hence precocious synthesis of ecdysone. I allowed developmentally staged larvae to proceed to pupariation and found that *dilp2>PKAR** and *dilp2>Creb2^{DN}* larvae pupariated and ultimately eclosed as adults about 36 hours before *dilp2>YFP* (Figure 2A and not shown). However, the duration of the pupal stage did not appear to be significantly affected by the PKAR* and Creb2^{DN} transgenes. We suggest that the 36 hour attenuation of the larval growing stages prevented *dilp2>PKAR** and *dilp2>Creb2^{DN}* from increasing pupal or adult size despite the increased growth rate. To determine if this precocious metamorphosis resulted from increased transcription of ecdysone biosynthetic genes and precocious ecdysone synthesis, I used Q-PCR on RNA prepared from developmentally staged larvae and found that *dilp2>PKAR** and *dilp2>Creb2^{DN}* larvae exhibited greatly increased transcript levels of *dib* (Figure 2C), and induced transcription of the ecdysone-inducible reporter genes *E74B* at least 12 hours before *dilp2>YFP* (Figure 2B). These results suggest that increased IIS advances the onset of metamorphosis by activating PI3K in the PG, thus potentiating transcription of *dib* and presumably other ecdysone biosynthetic genes, and causing precocious ecdysone synthesis.

The results described above demonstrate that PKA pathway inhibition in the IPCs both increases IIS and advances the onset of metamorphosis. To demonstrate that metamorphosis is advanced as a result of the increased IIS, and not an effect of PKA pathway inhibition distinct from increased IIS, we overexpressed *dilp2* in the IPCs by driving *UAS-dilp2* with *dilp2-Gal4*. This manipulation was previously demonstrated to increase IIS (Broughton et al.,

2008). We found that *dilp2>dilp2* larvae exhibited both an increased growth rate (Figure 1B) and a precocious metamorphosis (Figure 2A) similar to *dilp2>PKAR** and *dilp2>Creb2^{DN}*. Furthermore, adult weight and pupal length were not significantly different from wildtype in *dilp2>dilp2* (Figures 1E and 1F). These observations confirm that increased IIS advances the onset of metamorphosis.

It was previously reported that increased IIS, caused by transgenes-induced overexpression of *dilps*, increases body size (Ikeya et al., 2002; Lee et al., 2008). In particular, ubiquitous overexpression of *dilps*, accomplished by the *arm-Gal4* driver, increased adult weight by up to about 50% (Ikeya et al., 2002). I found that we were likewise able to increase adult weight by ubiquitous, *arm-Gal4*-dependent *dilp2* overexpression: male and female *arm>dilp2* adults (n≥7) weighed 28% and 30% greater, respectively than *arm>YFP* adults (not shown). Thus, it appears that ubiquitous *dilp* overexpression and IPC-specific *dilp* overexpression affect body size differently.

It was previously reported that *dilp2>dilp2* adults were about 25% longer than wildtype (Lee et al., 2008). In contrast, I found no significant differences in length between *dilp2>dilp2* pupae and controls (Figures 1E and 1F). The discrepancy between our results and those previously reported might involve differences in composition of the rearing media: it was previously reported that responsiveness of flies to altered IIS appears to be exquisitely sensitive to rearing media (Broughton et al., 2008). To test this possibility, I grew larvae on media containing a high yeast concentration (35 g/L) and low carbohydrate.

A — Percentage of Puparia vs. Time AEL (hrs), with curves for *dilp2>YFP*, *dilp2>PKAR**, *dilp2>Creb2DN*, and *dilp2>dilp2*.

B — Fold Activation of *E74B* vs. Time AEL (hrs), for *dilp2>YFP*, *dilp2>PKAR**, and *dilp2>Creb2DN*.

C — Fold Activation of *dib* vs. Time AEL (hrs), for *dilp2>YFP*, *dilp2>PKAR**, and *dilp2>Creb2DN*.
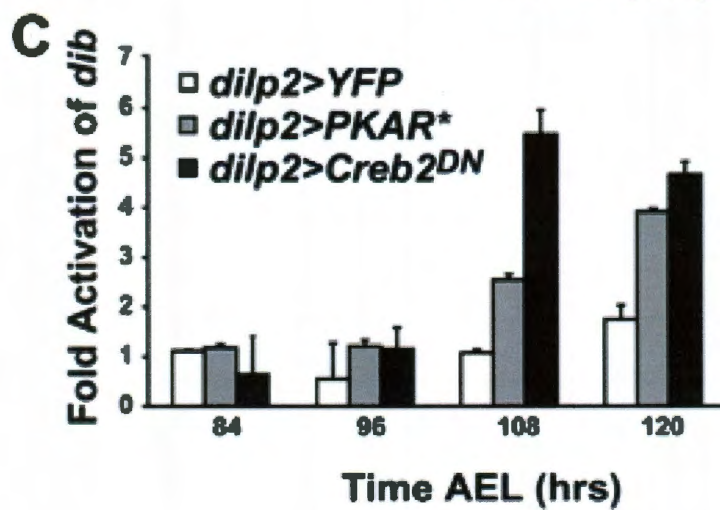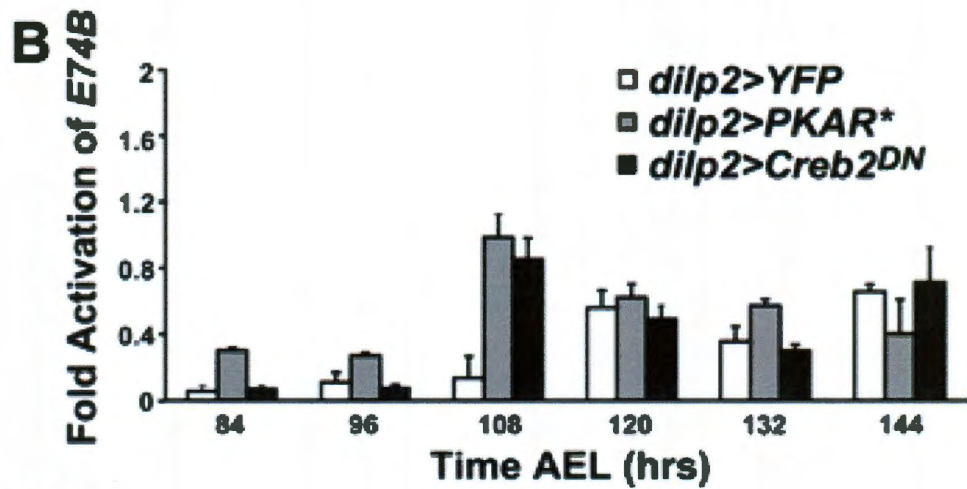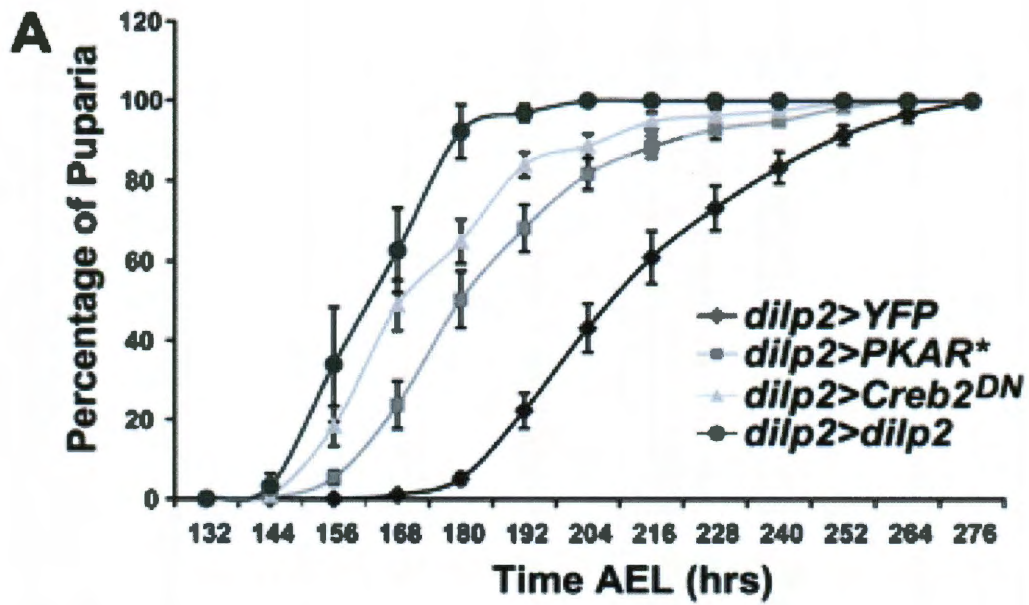
Figure 3.2 **Increasing IIS causes precocious metamorphosis.** (A) Mean fraction of developmentally staged larvae pupariating (y-axis) by the indicated time after egg laying (AEL, x-axis). Error bars represent SEM, genotypes are listed in the inset. At least six independent biological samples were measured for each data point. (B, C) Mean *E74B* and *dib* transcript levels (y-axis), respectively, were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (x-axis) and measured in triplicate at the indicated time AEL. Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A*.

I found that under these rearing conditions, *dilp2>dilp2* and *dilp2>PKAR** adult females were about 15% and 5%, heavier, respectively than *dilp2>YFP* (not shown). Furthermore, the advancement of metamorphosis reported in Figure 2A was maintained under these conditions. Thus, the effects of increased IIS on final body size are affected by the yeast/carbohydrate ratios in rearing media. However, even under these new growth conditions, we still do not observe final body size effects comparable to what was reported previously (Lee et al., 2008), suggesting that additional genetic or environmental variables affecting the responsiveness of larvae to increased IIS remain to be identified.

3.3 Increased IIS advances the onset of metamorphosis

Our results lead to three conclusions. First, IIS is a rate-limiting step for metamorphosis. It was previously shown that decreased IIS delays metamorphosis (Rulifson et al., 2002), whereas here we show that increased IIS is sufficient to advance metamorphosis even in the absence of direct changes to other hormone systems. Second, it was previously shown that transgenes-induced PI3K activation in the prothoracic gland (PG) advances metamorphosis by advancing the timing of ecdysone synthesis via transcriptional activation of ecdysone biosynthetic genes (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Here we show that increasing IIS by transgenes manipulation in the insulin producing cells confers a similar advancement of metamorphosis by a similar mechanism. Third, under conditions of extremely high growth rates, we

show that Drosophila larvae proceed through development rapidly, rather than form large pupae and adults.

3.4 A second pathway, mediated by prothoracicotropic hormone (PTTH), also regulates the timing of metamorphosis

The proper timing of metamorphosis requires a second hormone-mediated signaling pathway in addition to insulin. Genetic ablation of the neurons expressing *ptth* delays pupariation and metamorphosis as a consequence of attenuation of ecdysone biosynthetic genes transcription (McBrayer et al., 2007). It was suggested (McBrayer et al., 2007) that PTTH communicates circadian time to the PG, thus linking metamorphosis to the circadian clock. The pathways within the PG mediating the response to PTTH are not known. However, previous results demonstrating that the Ras/Raf pathway in the PG regulates the timing of metamorphosis (Caldwell et al., 2005), taken together with experiments from the tobacco hornworm *Manduca sexta* that show that PTTH application increases levels of phospho-Erk, a target of Ras/Raf, within the PG (Rybczynski et al., 2001), raise the possibility that PTTH activates ecdysone biosynthetic genes expression via the Ras/Raf pathway. In this view, full induction of ecdysone biosynthetic genes expression, and hence ecdysone synthesis and ultimately metamorphosis, requires both attainment of a specific body size and arrival at the proper time in the circadian clock, as communicated by IIS and PTTH regulating PI3K and Ras/Raf, respectively (Figure 3).
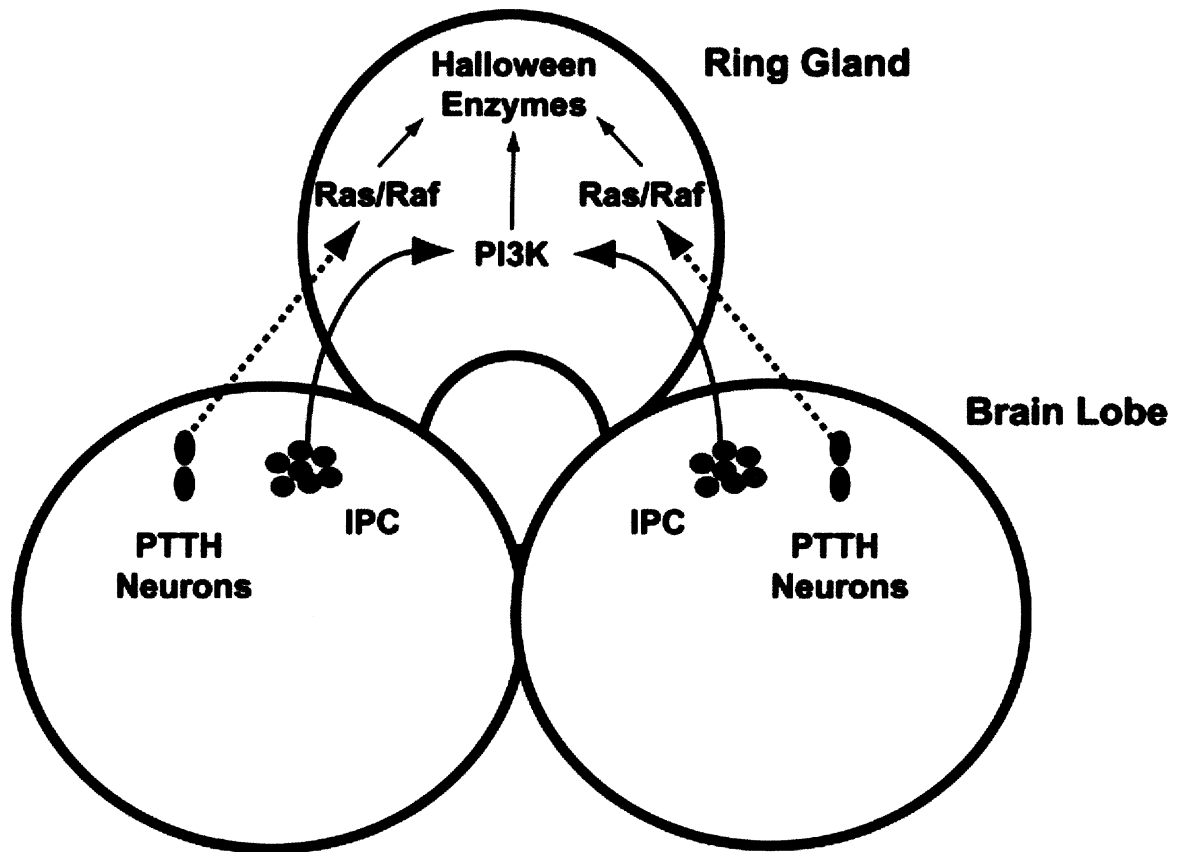
Figure 3.3 **The regulation of ecdysone synthesis by insulin and PTTH.**

Brain lobes and the ring gland are indicated. Ecdysone synthesis is triggered when insulin released from the IPCs and PTTH released from the PG-LPs activate the PI3K and Ras pathways, respectively, in the PG. These two pathways, acting together, activate transcription of the PG-specific "Halloween genes", which encode the ecdysone biosynthetic enzymes, ultimately triggering ecdysone synthesis. Solid arrows indicate activation pathways for which there is experimental evidence in Drosophila, hatched arrows indicate a speculative activation pathway.

