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Ras Signaling in Either Prothoracic Gland Cells or Cholinergic Neurons of Drosophila melanogaster Regulates Fly Size

by

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ABSTRACT

Ras Signaling in Either Prothoracic Gland Cells or Cholinergic Neurons of *Drosophila melanogaster* Regulates Fly Size

by

Philip E. Caldwell

Body size in multicellular organisms is determined by the integration of two factors: the rate of growth and the duration of growth. In most animals, the rate of growth is controlled cell autonomously by the insulin-stimulated Pi3 kinase (Pi3K) pathway. However, the duration of growth is controlled in a more complex manner that involves endocrine factors that act cell non-autonomously. For example, in insects such as *Drosophila*, the duration of each larval phase is regulated by the timing of release of the molting hormone ecdysone from the prothoracic gland (PG).

The molecular mechanisms by which the rate of growth and the duration of growth are integrated remain poorly understood. To help shed light in this area, I have investigated the intracellular signaling events that regulate ecdysone release in the *Drosophila* PG. I have found that expressing activated Ras, or the targets of Ras signaling Raf or Pi3K, in the PG reduces fly size and accelerates larval development via precocious synthesis and release of ecdysone. In contrast, expression of dominant-negative (dn) Ras, Raf, or Pi3K increases fly size and prolongs larval development via delayed synthesis and release of ecdysone. These results indicate that Ras-Raf and Pi3K signaling act in the PG to regulate the duration of growth by altering the timing of ecdysone synthesis and release.
Conversely, I have found that expressing dn-Ras or dn-Raf, but not dn-Pi3K, in cholinergic neurons increases fly size and prolongs larval development, whereas, expression of activated Ras or Raf, but not Pi3K, in cholinergic neurons decreases fly size, but delays larval development. Inhibition of insulin signaling in flies, via chromosomal loss-of-function mutations, also decreases fly size and delays development, raising the possibility that Ras-Raf signaling in cholinergic neurons may affect fly size by controlling the rate of growth via systemic insulin signaling.
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This dissertation is dedicated to my family, especially my wife Hayley.
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CHAPTER 1. INTRODUCTION AND BACKGROUND

One fundamental question of development remains mysterious – how is the size of an animal determined? How is it, for example, that an elephant grows to be larger than a mouse, and that the arms of a human grow to be the same length? We know that the size of an animal or tissue depends on the rate and duration of growth. However, the molecular mechanisms underlying these factors largely remain unknown and have historically been studied by different schools of biology. Mechanisms controlling the rate of growth have been elucidated largely by geneticists; whereas, mechanisms controlling the duration of growth have been studied principally by physiologists. Perhaps because of these differences, it is unclear how, or even if, these two factors interact.

1.1 Regulation of cell size

Both local and systemic controls can determine tissue size, but their relative importance can vary greatly. For example, if multiple fetal thymus glands are transplanted into a developing mouse, each thymus grows to its normal adult size, suggesting that their growth is mainly controlled by factors within the thymus (Metcalf, 1963). However, if the same experiment is performed with fetal spleens, the total mass of the transplanted spleens attains the mass of one normal adult spleen, suggesting that their growth is mainly controlled by factors outside the spleen (Metcalf, 1964). Similar transplantation experiments, as well as tissue culture experiments, indicate that most animal organs grow to a characteristic size under cell-autonomous controls (Bryant and Simpson, 1984; Goss, 1978).
Systemic factors can also influence final animal and tissue size. Growth hormone (GH), for instance, which the pituitary gland secretes under the control of the hypothalamus, plays a major role in stimulating postnatal mammalian growth: children deficient in GH become dwarfs, whereas those with excessive GH become giants. GH stimulates growth largely by inducing the liver and other organs to produce insulin-like growth factor 1 (IGF1) (Heyner and Garside, 1994). Although genes largely determine the size of an animal or tissue, environmental factors such as nutrition also play a part. During development, tissues deprived of adequate nourishment end up smaller than normal (Day and Lawrence, 2000).

Differences in size between animals of the same species can reflect differences in cell size, cell number, or both. Cell numbers depend on cell division, which, like cell size, depends on both intracellular signaling events and extracellular signaling molecules that regulate these events.

1.1.1 Pi3K signaling and the regulation of cell size

Growth factors activate intracellular signaling pathways that stimulate protein synthesis in the cell, so that the rate of macromolecular synthesis exceeds the rate of macromolecule degradation. One of the most important of these pathways operates through the binding of insulin-like peptides to the insulin receptor (InR) (Figure 1.1). This process activates phosphatidylinositol 3-kinase (Pi3K), by bringing it to the plasma membrane (Stocker et al., 2002). Pi3K can then phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP₂) at the 3’ position to create phosphatidylinositol 3,4,5-trisphosphate (PIP₃), which recruits proteins that contain a pleckstrin homology domain to the plasma membrane for activation (Corvera and Czech, 1998). Among these are the serine-
Figure 1.1 The insulin-Pi3K signaling pathway (adapted from Hay and Sonenberg, 2004). Insulin binding to its receptor activates Pi3K, simultaneously repressing its negative regulator PTEN, to increase PIP$_3$ concentrations at the cell membrane. PIP$_3$ attracts proteins with a pleckstrin homology domain, such as Akt and Pdk1, to the cell membrane where they are activated. Akt inhibits both the TSC1/2 complex and the forkhead transcription factor FOXO. TSC1/2 also inhibits the small GTPase Rheb, which activates TOR by releasing its negative regulator, Raptor. Activated TOR activates the translational activator S6K and inhibits the translational inhibitory factor 4E-BP, which combine to increase cellular protein synthesis. (+) Activation, (-) inhibition.

Threonine kinase Akt, as well as its activating kinases Pdk1 (3-phosphoinositide-dependent kinase 1) and possibly Pdk2 (Verdu et al., 1999). Recruitment of Akt to the membrane by PIP$_3$ results in activation, which is the predominant and essential mediator for the regulation of both growth and proliferation by Pi3K (Gray et al., 1999; Lietzke et al., 2000; Oatey et al., 1999). Akt inactivates the tuberous sclerosis complex (TSC1/2) via phosphorylation, which results in the inactivation of the GTPase, Rheb; thus, Akt signaling activates Rheb (Jaeschke et al., 2002; Li et al., 2004; Marygold and Leevers, 2002). Rheb-GTP can then dissociate the target of rapamycin (TOR) from its negative regulatory associated protein (Raptor) (Goberdhan and Wilson, 2003). TOR has multiple
and diverse functions, including the control of protein synthesis (Thomas et al., 2004). Activated TOR phosphorylates the translation inhibitory factor 4E-binding protein (4E-BP) and the translational activator, p70 S6-kinase (S6K) (Hay and Sonenberg, 2004), causing an increase in translation.

Additionally, InR-Pi3K signaling can regulate the the activity of the forkhead transcription factor FOXO. When activated, FOXO activates the transcription of 4E-BP, resulting in translational inhibition. However, activated Akt can triply phosphorylate FOXO, allowing 14-3-3 proteins to inactivate FOXO by sequestering it outside of the nucleus. FOXO inactivation results in decreased 4E-BP transcription and allows translation to proceed (Junger et al., 2003). Together, these effectors of insulin signaling can control transcription and translation, which integrate to control cell size (Zetterberg et al., 1984; Chen et al., 1996).

Mutations in Drosophila genes encoding insulin signaling molecules, such as InR, the InR substrate Chico, Pi3K, FOXO, and several additional targets of Pi3K activity, can create larger or smaller flies dependent on their promotion or inhibition of insulin signaling (Huang et al., 1999; Leevers et al., 1996; Verdu et al., 1999; Weinkove et al., 1999). At the organismal level, mutations in Pi3K are lethal and result in larval growth arrest or delay in the third instar (Weinkove et al., 1999). Loss of InR is embryonic lethal (Chen et al., 1996; Fernandez et al., 1995), but certain combinations of InR alleles, as well as mutations in Chico or S6K, cause delays in larval development, reduced cell size, and small adults (Bohni et al., 1999; Chen et al., 1996; Montagne et al., 1999).
1.1.2 Ras signaling and the regulation of cell size

Another important pathway that regulates cell size involves the oncoprotein Ras. Ras is a membrane-associated GTPase that is normally activated in response to the binding of extracellular signals, such as growth factors, to receptor tyrosine kinases (Carpenter, 2000; Willingham et al., 1980). These receptors include the epidermal growth factor receptor (EGFR) in mammals and its Drosophila homologs dEGFR/DER, Sevenless, and Torso (Duffy and Perrimon 1994). Phosphotyrosine residues on activated receptors bind the Src homology 2 (SH2) domains of adaptor proteins, which bind guanine-nucleotide exchange factors, such as Son of sevenless (Sos) (Egan et al., 1993; Medema et al., 1993). Sos catalyzes the conversion of Ras from a GDP-bound state to a GTP-bound active state, resulting in conformational changes in two so-called switch domains (Boriack-Sjodin et al., 1998). Ras is inactivated by Ras GTPase-activating proteins (Ras-GAPs), such as Neurofibromin (NF1), which function by providing an essential catalytic arginine for Ras-GTP hydrolysis (Scheffzek et al., 1997). GTP-bound mammalian Ras recruits several effector proteins including Raf, Pi3K, and the GEF for Ral to the cell membrane, in which they become activated by either Ras-induced conformational changes (Pacold et al., 2000), or other membrane-associated proteins (Morrison and Cutler, 1997). Mutations in Ras that cause constitutive GTP binding result in constitutive signaling to downstream effector proteins, and are frequently found in a variety of human tumors (Barbacid, 1987).

Activation of Ras signaling increases protein levels of the basic helix-loop-helix/leucine zipper transcription factor, Myc, and work in Drosophila has suggested that Myc at least partially mediates Ras-driven growth (Prober and Edgar, 2000; Sears et al.,
1999). Mutations that affect either Ras or Myc activity within the developing *Drosophila* wing can affect cell size (Johnston et al., 1999; Prober and Edgar 2000). Consistent with these results, constitutive Myc expression in B-lymphocytes of developing mice, or in a transformed human B cell line, increases rates of cellular growth, observed as increases in cell size and rate of protein synthesis (Irintani and Eisenman, 1999; Schumacher et al., 1999). Expression of activated Ras in mouse heart tissue also increases tissue size as a result of cell enlargement (Hunter et al., 1995). Thus, control of cell size appears to be a primary role of Ras and Myc in both flies and mice.

1.1.3 Regulation of cell division

Extracellular mitogens and inhibitory molecules regulate cell division to ensure that the cells of an organism only divide when more cells are needed. Mitogens stimulate the production and activity of components of the cell-cycle control system that promote progression through the cycle (Sherr, 1994). Conversely, proliferation inhibitors usually stimulate the production or activity of components of the cell-cycle control system that block progress through the cycle, usually in G1 (Sherr and Roberts, 1995).

Studies in *Drosophila* point to a role for Ras and Myc in control of the cell cycle. Ectopic expression of Myc, or constitutively active Ras, in wing imaginal discs promotes growth through cell division, which occurs through a shortening of the G1 phase by inducing the transcription of Cyclin E, the limiting regulator of the G1-S transition (Johnston et al., 1999).

1.1.4 Determination of total cell mass

Total cell mass is controlled both by extracellular signals and by intracellular mechanisms that limit cell size and division. In most cases these signaling cues lead
organisms and tissues to attain their correct cell mass; however, this is not always the case, as signaling pathways can be experimentally or accidentally perturbed. Excessive or deficient signaling along the PI3K pathway can lead to more and larger cells, or fewer and smaller cells, respectively. Signaling along the PI3K pathway may be especially important for the determination of total cell mass, as it regulates cell size and cell division. Interestingly, Ras signaling also regulates cell size and division, and recent studies in *Drosophila* have shown that constitutive activation of Ras can activate PI3K (Prober and Edgar, 2002). This observation indicates that the intracellular interactions between Ras and PI3K signaling may integrate extracellular signals and act as the driving factor behind the determination of total cell mass. However, the importance of Ras for the activation of PI3K under physiological conditions remains controversial (Johannessen et al., 2005; Prober and Edgar, 2002). PI3K can be activated by several Ras-independent mechanisms, raising the possibility that activated Ras might be sufficient but not necessary for PI3K activation. Furthermore, it has been suggested that in *Drosophila*, at least, wild-type Ras normally activates PI3K poorly, and thus activation might often be an artifact of the constitutively active Ras mutation used in these experiments (Prober and Edgar, 2002).

1.2 The duration of growth

A developing organism grows by controlling cell size and cell division, while simultaneously differentiating. However, this process does not continue indefinitely, as most organisms, including mammals and insects, stop growing at some point in
development. In humans, the cessation of growth occurs at the end of puberty; whereas in holometabolic insects, growth stops at the beginning of metamorphosis.

1.2.1 The duration of growth in mammals

In mammals, the duration of growth is chiefly controlled by the release of growth hormone (GH) from somatotrope cells of the anterior pituitary gland. In both mice and humans, a deficiency of GH produces dwarfism, and an excess leads to gigantism (Cummings and Merriam, 1999). GH is the most abundant pituitary hormone, but its synthesis and secretion depend on hypothalamic stimulation by GH-releasing hormone (GHRH), and inhibition by somatostatin (SRIF) (Gelato and Merriam, 1986). The interplay of stimulation by GHRH and inhibition by SRIF responds to higher physiologic stimuli, including nutrition, stress, and circadian rhythms, to generate to a pattern of daily episodic GH secretion (Giustina and Veldhuis, 1998). For example, mice with larger amounts of food, increased oxidative stress, or decreased daily light exposure are heavier and have increased levels of GH, whereas mice that eat less food, have lower oxidative stress, or are exposed to greater amounts of light weigh less and have decreased GH levels (Giustina and Veldhuis, 1998). Over a lifetime, GH levels are most abundant in juveniles and are essential for normal linear growth; however, GH levels decline gradually as puberty progresses and drop sharply once puberty has ended (Cummings and Merriam, 2003).

Within somatotropes of the anterior pituitary, GHRH binds to the GHRH receptor and activates a cAMP-mediated protein kinase A response that controls the activation of the POU-domain containing transcription factor Pit-1 (Godfrey et al., 1993; Lin et al., 1993). The POU-domain consists of an amino-terminal POU-specific domain separated
by a short linker from a carboxy-terminal POU homeodomain (Ingraham et al., 1988). Pit-1 was originally identified by analysis of Snell and Jackson dwarf mice that are deficient in GH release, and it was shown that Pit-1 is essential for the transcriptional regulation of GH in somatotropes (Camper et al., 1990; Li et al., 1990). Although cyclic adenosine monophosphate (cAMP) and PKA are known intracellular mediators of GHRH signaling on GH transcription, this area of GH biology, as well as the molecular mechanisms regulating SRIF signaling on GH synthesis, remain poorly understood (Dasen and Rosenfeld, 2001).

GH receptors are tyrosine kinase receptors that homodimerize upon GH binding and induce the secretion of insulin-like growth factor-1 (IGF1) primarily from the liver, which can have a growth-stimulating effect on a wide variety of tissues. Additionally, this growth effect causes the generation of supplementary IGF1 within target tissues, making GH apparently both an endocrine and an autocrine/paracrine hormone (Gelato et al., 1986). Recent experiments have shown that PI3K signaling is a molecular target of IGF1; unfortunately, little else is known about the roles of IGF1, although many have speculated that IGF1 also activates Ras signaling as it, along with PI3K, is a main intracellular regulator of the rate of growth (Dasen and Rosenfeld, 2001).

