ACTIVIN SIGNALING PROMOTES THE COMPETENCE OF THE PROTHORACIC GLAND DURING *DROSOPHILA* METAMORPHOSIS

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

YING YE GIBBENS

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

MICHAEL B. O'CONNOR, ADVISOR

AUGUST 2010

Acknowledgments

I would like to give my sincere gratitude to my mentor Dr. Mike O'Connor, a very insightful, liberal and generous scientist who is always passionate about science and courteous to people. I would like to thank everyone in the O'Connor lab for making a very helpful and friendly environment for my Ph.D training. Finally, without the great support from my loving family, I wouldn't have made it this far.

Table of Contents

List of Tables

List of Figures

Chapter 1

Chapter 2

Chapter 3

Abbreviations

Full name	abbreviations
ecdysone	**E**
20-hydroxyecdysone	**20E**
Prothoracic gland	**PG**
Prothoracicotropic hormone	**PTTH**
neverland	***nvd***
phantom	***phm***
disembodied	***dib***
shadow	***sad***
spook	***spo***
spookier	***spok***
shroud	***sro***
baboon	***babo***
dawdle	***daw***
Activinβ	***Actβ***
myoglianin	***myo***

Chapter 1: Introduction

How Developmental Timing is Controlled:

Lessons from Insects

Many metazoans undergo the juvenile-to-adult transition when specific signals alter gene expression to orchestrate the development of adult characteristics and the attainment of sexual maturity. In humans, for instance, puberty is a process during which an adolescent develops primary and secondary sexual traits and completes reproductive maturation. Frogs, toads, and newts also go through a series of extensive morphological changes including resorption of the tail and formation of limbs before becoming a sexually mature adult. Perhaps the most remarkable example of such developmental transitions can be found in the holometabolous insects whose larval forms undergo dramatic morphological transformations in a process known as metamorphosis to become an adult. Although these developmental transitions have been well characterized and described in detail, how the timing of these events is executed remains largely unknown. Considering that the onset of human puberty has advanced significantly in the last few decades (Kaplowitz and Oberfield, 1999) and that premature puberty has been linked to social and behavioral problems (Sonis et al., 1985), it is of particular importance to fully comprehend mechanisms of developmental timing in order to understand and prevent the causes of premature puberty.

Although juvenile-to-adult developmental transitions are seemingly quite different in various species, one common feature that they all share is involvement of hormonal regulation. In humans, the timing of the pubertal transition has been associated with the

release of kisspeptin, a neuropeptide encoded by the Kiss1 gene (for a review, see Oakley et al., 2009). Kisspeptin-producing neurons in the brain directly stimulate gonadotropin-releasing hormone (GnRH)-producing neurons and thus induce the release of GnRH. When transported to the anterior pituitary gland, GnRH binds to its receptor, a G-protein coupled receptor that induces the expression and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Ebling and Cronin, 2000). LH and FSH stimulate the gonads to synthesize sex steroid hormones by activating the cAMP pathway (LeMaire, 1989). Ultimately, the sex steroid hormones promote sexual maturation and reproductive function during puberty. This basic scheme of hormonal cascades in regulating the timing of the developmental transition appears to be conserved from humans to insects. In insects, metamorphosis is also initiated by the release of a brain-derived neuropeptide called prothoracicotropic hormone (PTTH) (Kataoka et al., 1991). PTTH is able to trigger acute synthesis of the insect steroid hormone ecdysone (E) in the prothoracic gland (PG), the primary source of E during the larval stage. Because E serves as the immediate precursor for the active steroid hormone 20-hydroxyecdysone (20E) which is able to trigger metamorphosis, the synthesis and release of E from the PG is essential for a successful initiation of the larval-to-pupal transition (for reviews, see Gilbert et al., 2002; Lafont et al., 2005).