3.5 Regulation of IIS by the PKA pathway

Our work suggests that PKA/Creb2 activity in the IPCs inhibits IIS. Although the extracellular ligands regulating IPC PKA activity are unknown, possible regulators include adipokinetic hormone (AKH), which has functional similarity to glucagon (Gronke et al., 2007; Lee and Park, 2004) and serotonin or another factor produced in serotonergic neurons, which regulates IIS in larvae (Kaplan et al., 2008). The AKH receptor is coupled to $G_\alpha s$ and activates adenylate cyclase and PKA (Gronke et al., 2007), whereas certain serotonin receptors inhibit adenylate cyclase (Noda et al., 2004). Because AKH signaling is increased by starvation (Gronke et al., 2007; Lee and Park, 2004), it seems possible that AKH might inhibit growth under nutrient limitation by activating PKA within the IPCs and hence downregulating insulin signaling.

The mechanism by which PKA/Creb2 inhibition activates IIS remains unknown. In mammals, Creb carries out both transcriptional activation and feedback inhibition of transcription at the insulin promoter (Hussain et al., 2000). Creb is also required for transcription of the insulin receptor substrate (IRS)-2 (Jhala et al., 2003). If the Drosophila IRS-2 within the IPCs is similarly decreased by $PKAR^*$ or $Creb2^{DN}$, then absence of IRS-2 could increase insulin signaling by blocking insulin responsiveness and thus the recently-described (Broughton et al., 2008) IIS-dependent negative feedback. Alternatively, PKA/Creb2 might regulate IIS by regulating the transcription of additional factors that potentiate insulin signaling, such as acid labile subunit (Arquier et al., 2008)

or ASNA-1 (Kao et al., 2007). Further experiments will be required to address these issues.

3.6 Regulating the timing of developmental transitions by growth rate in mammals

Altered growth rate, achieved by altered nutrition or endocrine disruptions, can also affect the timing of puberty in mammals. For example, genetic disruption of mouse growth hormone signaling, which decreases insulin growth factor (IGF) synthesis, delays puberty (Bartke and Brown-Borg, 2004). Similarly, delayed puberty observed in individuals with certain chronic diseases (for example, cystic fibrosis) has been attributed at least in part to difficulties in nutrient absorption, leading to disruptions in IGF signaling (Simon, 2002). The observation that improved nutrition and increased growth rate have advanced the onset of puberty in humans in the west raises the possibility that increased IIS might be sufficient accelerate developmental transitions in humans as well as flies.

Chapter 4: PI3 Kinase promotes metamorphosis by inducing competence of the prothoracic gland to respond to the PTTH metamorphic signal

4.1 PI3K and Raf control the onset of metamorphosis by regulating transcription of Halloween genes

Onset of metamorphosis is triggered by the steroid hormone ecdysone, which is synthesized within the prothoracic gland (PG) by a series of hydroxylation reactions catalyzed by a group of cytochrome P450 enzymes termed the "Halloween" enzymes (Chavez et al., 2000; Gilbert, 2004; Gilbert et al., 2002; Warren et al., 2002). Ecdysone synthesis is regulated in large part by transcriptional control of the genes encoding these Halloween enzymes: transcripts from at least two Halloween genes accumulate in the PG immediately prior to a molt (Warren et al., 2002) and every Halloween genes identified is strongly induced during the second half of the third larval instar stage (L3), immediately prior to metamorphosis (McBrayer et al., 2007). These observations lead to the hypothesis that ecdysone synthesis, and hence the onset of metamorphosis is controlled in large part by transcriptional regulation of these Halloween genes.

Both *Raf* and *PI3K* activities in the PG regulate the timing of onset of metamorphosis (developmental timing): expressing transgenes within the PG that inhibit Raf or PI3K activities delays metamorphosis, whereas expressing
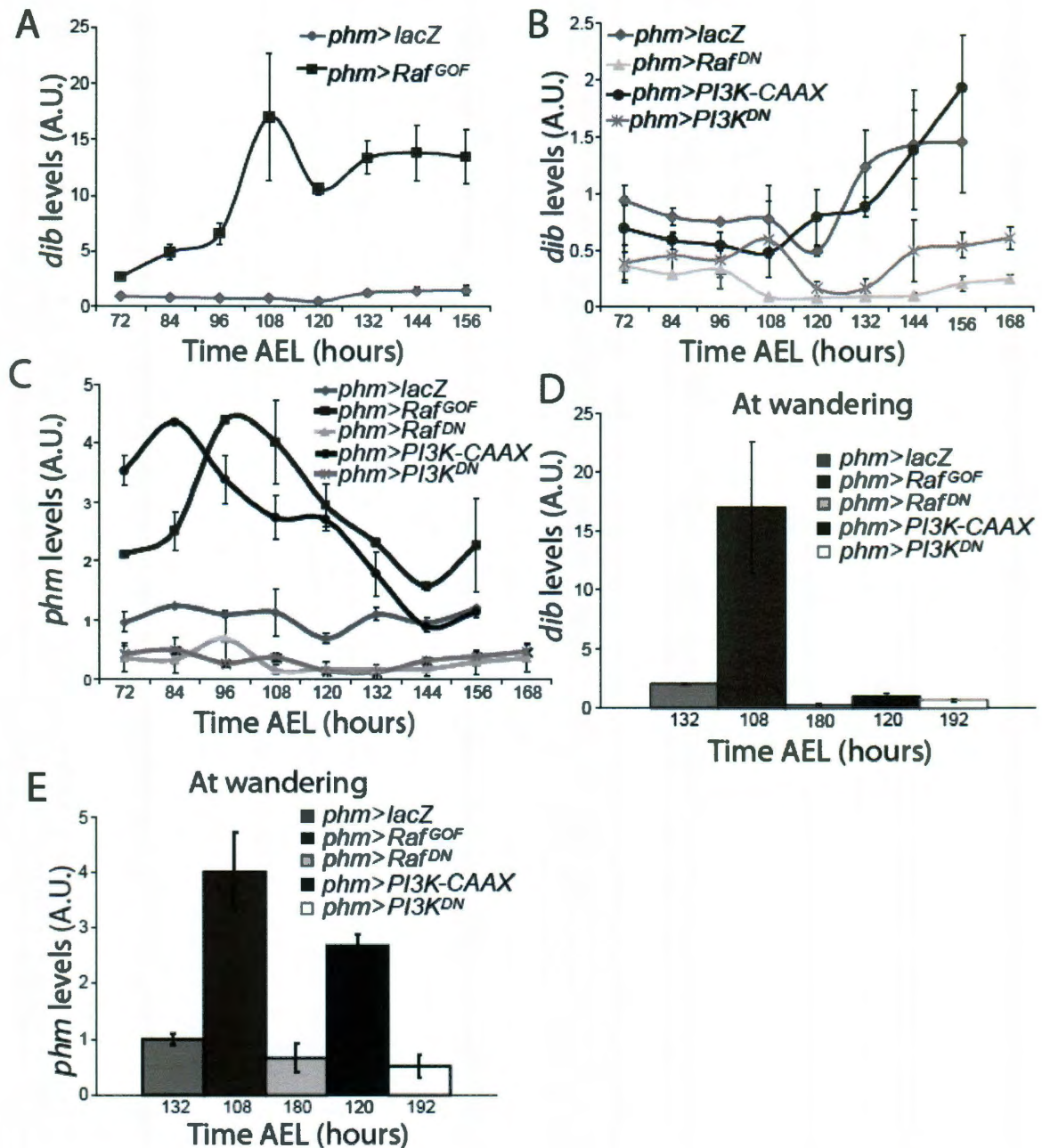
Figure 4.1 **PI3K and Raf regulate Halloween genes transcription in Drosophila.** (A, B,) Mean *dib* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at the indicated time AEL (x-axis). D) Mean *dib* transcript levels (y-axis), were measured from two biological samples

collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at wandering; the time AEL at which the larvae wander is also indicated (x-axis). Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A* and are presented in arbitrary units (A.U). C,) Mean *phm* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at the indicated time AEL (x-axis). E) Mean *phm* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at wandering; the time AEL at which the larvae wander is also indicated (x-axis). Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RPII140* (Applied Biosystems) and are presented in arbitrary units (A.U.).

transgenes within the PG that activate Raf or PI3K advances onset of metamorphosis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005).

To determine if these effects on the timing of metamorphosis reflect altered transcription of *Halloween* genes, we used the *phm-Gal4* driver (Mirth et al., 2005), which expresses specifically in the PG, to induce expression of the dominant-negative *Raf^{DN}* and *PI3K^{DN}* transgenes (Leevers et al., 1996; Roch et al., 1998) and the constitutively-active *Raf^{GOF}*, and *PI3K-CAAX* transgenes (Brand and Perrimon, 1993; Leevers et al., 1996). We then used quantitative RT-PCR (Q-PCR) to measure transcript levels from two Halloween genes (*dib* and *phm*) from developmentally staged larvae of these genotypes at several times during L3. We found that expression of either *Raf^{DN}* or *PI3K^{DN}* inhibited both *dib* and *phm* transcript levels, whereas expression of *Raf^{GOF}* increased *dib* and *phm* transcript levels (Figure 4.1A, 4.1B, and 4.1C).

It was previously reported that activating PI3K increased *dib* and *phm* transcript levels about two-fold (Colombani et al., 2005); we found that *PI3K-CAAX* increased *phm* levels but had no effect on *dib* levels (Figure 4.1B and 4.1C). These results are consistent with the possibility that the PI3K and Raf pathways regulate developmental timing by regulating *Halloween* transcript levels.

Halloween transcript levels are strongly affected by developmental stage (McBrayer et al., 2007); transcription of each gene is strongly induced during the second half of L3. Given that altering PI3K or Raf activities in the PG alters developmental timing (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al.,

2005) it is not clear if the altered *dib* and *phm* transcript levels in the genotypes above are the cause of or a consequence of altered developmental timing. To address this issue, we measured *dib* and *phm* transcript levels in the genotypes described above from larvae at the same developmental stage (wandering L3), rather than at the same time AEL. We found that inhibiting PI3K or Raf activities in the PG decreased, whereas activating Raf increased *dib* and *phm* transcript levels in larvae at this wandering stage compared to wildtype (Figure 4.1D and 4.1E). Activating PI3K increased *phm*, but not *dib* transcript levels (Figure 4.1D and 4.1E). These observations suggest that altered *Halloween* genes expression causes at least in part the altered developmental timing conferred by altered Raf and PI3K activities.