Only recently has it become apparent that GH synthesis does not cease at the end of puberty, but continues to serve many important functions throughout life. GH aids bone mineralization by increasing calcium retention, increases muscle mass, and promotes lipolysis. GH also plays a role in fuel homeostasis, as it reduces liver uptake of glucose, an effect that opposes that of insulin (Cummings and Merriam, 2003).
1.2.2 The duration of growth in insects

In insects, the duration of growth is controlled by the temporal release of the ecdysteroid class of steroid hormones, which comprise ecdysone and 20-hydroxyecdysone, hereafter known as ecdysone (Nijhout, 2003). Ecdysone is released during multiple phases of development (Figure 1.2) and activates a nuclear hormone-mediated signaling pathway that induces embryogenesis, larval molting, pupation, metamorphosis, and gonadogenesis (Gilbert et al., 2002). Ecdysone released during the larval stages is synthesized in the prothoracic gland (PG) to trigger molting, which

![Graph of Drosophila ecdysone levels during development.](image)

**Figure 1.2** Graph of *Drosophila* ecdysone levels during development (Riddiford, 1993). An increase in ecdysone content signals the onset of embryogenesis, larval molting, puparium formation, and metamorphosis in *Drosophila*. Ecdysone is also synthesized in the gonads of adult flies to signal for gondaogenesis (Gilbert et al., 2002).

temporarily stops growth (Henrich et al., 1999). A larger peak of ecdysone in the last instar stage causes pupariation, which halts the growth phase of fly development (Henrich et al., 1999). Therefore, the temporal pattern of larval ecdysone release itself can regulate the timing of larval molting and body size. For example, premature application of ecdysone to larvae mimics early ecdysone release and causes early
molting, small larvae, and small adults; whereas delayed application of ecdysone to larvae with ablated PG cells mimics late ecdysone release and causes late molting, large larvae, and large adults (Nijhout, 2003).

Larval ecdysone synthesis has been studied extensively in both the tobacco hornworm *Manduca sexta* and the fruit fly *Drosophila melanogaster*. The former is a favorable organism because of its large size and easily accessible paired PGs, while the latter has excellent molecular and genetic tools. The paired PGs of *M. sexta* each secrete ecdysone at similar rates and are composed of a single steroidogenic cell type (Gilbert et al., 2002). However in higher flies like *D. melanogaster*, the PG is a part of the ring gland (RG), a composite organ (Figure 1.3) that also includes the corpus allata, which secretes the ecdysone antagonist juvenile hormone (JH) (Zhou and Riddiford, 2002), and the corpus cardiaca, which secretes the energy mobilizing protein adipokinetic hormone (Kim and Rulifson, 2004).

![Figure 1.3 Anatomy of the *Drosophila* third instar central nervous system.](image)

**Figure 1.3** Anatomy of the *Drosophila* third instar central nervous system. **A**, The larval central nervous system is composed of the ventral ganglion (VG); two brain lobes; and the ring gland (RG) (Kim and Rulifson, 2004). **B**, The ring gland is a composite organ that contains three cell types (Kim and Rulifson, 2004). First, the prothoracic gland (PG) creates the “ring” around the aorta and contains about 40 relatively large cells that secrete ecdysone (Gilbert et al., 2002). Second, the corpora allata (CA) is restricted to an oval in an anterior section of the ring gland, contains about 20 relatively small cells, and secretes the ecdysone antagonist juvenile hormone (Zhou and Riddiford, 2002). Third, the corpora cardiaca (CC) is nestled in the junction between the ring gland and brain lobes, contains about 50 cells of similar size as CA cells, and secrete the energy mobilizing protein adipokinetic hormone (Kim and Rulifson, 2004). **C**, Prothoracic gland-lateral projection (PG-LP) neurons arise in the brain, decussate contralaterally, and synapse directly onto PG cells (Siegmund and Korge, 2001). Prothoracicotropic hormone secretion from PG-LP neurons activates ecdysone synthesis in PG cells (Siegmund and Korge, 2001).
In most insects, ecdysone synthesis is triggered by a prothoracicotropic hormone (PTTH) stimulated signaling cascade, which may be similar to intracellular signaling pathways that activate human adrenocorticotropic hormone and luteinizing hormone synthesis (Ishizaki and Suzuki, 1994; Kawakami et al., 1990; Kim et al., 1997; Saumann and Reppert, 1996). Studies with M. sexta indicate that PTTH is released by the corpus allota (Agui et al., 1979); however in D. melanogaster, PTTH may be released by prothoracic gland lateral projection (PG-LP) neurons that originate in the brain and synapse directly onto PG cells (Figure 1.3-C) (Siegmund and Korgé, 2001).

PTTH release in insects is regulated by a complex process that integrates both nutrition, through larval size (Nijhout, 1981), and photoperiod, through a circadian regulator (Truman, 1972; Truman and Riddiford, 1974). In Hemiptera, the true bugs, PTTH secretion is controlled by stretch receptors in the abdomen that are activated when the animal reaches a critical size (Nijhout, 1979). In the milkweed bug, Oncopeltus fasciatus, this size-monitoring mechanism can be fooled by artificially expanding the abdomen with an injection of saline (Nijhout, 1979). In the last instar larva, such an injection causes the animal to secrete ecdysone and initiate premature metamorphosis, resulting in a miniature adult. Thus, under normal growth, the abdominal stretch receptor is not activated until the larva has achieved a critical body mass (Nijhout, 1979).

In higher insects, the secretion of PTTH is inhibited by JH. In Manduca, if the CA is removed in the early instar stages, the larva secretes PTTH and ecdysone prematurely and metamorphoses into a miniature adult. Conversely, if JH is injected, PTTH secretion is delayed in a dose-dependent manner and metamorphosis begins at a much larger body size than normal (Nijhout and Williams, 1974; Rountree and
Bollenbacher, 1986). The inhibition of PTTH by JH only occurs in the last larval instar and prevents the secretion of molt-stimulating hormones until all the JH has been cleared from the hemolymph by the JH degrading enzyme, JH esterase. If metamorphosis occurs in the presence of JH, then the insect becomes a mosaic of larval/pupal or larval/adult traits (Nijhout, 1983; Wigglesworth, 1940; Williams, 1961). Thus, the disappearance of JH during the middle of the last instar disinhibits the secretion of ecdysone.

These findings reduce the problem of the control of PTTH secretion in higher insects to two independent questions: what causes JH secretion to stop, and what finally stimulates PTTH secretion? In both Manduca and Drosophila, the cessation of JH secretion is tightly associated with the attainment of critical weight, which is determined by the weight of the larva at the outset of the last larval instar (Nijhout, 1981, Mirth et al., 2005). In Manduca, the activity of JH esterase increases gradually over the course of the last larval instar and is essential for the effective clearance of JH from the hemolymph (De Kort and Granger, 1996; Hammock, 1985). In Manduca, the level of JH esterase is strongly affected by nutrition, and its activity drops to zero almost immediately if the larva is starved (Browder et al., 2001). It is likely that variation in nutrition modulates JH esterase activity as well as the secretion of JH, and the consequent persistence of JH accounts for the delay in PTTH secretion in insects that grow slowly or are periodically starved.

In Manduca, the timing of PTTH secretion is controlled by a photoperiodic clock once it is disinhibited by the disappearance of JH (Truman, 1972; Truman and Riddiford, 1974). PTTH secretion can only occur during a relatively brief “photoperiodic gate” that recurs daily. If the secretion of PTTH becomes disinhibited while this periodic gate is
open, PTTH secretion begins immediately, followed by ecdysone secretion and the cessation of growth. Otherwise, PTTH secretion is delayed until the next day's photoperiodic gate opens (Truman and Riddiford, 1974), and during this delay the larva can continue to feed and grow.

Pharmacological studies in Manduca indicate that PTTH stimulates ecdysone synthesis (Figure 1.4) by binding to an unknown receptor in PG cells that can open plasma membrane Ca\(^{2+}\) channels to increase the intracellular Ca\(^{2+}\) concentration (Birkenbeil, 1998; Birkenbeil, 2000; Girgenrath and Smith, 1996). Ca\(^{2+}\) may then bind calmodulin and activate adenylyl cyclase to synthesize cAMP (Gu et al., 1998; Meller et al., 1988; Smith et al., 1985). Increased cAMP levels activate protein kinase A (PKA), which in turn induces ecdysone synthesis through an unknown pathway (Gilbert et al., 2002; Smith and Gilbert, 1986; Smith et al., 1996). In Manduca, PTTH may also activate a mitogen activated protein kinase (MAPK) pathway that results in ecdysone synthesis through the phosphorylation of an extracellular receptor activated kinase (ERK) (Rybczynski et al., 2001). PTTH may also stimulate a potentially parallel pathway within the PG of Manduca, as PTTH signaling causes the phosphorylation of ribosomal protein S6 kinase (S6K; Gilbert et al., 1988; Song and Gilbert, 1994; Song and Gilbert 1995). S6K phosphorylation induces several trophic effects on PG cells, which increase cell size and the rate of translation (Hanton et al., 1993; Rybczynski and Gilbert, 1998).

The observance of these potential parallel pathways (PKA, MAPK, and S6K) indicates that ecdysone synthesis may be regulated in a variety of methods. For example, S6K, a target of insulin/Pi3K signaling, is known to increase general translation when
activated, whereas the targets of MAPK signaling are generally transcription factors (Gilbert et al., 2002).

![Diagram of PTTH signaling](image)

**Figure 1.4** A model for PTTH signaling in the PG of *M. sexta* (Gilbert et al., 2002). Signaling cascades are described in the text. It is hypothesized that ERK phosphorylation leads to basal ecdysone synthesis, which can be supplemented via an increase in translation caused by S6 kinase phosphorylation. Proteins and cellular processes highlighted in blue indicate well established PTTH-dependent intracellular events. Highlighting in red indicates the contribution of factors other than PTTH in the regulation of ecdysone synthesis. Solid arrows indicate characterized events, while dashed arrows indicate hypothetical relationships between signaling molecules. A question mark indicates the probable contribution of one or more unidentified molecules or events in a given pathway. R, unknown PTTH receptor; CaM, calmodulin; PKA, protein kinase A; S6, ribosomal protein S6; S6<sup>70</sup> kinase, 70-kDa S6 kinase; MEKK, MAP/ERK kinase kinase; MEK, MAP/ERK kinase; ERK, extracellular signal-regulated kinase.

However, in *Drosophila* fewer pharmacological studies have been performed because ecdysone levels are more difficult to measure, due to the small size of the ring gland and complexity of ring gland anatomy. Recently, cAMP levels and PKA activity
have been assayed for their importance in ecdysone synthesis, but these results are unclear (Henrich, 1995; Venkatesh et al., 2001). Additionally, roles for S6K and MAPK signaling in ecdysone synthesis have been more difficult to find in Drosophila than in Manduca, which only adds to the uncertainty surrounding PTTH-mediated ecdysone synthesis in insects.

In Drosophila ecdysone synthesis, the dehydrogenated form of cholesterol, 7-dehydrocholesterol, is converted into ecdysone and 20-hydroxyecdysone through a series of hydroxylation reactions (Figure 1.5) catalyzed by the “Halloween” family of cytochrome P450 enzymes (Gilbert et al., 2002). Recent RNA in situ hybridization analysis indicates that ecdysone synthesis may not only be regulated by the availability of 7-dehydroxyecdysone, but also by the transcriptional activation of cytochrome P450 enzymes, as the Halloween gene transcripts phantom, shadow, and disembodied only appear just prior to a molt (Gilbert, 2004; Niwa et al., 2004; Petryk et al., 2003; Warren et al., 2002).

![Diagram of ecdysone synthesis](image)

**Figure 1.5** Enzymology of ecdysone synthesis in Drosophila (adapted from Petryk et al., 2003). Cholesterol is ingested, because insects cannot synthesize this molecule, then converted to the active ecdysteroids, ecdysone and 20-hydroxyecdysone, by a series of hydroxylation reactions catalyzed by the Halloween family of cytochrome P450 enzymes.
In *Drosophila*, ecdysone travels through the hemolymph and binds to the ecdysone receptor (EcR) complex (Kozlova and Thummel, 2002). The EcR complex is a nuclear receptor heterodimer, composed of the retinoid X receptor family protein EcR, and the orphan nuclear receptor ultraspiracle (Usp) (Yao et al., 1993). The activated EcR complex can recognize certain DNA response elements, termed ecdysone response elements (EcREs), to trigger various transcription events.

The three *Drosophila* EcR splice variants (A, B1, and B2) have distinct expression patterns and elicit individual responses (Bender et al., 1997). EcR-A is primarily expressed in the embryo and is essential for embryonic development and larval salivary gland degeneration (Davis et al., 2005). EcR-B1 is expressed in almost all tissues of the larvae and triggers transcription of the early class of ecdysone responsive genes: *E74, broad complex, E75, E93*, and βFtz-F1 (Baehrecke and Thummel, 1995; Bender et al., 1997; Fletcher and Thummel, 1995; Henrich et al., 1999). In this class of early ecdysone responsive genes, the B isoform of E74 (E74B) is transcribed at the the lowest ecdysone concentrations (Fletcher et al., 1997). Then, as ecdysone titers rise, the A isoform of E74 (E74A) is transcribed along with the *broad complex, E75, E93*, and βFtz-F1 (Fletcher et al., 1997; Fletcher and Thummel, 1995). All of these early ecdysone responsive proteins act as transcription factors that induce the expression of late ecdysone responsive genes, such as *E78* and *DHR3*, which can then direct molting, pupariation, and metamorphosis (Fletcher and Thummel, 1995; Henrich et al., 1999). The early ecdysone responsive proteins also activate the transcription of *EcR-B1* to induce an autoregulatory loop that increases the level of receptor protein in response to
the hormone ligand (Kozlova and Thummel, 2002). EcR-B2 is expressed in the larval epidermis; however, its function remains unknown (Cherbas et al., 2003).

1.3 Determining final body size: Integrating the rate and duration of growth

The control of final body size is not so much a control of growth, but a control of when to stop growing (Conlon and Raff, 1999; Nijhout, 1994). This is true not only for insects, whose size increases exponentially with time, but also for mammals, as small changes in the timing of cessation of growth at the end of puberty can have large consequences for final body size (Cummings and Merriam, 2003). The molecular mechanisms underlying the cessation of growth have historically been studied by physiologists; whereas, mechanisms controlling the rate of growth have been elucidated largely by geneticists. Perhaps because of these differences, it is unclear how, or even if, the rate and duration of growth interact, as only recent experiments have begun to shed light on these complex processes.

1.3.1 Successes in integrating the rate and duration of growth

In mammals, elevated GH levels accelerate the rate of growth; however when puberty ends, GH levels decline dramatically because of higher somatostatin (SRIF) levels (Giustina and Veldhuis, 1998). This event decelerates the rate of growth and sets final body size. The mammalian integration of rate and duration of growth has been studied primarily in mice. The binding of GH to its receptor has been shown to induce the secretion of insulin-like growth factor-1 (IGF1), which can have various growth stimulatory effects. For example, binding of IGF1 to the IGF1 receptor in cultured rat glioblastoma cells activates Pi3K signaling and increases cell size and division (Resnicoff
et al., 1995). Conversely, when the IGF1 receptor is inhibited in cultured rat glioblastoma cells, via application of antisense oligonucleotides or expression of dominant-negative mutations, cell size and division decreases (D’Ambrosio et al., 1996; Resnicoff et al., 1994).

In insects, decreased levels of ecdysone increase the rate of growth; whereas, increased ecdysone concentrations rapidly decelerate growth and cause molting (Gilbert et al., 2002). Final body size is set during pupariation, a process that is activated when the ecdysone concentration is elevated past a certain threshold. The integration of the rate and duration of growth in insects has been studied in Manduca sexta by assaying the activity of insulin signaling under pharmacological applications of ecdysone to wandering larvae (Gilbert et al., 2002). This assay measured the activity of insulin signaling by measuring the phosphorylation states of the InR-Pi3K signaling target Akt. Premature application of ecdysone to larvae prematurely decreases Akt phosphorylation and insulin signaling activity; whereas, delayed application of ecdysone to larvae with ablated PG cells delays this decrease in Akt phosphorylation and insulin signaling activity (Nijhout, 2003). Additionally, total ablation of the PG cells in Manduca creates larvae that have increased insulin activity and do not molt or pupate, presumably because of a lack of ecdysone (Gilbert et al., 2002).