Because hormonal control of developmental timing decisions is widely conserved, insects provide an excellent system to study these processes due to their short life cycle and minimal space and cost requirement. The knowledge of the temporal regulation of the juvenile-to-adult transition gained from insect models will provide relevant insights into the timing mechanisms that control puberty in humans.

1. Insect models for developmental timing studies

The classic insect models for studying how metamorphosis timing is controlled are the tobacco hornworm *Manduca sexta* and the silkworm *Bombyx mori*, both of which are in the Lepidoptera order of insects. Because of their large size, *Manduca* and *Bombyx* have aided biochemical characterization of the players involved in developmental timing regulation. However, the lack of genetics in these two insect models has limited their use. Fortunately, the fruit fly *Drosophila melanogaster* in the order of Diptera has provided a great genetic system, given its classic genetics and the completed genome sequence. A number of genes critical for the hormonal regulation of metamorphosis have been identified and cloned in *Drosophila*. Added to this, the availability of mutants and the large number of genetic tools available to *Drosophila* researchers have enabled the functions of many of these genes to be characterized. More importantly, there is remarkable gene conservation between flies and humans: roughly 70% percent of *Drosophila* genes have human homologues and 50% of the *Drosophila* proteins have matching mammalian counterparts (Gilbert, 2004). In many cases, the *Drosophila* genes perform similar functions or are involved in similar physiological processes to their human counterparts, strongly suggesting that the findings in *Drosophila* will be relevant to humans. Therefore, my thesis project utilizes *Drosophila* as a model organism to investigate the mechanisms that control developmental transitions. Using *Drosophila* to study the hormonal signals in developmental transitions is more advantageous than other model organisms such as *Xenopus* and mice due to its short life cycle, well-defined genetics and low gene redundancy.

2. Insect ecdysteroids are essential for metamorphosis

Ecdysteroid hormones play critical roles in insect development and physiology. Among them, the principal hormone responsible for molting and metamorphosis is 20-hydroxyecdysone (20E). Although α-ecdysone (E) mainly serves as an immediate precursor for 20E and is therefore considered an inactive ecdysteroid, E may have its own functions in regulating gene expression during metamorphic events (Beckstead et al., 2007). In *Drosophila melanogaster*, E is generated from the prothoracic gland (PG), a larval endocrine organ that is composed of a single steroidogenic cell type. The PG of the fruit fly as well as other higher flies is part of a composite endocrine gland called the ring gland that also contains the corpus allatum and corpus cadiacum (Dai and Gilbert, 1991). Once E is released into the hemolymph, the insect equivalent of blood, it is rapidly converted to 20E in the target tissues. The synthesis of 20E in Lepidoptera as represented by the tobacco hornworm *Manduca sexta* and the silkworm *Bombyx mori* differs from *Drosophila* in that 3-dehydroecdysone (3dE) is the major ecdysteroid secreted from the PG. 3dE is immediately converted to E in the hemolymph and then to 20E at the target tissues (Gilbert et al., 2002).

In *Drosophila*, the acute synthesis of 20E at the end of the third instar stage elicits a cascade of gene activation events through the action of the ecdysone receptor (EcR) and its heterodimeric co-receptor Ultraspiracle (USP) (Yao et al., 1993). EcR and USP belong to the nuclear hormone receptor family which is characterized by a DNA-binding domain and a carboxyl terminal ligand-binding domain (Evans, 1988). EcR shows sequence homology to the vertebrate thyroid receptor (TR), vitamin D receptor (VDR) and retinoic acid receptor (RAR) whereas USP is homologous to the vertebrate retinoid X receptor (RXR) which functions as a co-receptor for TR, VDR and RAR