4.2 GSK-3 mediates the effects of PI3K on Halloween genes expression, developmental timing, and PG growth

Next we were interested in identifying the PI3K effector pathways responsible for the effects of PI3K activity on PG properties. Many of the pathways regulated by PI3K are mediated by the kinase Akt, which is phosphorylated and activated in the presence of activated PI3K (Marte and Downward, 1997). One critical target of PI3K and Akt is the kinase GSK-3: Activated Akt phosphorylates GSK-3 on serine 9 and inhibits GSK-3 activity (Cross et al., 1995). If GSK-3 mediates the effects of PI3K on PG properties, then the *GSK-3*^{S9A} mutation, which cannot be phosphorylated and inhibited by
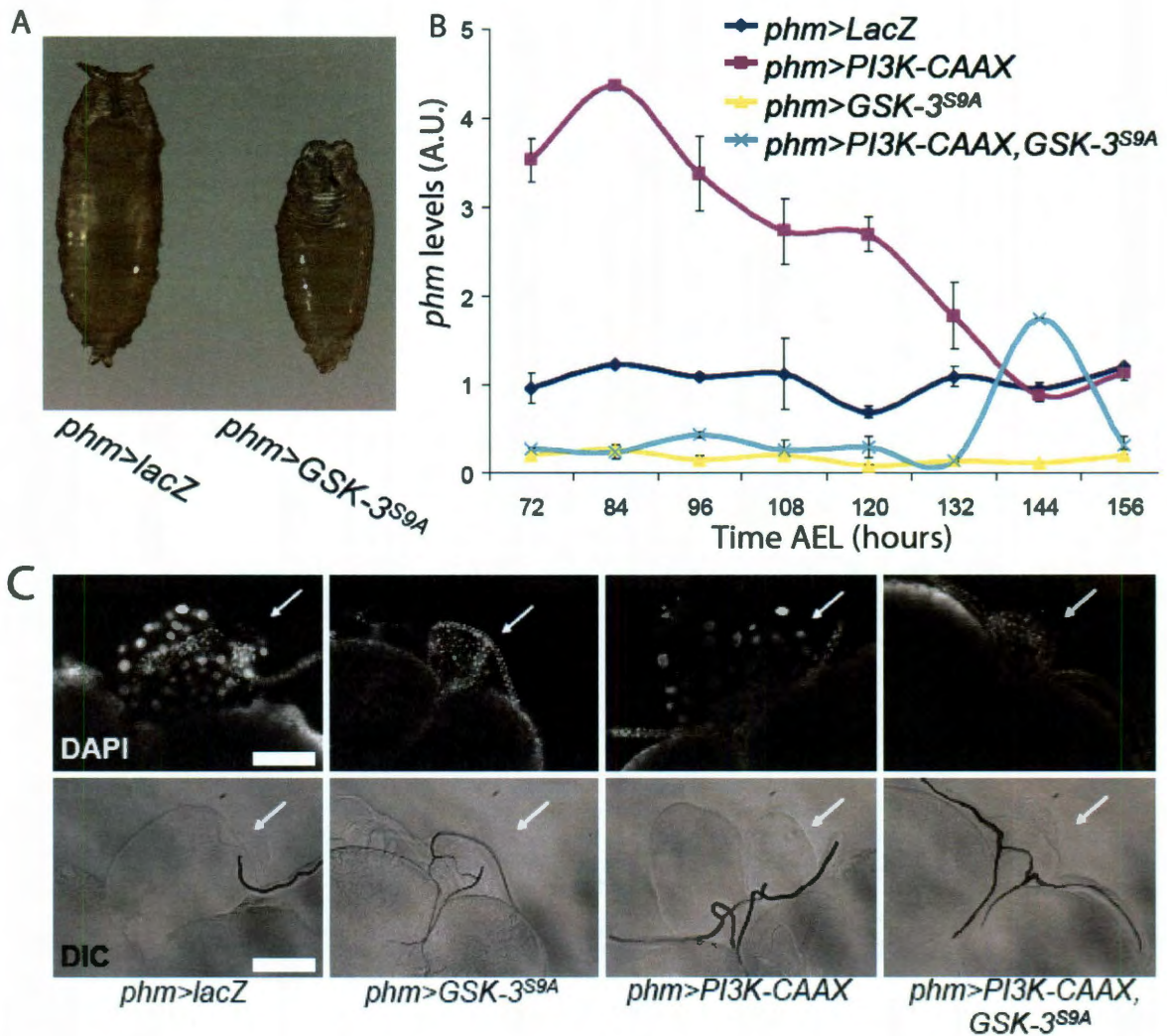
Figure 4.2. **GSK-3 controls Halloween genes expression, developmental timing, and PG growth in Drosophila.** **A)** Photographs of pupae of the indicated genotypes. B) Mean *phm* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at the indicated time AEL (x-axis). Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RPII140* (Applied Biosystems) and are presented in arbitrary units (A.U.). C) Pictures of brain lobes and ring gland of the indicated phenotypes. Top panel is the DAPI image and the bottom

panel is the DIC image. The arrows point to the RG. The scale bar is 100 μm.

PI3K/Akt, and hence is constitutively active, will confer phenotypes similar to $PI3K^{DN}$. To test this possibility, we expressed $GSK\text{-}3^{S9A}$ in the PG with both the phm $Gal4$ and amn^{c651} $Gal4$ driver (Waddell et al., 2000), which express specifically in the PG (Caldwell et al., 2005) but is slightly weaker than $phm\text{-}Gal4$. We found that $amn^{c651}{>}GSK3^{S9A}$ larvae exhibited strongly inhibited dib and phm transcription (data not shown) and strongly inhibited molting and metamorphosis: $GSK\text{-}3^{S9A}$-expressing larvae often failed to molt from second instar (L2) to L3; those that were able to molt to L3 failed to initiate pupariation and instead exhibited prolonged larval wandering (not shown). Some larvae that were unable to molt from L2 to L3 pupariated directly from L2. We also found that $phm{>}GSK3^{S9A}$ larvae exhibited strongly inhibited dib and phm transcription (data not shown and Figure 4.2B) and strongly inhibited molting and metamorphosis: $GSK\text{-}3^{S9A}$-expressing larvae failed to molt from second instar (L2) to L3; the L2 exhibited prolonged larval wandering (not shown). Some of the $phm{>}GSK3^{S9A}$ second instars larvae also pupariated directly from L2 (Figure 4.2A). These properties are often observed in larvae with profoundly decreased ecdysone titers (Bialecki et al., 2002; Mirth et al., 2005; Venkatesh and Hasan, 1997) and in fact have been reported for larvae in which the PI3K pathway is strongly inhibited (Mirth et al., 2005). These observations support the hypothesis that PI3K inhibition prevents metamorphosis by preventing the phosphorylation and inhibition of GSK-3.

To determine if GSK-3 activity mediates the effects of PI3K on dib and phm expression, we used Q-PCR to measure dib and phm transcript levels in

developmentally staged larvae. As we found for the effects of $PI3K^{DN}$, we found that $GSK-3^{S9A}$ expression blocked *dib* and *phm* transcript levels (data not shown and Figure 4.2B). Thus, constitutive GSK-3 activity prevents transcription of at least two Halloween genes.

In addition to regulating Halloween gene expression and developmental timing, PI3K activity in the PG regulates cell and nuclear size: PI3K activation greatly increases PG cell and nuclear size, whereas PI3K inhibition decreases PG cell and nuclear size (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). These effects of altered PI3K also appear to be mediated by GSK-3, as $GSK-3^{S9A}$ expression decreases PG cell and nuclear size to an extent similar to $PI3K^{DN}$ expression (Figure 4.2C).

If GSK-3 mediates the effects of PI3K on *dib* and *phm* transcription, PG cell and nuclear size, and developmental timing, then expressing the $GSK-3^{S9A}$ allele, which cannot be phosphorylated and inhibited by PI3K, is anticipated to prevent the constitutively-active *PI3K-CAAX* allele from exerting phenotypic effects. To test this prediction, we co-expressed $GSK-3^{S9A}$ and *PI3K-CAAX* and found that as predicted, the GSK-3^{S9A} phenotype, not the PI3K-CAAX, is exhibited: *phm>PI3K-CAAX*, $GSK-3^{S9A}$ larvae exhibit decreased PG size, inhibition of metamorphosis, and inhibition of *dib* and *phm* transcript levels characteristic of $GSK-3^{S9A}$ expression in an otherwise wildtype background (Figure 4.2B, 4.2C, and not shown). These results support the notion that PI3K regulates PG properties via GSK-3 inhibition.

4.3 PI3K and GSK3, but not Raf, are required for *br* expression in the PG

Null mutations in the *broad (br)* gene, which encodes a transcription factor, prevent metamorphosis and confer prolonged larval wandering, at least in part by preventing ecdysone synthesis (Kiss et al., 1980). It was recently found that chromosomal null *br* mutations or RNAi knockdown of *br* specifically in the PG significantly decreased transcript levels of *Halloween* genes, including *dib* and *phm* (Xiang et al., 2010). This effect on *Halloween* genes expression is presumably a secondary consequence of strongly inhibited transcript levels of *torso*, which encodes the receptor for the PTTH metamorphic signal and is required for *Halloween* genes expression (Rewitz et al., 2009). We wondered if the requirement for PI3K and GSK-3 in *dib* and *phm* transcription might reflect a requirement for PI3K and GSK-3 in *br* expression.

To test this possibility, we used immunocytochemistry to compare Br levels within the PG in *phm>PI3K^{DN}*, *phm>Raf^{DN}*, and *phm>lacZ* at specific times during L3 in developmentally staged larvae. It was previously reported that in wildtype, Br is first detected about 17 hours after the L2/L3 molt, and is maintained until pupariation (Zhou et al., 2004). We confirmed this finding: we detect Br in *phm>lacZ* PGs in larvae 17-24 hours after the L2/L3 molt, and in wandering larvae (Figure 4.3A and 4.3B). In contrast, Br is not detected in the PG of *phm>PI3K^{DN}* either in wandering larvae, or in larvae 17-24 hours following the L2/L3 molt (Figure 4.3A and 4.2B). Several lines of evidence suggest that this loss of *br* expression is a cell-autonomous consequence of PI3K inhibition in the

A ~24 hrs after L2/L3 molt

DAPI

Broad

phm>lacZ   *phm>Raf^DN*   *phm>PI3K^DN*

B At wandering

DAPI

Broad

phm>lacZ   *phm>Raf^DN*   *phm>PI3K^DN*

C   At wandering

DAPI

Broad

Gal80^{TS},   Gal80^{TS},
amn^{c651}>lacZ   amn^{c651}>GSK-3^{S9A}
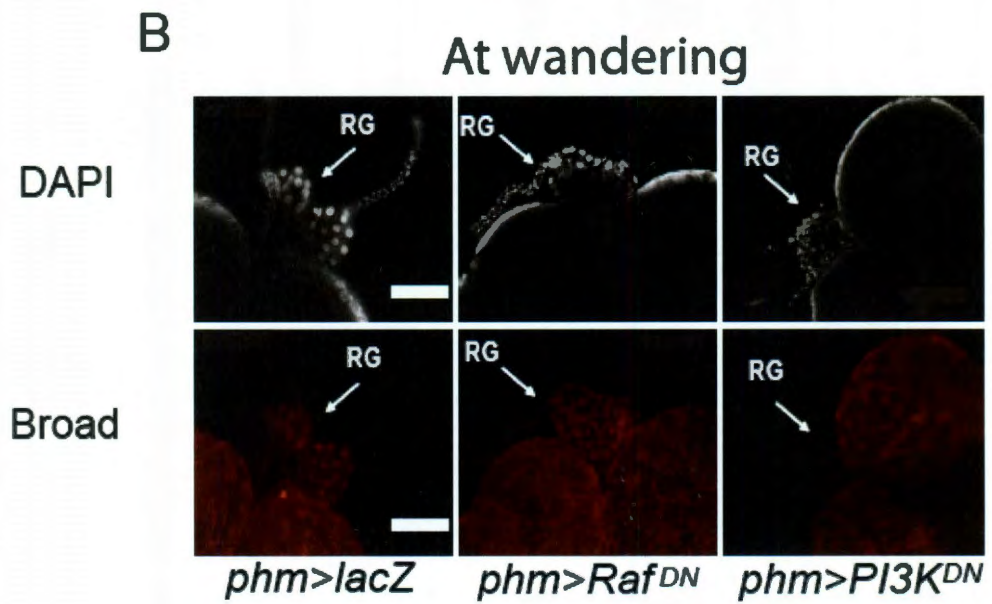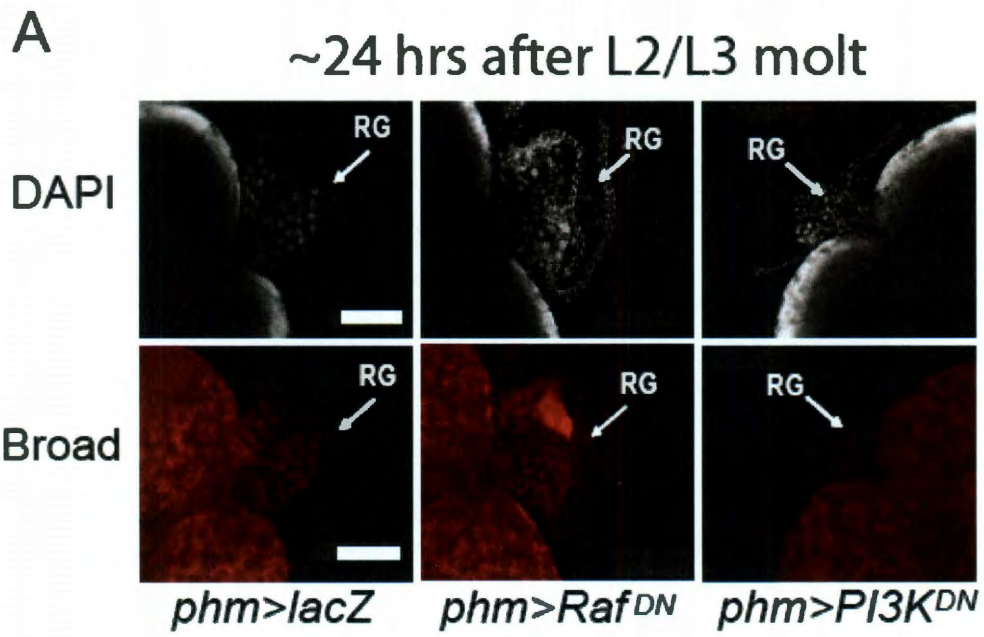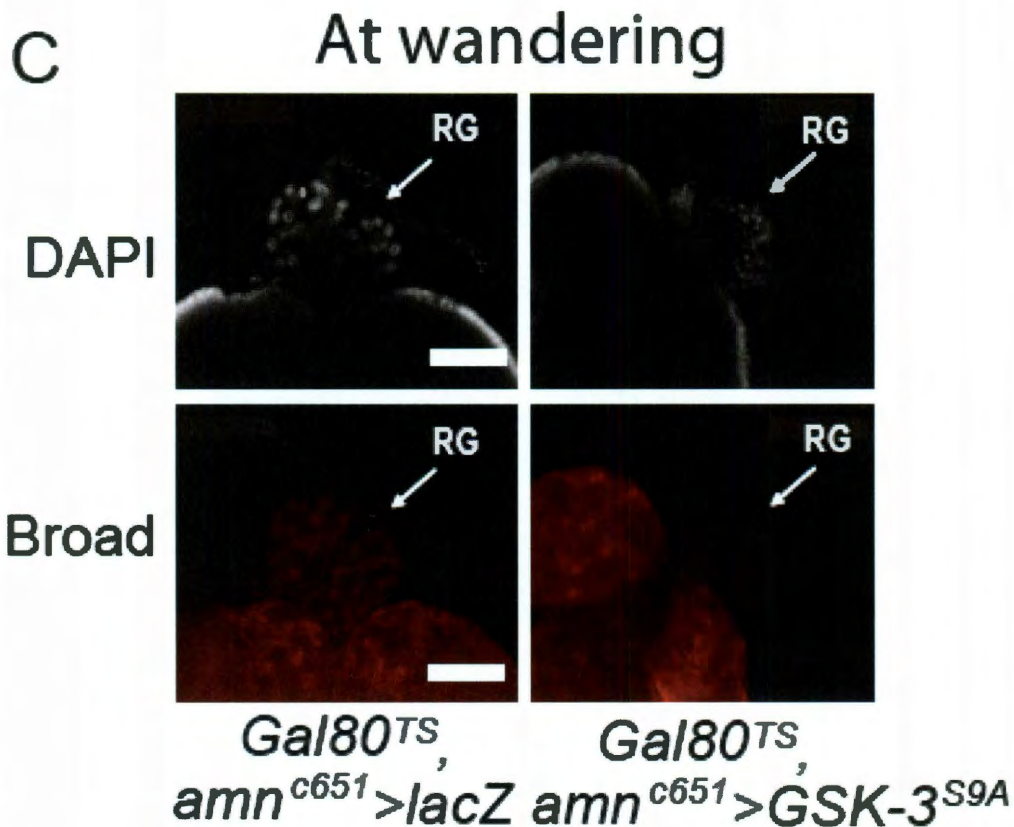
Figure 4.3 **PI3K and GSK-3 regulate *br* transcription in the PG.** A) Brain lobes and ring glands from larvae of indicated genotypes at ~24 hrs after the L2/L3 molt stained with DAPI (white; top panel) and with anti-Broad antibody (red, bottom panel). The arrows point to the RG. The scale bar is 100 µm. B) Brain lobes and ring glands from larvae of indicated genotypes at wandering stained with DAPI (white; top panel) and with anti-Broad antibody (red, bottom panel). The arrows point to the RG. The scale bar is 100 µm. C) Brain lobes and ring glands from larvae of indicated genotypes at wandering stained with DAPI (white; top panel) and with anti-Broad antibody (red, bottom panel). The arrows point to the RG. The scale bar is 100 µm.