Additionally, recent work in Drosophila has shown that ecdysone down-regulates the rate of growth and Pi3K signaling in the fat body (Rusten et al., 2004). The fat body, the functional equivalent of the vertebrate liver, functions as a nutrient sensor and is involved in the coordination of organismal growth. Importantly, amino acid starvation of the fat body alone not only causes a slight decrease in Pi3K activity there, but also shuts
down Pi3K activity in other tissues and reduces larval growth, through an unknown humoral mechanism (Colombani et al., 2003). Ecdysone-mediated suppression of Pi3K signaling in the fat body may therefore have a similar effect.

This hypothesis was confirmed as ecdysone-fed Drosophila larvae show a decrease in Pi3K activity in their fat body, whereas inhibiting the effects of ecdysone on the fat body, by silencing EcR-B1 there, removes these effects and leads to an increase in the larval growth rate (Colombani et al., 2005). However, the ecdysone suppression of growth rate is lost when insulin signaling is systemically activated (Colombani et al., 2005). Thus, ecdysone and insulin appear to counteract each other’s effects on growth throughout the Drosophila larvae (Colombani et al., 2005).

Despite these recent advances in both mammals and insects, little is known about the molecular mechanisms acting both upstream and downstream of GH or ecdysone. How do intracellular signaling events in somatotropes and PG cells respond to extrinsic cues (nutrition, stress, and photoperiod) and integrate them to regulate GH or ecdysone synthesis? Additionally, what are the intracellular signaling events that respond to GH and ecdysone receptor activity, and how do they regulate the rate of growth? These questions have not been answered to date because of several difficulties in analysis using the model organisms. For example, in mice GH is present in high levels until the end of puberty, which can last for weeks, and the timing of puberty can vary between littermates making specific measurements of GH and IGF-1 activity problematic (Hursting et al., 2003). These timing issues, along with difficult genetics, makes integrating the rate and duration of growth in mice challenging. Additionally, studying the interaction of
ecdysone and insulin in insects has been limited by the poor genetics of *Manduca*. This could be solved by utilizing the superior genetic tools of *Drosophila*.

1.3.2 *Drosophila* as a model organism to study the rate and duration of growth

Both GH and ecdysone are synthesized in response to similar extrinsic cues, are produced by similar endocrine tissues, and are the main regulators of the duration of growth. However, GH (a protein hormone) positively regulates the rate of growth; whereas, ecdysone (a steroid hormone) negatively regulates the rate of growth.

Despite these differences, *Drosophila* is an excellent model organism for studying the integration of the rate and duration of growth. It has excellent genetic and molecular tools, and the timing of ecdysone release can be measured effectively. I have used one of these genetic tools, the Gal4-UAS system (Brand and Perrimon, 1993) to regulate the synthesis of ecdysone in *Drosophila* PG cells. Gal4 is a transcriptional activator endogenous to *Saccharomyces cerevisiae* that induces expression of any *D. melanogaster* gene under control of an upstream activating sequence (UAS) (Figure 1.6). Gal4 expressing lines are created by inserting a P-element carrying the Gal4 gene (P\{Gal4\}) into the *D. melanogaster* genome, so that the regulatory elements surrounding the P\{Gal4\} insertion determines the spatial and temporal expression pattern of Gal4. Gal4 then binds to the UAS to drive expression of the downstream gene in a corresponding pattern (Brand and Perrimon, 1993). Thus, the Gal4-UAS system can be used to misexpress any desired gene by inserting it downstream of the UAS, or to report the spatial and temporal pattern of gene misexpression by inserting a reporter, such as GFP, downstream of the UAS. With this genetic tool, we can begin to integrate the rate and
duration of growth in *Drosophila* by specifically controlling the mechanisms that lead to ecdysone release from PG cells.

**Figure 1.6** Cartoon description of the Gal4-UAS system in *Drosophila* (Duffy, 2002). Male flies carrying RE-Gal4 are mated to females carrying UAS-GFP. Gal4 is produced in the spatial and temporal pattern defined by the regulatory elements, and because UAS is a Gal4 sensitive transcriptional activator, GFP will only be produced when Gal4 is present. In this case, Gal4 is present in an alternating segmental pattern within embryos. Gal4 binding to the UAS drives expression of the UAS responder gene, GFP, in a corresponding pattern.
CHAPTER 2. METHODS AND MATERIALS

2.1 Drosophila stocks

All fly stocks were maintained on standard cornmeal/agar Drosophila media (Lakovaara, 1969) at room temperature (22°C). The Drosophila Stock Center at Bloomington, IN provided UAS-Ras\textsuperscript{+}, UAS-Ras\textsuperscript{N17}, the third chromosome UAS-Ras\textsuperscript{V12}, UAS-Raf\textsuperscript{F20}, UAS-Raf\textsuperscript{K497M}, UAS-Akt\textsuperscript{+}, UAS-4E-BP\textsuperscript{+}, UAS-EcR\textsuperscript{R1-3C655-W650A}, ChaGal4, c179, c805, PDFGal4, DdcGal4, sevGal4, EcRGal4, c21, 11c, 43A, c135, c747, OK107, ninaEGal4, and c309. The second chromosome UAS-Ras\textsuperscript{V12} was provided by Andreas Bergmann and both UAS-Pi3K-CAXX and UAS-Pi3K\textsuperscript{D954A} were provided by Sally Leevers. UAS-TSC2\textsuperscript{S924A} was provided by Tian Xu, UAS-S6K\textsuperscript{ACT} was provided by Ping Shen, UAS-Ral\textsuperscript{20V} was provided by Hideyuki Okano, UAS-FOXO\textsuperscript{TM} was given by Marc Tatar, and UAS-TSC2\textsuperscript{+} was provided by Duojiya Pan. The ann\textsuperscript{c651} Gal4 line was provided by Doug Armstrong, the ann\textsuperscript{X8} and ann\textsuperscript{28A} Gal4 lines were provided by Ulrike Heberlein, and the dilp2-Gal4 line was given by Eric Rulifson. Gunther Korge provided the Jan229, Feb204, Feb211, Feb296, Kurs6, Kurs21, Kurs58, Mai179, Mai301, and Mai369 Gal4 lines.

2.2 Drosophila crosses

All crosses were performed on standard cornmeal/agar Drosophila media (Lakovaara, 1969) with at least 10 mating pairs at room temperature (22°C).

2.2.1 Crosses involving ann\textsuperscript{c651}

ann\textsuperscript{c651}/+; +/-; UAS-GFP(nls)/+ was created by mating ann\textsuperscript{c651}/ann\textsuperscript{c651}; +/-; +/- females with +/-; +/-; UAS-GFP(nls)/UAS-GFP(nls) males. ann\textsuperscript{c651}/+; UAS-Ras\textsuperscript{+}/+; +/-
was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; +/+; \textit{UAS-Ras}^{-}/\textit{UAS-Ras}^{+} males. \textit{amn}^{651}/+; +/+; \textit{UAS-Ras}^{V12}/+ was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; +/+; \textit{UAS-Ras}^{V12}/\textit{UAS-Ras}^{V12} males. \textit{amn}^{651}/+; \textit{UAS-Ras}^{V12}/+; +/+ was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; +/+; \textit{UAS-Ras}^{V12}/\textit{UAS-Ras}^{V12} males. Non-tubby \textit{amn}^{651}/+; +/+; \textit{UAS-Ras}^{V12}/\textit{UAS-GFP(nls)} females were chosen from the mating of +/+; +/+; \textit{UAS-Ras}^{V12}/\textit{UAS-Ras}^{V12} females with \textit{amn}^{651}/\tau; +/+; \textit{UAS-GFP(nls)}/\textit{TbTM6} males. Non-tubby \textit{amn}^{651}/+; +/+; \textit{UAS-Ras}^{+}/\textit{UAS-GFP(nls)} females were chosen from the mating of +/+; +/+; \textit{UAS-Ras}^{+}/\textit{UAS-Ras}^{+} females with \textit{amn}^{651}/\tau; +/+; \textit{UAS-GFP(nls)}/\textit{TbTM6} males. \textit{amn}^{651}/+; +/+; \textit{UAS-Raf}^{F20}/+ was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; +/+; \textit{UAS-Raf}^{F20}/\textit{UAS-Raf}^{F20} males. \textit{amn}^{651}/+; +/+; \textit{UAS-Raf}^{K497M}/+ was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; +/+; \textit{UAS-Raf}^{K497M}/\textit{UAS-Raf}^{K497M} males. \textit{amn}^{651}/+; \textit{UAS-Pi3K}^{D954A}/+; +/+ was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; \textit{UAS-Pi3K}^{D954A}/\textit{UAS-Pi3K}^{D954A}; +/+ males. \textit{amn}^{651}/+; +/+; \textit{UAS-Ral}^{V20}/+ was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; +/+; \textit{UAS-Ral}^{V20}/\textit{UAS-Ral}^{V20} males. \textit{amn}^{651}/\textit{UAS-Pi3K-CAAX}; +/+; +/+ females were chosen from the mating of \textit{UAS-Pi3K-CAAX}/\textit{UAS-Pi3K-CAAX}; +/+; +/+ females with \textit{amn}^{651}/\tau; +/+; +/+ males. \textit{amn}^{651}/\textit{UAS-Ras}^{N17}; +/+; +/+ females were chosen from the mating of \textit{UAS-Ras}^{N17}/\textit{UAS-Ras}^{N17}; +/+; +/+ females with \textit{amn}^{651}/\tau; +/+; +/+ males. \textit{amn}^{651}/+; +/+; \textit{UAS-FOXOTM}/+ was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; +/+; \textit{UAS-FOXOTM}/\textit{UAS-FOXOTM} males. \textit{amn}^{651}/+; +/+; \textit{UAS-TSC2}^{S924A}/+ was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; +/+; \textit{UAS-TSC2}^{S924A}/\textit{UAS-TSC2}^{S924A} males. \textit{amn}^{651}/+; \textit{UAS-S6K}^{ACT}/+; +/+ was created by mating \textit{amn}^{651}/\textit{amn}^{651}; +/+; +/+ females with +/\tau; \textit{UAS-
S6K^{ACT}/UAS-S6K^{ACT}; +/+ males. amn^{651}/+; UAS-Akt^{+}/+; +/+ was created by mating amn^{651}/amn^{651}; +/+; +/+ females with +/+; UAS-Akt^{+}/UAS-Akt^{+}; +/+ males. amn^{651}/+; +/+; UAS-TSC2^{+}/+ was created by mating amn^{651}/amn^{651}; +/+; +/+ females with +/+; UAS-TSC2^{+}/UAS-TSC2^{+} males. amn^{651}/+; UAS-4E-BP^{+}/+; +/+ was created by mating amn^{651}/amn^{651}; +/+; +/+ females with +/+; UAS-4E-BP^{+}/UAS-4E-BP^{+}; +/+ males.

2.2.2 Crosses involving amn^{X8}

amn^{X8}/+; +/+; UAS-GFP(nls)/+ was created by mating amn^{X8}/amn^{X8}; +/+; +/+ females with +/+; +/+; UAS-GFP(nls)/UAS-GFP(nls) males. amn^{X8}/+; +/+; UAS-Ras^{+}/+ was created by mating amn^{X8}/amn^{X8}; +/+; +/+ females with +/+; +/+; UAS-Ras^{+}/UAS-Ras^{+} males. amn^{X8}/+; +/+; UAS-Ras^{V12}/+ was created by mating amn^{X8}/amn^{X8}; +/+; +/+ females with +/+; +/+; UAS-Ras^{V12}/UAS-Ras^{V12} males. amn^{X8}/+; UAS-Ras^{V12}/+; +/+ was created by mating amn^{X8}/amn^{X8}; +/+; +/+ females with +/+; UAS-Ras^{V12}/UAS-Ras^{V12}; +/+ males. amn^{X8}/+; +/+; UAS-Raf^{F20}/+ was created by mating amn^{X8}/amn^{X8}; +/+; +/+ females with +/+; +/+; UAS-Raf^{F20}/UAS-Raf^{F20} males. amn^{X8}/+; +/+; UAS-Raf^{K497M}/+ was created by mating amn^{X8}/amn^{X8}; +/+; +/+ females with +/+; +/+; UAS-Raf^{K497M}/UAS-Raf^{K497M} males. amn^{X8}/+; UAS-Pi3K^{D954A}/+; +/+ was created by mating amn^{X8}/amn^{X8}; +/+; +/+ females with +/+; UAS-Pi3K^{D954A}/UAS-Pi3K^{D954A}; +/+ males. amn^{X8}/+; +/+; UAS-Ral^{V20}/+ was created by mating amn^{X8}/amn^{X8}; +/+; +/+ females with +/+; +/+; UAS-Ral^{V20}/UAS-Ral^{V20} males. amn^{X8}/UAS-Pi3K-CAAX; +/+; +/+ females were chosen from the mating of UAS-Pi3K-CAAX/UAS-Pi3K-CAAX; +/+; +/+ females with amn^{X8}/+; +/+; +/+ males. amn^{X8}/UAS-Ras^{N17}; +/+; +/+ females were chosen from the mating of UAS-Ras^{N17}/UAS-Ras^{N17}; +/+; +/+ females with amn^{X8}/+; +/+; +/+ males.
2.2.3 Crosses involving \textit{ChaGal4}

\textit{+/+; ChaGal4/+; UAS-GFP(nls)/+} was created by mating \textit{+/+; ChaGal4/ChaGal4; +/+ females with +/\tau; +/+; UAS-GFP(nls)/UAS-GFP(nls) males.} \\
\textit{+/+; ChaGal4/+; UAS-tau-GFP/+} was created by mating \textit{+/+; ChaGal4/ChaGal4; +/+ females with +/\tau; +/+; UAS-tau-GFP/UAS-tau-GFP males. +/+; ChaGal4/+; UAS-Ras\textsuperscript{+/+} was created by mating \textit{+/+; ChaGal4/ChaGal4; +/+ females with +/\tau; +/+; UAS-Ras\textsuperscript{+/+} males. +/+; ChaGal4/+; UAS-Ras\textsuperscript{1/12}/+} was created by mating \textit{+/+; ChaGal4/ChaGal4; +/+ females with +/\tau; +/+; UAS-Ras\textsuperscript{1/12}/UAS-Ras\textsuperscript{1/12} males. +/+; UAS-Ras\textsuperscript{1/12}/ChaGal4; +/+ was created by mating \textit{+/+; ChaGal4/ChaGal4; +/+ females with +/\tau; +/+; UAS-Raf\textsuperscript{F20}/UAS-Raf\textsuperscript{F20} males. +/+; ChaGal4/+; UAS-Raf\textsuperscript{K497M}/+} was created by mating \textit{+/+; ChaGal4/ChaGal4; +/+ females with +/\tau; +/+; UAS-Raf\textsuperscript{K497M}/UAS-Raf\textsuperscript{K497M} males. +/+; UAS-Pi3K\textsuperscript{D954A}/ChaGal4; +/+ was created by mating \textit{+/+; ChaGal4/ChaGal4; +/+ females with +/\tau; +/+; UAS-Pi3K\textsuperscript{D954A}/UAS-Pi3K\textsuperscript{D954A} males. +/+; ChaGal4/+; UAS-Raf\textsuperscript{V20}/+} was created by mating \textit{+/+; ChaGal4/ChaGal4; +/+ females with +/\tau; +/+; UAS-Raf\textsuperscript{V20}/UAS-Raf\textsuperscript{V20} males. UAS-Pi3K-CAAX/+; ChaGal4/+; +/+ was created by mating \textit{UAS-Pi3K-CAAX/ UAS-Pi3K-CAAX; +/+; +/+ females with +/\tau; ChaGal4/ChaGal4; +/+ males. UAS-Ras\textsuperscript{N17}/+; +/+; +/+ was created by mating \textit{UAS-Ras\textsuperscript{N17}/UAS--Ras\textsuperscript{N17}; +/+; +/+ females with +/\tau; ChaGal4/ChaGal4; +/+ males.} \\
\textit{+/+; ChaGal4/+; UAS-EcR\textsuperscript{B1-SC655-W650A}/+} was created by mating \textit{+/+; ChaGal4/ChaGal4; +/+ females with +/\tau; +/+; UAS- EcR\textsuperscript{B1-SC655-W650A}/UAS-EcR\textsuperscript{B1-SC655-W650A} males.}
2.2.4 Other crosses