(Cherbas and Cherbas, 1996). The conservation of the hormone receptors makes the study of hormonal responses in insects highly relevant to those in mammals. In the absence of the ligand, the EcR/USP heterodimer complexed with transcription co-repressors binds to the hormone response elements (EcREs) in the promoter regions of the 20E responsive genes and silences their expression. Once bound by the ligand 20E, the EcR/USP complex dissociates from the co-repressors and recruits co-activators, leading to derepression of the expression of these 20E responsive genes (for a review, see Nakagawa and Henrich, 2009). The 20E responsive genes were originally identified by studying the puffs on the polytene chromosomes, particularly in *Drosophila* salivary gland cells (Russell and Asuburner; 1996). The unique temporal pattern of puff formation and regression that reflects the sequential activation of gene transcription during the larval-to-pupal transition revealed a hierarchy of gene activation events provoked by 20E and its receptor complex (Russell and Asuburner; 1996; Thummel, 1996). The 20E inducible genes can be grouped into two categories, the early genes and the late genes. The transcription of early genes is under direct control of the 20E/EcR/USP complex because cycloheximde, a protein synthesis inhibitor, does not block their transcription (Ashburner, 1974). Several genes located in the early puffs including *Broad-Complex* (*BR-C*), E74 and E75 have been cloned and their functions have been extensively studied. These early genes invariably contain DNA-binding motifs and function as transcription factors that directly participate in the activation of the late genes (Farner and Lederis, 1980; Karim et al., 1990; Karim et al., 1993; Segraves and Hogness, 1990). Intriguingly, compared to the widely-expressed pattern of the early genes, the expression of many late 20E inducible genes is usually limited to one tissue. For instance, the late genes contained in the 71E puff are only detected in the salivary gland. Also, the late genes are turned on and off in a coordinated manner. In the case of the late genes in the

71E puff, the timing of the appearance and disappearance of their transcripts is almost identical (Andres et al., 1993). The restricted expression pattern as well as the temporal coordination of the late genes is thought to give rise to tissue- and stage-specific responses to the hormone 20E during metamorphosis (Thummel, 1996).

3. 20E biosynthetic pathway

Although much is known about the downstream events initiated by 20E, until recently comparatively little was known concerning more upstream events, particularly how acute 20E synthesis at the onset of metamorphosis is achieved. In order to understand this question, it is crucial to elucidate the biochemical nature of 20E biosynthesis and to identify the enzymes that are involved in this process.

3.1. Biochemical nature of 20E synthesis

Much of the 20E biosynthetic pathway has been uncovered through biochemical isolation and identification of ecdysteroid biosynthetic intermediates. Like the steroidogenic organs in vertebrates, the insect PG utilizes cholesterol as the starting material for steroid synthesis. However, in contrast to vertebrates which can synthesize cholesterol *de novo* from simple organic molecules (Clayton, 1964; Svoboda and Thompson, 1985), insects must either uptake cholesterol directly from diet in the case of carnivorous insects or modify ingested plant sterols into cholesterol in the case of herbivorous insects (Lafont et al., 1995). Inside the PG, the first step in 20E synthesis is the conversion of cholesterol to 7 dehydrocholesterol (7dC) by a 7,8-dehydrogenase

Figure 1-1:

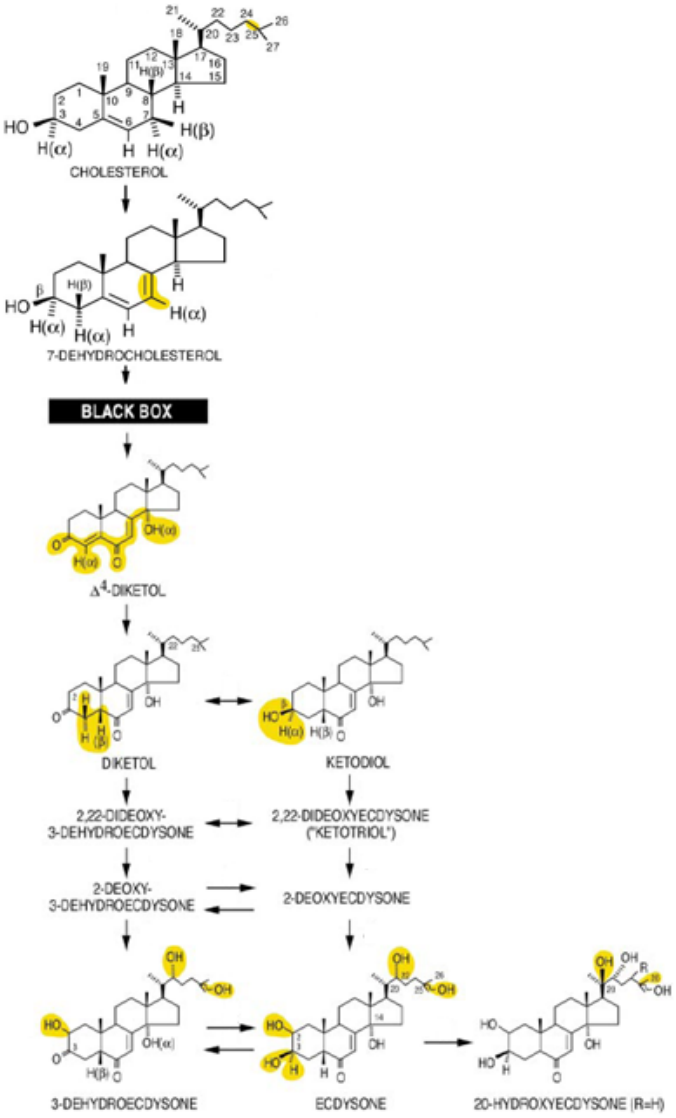

CHOLESTEROL
7-DEHYDROCHOLESTEROL
BLACK BOX
Δ4-DIKETOL
DIKETOL
KETODIOL
2,22-DIDEOXY-
3-DEHYDROECDYSONE
2,22-DIDEOXYECDYSONE
("KETOTRIOL")
2-DEOXY-
3-DEHYDROECDYSONE
2-DEOXYECDYSONE
3-DEHYDROECDYSONE
ECDYSONE
20-HYDROXYECDYSONE (R=H)
Manduca
Drosophila

Figure 1-1. The 20E biosthetic pathway.

The 20E pathway intermediates are shown in this figure. 20E synthesis starts from the conversion of cholesterol to 7dC. The step between 7dC and the next identifiable intermediate is known as the Black Box Step. In *Manduca*, the PG generates 3dE which is first converted to E and then to 20E, whereas in *Drosophila*, E is the product from the PG. Yellow highlights the molecular modifications that occurs after the reactions. (Adapted from Gilbert L.I., Rybczynski R., and Warren J. T., 2002 Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol. 47*, 883-916).

(Lafont et al., 1995, Figure 1-1). Subsequently, depending on the species, the next identifiable reaction intermediate is 5β-ketodiol (*Drosophila*) or 5β-diketol (*Manduca*). The modification from 7dC to 5β-ketodiol or 5β-diketol appears to require more than one reaction, yet no reaction intermediates have been detected; therefore, this step is referred to as the "Black Box" for a lack of understanding of the reaction mechanism (Figure 1-1). It is proposed that either the reaction intermediates are too unstable or the reactions are highly efficient (Lafont et al., 1995). The steps after the "black box" that modify 5β-ketodiol or 5β-diketol to E or 3dE respectively have been well studied. They basically are a series of hydroxylation reactions catalyzed sequentially by 25-hydroxylase, 22-hydroxylase and 2-hydroxylase (Figure 1-1). Finally, E, which is either directly released from PG in species like *Drosophila* or modified from 3dE in the case of *Manduca*, is converted immediately to the active hormone 20E at target tissues that express 20-hydroxylase (Figure 1-1).