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PG, rather than an indirect effect of decreased ecdysone titers in these *phm>PI3K^DN* larvae. First, the requirement for PI3K in *br* expression is apparently cell autonomous, because Br immunoreactivity remains detected in the brain lobes from *phm>PI3K^DN* (Figure 4.3A and 4.3 B). Secondly, *phm>Raf^DN* larvae exhibit no deficits in *br* expression at either wandering, or 17-24 hours after the L2/L3 molt (Figure 4.3A and 4.3B) despite exhibiting a lack of ecdysone synthesis similar to that observed in *phm>PI3K^DN* larvae. Third, the loss of *br* expression in *phm>PI3K^DN* larvae only 17-24 hours after the L2/L3 molt, when ecdysone titers are low, suggests that this early induction of *br* expression is not ecdysone-driven, which is a suggestion made previously (Zhou et al., 2004).

The observation that PI3K, but not Raf, activity is required for *br* expression, places Br after PI3K but before Raf in an epistasis hierarchy. This placement is completely consistent with the hypothesis that Raf acts after torso, and thus after Br, in the control of Halloween genes expression (Xiang et al., 2010). This result also suggests that PI3K inhibition decreases *dib* and *phm* expression and delays metamorphosis as a consequence of failure to express *br*, thus *torso*, which then prevents response to the PTTH metamorphic signal.

We were interested in determining if *GSK-3^{S9A}* expression would prevent *br* expression in a manner similar to *PI3K^DN*. However, we were unable to examine this question in *phm>GSK-3^{S9A}* because these larvae fail to molt to L3. Furthermore, PG size is so attenuated in the presence of GSK-3^{S9A} that Br detection might be problematic. To address these issues, we induced *GSK-3^{S9A}* with the weaker *amn^{c651}* driver. In addition, we included a transgene expressing

the temperature-sensitive $gal80^{ts}$ protein in these larvae: with this transgene, Gal4 activity is inhibited by Gal80 protein at the permissive temperature (18 degrees), which prevents the GSK-3^{S9A} phenotype from expression, but this inhibition is relieved when larvae are switched to the restrictive temperature (30 degrees), thus inducing expression of Gal4-regulated transgenes (McGuire et al., 2003). We reared amn^{c651}, $gal80^{ts}$>GSK-3^{S9A} at 18 degrees until approximately the L2/L3 molt, and then shifted to 30 degrees. We found that whereas Br protein was detectable in identically treated amn^{c651}, $gal80^{ts}$>$lacZ$ larvae Br protein was not detectable in amn^{c651}, $gal80^{ts}$>GSK-3^{S9A} larvae (Figure 4.3C). Thus, GSK-3 activity prevents *br* expression in the PG.

4.4 βFTZ-F1 is required for *br* expression in the PG

Next we wanted to identify a transcription factor responsible for the initiation of *br* expression in early L3. For three reasons, the nuclear hormone receptor βFTZ-F1 was a good candidate for a *br* transcription factor. First, βFTZ-F1 immunoreactivity appears in the PG about prior to or concurrently with Br (Parvy et al., 2005; Zhou et al., 2004), which is consistent with the possibility that βFTZ-F1 induces *br* expression. Second, βFTZ-F1 is required cell-autonomously for *dib* and *phm* expression: PGs mosaic for *βftz-f1* mutant and wildtype, the mitotic clones mutant for *βftz-f1* lack both Dib and Phm immunoreactivity (Parvy et al., 2005).
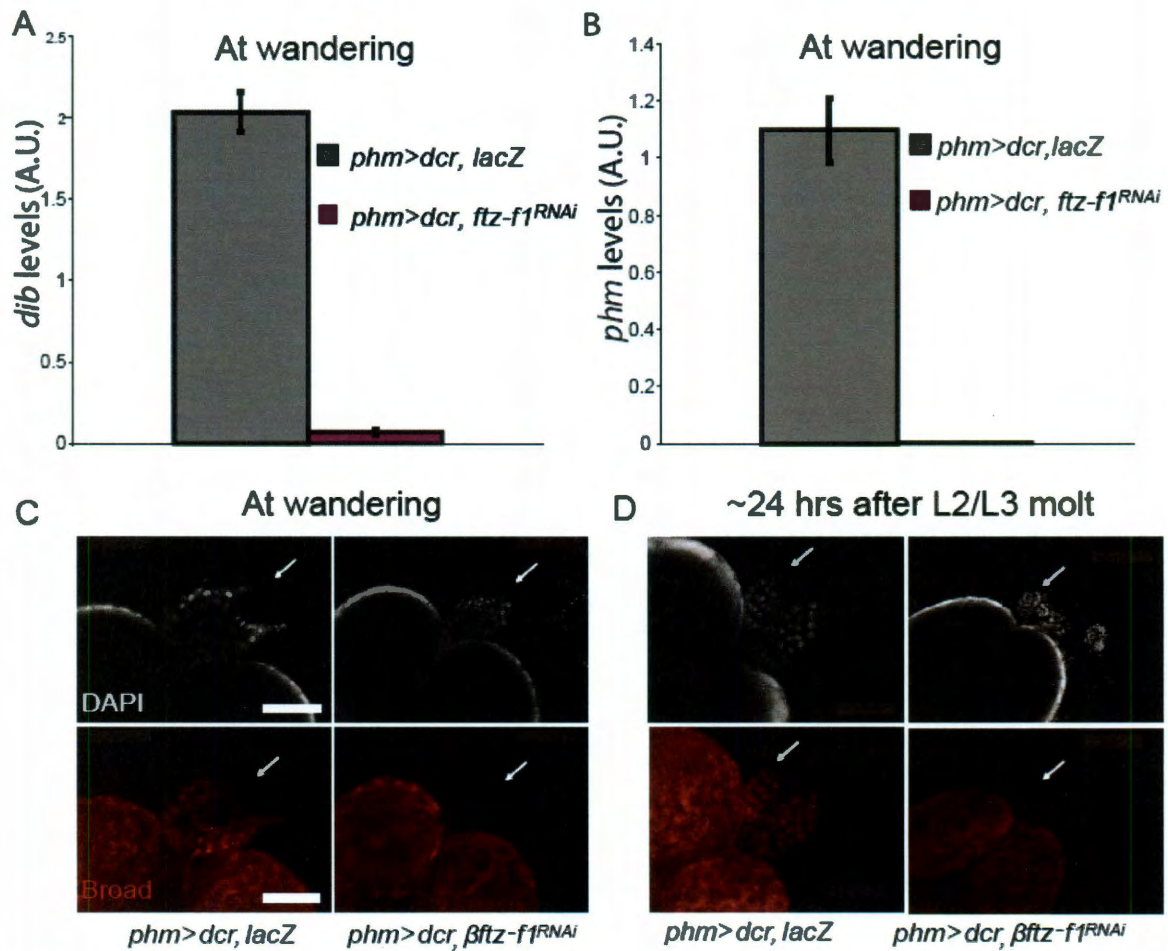
Figure 4.4 **Loss of βFTZ-F1 in the PG decreases *dib* and *phm* transcription and Br immunoreactivity.** A) Mean *dib* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at wandering. Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A* and are presented in arbitrary units (A.U.). B) Mean *phm* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at wandering. Error bars represent

SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RPII140* (Applied Biosystems) and are presented in arbitrary units (A.U.). C) Brain lobes and ring glands from larvae of indicated genotypes at wandering stained with DAPI (white; top panel) and with anti-Broad antibody (red, bottom panel). The arrows point to the RG. The scale bar is 100 µm. D) Brain lobes and ring glands from larvae of indicated genotypes at ~24hrs after the L2/L3 molt stained with DAPI (white; top panel) and with anti-Broad antibody (red, bottom panel). The arrows point to the RG. The scale bar is 100 µm.

This requirement of βFTZ-F1 for *dib* and *phm* expression is predicted if βFTZ-F1 is required for *br* expression. Third, in larval tissues distinct from the PG, βFTZ-F1 is required for re-initiation of *br* expression following the initial, ecdysone-dependent transcriptional induction (Broadus et al., 1999), and βFTZ-F1 might promote *br* expression by binding directly to the *βftz-f1* promoter (Kucharova and Farkas, 2002).

To test the prediction that *βftz-f1* activity in the PG is required for *br* expression, we examined Br protein levels in larvae in which *βftz-f1* levels were knocked down with RNAi. In addition, we determined if *βftz-f1* knockdown would prevent pupariation, and decrease *dib* and *phm* levels, as is predicted from previous results (Parvy et al., 2005; Xiang et al., 2010). We co-expressed *UAS-dicer (dcr)* with *UAS- βftz-f1^{RNAi}* to increase efficiency of knockdown. We found that *UAS- βftz-f1^{RNAi}* larvae arrested at either the L2 or L3 stages and failed to undergo metamorphosis; rather, knockdown larvae either pupariated from the L2 stage, or formed L3 larvae that exhibited prolonged wandering (not shown), as is observed in *br* null mutants and in larvae expressing *PI3K^{DN}* or *GSK3^{S9A}* (Caldwell et al., 2005; Kiss et al., 1980; Mirth et al., 2005). *βftz-f1^{RNAi}* larvae also failed to express *dib* and *phm* (Figure 4.4), which is consistent with the previous observation that *βftz-f1* mutant mitotic clones in the PG failed to express *dib* or *phm* (Parvy et al., 2005). Finally, we found that L3 *phm>dcr, βftz-f1^{RNAi}* larvae failed to produce detectable Br immunoreactivity in the PG both at wandering, and 17-24 hours after the L2/L3 molt (Figure 4.4), which suggests that *βftz-f1* is required for *br* expression. This observation further suggests that the lack of *dib*

and *phm* expression in *phm>dcr, βftz-f1^RNAi* larvae is a secondary consequence of lack of *br* expression.

4.5 Ectopic activation of the Torso pathway rescues the PG phenotypes conferred by *PI3K, GSK-3^S9A, br* and *βftz-f1* inhibition

Inhibition of *PI3K^DN, GSK-3^S9A* or *βftz-f1* or *br* knockdown each affect developmental timing and Halloween genes expression in similar manners. Given that *PI3K^DN, GSK-3^S9A* or *βftz-f1* knockdown each block Br expression, and that Br is required for expression of *torso*, the receptor for the PTTH metamorphic signal (Rewitz et al., 2009; Xiang et al., 2010), it seemed possible that *PI3K^DN, GSK-3^S9A* or *βftz-f1* knockdown each prevent Halloween genes expression by preventing *torso* expression. In this view, PI3K, βFTZ-F1, GSK-3, and Br activities are required to induce competence in the PG to respond to PTTH. If so, then ectopic (that is, PTTH- and Torso-independent) activation of theTorso pathway is predicted to restore both pupariation and Halloween gene expression to *PI3K^DN, GSK3^S9A* or *βftz-f1^RNAi* or *br* knockdown larvae.

Several lines of evidence suggest that PTTH and Torso activation promote Halloween genes expression and pupariation by activating Raf and its downstream MAP kinase Erk. First, Torso requires Raf activity for another Drosophila larval developmental process, induction of anterior and posterior structures (Ambrosio et al., 1989; Baek et al., 1996). Second, Torso activation by PTTH in S2 cells increases phosphorylated (active) Erk (Rewitz et al., 2009).

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Third, as shown above, activating Raf increases Halloween genes expression, whereas inhibiting Raf decreases Halloween genes expression (Figure 4.1), which is consistent with a role for Raf in transducing the PTTH/torso signal. Therefore we chose to activate the PTTH/Torso pathway by expressing the constitutively active Raf^{GOF} in the PG: Raf^{GOF} is active even in the absence of activation by receptor tyrosine kinases because the mutation deletes most of the N terminal regulatory region, leaving the remaining C terminal kinase domain constitutively active.