+/+; c805/+; UAS-Ras\textsuperscript{V12}/+ was created by mating +/++; c805/c805; +/+ females with +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/+; UAS-Ras\textsuperscript{V12}/dilp2Gal4; +/+ was created by mating +/-; dilp2Gal4/dilp2Gal4; +/- females with +/++; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/-; dilp2Gal4; +/+ was created by mating +/-; dilp2Gal4/dilp2Gal4; +/- females with +/++; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/-; dilp2Gal4; +/-; UAS-EcR\textsuperscript{B1-\Delta C655-W650A}/+ was created by mating +/-; dilp2Gal4/dilp2Gal4; +/- females with +/++; +/+; UAS-EcR\textsuperscript{B1-\Delta C655-W650A}/UAS-EcR\textsuperscript{B1-\Delta C655-W650A} males. +/-; UAS-Ras\textsuperscript{V12}/+; Feb211/+ was created by mating +/-; +/-; Feb211/Feb211 females with +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/-; +/-; UAS-Ras\textsuperscript{V12}/Feb211 was created by mating +/-; +/-; Feb211/Feb211 females with +/-; +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/-; +/-; +/+; UAS-EcR\textsuperscript{B1-\Delta C655-W650A}/Feb211 was created by mating +/-; +/-; Feb211/Feb211 females with +/-; +/-; UAS-EcR\textsuperscript{B1-\Delta C655-W650A}/UAS-EcR\textsuperscript{B1-\Delta C655-W650A} males. amn\textsuperscript{28a}/+; +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} females with +/-; +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/-; +/-; c179/+; UAS-Ras\textsuperscript{V12}/+ was created by mating +/-; c179/CyO; +/- females with +/-; +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/-; +/-; UAS-Ras\textsuperscript{V12}/PDFGal4 was created by mating +/-; +/-; PDFGal4/TM6 females with +/-; +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/-; DdcGal4/+; UAS-Ras\textsuperscript{V12}/+ was created by mating +/-; DdcGal4/CyO; +/- females with +/-; +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males.

sev2Gal4/+; +/-; UAS-Ras\textsuperscript{V12}/+ was created by mating sev2Gal4/FM7; +/-; +/- females with +/-; +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/-; EcRGal4/+; UAS-Ras\textsuperscript{V12}/+ was created by mating +/-; EcRGal4/CyO; +/- females with +/-; +/-; UAS-Ras\textsuperscript{V12}/UAS-Ras\textsuperscript{V12} males. +/-; c21/+; UAS-Ras\textsuperscript{V12}/+ was created by mating +/-; c21/CyO; +/-
females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; UAS-Ras^{V12}/11c was created by mating +/-; +/-; 11c/TM6 females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. 43a/++; +/- UAS-Ras^{V12}/+ was created by mating 43a/FM7; +/-; +/-; females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; UAS-Ras^{V12}/c135+ was created by mating +/-; +/-; c135/TM6 females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; c747/++; UAS-Ras^{V12}/+ was created by mating +/-; c747/CyO; +/- females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; OK107/++; UAS-Ras^{V12}/+ was created by mating +/-; OK107/CyO; +/- females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; UAS-Ras^{V12}/ninaEGal4 was created by mating +/-; +/-; ninaEGal4/TM6 females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; c309/++; UAS-Ras^{V12}/+ was created by mating +/-; c309/CyO; +/- females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; Jan229/++; UAS-Ras^{V12}/+ was created by mating +/-; Jan229/CyO; +/- females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; UAS-Ras^{V12}/Feb296 was created by mating +/-; +/-; Feb296/TM6 females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; Feb204/++; UAS-Ras^{V12}/+ was created by mating +/-; Feb204/CyO; +/- females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; Kurs6/++; UAS-Ras^{V12}/+ was created by mating +/-; Kurs6/CyO; +/- females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; Kurs21/++; UAS-Ras^{V12}/+ was created by mating +/-; Kurs21/TM6; +/- females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; Kurs58/++; UAS-Ras^{V12}/+ was created by mating +/-; Kurs58/CyO; +/- females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; Mai179/++; UAS-Ras^{V12}/+ was created by mating +/-; Mai179/CyO; +/- females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males. +/-; +/-; UAS-Ras^{V12}/Mai301 was created by mating +/-; +/-; Mai301/TM6 females with +/-; +/-; UAS-Ras^{V12}/UAS-Ras^{V12} males.
+/+; Mai369/+; UAS-Ras^{V12}/+ was created by mating +/+; Mai369/CyO; +/+ females with +/+; UAS-Ras^{V12}/UAS-Ras^{V12} males.

2.3 Imaging

For fluorescent imaging, tissues were fixed in 4% paraformaldehyde dissolved in phosphate-buffered saline containing 0.2% Triton X-100 (PBS-T, pH 7.2). After rinsing in PBS-T, GFP containing larval tissues and whole larvae were mounted in Vectashield medium (Vector Laboratories), while some tissues used to determine prothoracic gland cell size were mounted in Vectashield medium (Vector Laboratories) containing 0.1% Hoechst stain (Molecular Probes). All larvae and tissues were viewed on a Zeiss Axioplan 2 with Metamorph deconvolution software. For light microscopy, flies were viewed on a Leica MZFLIII dissecting microscope.

2.4 Growth measurements

The average length was determined for each genotype from at least 10 male and female pupae and adults. P-values for these length measurements were determined by two-tailed t-tests. The area of one female wing was determined for each genotype by multiplying the product of the vertical and horizontal radii by pi (Griffiths et al., 2005). The density of wing hair cells was determined by calculating the number of wing hair cells in three separate 100 μm² regions of the female wing between veins L3 and L4, averages and standard errors were then calculated from these three measurements.
2.5 Fly staging and molting profiles

At least 10 mating pairs were crossed in a vial containing standard media for 3 days then transferred to a new vial every 12 hours. Each time point was obtained from a minimum of two separate crosses. The larvae were washed with phosphate-buffered saline solution containing 0.2% Triton X-100 (PBS-T, pH 7.2) and screened for developmental stage, via mouth hook and anterior spiracle morphology, at each appropriate time point. All staging experiments were performed at room temperature (22°C).

2.6 Quantitative PCR

Optimized taqman primers and probes for *dib, phm, thor*, and *RNA polymerase II 140kD* (*RPII-140*) were obtained from Applied Biosystems. Taqman primers and probes for *E74A, E74B*, and *RpL13A* were designed using the PrimerExpress software (Applied Biosystems) according to their published sequences. *E74A* forward primer (GTTGCCGGAACATTATGGATATA), *E74A* reverse primer (GCCCTATGTCGGCTTGCT), *E74A* probe (FAM-CTTGAGATGAGGCGCA-MGB). *E74B* forward primer (ATCGCCGCGCTACAAGAAG), *E74B* reverse primer (TCGATTGGACCAATAGGAATTTC), *E74B* probe (FAM-TTGATGAAGCGATATTACAC-MGB). *RpL13A* forward primer (TCCGTGCGGTTCGAAAAAT), *RpL13A* reverse primer (TGGCCGCGACCATCA), *RpL13A* probe (VIC-TGGGTGACAGACC-MGB). Taqman Q-PCR reactions for *E74A, E74B*, and *RpL13A* primer-probe sets are of equal efficiency, as determined by a 3.3-fold (± 5%) decrease in Q-PCR fluorescence for each 10X RNA or DNA serial
dilution (Livak and Schmittgen, 2001). Total RNA was extracted from frozen tissues with TRIzol reagent (Invitrogen). The yield of RNA was estimated spectrophotometrically by absorbance at 260 nm ($A_{260}$). $A_{260}/A_{280}$ ratio was 1.8-2.

SuperScript II RNase H-Reverse Transcriptase (Invitrogen) treatments were carried out with an oligo-d(T) primer in accordance with the manufacturer's instructions. Reverse-transcribed cDNA from tissues was subsequently examined in a 50 µL PCR reaction by the ABI Prism 7000 system (Applied Biosystems) using the universal conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Two separate samples were collected from each genotype, and triplicate measures of each sample were conducted. $\Delta\Delta$Ct = (Ct of the experimental – Ct of the control) – (Ct of the calibrator – Ct of the control). The control was either RpLI3A or RPII-140. The calibrator was from wild-type larvae at 192 hours AEL. Fold activation of a target transcript = $2^{\Delta\Delta$Ct} - (average of all 6 $\Delta\Delta$Ct values). Positive error of the fold = fold activation of target transcript - $2^{\Delta\Delta$Ct} - (average of all 6 $\Delta$Ct values – standard error of the average $\Delta$Ct). Negative error of the fold = $2^{\Delta\Delta$Ct} - (average of all 6 $\Delta$Ct values + standard error of the average $\Delta$Ct) – fold activation of target transcript.
CHAPTER 3. EFFECTS OF RAS SIGNALING IN THE PROTHORACIC GLAND (PG)

Parts of this chapter have been published in Caldwell et al., 2005.

3.1 Two Gal4 lines, amn^{651} and amn^{X8}, are expressed in the PG

The Drosophila amnesiac (amn) gene, which is thought to encode three neuropeptides (Feany and Quinn, 1995) is essential for a number of physiological processes, including learning and memory, normal ethanol sensitivity and proper growth control of peripheral nerves (Feany and Quinn, 1995; Moore, et al., 1998; Yager, et al., 2001). Two independent insertions of P(Gal4) elements into amn have been reported: amn^{651} (Waddell, et al., 2000) and amn^{X8} (Moore, et al., 1998), which is a deletion of amn caused by an imprecise excision of the P(Gal4) insertion amn^{26a} that retains full Gal4 activity (V. Hall and M. Stern, unpublished; S. Waddell, personal communication).

The Gal4 expression pattern of amn^{651} within adult heads has been reported (Waddell, et al., 2000). To visualize the cells expressing Gal4 within developing larvae, we crossed amn^{651} and amn^{X8} flies to flies carrying a nuclear localized GFP expressed under UAS control, UAS-GFP(nls) (Shiga, et al., 1996). We found that amn^{651} first produces detectable Gal4 activity in the ring gland of first instar larvae (Figure 3.1-A). In third instar larvae, Gal4 activity is detected in prothoracic gland (PG) cells of the ring gland (Figure 3.1-A and Figure 3.1-B) as well as five cells within each brain lobe, and about 2 cells per hemisegment in the ventral ganglion (Figure 3.1-B). The amn^{X8} Gal4 element is also expressed strongly in the PG, but is expressed more widely, both outside of the central nervous system (CNS) (Figure 3.1-A) and within the CNS (Figure 3.1-B) than the amn^{651} Gal4 element. The non-specific, non-nuclear-localized fluorescence within the gut is present in wild-type larvae (Figure 3.1-A), which lack GFP, and thus
**Figure 3.1** Temporal and spatial expression patterns of \textit{amn}\textsuperscript{c651} and \textit{amn}\textsuperscript{X8}. A, expression patterns of wild-type, \textit{amn}\textsuperscript{c651} and \textit{amn}\textsuperscript{X8} during the first, second, and third instar stages visualized with nuclear localized GFP. Larvae from wild-type flies do not carry GFP and thus background fluorescence in the intestinal lumen is GFP-independent. Both \textit{amn}\textsuperscript{c651} and \textit{amn}\textsuperscript{X8} are expressed in the ring gland during each larval stage (shown by arrows in panels e, f, i and j; magnified images of the CNS are shown in panels c, g and k); however, \textit{amn}\textsuperscript{X8} is expressed in many other cell types. For example, the far-right panels show a magnified image of the gut in which specific, nuclear-localized GFP expression can clearly be seen over background fluorescence in \textit{amn}\textsuperscript{X8} larvae but not in wild-type or \textit{amn}\textsuperscript{c651} larvae. Scale bars are 200 \textmu m for a, b, c, f, i and j and 50 \textmu m for c, d, g, h, k and l. The fluorescent nuclei in panel k appear blurry because they were photographed through the cuticle. B, expression patterns of \textit{amn}\textsuperscript{c651} and \textit{amn}\textsuperscript{X8} in the CNS of wandering third instar larvae. m-p, \textit{amn}\textsuperscript{c651} is expressed in prothoracic gland cells of the ring gland and is expressed in very few cells within the brain and ventral ganglion. q-t, \textit{amn}\textsuperscript{X8} is expressed strongly in prothoracic gland cells of the ring gland and is expressed in many more cells of the brain and ventral ganglion than \textit{amn}\textsuperscript{c651}. Scale bars are 50 \textmu m for m, o, p, r, s and t, and 200 \textmu m for m and q. The dashed line in p represents the edge of the ventral ganglion.
represents GFP-independent background fluorescence. However, we are able to visualize specific, nuclear-localized GFP over this background. For example, higher magnification images (Figure 3.1-A, far right panels) show specific, nuclear-localized GFP within a subset of cells of the gut from amn$^{X8}$, but not amn$^{c651}$ or wild-type larvae. amn$^{X8}$ is expressed in a few other tissues in addition to CNS and gut (for example, fat body and salivary gland, not shown). In contrast, we are unable to detect amn$^{c651}$ expression outside of the nervous system (Figure 3.1-A and not shown). This extremely restricted expression pattern is similar to what was observed for amn$^{c651}$ expression in the adult head (Waddell, et al., 2000).

3.2 Ras signaling in the PG regulates body size

We used amn$^{c651}$ and amn$^{X8}$ to test the effects of altering the activity of Ras or downstream effectors within the PG. Ras is a small cytosolic GTPase that exists in either an inactive GDP-bound, or an active GTP-bound form. A G12V mutation to Ras, Ras$^{V12}$, eliminates the Ras GTPase and constitutively activates Ras by locking it in the GTP-bound state (Lee et al., 1996).