3.2. *Halloween* genes encode 20E biosynthetic enzymes

3.2.1. Identification of 20E biosynthetic enzymes

The identification and characterization of the 20E biosynthetic enzymes would be instrumental in gaining a more comprehensive and thorough understanding of the 20E biosynthetic pathway. Based on their biochemical characteristics, it was found that the hydroxylases involved in 20E biosynthesis are cytochrome P450 proteins (CYPs) (Grieneisen, 1994), however, cloning those CYP genes for further molecular studies in *Manduca* was difficult due to the lack of the classical genetics and the unavailability of the genome sequence. Fortunately, the advancement in *Drosophila* molecular genetics

has aided researchers in identifying and cloning important players in 20E biosynthesis. Since cuticle synthesis requires ecdysteroids, one predicted phenotype of loss-of-function mutations in the enzymes involved in 20E biosynthesis is a failure to produce a cuticle. The earliest manifestation of this phenotype occurs at the second half of the embryonic stage when cuticle synthesis takes place. Based on this prediction, a gene known as *disembodied* (*dib*) whose loss-of-function mutations result in abnormal cuticle formation and low ecdysteroid titers was cloned (Chavez et al., 2000). Sequence analysis showed that *dib* indeed codes for a CYP (Chavez et al., 2000). In fact, *dib* belongs to a family of genes known as the *Halloween* genes which were recognized among a group of embryonic lethal mutations first identified in the pioneering screens conducted by Nüsslein-Volhard, Weischaus and their colleagues for lethal mutations that affected cuticular pattern formation (Jürgens et al., 1984; Wieschaus et al., 1984; Nüsslein-Volhard et al., 1984). The *Halloween* mutants are characterized by their defects in cuticle differentiation during the embryonic stage. The finding that *dib* encodes a CYP that directly participates in 20E synthesis led to the subsequent characterization of other *Halloween*-family genes including *phantom* (*phm*), *spook* (*spo*), *shadow* (*sad*) and *shade* (*shd*). All these genes have been shown to code for CYP enzymes and biochemical analyses have demonstrated that Phm, Dib, Sad and Shd function as the C25-, C22-, C2- and C20-hydroxylases, sequentially catalyzing the last four reactions of the E biosynthetic pathway (Chavez et al., 2000; Ono et al., 2006; Petryk et al., 2003; Warren et al., 2002; Warren et al., 2004) (Figure 1-2). Although the precise enzymatic function of Spo remains unassigned, it has been implicated in the “Black Box” reactions based on the observation that *spo* mutants can be rescued by ecdysteroid intermediates upstream of the Black Box step (Ono et al., 2006). Intriguingly, *Drosophila* contains a *spo* paralog named *spookier* (*spok*) which is functionally equivalent to Spo but which functions during

Figure 1-2:

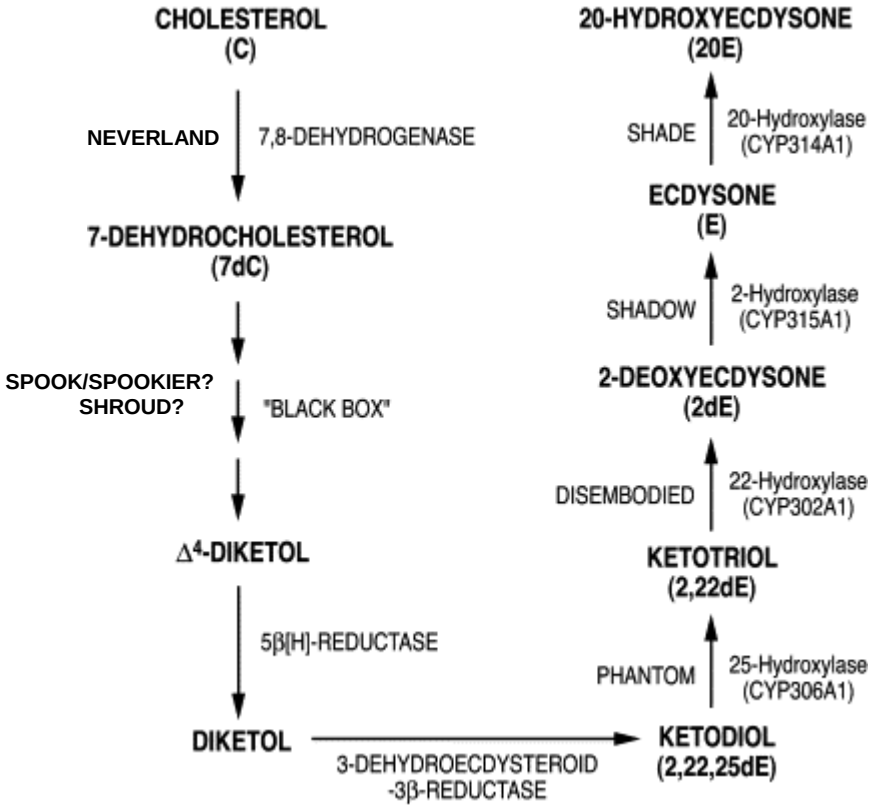

CHOLESTEROL
(C)
NEVERLAND
7,8-DEHYDROGENASE
7-DEHYDROCHOLESTEROL
(7dC)
SPOOK/SPOOKIER?
SHROUD?
"BLACK BOX"
Δ4-DIKETOL
5β[H]-REDUCTASE
DIKETOL
3-DEHYDROECDYSTEROID
-3β-REDUCTASE
KETODIOL
(2,22,25dE)
PHANTOM
25-Hydroxylase
(CYP306A1)
KETOTRIOL
(2,22dE)
DISEMBODIED
22-Hydroxylase
(CYP302A1)
2-DEOXYECDYSONE
(2dE)
SHADOW
2-Hydroxylase
(CYP315A1)
ECDYSONE
(E)
SHADE
20-Hydroxylase
(CYP314A1)
20-HYDROXYECDYSONE
(20E)

Figure 1-2. *Halloween* genes encode cytochrome P450 proteins that direct 20E biosynthesis.

The synthesis of 20E is catalyzed by a group of cytochrome P450 enzymes that are encoded by the *Halloween* genes including *shroud*, *spook/spookier*, *phantom, disembodied, shadow and shade*. Phantom, Disembodied, Shadow and Shade function as hydroxylases that catalyze the last four reactions in the pathway. Spook/Spookier, although their specific enzymatic action is still unknown, have been implicated in the black box step. Also involved in the black box step is Shroud, a side chain dehydrogenase, which may function in conjugation with Spook/Spookier. The 7,8-dehydrogenase has been identified as Neverland, a Reiske-domain containing protein. (Modified from Gilbert L.I., Warren J.T., **2005** A molecular genetic approach to the biosynthesis of the insect steroid molting hormone. *Vitam. Horm.73*, 31-57).

larval stages whereas Spo is required during embryogenesis (Ono et al., 2006). Quite recently, another *Halloween* gene *shroud* (*sro*), which encodes a short-chain dehydrogenase, has been identified and demonstrated to function at the Black Box step, possibly by working with Spo/Spok to convert 7dC to the 3β,5β [H] ketodiol (Niwa et al., 2010). In addition to the *Halloween* genes, the 7,8-dehydrogenase, the first enzyme in the pathway, has been proposed to be a highly conserved Rieske domain- and non-heme iron binding domain-containing oxygenase known as Neverland (Nvd) (Yoshiyama et al., 2006). Nonetheless, whether Nvd is indeed the 7,8-dehydrogenase awaits additional biochemical characterization that explicitly demonstrates its enzymatic activity.