First, to test the prediction that Raf^{GOF} would restore pupariation and Halloween genes expression to *br* mutants, we compared *dib* and *phm* transcript levels in the *br* null mutant *br^{npr}* in which *phm-Gal4* drove either *Raf^{GOF}* or *lacZ*. We found that both *dib* and *phm* transcript levels were strongly decreased in *br^{npr}*, *phm>lacZ* (Figure 4.5A and data not shown), consistent with what was reported previously (Xiang et al., 2010). Furthermore, as previously reported (Kiss et al., 1980), *br^{npr}*, *phm>lacZ* L3 larvae failed to initiate pupariation, but rather exhibited prolonged wandering behavior (not shown). In contrast, *dib* and *phm* transcript levels were strongly increased in *br^{npr}*, *phm>Raf^{GOF}* (Figure 4.5A and data not shown). Furthermore, *br^{npr}*, *phm>Raf^{GOF}* mutant larvae initiated pupariation at the same small size characteristic of larvae in which *Raf^{GOF}* is expressed in the PG (Figure 4.5B). Given that initiation of pupariation requires ecdysone, this observation suggested that *Raf^{GOF}* expression restored ecdysone synthesis to *br^{npr}*. Pupariation arrested at an early stage, most likely because *br^{npr}* eliminates *br* product from all larval tissues, thus preventing target tissues

from responding properly to ecdysone. These results suport the notion that *br* mutants fail to pupariate because they fail to activate the PTTH/Torso pathway. These results also argue against the notion that *br* activates Halloween genes expression directly, because Br function is dispensable when the PTTH pathway is ectopically activated.

We found that Raf^{GOF} expression similarly rescued the Halloween gene expression defect and pupariation defect conferred by $\beta ftz\text{-}f1^{RNAi}$. In particular, co-expression with Raf^{GOF}, but not *lacZ*, restored L3 pupariation and restored *dib* and *phm* transcript levels to larvae expressing $\beta ftz\text{-}f1^{RNAi}$ (Figure 4.5C and 4.5D). As was the case in br^{npr}; $phm{>}Raf^{GOF}$, *phm>dcr*, $\beta ftz\text{-}f1^{RNAi}$, Raf^{GOF} exhibited both the small puparia and elevated *dib* and *phm* transcript levels characteristic of $phm{>}Raf^{GOF}$ in an otherwise wildtype background. These results indicate that loss of βFTZ-F1 prevents Halloween genes expression by preventing the PG from responding to the PTTH metamorphic signal.

The observation that PI3K^{DN} blocks *br* expression in a manner similar to $\beta ftz\text{-}f1^{RNAi}$ suggested that ectopic activation of the PTTH pathway by Raf^{GOF} expression would suppress the PI3K^{DN} phenotypes in a manner similar to the suppression of to $\beta ftz\text{-}f1^{RNAi}$. It was previously reported that PG expression of Raf^{GOF} decreases body size, whereas PG expression of $PI3K^{DN}$ increases body size (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005), at least in part as a consequence of altered developmental timing. We found that pupae expressing both Raf^{GOF} and $PI3K^{DN}$ showed the small pupal size characteristic of Raf^{GOF} in an otherwise wildtype background, rather than the large body size of

A

At wandering

- ■ phm>lacZ
- ■ phm>Raf^{GOF}
- □ br^{npr},phm>lacZ
- ▨ br^{npr},phm>Raf^{GOF}

B

br^{npr},phm>Raf^{GOF}

C

phm>lacZ
phm>Raf^{GOF}
phm>βftz-f1^{RNAi}, Raf^{GOF}

D

At wandering

- ■ phm>dcr, lacZ
- ▨ phm>dcr, Raf^{GOF}
- ■ phm>dcr, βftz-f1^{RNAi}
- ■ phm>dcr, βftz-f1^{RNAi}, Raf^{GOF}
- ■ phm>dcr, βFtz-F1^{RNAi}, lacZ

E

phm>LacZ
phm>Raf^{GOF}
phm>PI3K^{DN}
phm>Pi3K^{DN},Raf^{GOF}

F

G

H

phm>lacZ *phm>PI3K-CAAX* *phm>Raf^DN* *phm>Raf^DN,PI3K-CAAX*

I
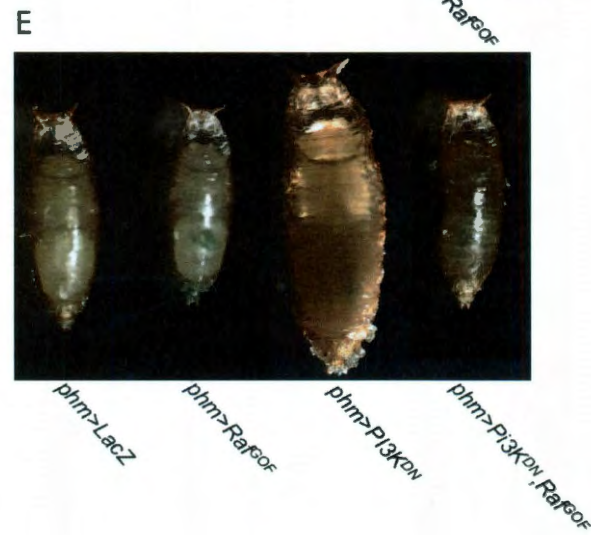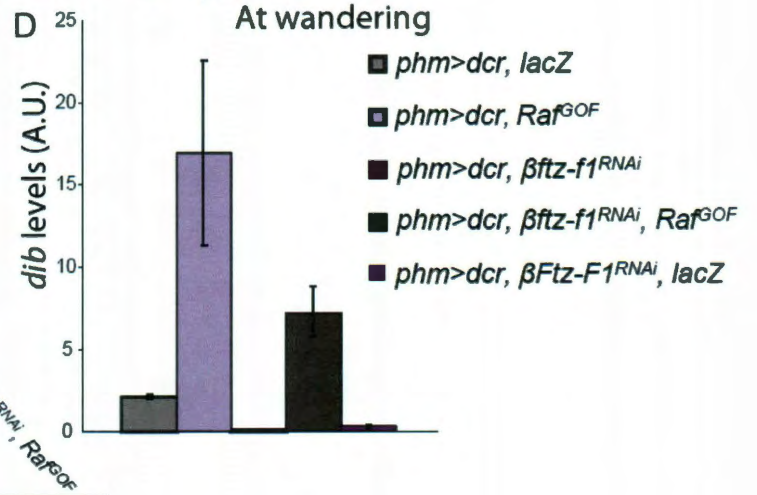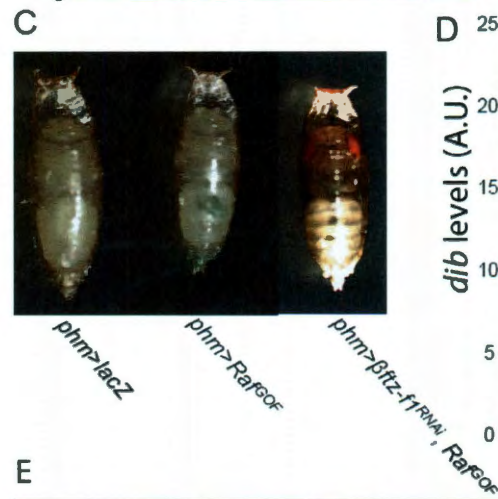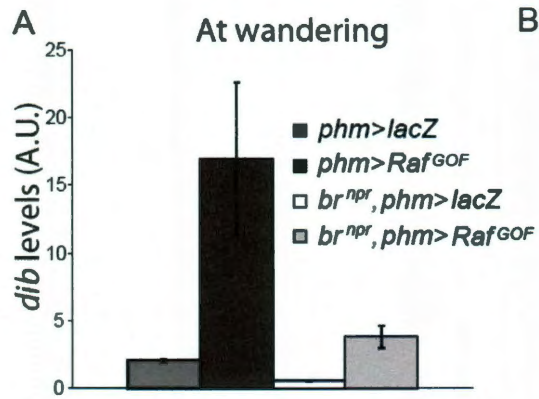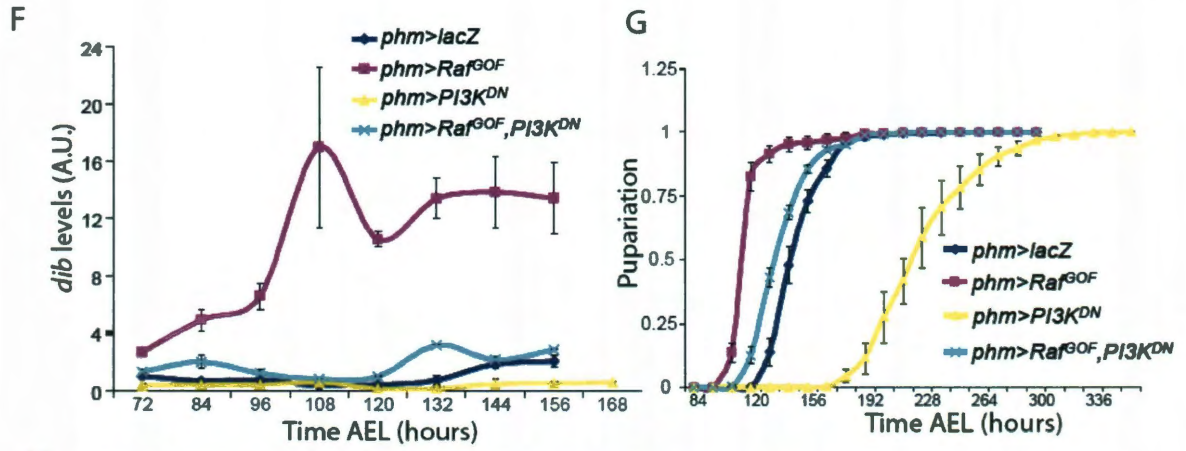
Figure 4.5. **Raf^GOF^ overexpression in the PG rescues the loss of *PI3K^DN^*, *βftz-f1* and *br* phenotype.** A) Mean *dib* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at wandering. Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A* and are presented in arbitrary units (A.U.). B) Mean fraction of developmentally staged larvae pupariating (y-axis) by the indicated time after egg laying (AEL, x-axis). Error bars represent SEM, genotypes are listed in the inset. At least three independent biological samples were measured for each data point. C) Photographs of pupae of the indicated genotypes. D) Mean *dib* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at wandering. Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A* and are presented in arbitrary units. E) Photographs of pupae of the indicated genotypes. F) Mean *dib* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at indicated time AEL (x-axis). Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A* and are presented in arbitrary units (A.U.). G) Mean fraction of developmentally staged larvae of indicated genotypes. pupariating (y-axis) by the indicated time after egg laying (AEL, x-axis). Error bars represent SEM,

genotypes are listed in the inset. At least three independent biological samples were measured for each data point. H) Photographs of pupae of the indicated genotypes. I) Mean *dib* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at indicated time AEL (x-axis). Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A* and are presented in arbitrary units (A.U.).

However, the ability of Raf^{GOF} to rescue other PI3K^{DN} phenotypes is incomplete *dib* and *phm* transcript levels are decreased relative to *br^{npr}*; *phm>Raf^{GOF}* and *phm> βftz-f1^{RNAi}*, *Raf^{GOF}* in *phm>PI3K^{DN}*, *Raf^{GOF}* (Figure 4.6E and data not shown). In addition, the time to onset of metamorphosis is intermediate in *phm>PI3K^{DN}*, *Raf^{GOF}* compared to *phm>PI3K^{DN}*, and *phm>Raf^{GOF}* (Figure 4.6F). These results suggest that only part of the PI3K^{DN} phenotypes can be rescued by Raf^{GOF}, further suggesting although part of the deficits in Halloween genes expression and onset of metamorphosis conferred by PI3K^{DN} result from inability to activate the torso pathway, an additional part of the PI3K^{DN} phenotypes are torso-independent. These torso-independent effects of PI3K^{DN} might involve effects of PI3K effector pathways on mRNA translation (Hay and Sonenberg, 2004) or on PG cell and nuclear size, which might affect the ability of the PG to synthesize ecdysone independently of effects on the torso pathway. Larvae expressing *GSK3^{S9A}* are even more refractory to the rescue effects of *Raf^{GOF}*: *Raf^{GOF}* expression restores neither pupariation nor *dib* and *phm* transcription to GSK3^{S9A}-expressing larvae (not shown).

Further evidence that PI3K-βFTZ-F1 pathway activity enables activation of the PTTH/torso pathway comes from epistasis tests between the dominant-negative Raf^{DN} and the constitutively active PI3K-CAAX. Just as Raf^{GOF} is epistatic to PI3K^{DN}, so is Raf^{DN} epistatic to PI3K-CAAX. In particular, *phm>Raf^{DN}, PI3K-CAAX* pupae exhibit the large body size characteristic of Raf^{DN} in an otherwise wildtype background, and not the small size phenotype of PI3K-CAAX (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005), Figure

4.6 G). Similarly, *phm>Raf^{DN}, PI3K-CAAX* larvae exhibit the low levels of *dib* and *phm* transcripts characteristic of *phm>Raf^{DN}*, and show a developmental delay similar to, although less extreme than, the delay observed in *phm>Raf^{DN}*, rather than the advanced onset of metamorphosis observed in *phm>PI3K-CAAX*.

4.6 Evidence that the nuclear hormone receptors E75 and HR3 regulate *br* expression via βFTZ-F1

βFTZ-F1 immunoreactivity appears in the PG during L3 about 16 hours after the L2/L3 molt (Parvy et al., 2005). The factors responsible for this induced expression are not known; however, in other Drosophila tissues, *βftz-f1* transcription is regulated by the relative amounts of two additional nuclear hormone receptors, HR3 and E75; HR3 activates *βftz-f1* transcription both in vitro and in cell culture by directly binding to the *βftz-f1* transcription regulatory region, whereas E75 dimerizes with and prevents HR3 from activating *βftz-f1* (White et al., 1997). In vivo expression patterns of these geness during larval development are consistent with these transcriptional regulatory pathways (Sullivan and Thummel, 2003). If *βftz-f1* transcription in the PG is regulated by this pathway, then either *E75* overexpression or RNAi-mediated *HR3* knockdown in the PG is predicted to prevent *βftz-f1* transcription and hence confer phenotypes similar to RNAi-mediated *βftz-f1* knockdown in the PG: a block in *br* expression, and a consequent inhibition of *dib* and *phm* transcription and a block in pupariation.