We found that adults bearing amn$^{c651}$ and either of two transgenes expressing the constitutively active Ras$^{V12}$ under UAS control, denoted UAS-Ras$^{V12}$ (II) and UAS-Ras$^{V12}$ (III) (Karim and Rubin, 1996; Lee, et al., 1996) are morphologically normal but significantly reduced in size (Figure 3.2 and Table 3.1). This phenotype is not simply a consequence of Ras overexpression, because flies carrying amn$^{c651}$ and UAS-Ras$^{+}$ exhibit normal body size (Figure 3.2 and Table 3.1). To determine if this size phenotype was a result of reduced cell size or cell number, we measured total wing hair cell number and
Figure 3.2 Altered Ras signaling in the PG affects fly size. A, photographs of pupal and adult size. a/b, expression of Ras<sup>v12</sup> under amn<sup>651</sup> control decreases pupal and adult size. c/d, expression of Ras<sup>+</sup> under amn<sup>651</sup> control produces pupae and adults of normal size. e/f, expression of P<sup>i</sup>3K<sup>2985/41</sup> under amn<sup>651</sup> control increases pupal and adult size. All scale bars are 1 mm. B, photographs of wing size and wing hair cell density. g/h, expression of Ras<sup>v12</sup> under amn<sup>651</sup> control decreases wing size, but increases wing hair cell density. i/j, expression of Ras<sup>+</sup> under amn<sup>651</sup> control produces wings of normal size with normal wing hair cell density. Scale bars for g and h are 200 μm. C/D, graphs depicting the effects of Ras signaling under amn<sup>651</sup> and amn<sup>Ys</sup> control on female pupal size. Wild-type flies were w<sup>1118</sup> and isogenic for the second and third chromosome. Analysis is described in the text; n ≥ 10; mean ± SEM shown; a * represents a genotype with a p-value ≤ 0.01 compared to wild-type analyzed by a two-tailed t-test; embryonic lethality (EL).
<table>
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<th></th>
<th>Pupal Length (mm)</th>
<th>Adult Length (mm)</th>
<th>Average Weight (mg)</th>
<th>Wing Area (mm^2)</th>
<th>Wing Hair Cell Number/100 µm^2</th>
<th>Average Hair Cells/Wing</th>
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<td>Male</td>
<td>Female</td>
<td>Male</td>
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<tr>
<td>Wild-type</td>
<td>3.09 ± 0.09</td>
<td>2.95 ± 0.11</td>
<td>2.62 ± 0.09</td>
<td>2.51 ± 0.10</td>
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<td>2.94 ± 0.07</td>
<td>2.66 ± 0.07</td>
<td>2.53 ± 0.07</td>
<td>0.97 ± 0.04</td>
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<td>1.99 ± 0.07</td>
<td>0.41 ± 0.03</td>
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<td>2.55 ± 0.05</td>
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<td>PL</td>
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<td>2.84 ± 0.04</td>
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<td>N.A</td>
</tr>
</tbody>
</table>

Table 3.1 Altered Ras signaling in the PG affects fly size. Wild-type flies were w^1118 and isogenic for the second and third chromosome. Pupal lethal (PL), embryonic lethal (EL). Mean ± SEM indicated, all relevant p-values = 0.01 analyzed by a two-tail t-test. Pupal and adult lengths, n ≥ 10; average adult weight, n ≥ 50; wing area, n = 1; wing hair cell number/100 µm^2, n = 3. Anushree Kumar and Tina Chou contributed to this figure by counting wing hair cells.
wing hair cell density, and found that the reduced wing area was accompanied by a reduction in both cell number and cell size (Table 3.1). A similar property was previously observed in other fly mutants exhibiting reduced size (Bohni et al., 1999; The et al., 1997). Although flies bearing amn<sup>c631</sup> and UAS-Ras<sup>v12 (II)</sup> are significantly reduced in size (Figure 3.2 and Table 3.1), the presence of escapers of normal size prevents the growth reduction by Ras<sup>v12 (II)</sup> from reaching the same extent as the reduction conferred by Ras<sup>v12 (III)</sup>. Thus, expression of either activated Ras transgene within the PG reduces fly size.

We found that flies bearing amn<sup>x8</sup> and UAS-Ras<sup>v12 (II)</sup> or UAS-Ras<sup>v12 (III)</sup> produce very small but morphologically normal third instar larvae and pupae (Figure 3.2 and Table 3.1), which fail to eclose. In contrast, flies bearing amn<sup>x8</sup> and UAS-Ras<sup>+</sup> are normal in size and viability. Thus, both Gal4 insertions into amn confer small body size in the presence of UAS-Ras<sup>v12</sup>.

3.2.1 Raf and PI3K, but not Ral, activity controls fly size

Ras<sup>v12</sup> can signal onto several targets including the Raf/MAPK pathway (Halfar et al., 2001; Wood et al., 1992), phosphatidylinositol 3-kinase (Pi3K; Kauffman-Zeh et al., 1997; Prober and Edgar, 2002; Rodriguez-Viciana et al., 1997; Rubio et al., 1997), and the Ral-GDP dissociation stimulator, which in turn activates another small GTPase, Ral (Sawamoto et al., 1999). Activation of the Raf/MAPK cascade by Ras triggers consecutive tyrosine-serine/threonine phosphorylation events from Raf to Mek to Erk, which then regulates the activity of transcription factors, such as Yan (Lai and Rubin 1992; O’Neill et al. 1994), c-Fos and Creb (Karim, 1996; Roy et al., 2002). Pi3K is an enzyme required for the phosphorylation of inositol phospholipids at their 3 position and
can be activated by receptor tyrosine kinases, phospholipase C-γ, or GTP-bound Ras (Corvera and Czech, 1998; Prober and Edgar, 2002; Stocker et al., 2002). Pi3K is a central signaling molecule in the insulin receptor signaling pathway (Britton et al., 2002). Activation of Pi3K leads to increased protein synthesis and cell size via activation of the translational activator p70 S6-kinase (S6K), and repression of the translation inhibitory factor 4E-binding protein (4E-BP) (Hay and Sonenberg, 2004). Ral activity can also regulate gene expression and protein activity, but its mechanism of activation and effects remain unclear (Mirey et al., 2003).

To identify the effector(s) responsible for the small size phenotype seen with Ras activation in the PG, we introduced the gain-of-function transgenes Raf<sup>V12</sup>, Raf<sup>F20</sup>, and PI3K-CAAX (Levers, et al., 1996; Martin-Blanco, et al., 1999; Sawamoto, et al., 1999) under UAS control into flies bearing amn<sup>651</sup>. Raf<sup>F20</sup> activates Raf by deleting the N-terminal negative regulatory region (Martin-Blanco et al., 1999); whereas, PI3K-CAAX has a 3' fusion with the CAAX farnesylation signal to recruit Pi3K to the plasma membrane where it is activated (Annette Parks, personal communication); additionally, the G20V mutation in Raf<sup>V12</sup> eliminates the Ral-GAP activity and locks Ral in the active state (Sawamoto et al., 1999). We found that expression of Raf<sup>V12</sup> had no effect on body size. In contrast, expression of either Raf<sup>F20</sup> or PI3K-CAAX reduced body size to about half of the extent of Ras<sup>V12</sup> (Figure 3.2 and Table 3.1). These results suggest that Ras<sup>V12</sup> exerts its effects via both Raf and PI3K. PI3K-CAAX or Raf<sup>F20</sup> expressed under amn<sup>X8</sup> control conferred embryonic lethality.

The experiments described above demonstrate that activated Ras, PI3K, or Raf within the PG are sufficient to reduce fly size. To determine if these activities are
necessary for size regulation, we introduced the dominant-negative \( \text{Ras}^{N17} \), \( \text{PI3K}^{D954A} \), and \( \text{Raf}^{K497M} \) (Lee, et al., 1996; Leevers, et al., 1996; Roch, et al., 1998) alleles under \( UAS \) control into flies bearing \( amn^{c651} \). \( \text{Ras}^{N17} \) locks Ras in an inactive GDP-bound state by binding Ras-GEF and blocking its activity (Lee et al., 1996), whereas \( \text{Raf}^{K497M} \) encodes a kinase defective form of Raf that can still bind Ras, MEK, and ERK (Roch et al., 1998), and \( \text{PI3K}^{D954A} \) inhibits PI3K activity by placing a mutant residue in the putative ATP binding site while still binding PIP\(_2\) (Leevers et al., 1996).

Expression of any of these three constructs increased pupal and adult size (Figure 3.2 and Table 3.1). The increased size of the wing epidermal cells in flies expressing \( \text{PI3K}^{D954A} \) and \( \text{Raf}^{K497M} \) constructs suggests that most, if not all, of this increased size results from increased cell size rather than cell number (Table 3.1). We found a similar increase in pupal length when \( \text{Ras}^{N17} \), \( \text{PI3K}^{D954A} \) or \( \text{Raf}^{K497M} \) expression was driven by \( amn^{Y8} \) (Figure 3.2 and Table 3.1). These results demonstrate that a reduction in Ras signaling in the PG increases fly size.

### 3.2.2 Signaling molecules downstream of PI3K can affect body size

Activation of PI3K by insulin binding to its receptor enables PIP\(_2\) to be converted into PIP\(_3\), which recruits proteins that contain a plekstrin homology domain to the plasma membrane for activation (Corvera and Czech, 1998). Among these are the serine-threonine kinase Akt, as well as its activating kinases Pdk1 (3-phosphoinositide-dependent kinase 1) and possibly Pdk2 (Verdu et al., 1999). Recruitment of Akt to the membrane by PIP\(_3\) results in activation, which is the predominant and essential mediator for the regulation of both growth and proliferation by PI3K (Gray et al., 1999; Lietzke et al., 2000; Oatey et al., 1999). Akt inactivates the tuberous sclerosis complex (TSC1/2)
via phosphorylation, which results in the inactivation of the GTPase, Rheb; thus, Akt signaling activates Rheb (Jaeschke et al., 2002; Li et al., 2004; Marygold and Leevers, 2002). Rheb-GTP can then dissociate the target of rapamycin (TOR) from its negative regulatory associated protein (Raptor; Goberdhan and Wilson, 2003). TOR has multiple and diverse functions, including the control of protein synthesis (Thomas et al., 2004). Activated TOR phosphorylates the translation inhibitory factor 4E-binding protein (4E-BP) and the translational activator, p70 S6-kinase (S6K; Hay and Sonenberg, 2004) causing an increase in translation (Figure 1.1).

Additionally, InR-Pi3K signaling can regulate the activity of the forkhead transcription factor FOXO. When activated, FOXO activates the transcription of 4E-BP, resulting in translational inhibition. However, activated InR-Pi3K signaling can triply phosphorylate FOXO, allowing 14-3-3 proteins to inactivate FOXO by sequestering it outside of the nucleus. This results in decreased 4E-BP transcription and allows translation to proceed (Junger et al., 2003). Together, these effectors of insulin signaling can control transcription and translation, which integrate to control cell size and cell division (Chen et al., 1996; Zetterberg et al., 1984).

To identify the effector(s) responsible for the small size phenotype seen with Pi3K activation in the PG, we introduced the gain-of-function transgenes $TSC_2^{S924A}$, $S6K^{AC}$, and $FOXO^{TM}$ (Barecelo and Stewart, 2002; Junger et al., 2003; Potter et al., 2002) under $UAS$ control into flies bearing $amn^{651}$. The $TSC_2^{S924A}$ mutation prevents phosphorylation and inhibition of TSC2 by Akt (Potter et al., 2002); $S6K^{AC}$ attains constitutive activity via inhibition of the S6K autoinhibitory domain (Barecelo and
Stewart, 2002); whereas, FOXO$^{TM}$ is constitutively active because it contains mutations that block the 3 Akt phosphorylation sites of FOXO (Junger et al., 2003).

We found that expression of $TSC2^{S924A}$ and $S6K^{CT}$ under the control of $amm^{c651}$ had no effect on body size. In contrast, $amm^{c651}$-dependent expression of FOXO$^{TM}$ increased fly size, but not to the same extent as $Pi3K^{D954A}$ (Figure 3.2 and Table 3.1). These results suggest that Pi3K-CAAX may partially exert its effects by inhibiting FOXO. We also overexpressed endogenous forms of Akt, TSC2, and 4E-BP under the control of $amm^{c651}$, but these manipulations had no effect on fly size.

The experiments described above demonstrate that expression of activated FOXO within the PG is sufficient to increase fly size; however, we could not determine if this activity is necessary for size regulation because loss-of-function and dominant-negative forms of FOXO were not available.

3.3 Ras signaling in the PG affects the duration of growth

We have found that activated Ras-Raf and Pi3K signaling in the PG leads to increased final body size. Because these transgenes were expressed in the PG, a tissue whose only known function is to synthesize and secrete ecdysone, we were interested in determining if altering Ras activity within the PG would also affect the duration of growth.

3.3.1 Inhibition of Ras signaling prolongs development

The large flies produced by $amm^{N8}$- or $amm^{c651}$-driven $Ras^{N17}$, $Pi3K^{D954A}$ and $Raf^{K497M}$ develop (proceed through larval stages) more slowly than normal, consistent with their increased size. In particular, we found that in developing larvae staged at 12
hour intervals, flies heterozygous for amn$^X8$ or amn$^{c651}$ and either UAS-PI3K$^{D954A}$ or UAS-Raf$^{K497M}$ pupariate about 36 hours after wild-type controls (Figure 3.3-A and Figure 3.3-B), which results mostly from a prolonged third instar stage. Larvae in which Ras$^{N17}$ expression is driven by amn$^X8$ or amn$^{c651}$ also undergo delayed pupariation (about 48 hours after wild-type controls, Figure 3.3-A and Figure 3.3-B). However, most of this developmental delay is the result of a prolonged second instar stage. It is unclear why Ras$^{N17}$ appears to act earlier than PI3K$^{D954A}$ or Raf$^{K497M}$. It is possible that the Ras$^{N17}$ transgene is expressed at a higher level relative to endogenous Ras than are the PI3K$^{D954A}$ or Raf$^{K497M}$ transgenes relative to endogenous PI3K and Raf, respectively.

3.3.2 Activation of Ras signaling accelerates development

In contrast, larvae heterozygous for amn$^X8$ and UAS-Ras$^{V12}$ develop more rapidly than normal, and begin pupariation about 18 hours before wild-type or controls in which amn$^X8$ drives UAS-Ras$^+$ (Figure 3.3-B). In addition, amn$^{c651}$-driven Ras$^{V12}$ larvae begin wandering and pupariate about 24 hours before controls (data not shown). However, the time to pupariation of the amn$^{c651}$-driven Ras$^{V12}$ larvae is much more variable than in wild-type controls: the bulk (at least 80%) of control third instar larvae pupariate within a 24 hour time period, whereas the amn$^{c651}$-driven Ras$^{V12}$ third instar larvae require 36-48 hours for 80% of the larvae to pupariate, suggesting that variations in the "photoperiodic gate" and PTTH release may be involved. These results, taken together, indicate that the altered size conferred by alterations in Ras signaling might occur by alterations in the duration of the larval phases (rate of development), and hence the duration of the growth phase.
Figure 3.3 Ras signaling in the PG affects the rate and duration of growth. A, expression of the indicated transgenes under amn$^{657}$ control. B, expression of the indicated transgenes under amn$^{98}$ control. Wild-type flies were w$^{118}$ and isogenic for the second and third chromosome. Larvae were staged at 12 hour intervals after egg laying (AEL) as described in the text. The developmental stage of an approximately equal number of males and females were determined by morphology of mouth hooks and anterior spiracles. Vertical bars indicate that 50% of flies have transitioned to the next stage. Larvae were generated from 10 mating pairs of the appropriate cross. C, comparison of the length of wild-type larvae, or larvae that express Ras$^{17}$ or Ras$^{712}$ under the control of amn$^{657}$ at the indicated time points (AEL). Wild-type larvae were w$^{118}$ and isogenic for the second and third chromosomes. Scale bar is 1 mm.
3.4 Ras signaling in the PG affects the rate of growth

The growth rate (rate of size increase) is also affected in larvae expressing Ras\(^{V12}\) in the PG. For example, until about 84 hours after egg laying (AEL), larvae in which Ras\(^{V12}\) is driven by amn\(^{e651}\) are similar in size to wild-type or larvae in which Ras\(^{+}\) is driven by amn\(^{e651}\) (Figure 3.3-C). At this time about 1/3 of the Ras\(^{V12}\)-expressing larvae appear to stop growing (Figure 3.3-C). These larvae begin to pupariate but fail to develop and ultimately die. The other 2/3 of these larvae grow, although more slowly than wild-type, and can form the small pupae reported in Figure 3.2 and Table 3.1. All such pupae that we have observed are able to form viable adults. These results suggest that expressing activated Ras in the PG might reduce the growth rate, and thus that the reduced final body size in these larvae might reflect both a reduced growth rate and an increased rate of development.