To test these predictions, we overexpressed the *E75A* isoform (kindly

A

At wandering

DAPI

Broad

phm>dcr, lacZ   *phm>dcr, HR3^RNAi*

B

At wandering

dib levels (A.U.)

■ *phm>dcr, lacZ*
■ *phm>dcr, HR3^RNAi*
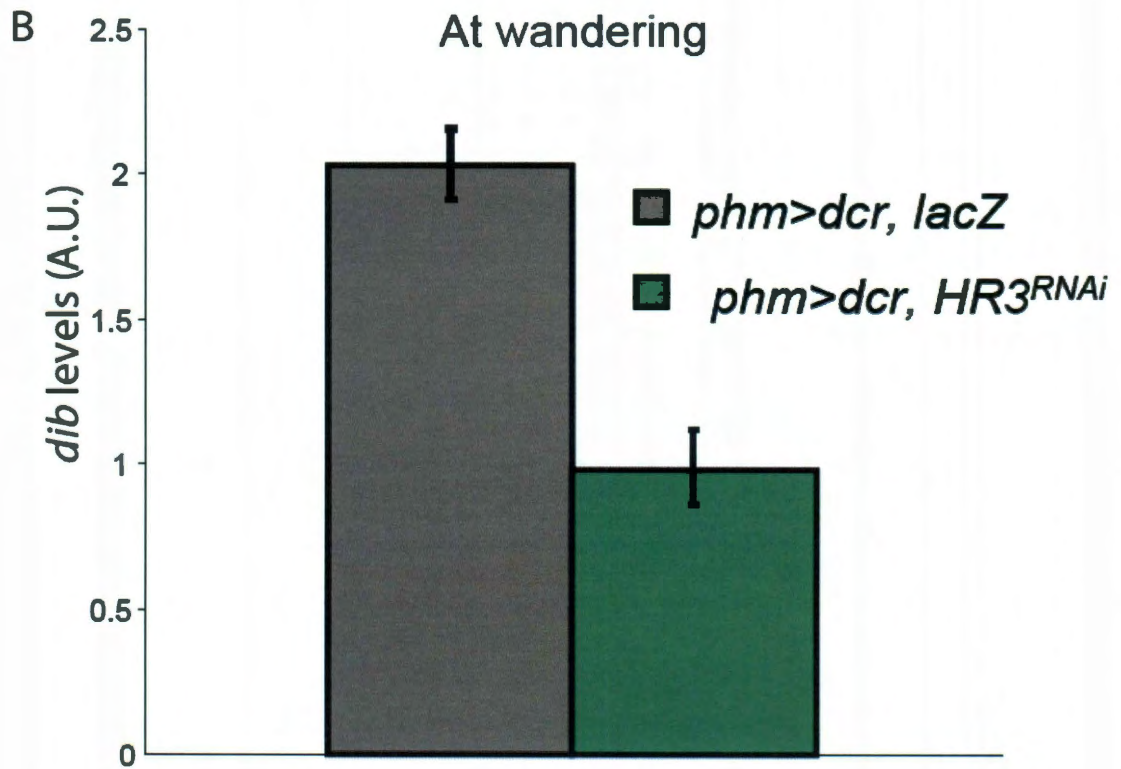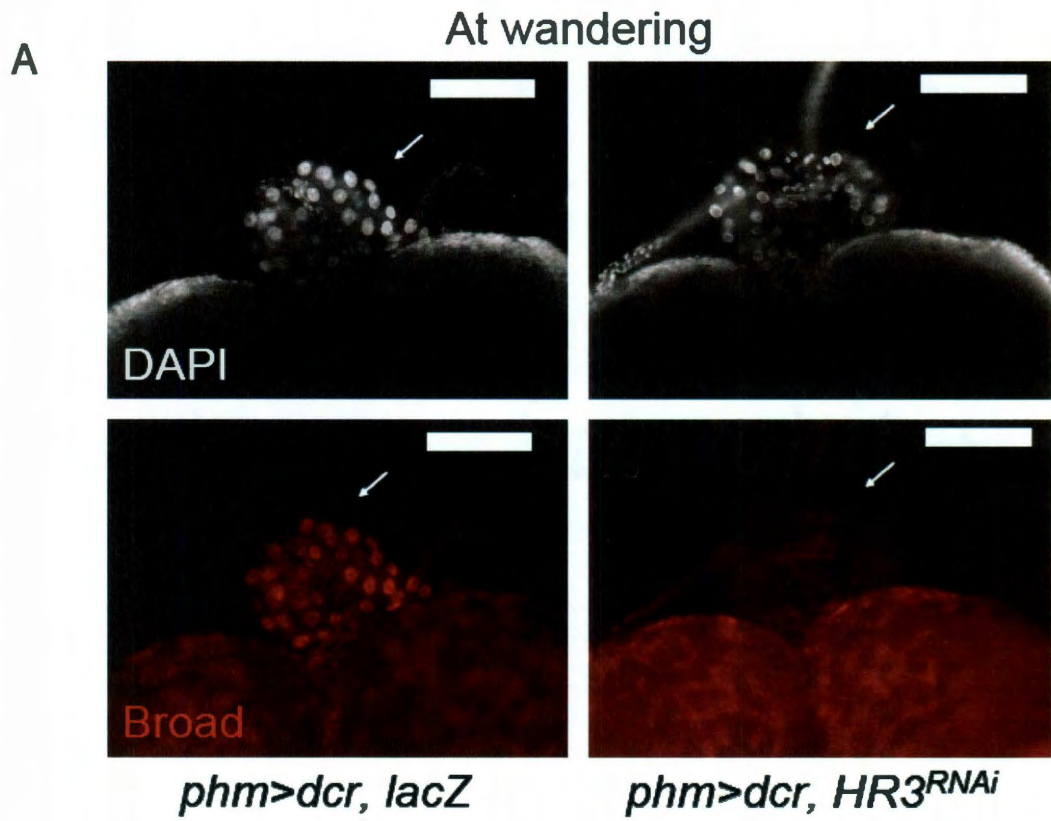
Figure 4.6. **Loss of *HR3* in the PG decreases Br immunoreactivity and *dib* levels.** A) Brain lobes and ring glands from larvae of indicated genotypes at wandering stained with DAPI (white; top panel) and with anti-Broad antibody (red, bottom panel). The arrows point to the RG. The scale bar is 100 μm. B) Mean *dib* transcript levels (y-axis) were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at wandering. Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A* and are presented in arbitrary units (A.U.).

provided by Henry Krause) and the *HR3^RNAi* with the *phm-Gal4* driver and found that, as predicted, Br protein levels in the PG were blocked both at wandering and 17-24 hours after the L2/L3 molt (Figure 7). In addition, onset of pupariation was blocked in both *phm>E75A^+* and *phm> HR3^RNAi*: larvae either exhibited prolonged, L3 wandering, or pupariated directly from L2 (not shown); as described above, both properties are indicative of profound deficits in ecdysone synthesis. Finally, transcript levels from both *dib* and *phm* were significantly decreased when measured at the wandering stage (Figure 7B and data not shown). These observations support the notion that HR3 and E75, as activator and repressor of *βftz-f1* transcription, regulate *br* expression and Halloween genes expression.

4.7 Ectopic expression of βFTZ-F1 rescues deficits in br and Halloween genes expression conferred by E75A

If *E75A* overexpression inhibits *br*, *dib* and *phm* transcription because of failure to transcribe *βftz-f1*, then ectopic βFTZ-F1 expression is predicted to restore *br*, *dib* and *phm* expression to this genotype. To express *βFTZ-F1* ectopically, we used flies bearing a Mae-UAS insertion upstream of the endogenous *βFTZ-F1* genes (constructed by John Merriam and provided by Craig Woodard). Because *phm*-driven *βFTZ-F1* misexpression appears to be toxic (larvae arrest at L1), we drove *βFTZ-F1* expression with the weaker *amn^c651* driver and further utilized the *gal80^ts* transgene (described above) to enable

Figure 4.7 **E75A regulates Halloween genes and *br* transcription in the PG.**
A) Mean *dib* transcript levels (y-axis) were determined from at least two biological
samples collected from each genotype (inset) and measured in triplicate at ~24
hrs after temperature shift. Error bars represent SEM. Data were obtained by
quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A*
and are presented in arbitrary units (A.U.). B) Brain lobes and ring glands from
larvae of indicated genotypes at ~24hrs after temperature shift stained with DAPI
(white; top panel) and with anti-Broad antibody (red, bottom panel). The arrows
point to the RG. The scale bar is 100 μm.

transgenes induction at the L2/L3 molt. We found that co-expression with *βftz-f1*, but not *lacZ*, was able to restore *br*, *dib* and *phm* expression to larvae overexpressing E75 (Figure 8). This observation supports the possibility that E75 inhibits *br, dib* and *phm* expression via HR3 and βFTZ-F1.

4.8 Ectopic expression of βFTZ-F1 rescues deficits in *br* and Halloween genes expression conferred by GSK3^{S9A}

In mammalian cells, activity of Rev-erb, the orthologue of E75, is maintained by GSK-3: Rev-erb is protected from proteolysis by GSK3-mediated phosphorylation (Wang et al., 2006). Given that GSK3 activity is decreased by PI3K/Akt, this observation raises the possibility that in the Drosophila PG, GSK-3 is required to maintain activity of E75, thus preventing premature HR3 activation (Figure 1.4). As insulin-mediated PI3K/Akt activity reaches threshold, GSK-3 is inactivated, thus permitting E75 dephosphorylation and destabilization, consequently releasing HR3 from inhibition and enabling transcription of *βftz-f1*. If so, then ectopic *βftz-f1* expression is predicted to restore *br, dib* and *phm* expression to GSK3^{S9A}- expressing larvae. To test this prediction, we compared *br* expression and *dib* and *phm* transcript levels in *gal80^{ts}; amn^{c651}>GSK-3^{S9A}*, *βftz-f1* and *gal80^{ts}; amn^{c651}>GSK-3^{S9A}, lacZ*. We found that as predicted, *βftz-f1* co-expression, but not *lacZ* co-expression, was able to restore both *br* expression and *dib* and *phm* transcript levels to *amn^{c651}>GSK-3^{S9A}* (Figure 4.8A and 4.8B).
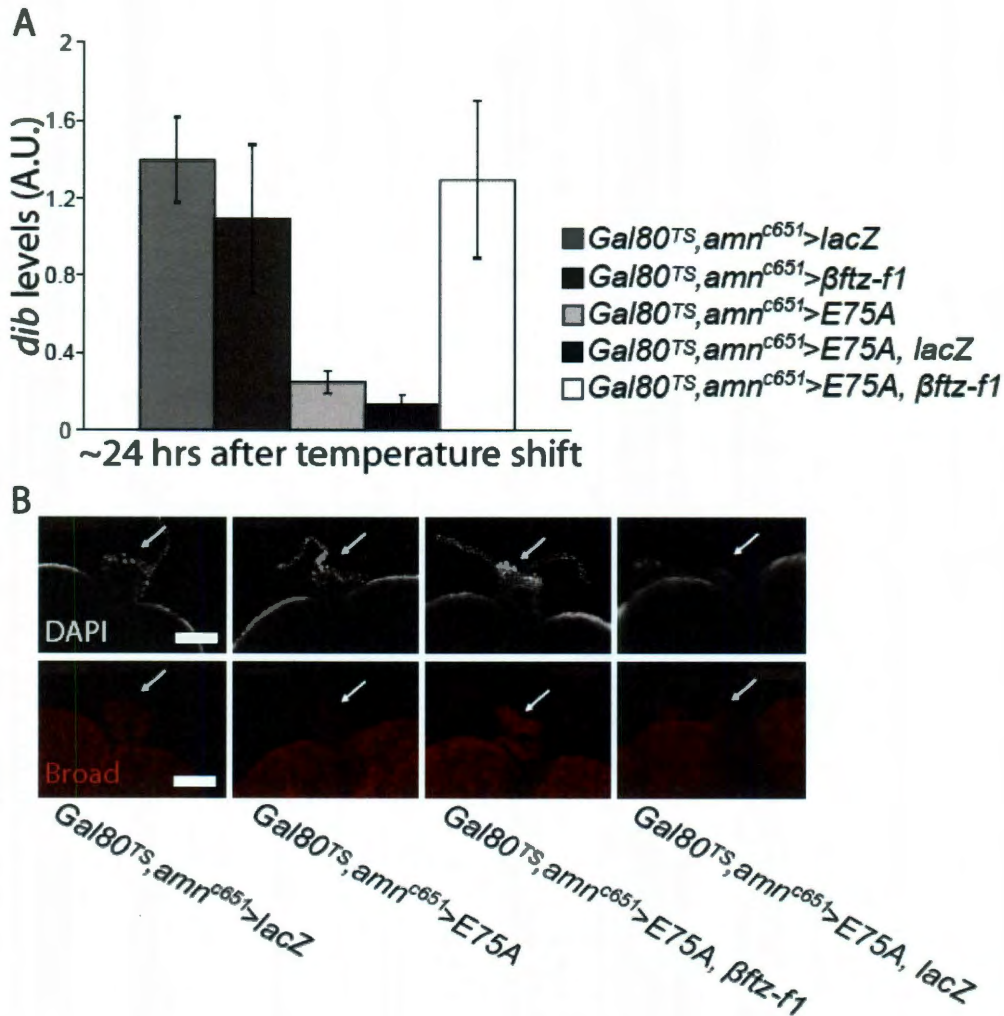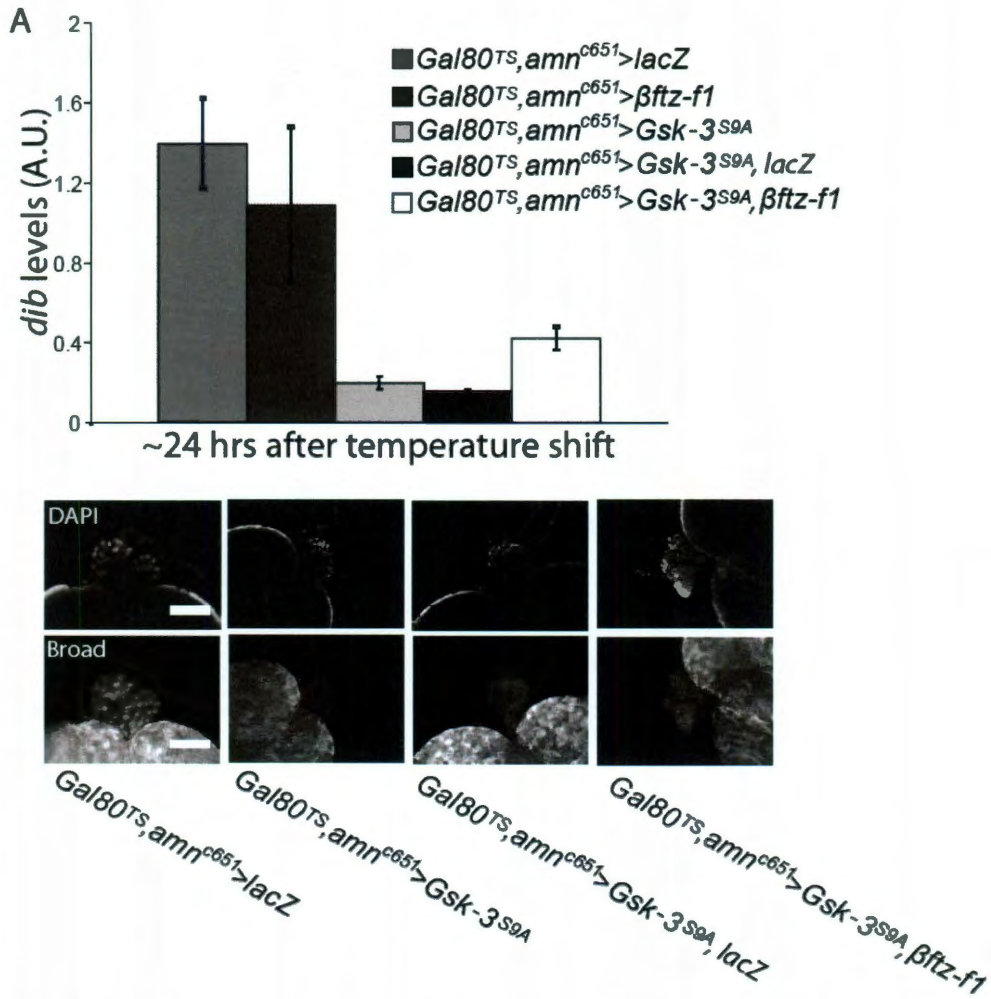
Figure 4.8 **GSK-3 regulates Halloween genes and *br* transcription in the PG.**
A) Mean *dib* transcript levels (y-axis) were determined from at least two biological
samples collected from each genotype (inset) and measured in triplicate at ~24
hrs after temperature shift. Error bars represent SEM. Data were obtained by
quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A*.
B) Brain lobes and ring glands from larvae of indicated genotypes at ~24hrs after
temperature shift stained with DAPI (white; top panel) and with anti-Broad
antibody (red, bottom panel). The arrows point to the RG. The scale bar is 100
μm.

Taken together, these observations suggest that the loss of *dib* and *phm* transcription conferred by $GSK3^{S9A}$ reflects at least in part insufficient levels of βFTZ-F1 protein and is consistent with the hypothesis that GSK3 activity prevents *br* expression by preventing relief of E75-mediated inhibition of *βftz-f1* expression.

4.9 An ecdysone-mediated feed forward is required for *br* expression and *dib* and *phm* transcription

Metamorphosis is an example of a phenomenon that proceeds in an "all or none", rather than in a graded fashion: once a larva has committed to metamorphosis, it must proceed to completion because a partial metamorphosis would not be effective. Often, all-or-none phenomena are mediated by positive feedback mechanisms (for example, the neuronal action potential). An ecdysone-mediated positive feedback has been observed in non-Drosophila insects (Sakurai and Williams, 1989), and some lines of evidence suggest that ecdysone-mediated positive feedbacks occur in Drosophila as well (Bialecki et al., 2002). Furthermore, the requirement for *br*, an ecydosone-induced gene, in ecdysone synthesis is consistent with a br-mediated positive feedback, as suggested previously (Xiang et al., 2010). To test if ecdysone might mediate a positive feedback in Drosophila, we blocked ecdysone signaling in the PG by driving expression of the dominant-negative ecdysone receptor EcR^{DN} (Cherbas et al., 2003) with amn^{c651}. We found that EcR^{DN} expression prevented *br*

expression in the PG at the wandering L3 stage (Figure 4.9A), and decreased both *dib* and *phm* transcript levels about two-fold (Figure 4.9B and data not shown), which is similar to the decrease in *dib* and *phm* transcription conferred by $PI3K^{DN}$ (Figure 4.1, Colombani at all., 2005). Thus, ecdysone-signaling in the PG is required for *br* expression and hence Halloween genes expression.

These results predict that EcR^{DN} expression attenuates Halloween genes expression by preventing *torso* expression, and hence ability to activate the torso pathway. If so, then ectopic activation of the torso pathway by Raf^{GOF} expression would be predicted to suppress these deficits in Halloween genes expression, as we observed for br^{npr}, $PI3K^{DN}$, and $\beta ftz-f1^{RNAi}$. Unexpectedly, however, we found that Raf^{GOF} failed to restore normal or elevated *dib* or *phm* transcript levels to EcR^{DN} (not shown). This result raises the possibility that EcR has a function in Halloween genes expression downstream of Raf, in addition to a role in promoting *br* expression.

A

At wandering

DAPI

Broad

phm>lacZ

phm>EcR^{DN}

B

dib levels (A.U.)

amn^{c651}>lacZ

amn^{c651}>EcR^{DN}
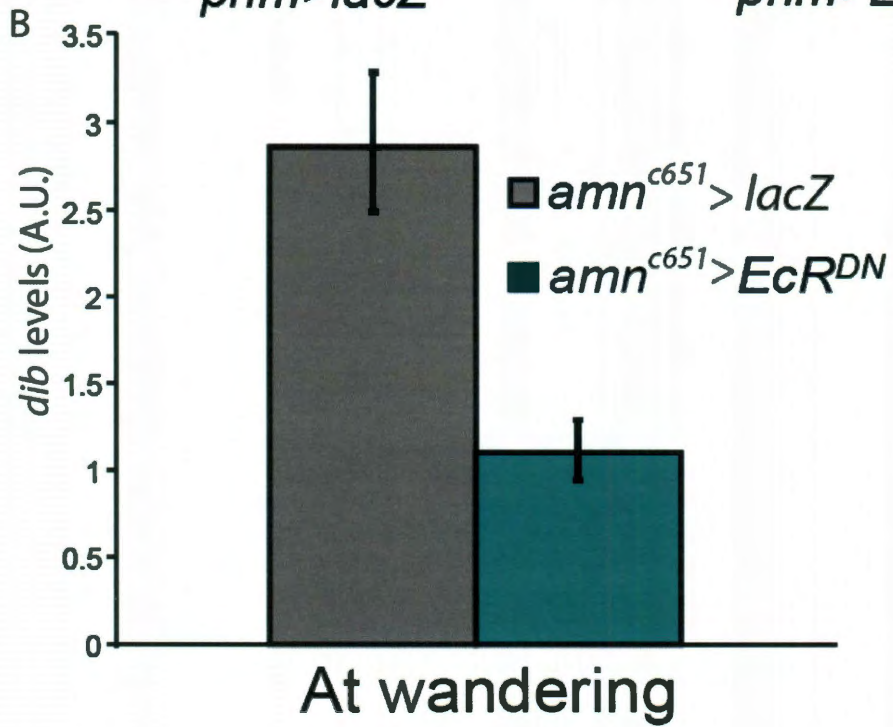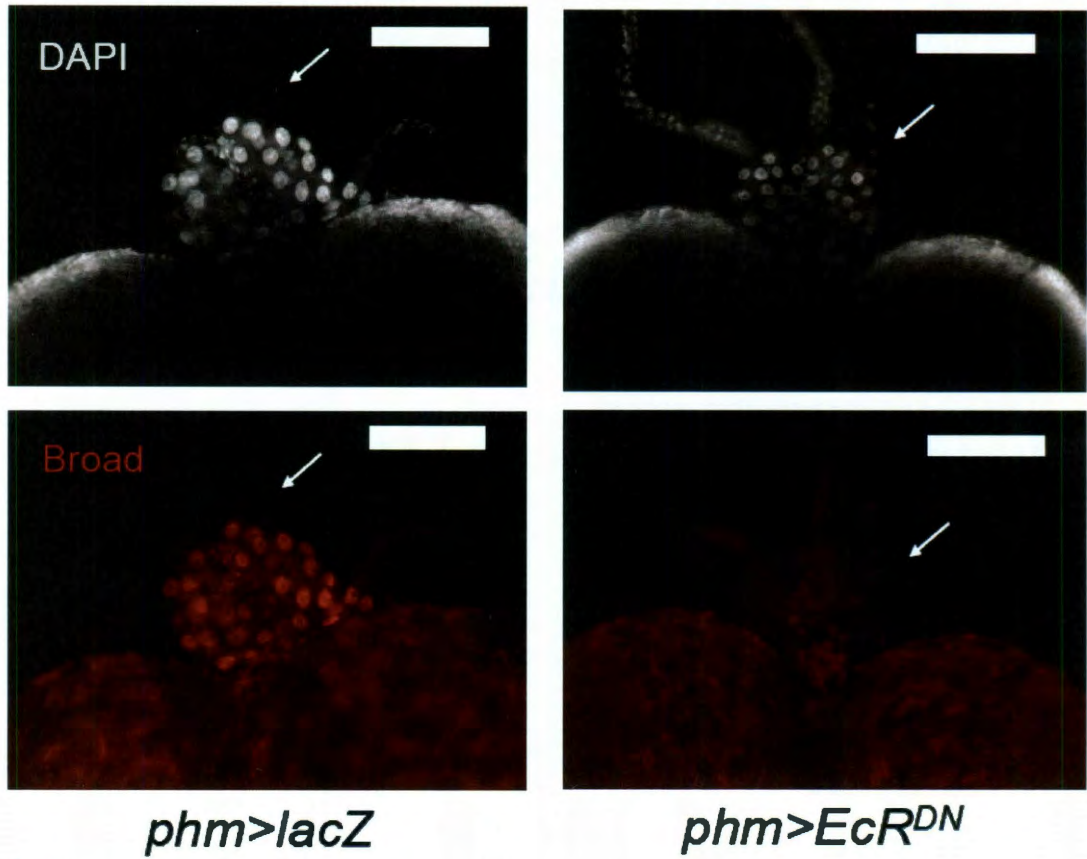
At wandering

Figure 4.9. **Loss of EcR activity in the PG decreases Br immunoreactivity and *dib* levels.** A) Brain lobes and ring glands from larvae of indicated genotypes at wandering stained with DAPI (white; top panel) and with anti-Broad antibody (red, bottom panel). The arrows point to the RG. The scale bar is 100 µm. B) Mean *dib* transcript levels (y-axis), were measured from two biological samples collected from developmentally staged larvae of the indicated genotype (inset) and measured in triplicate at wandering. Error bars represent SEM. Data were obtained by quantitative RT-PCR with the relative $2^{-\Delta\Delta Ct}$ method and normalized to *RpL13A* and are presented in arbitrary units (A.U.).

Chapter 5: Summary

5.1 The role of IIS on timing the onset of metamorphosis

We have shown that increasing IIS in Drosophila advances the onset of metamorphosis in Drosophila (Walkiewicz and Stern, 2009). Our results are consistent with previous observations that manipulations of the PI3K pathway, a known target of IIS, specifically in the PG also advance the onset of metamorphosis (Caldwell et al., 2005; Mirth et al., 2005) In addition, the mechanism by which the advancement in the onset of metamorphosis is achieved is the same; in both studies ecdysone synthesis was accelerated as a consequence of precocious transcriptional activation of at least two of the Halloween genes (Colombani et al., 2005; Walkiewicz and Stern, 2009). It was also previously reported that loss PI3K activity in the PG delays the onset of metamorphosis by decreasing ecdysone synthesis and hence Halloween genes transcription (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). These observations together strongly suggest that IIS is the rate limiting step for metamorphosis. Moreover, we also show that in Drosophila increasing IIS accelerates growth rate which results in very rapid development rather then increased body size (Walkiewicz and Stern, 2009). By what mechanism does IIS control Halloween genes transcription?

5.2 A mechanistic link between body size and activation of Halloween genes expression

It has been demonstrated that in Drosophila larvae, insulin growth factor (Dilp) signaling advances the onset of metamorphosis by increasing expression of at least certain "Halloween" genes, which encode ecdysone biosynthetic genes (Walkiewicz and Stern, 2009). Furthermore, evidence indicates that activity of PI3K, the major intracellular target of insulin growth factors, within the PG, mediates these effects of Dilp signaling: transgene-induced activation of PI3K in the PG advances the onset of metamorphosis and increases transcription of at least two *Halloween* genes (*dib* and *phm*), which are effects similar to those conferred by activating Dilp signaling (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Walkiewicz and Stern, 2009). In contrast, PI3K inhibition in the PG confers opposite phenotypes: delayed onset of metamorphosis and decreased expression of *dib* and *phm (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Walkiewicz and Stern, 2009).* However, the mechanism linking PI3K activity and *Halloween* genes expression was not elucidated.