3.5 Ras signaling in the PG affects PG cell size

Because activated Ras can cause increased growth and proliferation, we were interested in determining if altering Ras activity within the PG would affect PG cell size or cell number.

3.5.1 Ras or PI3K, but not Raf, activity affects prothoracic gland cell size

We found that driving either Ras\(^{V12}\) or PI3K-CAAX with amn\(^{e651}\) increased PG cell size, with no effect on cell number, whereas driving PI3K\(^{D954A}\) with amn\(^{e651}\) reduced PG cell size (Figure 3.4). Expression of Ras\(^{N17}\), Raf\(^{F20}\) or Raf\(^{K497M}\) had no effect on PG cell size (Figure 3.4). These data indicate that Ras\(^{V12}\) regulates fly size and developmental rate by two mechanisms: a PI3K-dependent effect on PG size, and a Raf-dependent step that does not involve PG growth.
Figure 3.4 Altered Ras signaling in the PG affects PG cell size. a/b, the PG of a wild-type larva (w^{1118} and isogenic for the second and third chromosomes). c/d, expression of both Ras^{-} and nuclear localized GFP under amn^{-} control produces 50-60 normal sized PG cells. e/f, expression of both Ras^{V12} and nuclear localized GFP under amn^{-} control produces 50-60 enlarged PG cells. g/h, expression of Ras^{N7} under amn^{-} control has no effect on PG cell size. i/j, expression of Pi3K-CAAX under amn^{-} control increases PG, but not CA or CC, cell size to the same extent as Ras^{V12}. k/l, expression of Pi3K^{Q954L} under amn^{-} control decreases PG, but not CA or CC, cell size. m/n, expression of Raf^{P20} under amn^{-} control has no effect on PG cell size. o/p, expression of Raf^{K497M} under amn^{-} control has no effect on PG cell size. q/r, expression of FOXO^{TM} under amn^{-} control has no effect on PG cell size. Arrows point to the ring gland in a, c, e, g, i, k, m, o and q, PG cell nuclei in b, h, j, l, n, p and r are visualized with Hoechst stain, scale bars for a, c, e, g, i, k, m, o and q are 200 μm, scale bars for b, d, f, h, j, l, n, p and r are 50 μm.
3.5.2 Pi3K and Ras-Raf signaling may act as parallel signaling pathways

The observation that Ras\textsuperscript{N17}, which inhibits Ras\textsuperscript{+} activity by preventing Ras activation (Feig and Cooper, 1988), fails to reduce PG cell size is consistent with the possibility that although Ras\textsuperscript{V12} is sufficient to activate PI3K, endogenous Ras activity in the PG is not necessary for PI3K activity under normal physiological conditions, presumably because PI3K can be activated in Ras-independent ways. A similar finding was reported previously in studies of the Drosophila wing (Prober and Edgar, 2002). However, an alternative possibility is that amn\textsuperscript{c651} fails to drive Ras\textsuperscript{N17} levels sufficiently to inhibit Ras\textsuperscript{+} completely.

3.5.3 Signaling molecules downstream of PI3K can affect PG cell size

We found that driving FOXO\textsuperscript{TM} with amn\textsuperscript{c651} had no effect on PG cell size (Figure 3.4). This observation indicates that FOXO regulates fly size independent of PG cell growth. Additionally, expression of TSC\textsuperscript{2S924A}, S6K\textsuperscript{ACT}, Akt\textsuperscript{+}, TSC2\textsuperscript{−}, or 4E-BP\textsuperscript{+} under the control of amn\textsuperscript{c651} had no effect on PG cell size.

3.6 Ras signaling in the PG affects the timing of ecdysone release

PG cells release the ecdysone class of molting hormones in response to PTTH release from neurosecretory cells within the brain (Gilbert, et al., 2002). The Ras\textsuperscript{V12} - or PI3K-CAAX -induced PG hypertrophy, and the PI3K\textsuperscript{D954A} -induced hypotrophy, raised the possibility that altered fly size and duration of the larval phases resulted from altered ecdysone release. In this view, enhanced or precocious ecdysone release would cause premature reduction of growth rate and premature pupariation, and hence the formation of small pupae and adults. In contrast, delayed or reduced ecdysone release would delay
pupariation and thus prolong the growth phase, and enable the delayed formation of large pupae and adults. To test this possibility, we collected larvae with altered Ras signaling, staged developmentally at 12 hour intervals, and measured ecdysone activity in these larvae as a function of both genotype and stage of development. To measure ecdysone activity, we used an indirect reporter system: we prepared RNA from the staged larvae, and used quantitative RT-PCR to measure transcriptional activation of the ecdysone signaling targets E74A and E74B (Karim and Thummel, 1991), as well as an internal control gene, RpL13A. E74A and E74B were chosen because both genes are direct targets of ecdysone signaling, but respond differentially to ecdysone concentrations. In particular, E74B is abundant at intermediate ecdysone levels, but inhibited at high ecdysone concentrations, whereas E74A transcripts are present only at high ecdysone concentrations (Fletcher, et al., 1997). These properties predict that a rise in E74B levels will precede a rise in E74A levels, when ecdysone titers rise during the third larval instar and metamorphosis (Fletcher, et al., 1997).

We found that in wild-type control larvae (either amn$^{c631}$ or amn$^{X8}$ crossed to UAS-Ras$^+$ (Figure 3.5) or flies bearing each UAS-transgene crossed to mothers lacking Gal4 (not shown)), E74B levels began increasing late in the second instar stage (at about 96 hours after egg laying (AEL), peaked at 120 hours AEL, and then dropped to low levels at 144 hours AEL (Figure 3.5-A), which corresponds to the onset of pupariation. E74A levels began to rise at 132 hours AEL and were maintained at high levels for at least the next 36 hours (Figure 3.5-B).
Figure 3.5 Altered Ras signaling in the PG affects ecdysone release. A, altered Ras signaling under the control of amn\textsuperscript{c651} affects the transcriptional activation of E74B. B, altered Ras signaling under the control of amn\textsuperscript{x9} affects the transcriptional activation of E74A. Fold activation ± error of the fold was determined from a total of 6 reactions. Two samples were collected from each genotype and triplicate measures of each sample were conducted using the relative 2\textsuperscript{-\Delta\Delta C_{T}} method (Livak and Schmittgen, 2001). Y-axis: E74A or E74B transcript levels normalized to Rpl13A (arbitrary units). X-axis: hours AEL at which larvae were collected. Magdalena Walkiewicz contributed to this figure by isolating RNA from larvae, reverse transcribing RNA to cDNA, and performing the majority of Q-PCR reactions.
3.6.1 Inhibition of Ras signaling delays ecdysone release

Ecdysone levels rose abnormally slowly during the third instar period in larvae in which Ras signaling in the PG was inhibited through amn<sup>c651</sup> or amn<sup>x8</sup>-driven expression of either PI3K<sup>D954A</sup> or Raf<sup>K497M</sup>. In particular, E74B levels were significantly lower in these larvae than in wild-type from 84 to 132 hours AEL (Figure 3.5-A). The appearance of the E74A spike was also delayed about 36 hours, which corresponds to the 36 hour delay in the onset of pupariation (Figure 3.5-B). We suggest that this abnormally slow increase in ecdysone release is responsible for prolonging the third instar stage, which in turn enables excessive growth to occur, and hence the delayed appearance of large adults.

3.6.2 Ras activity causes premature ecdysone release

Expression of activated Ras in the PG conferred a precocious rise in ecdysone activity. In particular, between 72 and 108 AEL in larvae in which amn<sup>c651</sup> drove Ras<sup>V12</sup> expression, E74B transcript levels were significantly higher than in wild-type (Figure 3.5-A). Furthermore, in larvae in which Ras<sup>V12</sup> expression was driven by amn<sup>x8</sup>, we observed that high levels of E74A transcripts appeared at 108 hours AEL, about 24 hours before comparable levels appeared in wild-type (Figure 3.5-B). These results indicate that Ras<sup>V12</sup> driven by either amn<sup>c651</sup> or amn<sup>x8</sup> cause ecdysteroid levels to rise 24 hours prematurely. We suggest that this precocious ecdysone burst causes the cessation of growth and the premature pupariation that we observe.
3.7 Ras signaling in the PG regulates Halloween gene transcription

In *D. melanogaster* ecdysone synthesis, the dehydrogenated form of cholesterol, 7-dehydrocholesterol, is converted into ecdysone and 20-hydroxyecdysone through a series of hydroxylation reactions (Figure 1.5) catalyzed by the "Halloween" family of cytochrome P450 enzymes (Gilbert et al., 2002). Recent RNA *in situ* hybridization analysis indicates that ecdysone synthesis may not only be regulated by the availability of 7-dehydroxyecdysone, but also by the transcriptional activation of cytochrome P450 enzymes, as the Halloween gene transcripts *phantom, shadow, and disembodied* only appear just prior to a molt (Gilbert, 2004; Niwa et al., 2004; Petryk et al., 2003; Warren et al., 2002).

To test if altered Ras signaling in the PG regulates the timing of ecdysone synthesis via the regulation of Halloween gene transcription, we collected larvae with altered Ras signaling in the PG, staged developmentally at 12 hour intervals, and used quantitative RT-PCR (Q-PCR) to measure transcript levels of *disembodied (dib)* and *phantom (phm)* as a function of both genotype and stage of development. *dib* and *phm* encode cytochrome P450 enzymes that synthesize the ecdysone precursors 2-deoxyecdysone and ketotriol, respectively. Our preliminary data shows that in wild-type control larvae (flies expressing a wild-type form of Ras, *Ras*⁺, in the PG), both *dib* and *phm* levels begin to rise in the late second instar stage at 108 hours after egg laying (AEL) and peak in the third instar stage at 120 hours AEL (Figure 3.6-A and Figure 3.6-B). Larvae that express activated Ras, *Ras⁺¹⁄₂*, in the PG confer not only a precocious rise, but also an increased amount of *dib* and *phm* transcripts (Figure 3.6-A and Figure 3.6-B). We suggest that this precocious Halloween gene transcript burst causes
Figure 3.6 Altered Ras signaling in the PG affects Halloween gene transcription. A, altered Ras signaling under the control of amn^{dos} affects the transcriptional activation of phantom (phm). B, altered Ras signaling under the control of amn^{dos} affects the transcriptional activation of disembodied (dib). Fold activation ± error of the fold was determined from a total of 6 reactions. Two samples were collected from each genotype and triplicate measures of each sample were conducted using the relative 2^{ΔΔCt} method (Livak and Schmittgen, 2001). Y-axis: phm or dib transcript levels normalized to RpIII-140 (arbitrary units). X-axis: hours AEL at which larvae were collected. Magdalena Walkiewicz contributed to this figure by isolating RNA from larvae, reverse transcribing a portion of the RNA to cDNA, and performing a third of the Q-PCR reactions,
premature ecdysone synthesis, the cessation of growth, and small flies. In contrast, in larvae in which Ras signaling in the PG was inhibited through expression of PI3K<sup>D954A</sup> or Raf<sup>K49?M</sup>, dib and phm transcript levels did not rise from 60 to 132 hours AEL (Figure 3.6-A and Figure 3.6-B). We suggest that this inhibition of Halloween gene transcription results in delayed ecdysone release, a prolonged third instar stage, and the appearance of large adults.

3.8 Model for Ras and PI3K signaling in the PG

The insect prothoracic gland (PG) releases the ecdysone class of steroid hormones, which triggers molting and ultimately metamorphosis in response to PTTH release from neurosecretory cells in the brain. Here we describe the effects of manipulating the activity of Ras and PI3K signaling in the PG of Drosophila larvae. We find that inhibiting Ras or PI3K signaling delays the second or third instar stages (reduces the developmental rate), allowing increased growth to occur, and ultimately leads to the delayed appearance of large flies. In contrast, activating Ras or PI3K signaling in the PG shortens the second and third instar stages (increases developmental rate), leading to premature pupariation and the generation of small pupae and adults. These effects appear to be mediated by altered Halloween gene transcription and ecdysone release: inhibition of Ras or PI3K signaling in the PG greatly attenuates the increase in both Halloween gene and ecdysone titers that occur during the third instar stage, whereas activating Ras or PI3K signaling in the PG causes a precocious increase in these titers. We conclude that altered Ras or PI3K signaling in the PG alters both the duration of the larval stages, and
final body size via the timing of Halloween gene transcription and ecdysone release. A model summarizing these conclusions is shown in Figure 3.7.

Our results suggest that both Ras and Pi3K signaling participate in the regulation of body size, developmental rate, the timing of Halloween gene transcription and ecdysone release. In particular, activation of Ras or Pi3K signaling in the PG generates small flies, whereas inhibition of either Ras or Pi3K signaling attenuates ecdysone release.

![Figure 3.7 Model for altered Ras signaling in the PG.](image)

In this view, Halloween gene transcription in the PG is regulated by three mechanisms: a PI3K-dependent cell autonomous growth effect on PG cells, a PI3K-FOXO-dependent step that does not involve the cell-autonomous growth of PG cells, and a Ras/Raf-dependent step. These Halloween enzymes are then able to synthesize ecdysone, which affects both the rate and duration of growth, and regulates final body size. Solid lines indicate direct events, whereas, dashed lines indicate that multiple steps are involved. A ? indicates a hypothetical relationship.
and confers the delayed appearance of large flies. However, Ras and Pi3K signaling mediate their size effects by distinct mechanisms. Activating Pi3K, but not Raf or FOXO, in the PG, increases PG cell size, whereas expression of dn-Pi3K, but not dn-Raf, in the PG reduces PG cell size. We suggest that the inhibition of PG growth conferred by the inhibition of canonical Pi3K signaling is responsible for the reduced ecdysone release that we observed; presumably, the hypertrophic PG caused by the activation of canonical Pi3K signaling promotes precocious ecdysone release. PG hypertrophy is also observed in mutants that exhibit reduced ecdysone release (Mirth et al., 2005), raising the possibility that ecdysone inhibits PG growth via inhibition of Pi3K signaling.

Because PG hypertrophy is caused by either activated Ras or Pi3K, but not activated Raf, we suggest that RasV12 is sufficient to activate Pi3K, as has been observed previously. However, the importance of Ras in the activation of Pi3K under physiological conditions remains controversial (Prober and Edgar, 2002; Warren et al., 2002); Pi3K can be activated by several Ras-independent mechanisms, raising the possibility that activated Ras might be sufficient but not necessary for Pi3K activation. Furthermore, it has been suggested that in Drosophila, at least, wild-type Ras normally activates Pi3K poorly, and thus activation might often be an artifact of RasV12 overexpression (Prober and Edgar, 2002). We present results that are consistent with this suggestion: inhibiting endogenous Ras activity within the PG by expression of the dominant-negative RasN17 conferred the delayed appearance of large flies, but did not detectably reduce larval PG size, suggesting that Pi3K is not inhibited by RasN17 expression. Taken together, these observations are most consistent with the possibility that inhibiting endogenous Ras inhibits Raf, but not Pi3K. In this view, Pi3K in the PG is
normally activated at least in part by Ras-independent factors (for example, by insulin-like peptides). Alternatively, it is possible that the inhibition of endogenous Ras by Ras\textsuperscript{N17} expression was not complete, and enough residual endogenous Ras remained active to permit Pi3K-dependent PG growth. In either case, the observation that Ras\textsuperscript{N17}, like Raf\textsuperscript{K497M}, can slow the developmental rate and increase body size without an effect on PG size suggests that Ras is necessary for Raf activity.