Here we identify several molecular intermediates linking PI3K activity and *Halloween* genes expression (Figure 5.1). First we identify the BTB transcription factor *broad (br)* as a critical link between PI3K and Halloween genes expression. *br* was previously shown to be required for ecdysone synthesis, and more recently, to be required for transcription of Halloween genes, presumably
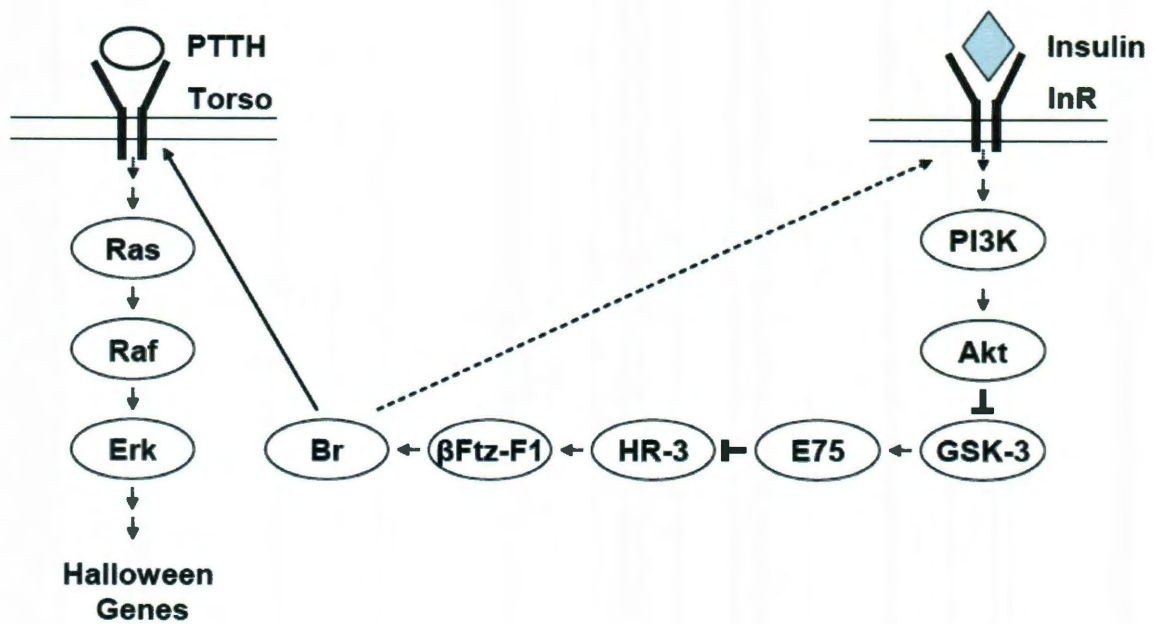
Figure 5.1 **A model for activation of Halloween gene transcription.** Insulin binds and activates the insulin receptor, which in turn activates PI3K. Activation of PI3K leads to AKT activation, which phosphorylates and inhibits GSK3. When inhibited GSK3 can not phosphorylate E75 resulting in degradation of the protein. Once E75 is degraded it can not inhibit *HR3*; HR3 then activates *βftz-f1*. βFTZ-F1 activates *br* transcription. Once present, Br activates *torso*. Torso is then activated by PTTH, which results in activation of Ras. Ras activates Raf, which activates Erk. Erk activates and unknown transcription factor(s). The unknown transcription factor(s) activate the Halloween gene transcription, which leads to ecdysone synthesis.

as a secondary consequence of a requirement for transcription of *torso*, which encodes the tyrosine kinase receptor for the PTTH metamorphic signal (chapter 4). We found that inhibiting PI3K prevents *br* expression, which provides an immediate mechanism for the activation of Halloween genes expression by PI3K (chapter 4). Second, we find that PI3K promotes *br* expression by inhibiting the activity of the downstream kinase GSK-3 (chapter 4): expressing a constitutively active $GSK-3^{S9A}$ prevents *br* expression and *dib* and *phm* transcription (chapter 4). Third, we show that the nuclear hormone receptor *βftz-f1* is required for *br* expression (chapter 4). A requirement for *βftz-f1* in *dib* and *phm* expression had previously been shown; our results suggest that failure to express *br* underlies this requirement (Parvy et al., 2005). Fourth, we find that the deficits in *dib* and *phm* expression conferred by *PI3K* inhibition, or by loss of *br* or *βftz-f1*, reflect failure to activate the Torso pathway because ectopic expression of the constitutively-active Raf^{GOF}, which is predicted to confer torso-independent activation of this pathway, suppresses these deficits (chapter 4). Fifth, we provide evidence that additional hormone receptors, E75 and HR3, which had previously been characterized as regulators of *βftz-f1* expression, also regulate *br* expression (chapter 4). Sixth, we find that lack of *βftz-f1* expression underlies these deficits conferred by *E75A* or $GSK-3^{S9A}$ expression, because ectopic *βftz-f1* expression at least partially rescues these deficits (chapter 4). Finally, the observation that mammalian GSK-3 stabilizes from proteolysis the E75 orthologue Rev-erb (Wang et al., 2006) raises the possibility that in the Drosophila PG, $GSK-3^{S9A}$ prevents *βftz-f1* expression by similarly stabilizing E75,

which then prevents HR3 from activating *βftz-f1*expression (chapter 4). .

These results are consistent with a model (Figure 5.1) in which growth rate, and hence Dilp signaling, promote Halloween genes expression and the consequent transition to adulthood by activating PI3K within the PG (Walkiewicz and Stern, 2009). When PI3K activity reaches a threshold, the consequent GSK3 inhibition destabilizes E75 and enables HR3-dependent expression of *βftz-f1*. βFTZ-F1, in turn, promotes *br* expression, followed by *torso* expression (chapter 4). Following the appearance of torso, the PG becomes competent for the first time to respond to the PTTH and permits the Raf-dependent activation of Halloween genes expression (chapter 4). This initial burst of Halloween enzymes generates an initial synthesis of ecdysone, which induces additional *br* expression, additional *torso* expression, and thus further increases sensitivity of the PG to PTTH (ecdysone feed forward mechanism.). Ultimately, ecdysone reaches high enough levels to promote the onset of metamorphosis (chapter 4).

5.3 A molecular correlate of critical size

Previous work in Manduca identified a larval size (termed "critical size") that once attained enabled metamorphosis even in the absence of further nutrition (Davidowitz et al., 2003). Attainment of critical size therefore meant successful completion of a nutritional checkpoint for metamorphosis. Although a molecular and cellular correlate to critical size was not specified, given the close connection between nutrition, Dilp signaling and PI3K activity, it would not be

surprising if PI3K activity played a role in critical weight. In fact, Mirth et al. (2005) reported that transgene-induced activation of *PI3K* within the PG significantly decreased critical size in Drosophila and thus enabled larvae to reach critical size earlier than wildtype: whereas wildtype reached critical size at about 12 hours after the molt from second to third instar (L2/L3), larvae in which the activated *PI3K-CAAX* was expressed in the PG reached critical size only 4 hours after the L2/L3 molt . Thus, PI3K activity in the PG specifies critical size, and this work identified a period about 12 hours after the L2/L3 molt as critical one for PI3K to promote competence to undergo metamorphosis (chapter 4).

We propose that the PI3K-βFTZ-F1 pathway described in Figure 5.1 represents the molecular correlate of critical size. In this view, in larvae prior to attainment of critical size, active GSK3 prevents expression of *βftz-f1* expression and thus prevents *br* and ultimately *torso* expression (chapter 4). Thus, prior to attainment of critical size, the PG lacks the receptor required to respond to the PTTH metamorphic signal. When critical size is attained, PI3K activity reaches a level that inactivates GSK3 sufficiently to destabilize E75, and thus permit *βftz-f1* expression, and hence *br* and ultimately *torso* expression (chapter 4). Thus, the following attainment of critical size, the PG attains competence to respond to PTTH. The consequent PTTH-dependent activation of Raf, and then Erk, enables induction of *Halloween* genes expression (Rewitz et al., 2009) by transcription factors that have not yet been identified .

The expression patterns of *βftz-f1* and *br* support this proposal. βFTZ-F1 immunoreactivity first becomes detectable in the PG 4 hours after critical size is

attained (Parvy et al., 2005), whereas Br immunoreactivity first appears 5 hours after critical size (Zhou et al., 2004). These results are consistent with the proposed requirement of PI3K for expression of *βftz-f1*, and of βFTZ-F1 for expression of *br* (chapter 4).

5.4 Participation of an ecdysone feed forward in Halloween genes expression

Although βFTZ-F1 immunoreactivity becomes undetectable in the PG about 12 hours after critical size is attained (Parvy et al., 2005), which might reflect ecdysone-mediated inhibition of *βftz-f1* expression (Woodard et al., 1994), Br levels are maintained until pupariation and beyond (Zhou et al., 2004). Given that *br* expression is inducible directly by ecdysone-EcR (Karim and Thummel, 1992), the maintenance of *br* expression following the disappearance of βFTZ-F1 might reflect a switch of *br* expression from βFTZ-F1-dependent to ecdysone-dependent. Because Br protein is required for ecdysone synthesis (Kiss et al., 1980; Xiang et al., 2010), such a process could represent a mechanism underlying the ecdysone-mediated positive feedback on ecdysone synthesis observed in Drosophila and other insects.

It was previously reported that small increases in both *ptth* transcripts and ecdysone titers occurs at specific intervals during the first half of L3 (McBrayer et al., 2007; Warren et al., 2006). We suggest that these periodic small increases in ecdysone levels serve to increase *br* expression; this increase in Br levels then

activates *torso* transcription and thus sensitizes the PG to the next release of PTTH release. Ultimately, this feed forward generates a burst of ecdysone sufficient to initiate pupariation. The period of time following attainment of critical size until onset of metamorphosis is termed the "terminal growth period" (TGP). We propose that this duration reflects the time required for the ecdysone feed forward once initiated to induce sufficient ecdysone to promote metamorphosis.

Previous investigators have studied the role of nutrition and insulin signaling in specifying the duration of the TGP, and have reported conflicting results (Layalle et al., 2008; Stieper et al., 2008). The model shown in Figure 5.1 provides no avenue for nutrition, Dilp signaling, or PI3K activity to affect developmental timing once critical size is attained, suggesting that the duration of the TGP is insulin independent. However, our results that altered PI3K and GSK3 strongly affect both developmental timing and *dib* and *phm* transcript levels even when the PTTH/Torso pathway is ectopically activated are consistent with the possibility that PI3K and GSK3, and hence nutrition, might affect duration of the TGP via pathways distinct from the PI3K-βFTZF1 pathway described here (for example, PG cell and nuclear size, which are affected by both PI3K and GSK-3, as well as PI3K-dependent effects on mRNA translation) (chapter 4). These possibilities require additional testing.

5.5 Pupariation in the absence of PTTH

Genetic ablation of the neurons that express PTTH delays development

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and significantly increases critical size (McBrayer et al., 2007). Given our hypothesis that critical size is sensed by Dilp signaling within the PG, we interpret this result to mean that larvae lacking PTTH require more insulin signaling than wildtype to induce pupariation. One possible mechanism underlying this interpretation is that Dilps, like PTTH, activate the Ras/Raf pathway and thus are capable of inducing PTTH- and torso- independent Raf activation and hence *Halloween* genes expression, further suggesting that in the complete absence of PTTH, pupariation is driven by Dilp and insulin receptor (InR)-driven Raf activation. In this view, the Dilp-InR activity sufficient to induce pupariation via βFTZF1 is not adequate to induce pupariation via direct activation of Ras/Raf. The increased Dilp-InR activity needed to induce pupariation via Ras/Raf is revealed as an increase in critical size for pupariation. The ability of InR or insulin growth factor receptors to activate Ras and Raf is well established (Goalstone and Draznin, 1998). In addition, we observed that increasing insulin signaling via expressing transgenes in the insulin producing cells confers a stronger increase in *dib* transcription than expressing the constitutively-active *PI3K-CAAX* in the PG (Walkiewicz and Stern, 2009). These observations support the possibility that insulin signaling has PI3K-independent effects on *dib* expression, consistent with an ability of Dilp peptides to activate *Halloween* genes expression via Ras/Raf.

Although larvae with PTTH neurons ablated are able to pupariate, *br* null mutants are not. It is possible that this complete block in pupariation is a consequence of the observed decrease in *InR* transcript levels in *br^{npr}* (Xiang et

al., 2010). This decrease in InR, combined with the stronger decrease in torso, might prevent sufficient activation of Ras/Raf by dilp-InR to support pupariation.

5.6 Other checkpoints for onset of metamorphosis

The studies reported here most likely deal with a nutritional check point that must be completed for the larva to become competent to undergo metamorphosis. However there are other, nutrition-independent, checkpoints required for metamorphosis. For example, damage or slow growth within imaginal discs delays metamorphosis (Halme et al., 2010; Stieper et al., 2008). This delay is mediated by retinoic acid (Halme et al., 2010). Part of this developmental delay reflects decreased expression of *PTTH;* thus, PTTH release onto the PG communicates the completion of a "disc damage" checkpoint. Damaged discs also delay development in a PTTH-independent manner; this delay might reflect inhibited insulin growth factor signaling, or direct effects on signaling pathways within the PG that regulate competence to respond to PTTH.

5.7. Concluding remarks and future experiments

Our work provides an insight to molecular events that govern the initiation of metamorphosis in Drosophila. We propose that the PI3K-βFTZ-F1 pathway (Figure 5.1), which we propose, is activated when the Drosophila larvae obtain "critical size" allows *br* activation, which then activates *torso* transcription.

Activation of *br* and consequently *torso* prime the PG to receive the incoming PTTH signal. Once the PTTH signal is received it leads to activation of Ras and the MAPK pathway, which activates transcription oh the at least two of the Halloween genes; *dib* and *phm*.

This model further suggests that nutrition plays a critical role in regulating initiation of metamorphosis. If this hypothesis is correct then larvae with ablated IPC, which has been shown delay the onset of metamorphosis (Rulifson et al., 2002) will exhibit decreased Br immunoreactivity in the PG. Consequently, I will predict that larvae reared on a diet with a poor nutritional content, will also exhibit decreased Br levels in the PG and the loss of Br immunoreactivity in the PG is the direct cause of the delay of initiation of metamorphosis previously observed (Robertson, 1960, Tu and Tatar, 2003).

Furthermore, our model also predicts that transcription of the Halloween genes is regulated by an unknown transcription factor(s). Additional work needs to be performed to elucidate these molecules. I propose a screen of known Erk targets using RNAi. I predict that loss of the transcription factor(s) in the PG will eliminate the Halloween enzymes form the PG.

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