A requirement for Raf supports the notion that ecdysone release is regulated differently in Drosophila than Manduca. Although in Manduca, PTTH induces Erk activation (Rybczynski et al., 2001), possibly via Raf, the role of this activation in regulating ecdysone release is unclear. Rather, a PTTH-induced increase in [cAMP] is both necessary and sufficient for ecdysone release in Manduca (Smith et al., 1984; Smith et al., 1996). In constrast, increases in [cAMP] within the Drosophila PG does not directly trigger ecdysone release (Henrich, 1995). However, there is recent evidence that Raf signaling might alter [cAMP] and regulate ecdysone release in Drosophila. Mutations in NF1 cell nonautonomously reduces body size in Drosophila by inhibiting Ras-GAP activity because the phenotype is completely rescued by expressing a functional NF1-GAP catalytic domain in larval neurons (Walker et al., 2006). Additionally, mutations in NF1 create small flies by reducing [cAMP] because the phenotype is completely rescued by induced expression of a constitutively active (cAMP-independent) PKA (The et al., 1997). These results suggest that NF1 alters body size by regulating both Ras activity and cAMP levels; however, it is not known if these effects of NF1 regulate body size via ecdysone release, but if so, a role for cAMP in regulating Drosophila ecdysone release would be demonstrated.
In this regard, it is noteworthy that two Gal4 elements inserted into ann each express in the PG. The ann<sup>c651</sup> expression pattern was suggested to mirror the endogenous ann expression pattern most closely (Waddell et al., 2000), raising the possibility that ann is normally expressed in the PG. The ann gene is predicted to encode three neuropeptides, homologous to growth hormone releasing hormone, the pituitary adenylate cyclase activator peptide called PACAP, and a novel neuropeptide (Feany and Quinn, 1995). At least one of these neuropeptides activates adenylate cyclase in Drosophila in vivo (Moore et al., 1998). Additionally, Drosophila NF1 is an essential mediator of PACAP-induced signalling at the Drosophila neuromuscular junction (Kiger and O’Shea, 2001). These observations raise the possibility that an Amn neuropeptide might actively participate in regulating ecdysone synthesis, rather than merely serving as a host for Gal4 elements. However, an ann mutant genotype is not required for altered Ras signalling in the PG to alter body size: the c805 Gal4 element confers Ras<sup>v12</sup>-dependent reductions in body size even in an ann<sup>+</sup> background, and ectopic expression of ann does not rescue the body size phenotypes conferred by ann<sup>c651</sup> or ann<sup>x8</sup>-driven Ras<sup>v12</sup> expression. Further investigation will be required to determine if the Amn neuropeptides play a role in regulating ecdysone synthesis.
CHAPTER 4. EFFECTS OF RAS SIGNALING IN CHOLINERGIC NEURONS

Regulation of Ras signaling in the PG controls the timing of ecdysone synthesis, the duration of growth, and fly size. To identify additional Gal4 lines that can create small flies when driving the expression of Ras$^{V12}$, we selected 27 Gal4 lines (Table 4.1) that were reported to be expressed in the central nervous system (Bloomingston; Siegmund and Korgé, 2001). We then crossed these Gal4 lines to flies bearing UAS-Ras$^{V12}$, and assayed the progeny for changes in fly size. We found that flies bearing Ras$^{V12}$ under the control of ChaGal4 are morphologically normal but significantly reduced in size.

4.1 ChaGal4 is expressed in cholinergic neurons

In ChaGal4, expression of Gal4 is regulated by 7.4 kb of 5' flanking DNA from the "cholinergic" locus. This locus contains genetic functions for both choline acetyltransferase (Cha) (Kitamoto et al., 1998) and the vesicular acetylcholine transporter (Vacht) (Erickson et al., 1994). Thus, ChaGal4 is expected to be expressed in all cholinergic neurons, and this possibility was confirmed in adult flies (Salvaterra and Kitamoto, 2001). However, these experiments did not determine whether ChaGal4 is expressed in the PG of larvae (Salvaterra and Kitamoto, 2001).

To test if ChaGal4 is expressed in the larval PG, we expressed a nuclear localized form of GFP under the control of ChaGal4. We found that ChaGal4 expresses within many cells of the brain and ventral ganglion, but is not expressed in PG cells (Figure 4.1). Additionally, we expressed a microtubule associated form of GFP under the control of ChaGal4 to determine if ChaGal4 is expressed in neurons that innervate the PG. These
<table>
<thead>
<tr>
<th>Gal4 Line</th>
<th>Reported Expression Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>amn&lt;sup&gt;68A&lt;/sup&gt;</td>
<td>Kenyon Cell</td>
</tr>
<tr>
<td>ChaGAL4</td>
<td>Cholinergic Neurons</td>
</tr>
<tr>
<td>c179</td>
<td>Mesoderem</td>
</tr>
<tr>
<td>PDF-GAL4</td>
<td>A Subset of Peptidergic Neurons</td>
</tr>
<tr>
<td>Ddc-GAL4</td>
<td>Dopaminergic Neurons</td>
</tr>
<tr>
<td>sev-GAL4</td>
<td>R7 Photoreceptor</td>
</tr>
<tr>
<td>EcR-GAL4</td>
<td>Mushroom Body, Salivary Gland, Fat Body</td>
</tr>
<tr>
<td>c21</td>
<td>Peptidergic Neurons</td>
</tr>
<tr>
<td>11c</td>
<td>Hemocytes, Fat Bodies, Testes, Salivary Gland</td>
</tr>
<tr>
<td>43A</td>
<td>Ecdysone-dependent Neurons, Imaginal Discs, Salivary Gland</td>
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<tr>
<td>c135</td>
<td>Larval Brain, Salivary Gland, Fat Body, Optic Lobe, Trachea, Malpighian Tubule</td>
</tr>
<tr>
<td>c747</td>
<td>Adult Brain, Pars Inter cerebralis, Thoracic Ganglion</td>
</tr>
<tr>
<td>OK107</td>
<td>Mushroom Body</td>
</tr>
<tr>
<td>ninaE-GAL4</td>
<td>Bolwig’s Organ, Adult Head (eye)</td>
</tr>
<tr>
<td>c309</td>
<td>Adult Brain, Pars Inter cerebralis, Thoracic Ganglion, Corpora Pedunculata</td>
</tr>
<tr>
<td>Jan229</td>
<td>CC-PI, CC-MS</td>
</tr>
<tr>
<td>Feb204</td>
<td>PG-LP, CC-MS</td>
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<td>Feb211</td>
<td>PG-LP, CC-LP</td>
</tr>
<tr>
<td>Feb296</td>
<td>PG-LP, CA-LP, CC-PI</td>
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<td>Kurs6</td>
<td>CC-LP, CC-PI, CC-MS</td>
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<td>Kurs21</td>
<td>CA-LP, CC-MS</td>
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<td>Kurs58</td>
<td>CC-PI, CC-MS</td>
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<tr>
<td>Mai301</td>
<td>PG-LP, CC-PI, CC-MS</td>
</tr>
<tr>
<td>Mai369</td>
<td>CC-LP</td>
</tr>
</tbody>
</table>

Table 4.1 Gal4 lines reported to express in the *Drosophila* CNS. Expression of Ras<sup>V12</sup>(III) under the control of these Gal4 lines was assayed for effects on fly size. Prothoracic gland innervating neurosecretory neurons of the lateral protocerebrum (PG-LP), corpora cardiaca and aorta innervating neurosecretory neurons of the pars intercerebralis (CC-PI), corpora cardiaca innervating neurosecretory neurons of the medial subesophageal ganglion (CC-MS), corpora cardiaca innervating neurosecretory neurons of the lateral protocerebrum (CC-LP), corpus allatum innervating neurosecretory neuron of the lateral protocerebrum (CA-LP).

Images show that ChaGal4 is expressed in neurons near the PG, but is not expressed in PG-LP neurons, or other neurons that may innervate the PG (Figure 4.1-e). Thus, Ras signaling under the control of ChaGal4 may affect fly size without altering the timing of ecdysone release, or may regulate ecdysone synthesis indirectly by changing the properties of PG innervated neurons.
Figure 4.1 Spatial expression pattern of ChaGal4. ChaGal4 expression in the central nervous system of wandering third instar larvae was visualized via nuclear localized GFP, GFP(nls), and cytosolic GFP, tau-GFP. a, Cha>tau-GFP shows that Cha is expressed throughout the ventral ganglion, but not as widely in the brain lobes. (b-d), Cha>GFP(nls) shows that ChaGal4 is expressed throughout the brain and ventral ganglion, but is not expressed in cells of the ring gland. (e-g), Cha>tau-GFP shows that ChaGal4 is expressed in neurons of the brain and ventral ganglion, but not in neurons that innervate the ring gland. Scale bar is 50 μm for a, and 200 μm for b-g.

4.2 Altered intracellular signaling in cholinergic neurons affects body size

4.2.1 Ras signaling in cholinergic neurons affects body size

We used ChaGal4 to test the effects of altering the activity of Ras signaling within cholinergic neurons. We found that flies bearing ChaGal4 and either of two transgenes expressing the constitutively active Ras^{V12} under UAS control, denoted UAS-Ras^{V12} (II) and UAS-Ras^{V12} (III) (Lee et al., 1996; Karim and Rubin, 1996) are morphologically normal but significantly reduced in size (Figure 4.2 and Table 4.2). This phenotype is not simply a consequence of Ras overexpression, because flies carrying ChaGal4 and UAS-Ras^{*} exhibit normal body size (Figure 4.2 and Table 4.2). To determine if this size phenotype was a result of reduced cell size or cell number, we measured total wing hair cell number and wing hair cell density, and found that the reduced wing area was accompanied by a reduction in both cell number and cell size (Table 4.2). A similar property was previously observed in other fly mutants exhibiting reduced size (Bohni et al., 1999; Caldwell et al., 2005; The et al., 1997). Although flies
Figure 4.2 Altered Ras and ecdysone signaling under the control of ChaGal4 affects pupal size. Wild-type flies were w^{118} and isogenic for the second and third chromosome. Graphs depict the mean ± SEM of female pupae; analysis is described in the text; n ≥ 10; a * represents a genotype with a p-value ≤ 0.01 compared to wild-type analyzed by a two-tailed t-test.

bearing ChaGal4 and UAS-Ras^{V12}(III) are significantly reduced in size (Figure 4.2 and Table 4.2), the presence of escapers of normal size prevents the growth reduction by Ras^{V12}(III) from reaching the same extent as the reduction conferred by Ras^{V12}(II). Thus, expression of either activated Ras transgene within cholinergic neurons reduces fly size.

4.2.2 Raf, but not Pi3K or Ral, activity in cholinergic neurons controls fly size

Ras^{V12} exerts its effects through several targets, including Raf, Pi3 kinase (Pi3K), and Ral (Kolch, et al., 1991; Rodriguez-Viciana, et al., 1994; Sawamoto et al., 1999). To
<table>
<thead>
<tr>
<th></th>
<th>Pupal Length (mm)</th>
<th>Adult Length (mm)</th>
<th>Average Weight (mg)</th>
<th>Wing Area (mm²)</th>
<th>Wing Hair Cell Number/100 μm²</th>
<th>Average Hair Cells/Wing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>3.09 ± 0.09</td>
<td>2.95 ± 0.11</td>
<td>2.62 ± 0.09</td>
<td>2.51 ± 0.10</td>
<td>0.84 ± 0.03</td>
<td>1.72</td>
</tr>
<tr>
<td>Cha&gt;Ras⁺</td>
<td>3.01 ± 0.05</td>
<td>3.04 ± 0.04</td>
<td>2.65 ± 0.04</td>
<td>2.56 ± 0.06</td>
<td>0.92 ± 0.03</td>
<td>1.74</td>
</tr>
<tr>
<td>Cha&gt;Ras⁺¹² (II)</td>
<td>2.44 ± 0.04</td>
<td>2.36 ± 0.06</td>
<td>2.29 ± 0.06</td>
<td>2.17 ± 0.06</td>
<td>0.54 ± 0.02</td>
<td>1.12</td>
</tr>
<tr>
<td>Cha&gt;Ras⁺¹² (III)</td>
<td>2.78 ± 0.04</td>
<td>2.73 ± 0.05</td>
<td>2.59 ± 0.07</td>
<td>2.52 ± 0.05</td>
<td>0.81 ± 0.05</td>
<td>1.55</td>
</tr>
<tr>
<td>Cha&gt;Ras⁺¹⁷</td>
<td>3.18 ± 0.04</td>
<td>3.06 ± 0.05</td>
<td>2.84 ± 0.04</td>
<td>2.74 ± 0.06</td>
<td>1.02 ± 0.04</td>
<td>1.94</td>
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<tr>
<td>Cha&gt;Raf²₀</td>
<td>2.44 ± 0.05</td>
<td>2.34 ± 0.04</td>
<td>2.30 ± 0.07</td>
<td>2.19 ± 0.05</td>
<td>0.56 ± 0.03</td>
<td>1.18</td>
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<tr>
<td>Cha&gt;Raf²₀⁻⁰⁵</td>
<td>3.39 ± 0.05</td>
<td>3.35 ± 0.03</td>
<td>2.80 ± 0.04</td>
<td>2.65 ± 0.04</td>
<td>1.16 ± 0.04</td>
<td>2.09</td>
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<tr>
<td>Cha&gt;EcR⁺</td>
<td>3.79 ± 0.05</td>
<td>3.59 ± 0.04</td>
<td>PL</td>
<td>PL</td>
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</tr>
</tbody>
</table>

Table 3.1 Altered Ras or ecdysone signaling under the control of ChaGal4 affects fly size. Wild-type flies were w¹¹¹⁸ and isogenic for the second and third chromosome. Pupal lethal (PL), embryonic lethal (EL). Mean ± SEM indicated, all relevant p-values = 0.01 analyzed by a two-tail t-test. Pupal and adult lengths, n ≥ 10; average adult weight, n ≥ 50; wing area, n = 1; wing hair cell number/100 μm², n = 3. Anushree Kumar and Tina Chou contributed to this figure by counting wing hair cells.
identify the effector(s) responsible for the decreased fly size, we introduced the gain-of-function transgenes Raf$^{F20}$, PI3K-CAAAX, and Raf$^{V20}$ (Leevers, et al., 1996; Martin-Blanco, et al., 1999; Sawamoto et al., 1999) under UAS control into flies bearing ChaGal4. We found that expression of PI3K-CAAAX or Raf$^{V20}$ had no effect on body size. In contrast, expression of Raf$^{F20}$ reduced body size to the same extent as Ras$^{V12}(II)$ (Figure 4.2 and Table 4.2). These results suggest that Ras$^{V12}$ exerts its effects only through Raf.

The experiments described above demonstrate that activated Ras and Raf within cholinergic neurons is sufficient to reduce fly size. To determine if these activities are necessary for size regulation, we introduced the dominant-negative Ras$^{N17}$ and Raf$^{K497M}$ (Lee, et al., 1996; Roch, et al., 1998) alleles under UAS control into flies bearing ChaGal4. Expression of Raf$^{K497M}$ increased fly size to a statistically significant degree, but expression of Ras$^{N17}$ only produced qualitatively larger flies because of an increased number of escapers of normal body size (Figure 4.2 and Table 4.2). These results demonstrate that a reduction in Ras-Raf signaling in cholinergic neurons increases fly size.

4.2.3 Ecdysone signaling in cholinergic neurons controls fly size

We inhibited EcR activity by introducing the dominant-negative transgene EcR$^{B1-AC655-W6504}$ (Cherbas et al., 2003) under UAS control into flies bearing ChaGal4. EcR$^{B1-AC655-W6504}$ encodes a truncated form of EcR that can bind Usp but not ecdysone (Cherbas et al., 2003). We found that expression of EcR$^{B1-AC655-W6504}$ significantly increased pupal size (Figure 4.2 and Table 4.2) and adults failed to emerge.
4.2.4 No effect on fly size of altered Ras-Raf or ecdysone signaling in other neurons that regulate ecdysone or insulin signaling

The *Drosophila* genome contains seven insulin-like peptide genes (*dilp*-1 through -7) that are expressed in small clusters of larval brain neurons known as insulin producing cells (IPCs) (Brogiolo et al., 2001). *dilp2-Gal4*, an 859-base pair promoter fragment comprised of sequences immediately 5' of *dilp2*, expresses in IPCs that express *dilp1*, -2, -3, and -5. Expression of the cell death-promoting factor Reaper under the control of *dilp2-Gal4* ablated larval brain IPCs, and conferred a similar decrease in fly size (Rulifson et al., 2004) as expression of activated Ras in cholinergic neurons, or partial loss-of-function mutations to the *Drosophila* InR (Britton et al., 2002; Goberdhan and Wilson, 2003). These results raise the possibility that a subset of cholinergic neurons might be *dilp2-Gal4* expressing neurons, and that regulation of Ras-Raf and EcR signaling in these cells may control insulin production. However, we found that expression of *Ras*$_{V12}$ and *EcR*$_{B1-SC655-W650A}$ under the control of *dilp2-Gal4* had no effect on fly size (data not shown). Similarly, expression of *Ras*$_{V12}$ and *EcR*$_{B1-SC655-W650A}$ in PG-LP neurons under the control of *Feb211* (Siegmund and Korge, 2001) had no effect on fly size (data not shown). Therefore, we conclude that the effects of Ras-Raf or ecdysone signaling in *ChaGal4* expressing neurons do not occur via neurons reported to regulate insulin or ecdysone synthesis.

4.3 Ras-Raf and ecdysone signaling in cholinergic neurons prolongs the duration of growth

The large flies produced by *ChaGal4*-driven *Ras*$_{N17}$ and *Raf*$_{K497M}$ develop (proceed through larval stages) more slowly than normal, consistent with their increased size. In particular, we found that in developing larvae staged at 12 hour intervals, flies
that express $\text{Raf}^{K497M}$ or $\text{EcR}^{B1-\Delta C655-W650A}$ under the control of ChaGal4 pupariate about 36 hours after wild-type controls (Figure 4.3-A), which results mostly from a prolonged third instar stage. Larvae in which $\text{Ras}^{N17}$ expression is driven by ChaGal4 also undergo delayed pupariation (about 12 hours after wild-type controls, Figure 4.3-A).

Interestingly, larvae heterozygous for ChaGal4 and either UAS-\text{Ras}^{V12}(II) or UAS-\text{Raf}^{F20} do not develop more rapidly than normal, but instead begin pupariation about 24 hours after wild-type or control larvae in which ChaGal4 drives UAS-\text{Ras}^{N}$ (Figure 4.3-A).

This decrease in size and delay in the duration of growth is similar to that of larvae with inhibited insulin signaling (Goberdhan and Wilson, 2003).

These results raise the possibility that Ras-Raf and ecdysone signaling in cholinergic neurons could affect fly size in one of two ways. First, ecdysone could act in a positive feedback loop within cholinergic neurons. Ecdysone signaling could activate Ras-Raf signaling allowing cholinergic neurons to signal onto PG-LP neurons resulting in the release of PTTH and activation of ecdysone synthesis in PG cells. Alterations to this ecdysone-dependent positive feedback loop would then lead to changes in the duration of growth and final body size. Second, ecdysone could negatively regulate insulin signaling. Ecdysone signaling in cholinergic neurons could activate Ras-Raf signaling allowing these neurons to inhibit insulin release from IPCs. Insulin could then act throughout the larval body to affect final body size by controlling the rate of growth through the classic insulin signaling pathway.
Figure 4.3 Altered Ras and ecdysone signaling under the control of ChaGal4 affects the rate and duration of growth. **A**, expression of the indicated transgenes under ChaGal4 control. Wild-type flies were w^{1118} and isogenic for the second and third chromosome. Larvae were staged at 12 hour intervals after egg laying (AEL) as described in the text. The developmental stage of an approximately equal number of males and females were determined by morphology of mouth hooks and anterior spiracles. Vertical bars indicate that 50% of flies have transitioned to the next stage. Larvae were generated from 10 mating pairs of the appropriate cross. **B**, comparison of the length of wild-type larvae, or larvae that express Ras', Ras^{V12}(II), Raf^{K497M} or EcR^{ON} under the control of ChaGal4 at the indicated time points (AEL). Wild-type larvae were w^{1118} and isogenic for the second and third chromosomes. EcR^{ON} represents EcR^{1-480S-495A}. Scale bar is 1mm.
4.4 Ras-Raf and ecdysone signaling in cholinergic neurons affects the rate of growth

The rate of growth is also affected in larvae expressing Ras$^{V_{12}}$ in cholinergic neurons. For example, at 84 hours after egg laying (AEL), larvae in which Ras$^{V_{12}}$ is driven by ChaGal4 are smaller than wild-type or larvae in which Ras$^{+}$ is driven by ChaGal4 (Figure 4.3-B). Similarly, at 144 hours AEL, larvae expressing Ras$^{V_{12}}$ in cholinergic neurons are smaller, and have grown more slowly, than wild-type larvae (Figure 4.3-B). Conversely, larvae that express Raf$^{K497M}$ or EcR$^{B1-3C655-W650A}$ in cholinergic neurons are larger and develop more rapidly than wild-type larvae (Figure 4.3-B). These results suggest that activated Ras-Raf signaling in cholinergic neurons creates small flies by reducing the rate of growth; whereas, inhibited Ras-Raf or ecdysone signaling in cholinergic neurons increases fly size via an increased rate of growth.

4.5 Ras-Raf or ecdysone signaling in cholinergic neurons has no effect on PG cell size

We have previously shown that Pi3K signaling in the PG alters PG cell size and the timing of ecdysone release (Figure 3.4). This observation led us to investigate whether Ras-Raf or ecdysone signaling in cholinergic neurons could affect the timing of ecdysone release via insulin-Pi3K signaling in the PG. To test this possibility, we determined the size of PG cells in larvae with altered Ras-Raf signaling in cholinergic neurons. Expression of activated Ras or Raf in cholinergic neurons does not alter the PG cell size of wandering third instar larvae (Figure 4.4). Similarly, expression of $dn$-Ras, $dn$-Raf, or $dn$-EcR under the control of ChaGal4 has no effect on PG cell size (Figure 4.4). Thus, Ras-Raf and ecdysone signaling in cholinergic neurons do not affect Pi3K
signaling in PG cells, and may not affect fly size by altering the timing of ecdysone synthesis.

**Figure 4.4** Altered Ras or ecdysone signaling under the control of ChaGal4 does not affect PG cell size. (a, b), wild-type larvae have 25-30 PG cells. (c, d), expression of Ras<sup>v12</sup> under the control of ChaGal4 produces 25-30 normal sized PG cells. (e, f), expression of Ras<sup>v12(II)</sup> under the control of ChaGal4 produces 25-30 normal sized PG cells with respect to the overall size of the larvae. (g, h), expression of Raf<sup>F20</sup> under the control of ChaGal4 produces 25-30 PG cells of normal size with respect to the overall size of the larvae. (i, j), expression of Ras<sup>N17</sup> under the control of ChaGal4 produces 25-30 PG cells of normal size with respect to the overall size of the larvae. (k, l), expression of Raf<sup>K497M</sup> under the control of ChaGal4 produces 25-30 PG cells of normal size with respect to the overall size of the larvae. (m, n), expression of EcR<sup>N</sup> under the control of ChaGal4 produces 25-30 PG cells of normal size with respect to the overall size of the larvae. Wild-type larvae were w<sup>1118</sup> and isogenic for the second and third chromosomes. EcR<sup>N</sup> represent EcR<sup>EcR<sup>B</sup>-K<sup>EcR<sup>B</sup>-K<sup>EcR<sup>B</sup>-K<sup>EcR<sup>B</sup>-K<sup>B</sup></sup></sup></sup></sup>. Arrows point to the ring gland in a, c, e, g, i, k, and m, PG cell nuclei in b, h, j, l, and n are visualized with Hoechst stain, scale bars for a, c, e, g, i, k, and m are 200 µm, scale bars for b, d, f, h, j, l, and n are 50 µm.
4.6 Ras-Raf signaling in cholinergic neurons affects insulin signaling

The observation that larvae that express activated Ras-Raf signaling in cholinergic neurons are small, grow for longer periods of time, develop more slowly than normal, and have PG cells of normal size and number suggests that activated Ras-Raf signaling in cholinergic neurons inhibits insulin signaling throughout the larva.

To test this possibility, we collected larvae with altered Ras-Raf signaling in cholinergic neurons, staged developmentally at 12 hour intervals, and used quantitative RT-PCR (Q-PCR) to measure transcript levels of the insulin signaling target 4E-BP (thor) as a function of both genotype and stage of development. Systemic insulin signaling regulates the the activity of the forkhead transcription factor FOXO. When activated, FOXO activates the transcription of thor, resulting in translational inhibition. However, activated insulin signaling can triply phosphorylate FOXO, allowing 14-3-3 proteins to inactivate FOXO by sequestering it outside of the nucleus. This results in decreased thor transcription and allows translation to proceed (Junger et al., 2003). Thus, when activated insulin signaling inhibits FOXO, thor transcription is decreased; this decrease results in increased translation, because Thor levels are insufficient to inhibit translation.

We found that in control larvae (flies expressing a microtubule associated form of GFP, tau-GFP, in cholinergic neurons or flies bearing each UAS-transgene crossed to mothers lacking Gal4 (not shown)), thor levels remained consistent from 96 to 168 hours after egg laying (AEL) (Figure 4.5). Similarly, larvae that express Ras\textsuperscript{N17} or Raf\textsuperscript{K497M} in cholinergic neurons exhibit consistent thor transcript levels (Figure 4.5). However, larvae that express Ras\textsuperscript{V12} or Raf\textsuperscript{F20} in cholinergic neurons exhibit increased amounts of
thor transcripts (Figure 4.5). We suggest that this thor transcript burst is the result of decreased insulin signaling throughout the larva, which decreases the rate of growth and creates small flies.

![Graph](image)

**Figure 4.5** Altered Ras-Raf signaling under the control of ChaGal4 affects thor transcription. Fold activation ± error of the fold was determined from a total of 6 reactions. Two samples were collected from each genotype and triplicate measures of each sample were conducted using the relative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Y-axis: thor transcript levels normalized to RPII-140 (arbitrary units). X-axis: hours AEL at which larvae were collected. Magdalena Wałękiewicz contributed to this figure by isolating RNA from larvae.

4.7 Model for Ras-Raf and ecdysone signaling in cholinergic neurons

We find that inhibiting Ras-Raf and ecdysone signaling in cholinergic neurons of Drosophila larvae increases the rate of larval growth leading to the delayed appearance of large flies. In contrast activating Ras-Raf signaling in cholinergic neurons decreases the rate of growth and increases thor transcript levels leading to the delayed appearance of small flies. These results suggest that Ras-Raf and ecdysone signaling in cholinergic
neurons regulate the rate of growth and final body size via insulin signaling throughout the larval body. These effects seem to be independent of the alterations to the timing of ecdysone synthesis and the duration of growth seen with activation of Ras and PI3K in the PG because of three factors. First, ChaGal4 is not expressed in PG cells or neurons that may innervate the PG. Second, expression of activated Ras in PG-LP neurons has no effect on fly size. Third, the regulation of Ras-Raf or ecdysone signaling in cholinergic neurons has no effect on PG cell size. Thus, we conclude that Ras-Raf signaling in cholinergic neurons alters both the rate of growth and final body size by regulating insulin activity throughout the larval body (Figure 4.6). Additionally, we suggest that ecdysone signaling in cholinergic neurons affects the rate of growth and final body size by regulating systemic insulin signaling (Figure 4.6). However, to more conclusively link the antagonistic actions of ecdysone upon insulin signaling, we must test thor transcript levels in flies that express EcR<sup>B1</sup>-I<sup>C655-W650A</sup> in cholinergic neurons.
Figure 4.6 Model for Ras-Raf and ecdysone signaling in cholinergic neurons. In this view, ecdysone signaling in cholinergic neurons could lead to the activation of Ras-Raf signaling, resulting in decreased insulin levels, rate of growth, and fly size. Thus, a high ecdysone titer at pupariation could halt growth, set final body size, and end the duration of growth all by decreasing insulin levels. Ecdysone signaling is activated by ecdysone binding to the EcR. Insulin levels are presumably affected by regulation of insulin producing cells. Solid lines indicate direct events, whereas, dashed lines indicate that multiple steps are involved.
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LIST OF COMMON ABBREVIATIONS

4E-binding protein (4E-BP or Thor)

After Egg Laying (AEL)

Amnesiac (Amn)

Choline Acetyltransferase (Cha)

Cyclic Adenosine Monophosphate (cAMP)

Disembodied (Dib)

Dominant-negative (dn)

*Drosophila* Insulin-like Peptide (Dilp)

Ecdysone Receptor (EcR)

Ecdysone Receptor Element (EcRE)

Epidermal Growth Factor Receptor (EGFR)

Growth Hormone (GH)

Growth Hormone Releasing Hormone (GHRH)

Insulin-like Growth Factor 1 (IGF1)

Insulin Producing Cell (IPC)

Insulin Receptor (InR)

Juvenile Hormone (JH)

Mitogen-activated Protein Kinase (MAPK)

Neurofibromin (NF1)

p70 S6-kinase (S6K)

Phantom (Phm)

Phosphatidylinositol 3-kinase (Pi3K)
Phosphatidylinositol 3,4,5-trisphosphate (PIP₃)
Phosphatidylinositol 4,5-bisphosphate (PIP₂)
Pituitary Adenylate Cyclase Activating Peptide (PACAP)
Protein Kinase A (PKA)
Prothoracic Gland (PG)
Prothoracic Gland Lateral Projection (PG-LP)
Prothoracicotropic Hormone (PTTH)
Quantitative RT-PCR (Q-PCR)
Ras GTPase-activating Proteins (Ras-GAPs)
Ring Gland (RG)
Somatostatin (SRIF)
Son of Sevenless (Sos)
Src Homology 2 (SH2)
Target of Rapamycin (TOR)
Tuberous Sclerosis Complex (TSC)
Ultraspiracle (Usp)
Upstream Activating Sequence (UAS)
LIST OF COMMON TRANSGENES

UAS-Ras\textsuperscript{+} - expresses wild-type Ras under UAS control

UAS-Ras\textsuperscript{V12} (II) - expresses constitutively active Ras under UAS control

UAS-Ras\textsuperscript{V12} (III) - expresses constitutively active Ras under UAS control

UAS-Ras\textsuperscript{N17} - expresses dominant-negative Ras under UAS control

UAS-Raf\textsuperscript{F20} - expresses constitutively active Raf under UAS control

UAS-Raf\textsuperscript{K497M} - expresses dominant-negative Raf under UAS control

Pi3K-CAAX - expresses constitutively active Pi3K under UAS control

UAS-Pi3K\textsuperscript{D954A} - expresses dominant-negative Pi3K under UAS control

UAS-Akt\textsuperscript{+} - expresses wild-type Akt under UAS control

UAS-TSC2\textsuperscript{+} - expresses wild-type TSC2 under UAS control

UAS-TSC2\textsuperscript{S924A} - expresses constitutively active TSC2 under UAS control

UAS-4E-BP\textsuperscript{+} - expresses wild-type 4E-BP under UAS control

UAS-S6K\textsuperscript{ACT} - expresses constitutively active S6K under UAS control

UAS-FOXO\textsuperscript{TM} - expresses constitutively active FOXO under UAS control

UAS-EcR\textsuperscript{B1-ΔC655-W650A} - expresses dominant-negative EcR under UAS control