3.2.2. Temporal and spatial expression pattern of 20E biosynthetic enzymes

The stage- and tissue-specific expression patterns of the *Halloween* genes *phm*, *dib*, *sad*, *shd* and *spo/spok* are consistent with their roles in ecdysteroid synthesis. In early embryos, *phm*, *dib*, *sad* and *shd* are expressed in embryonic epidermis which may be a source for 20E before the formation of the PG (Petryk et al., 2003; Warren et al., 2002; Warren et al., 2004), while *spo* is detected in amnioserosa (Ono et al., 2006), a sheet of extra-embryonic tissue which has also been proposed to be a primary endocrine organ responsible for 20E generation at the embryonic stage (Palanker et al., 2006). During the larval stage, *phm*, *dib* and *sad* transcripts are predominantly found in the PG, whereas *shd*, which is required for the final step of the 20E biosynthesis that converts E to 20E, is detected in the peripheral tissues such as the midgut, Malphighian tubules, epidermis and fat bodies (Petryk et al., 2003; Warren et al., 2002; Warren et al., 2004). *Spo* and *spok* also show specific temporal expression and are involved in the

synthesis of 20E at different developmental stages. While the *spo* transcript is present during early embryogenesis and at the adult stage, *spok* is turned on in the PG during the larval stage and is responsible for synthesizing 20E required for the larval molts and metamorphosis (Ono et al., 2006). Likewise, *sro* is found predominantly in the PG during the larval stage (Niwa et al., 2010). Nvd also exhibits prominent expression in the PG during larval growth (Yoshiyama et al., 2006). During the adult stage, expression of the *Halloween* genes and *nvd* is confined to the nurse cells or follicle cells of the ovary, the main steroidogenic organ after the degeneration of the PG during pupal development.

CYP enzymes are membrane-bound proteins located in either mitochondria or the ER. The specificity of the intracellular localization is determined by a signal sequence located in the N-terminus of each protein (Omura and Ito, 1991; Van den Broek et al., 1996). The Dib, Sad and Shd proteins contain a conserved N-terminal amphipathic mitochondria-importing sequence and they are indeed found to co-localize with mitochondria markers (Petryk et al., 2003; Warren et al., 2002). Phm and Spo, which have an ER signal sequence, a stretch of hydrophobic amino acids at the N terminus, are also detected in ER (Ono et al., 2006; Warren et al., 2004).

3.2.3. Other factors directly involved in 20E synthesis

The specific intracellular compartmentalization of these enzymes requires trafficking machinery that can transport the precursor cholesterol as well as the ecdysteroid intermediates to the designated compartments where the enzymes reside. One potential cholesterol transferase encoded by the gene *start1* was identified in *Drosophila* (Roth et al., 2004) based on sequence homology to the vertebrate metastatic

lymph node 64 (MLN64) protein, which is a vertebrate cholesterol transporter that mobilizes cholesterol from the outer mitochondrial membrane to the inner one (Moog-Lutz et al., 1997; Stocco and Clark, 1996). Still, it remains a question whether Start1 possesses transferase activity due to the lack of functional studies. Additionally, the *Drosophila* homologs of Niemann-Pick type C 1 and 2 (NPC1 and NPC2), which are cholesterol-binding proteins mutated in human disease, are implicated in the proper transportation of cholesterol to its destined organelles. In humans, mutations in either *NPC1* or *NPC2* cause abnormal accumulation of cholesterol, which leads to severe neurodegeneration (Carstea et al., 1997; Naureckiene et al., 2000). Similarly, mutations in *Drosophila* NPC-like genes (*dnpc1a* and *dnpc2a/dnpc2b*) result in low ecdysteroid titers which can be rescued by high levels of dietary cholesterol, indicating that these genes likely regulate transportation of cholesterol in PG cells (Huang et al., 2005; Huang et al., 2007). However, the exact molecular actions of DNPC proteins in transporting cholesterol have yet to be investigated.

4. Regulation of acute 20E biosynthesis

Because the 20E titer at the end of the third instar stage triggers the onset of metamorphosis, understanding how acute 20E synthesis is regulated is vital to unraveling the mechanisms by which the developmental timing is set. Numerous efforts have been made in the past few decades to elucidate the factors that can influence ecdysteroidogenesis within the PG. There are two primary regulatory mechanisms that together fine tune the timing of the 20E titer - transcription factors and extracellular signaling molecules.

4.1. Transcription factors involved in regulating 20E biosynthesis

Several transcription factors have been implicated in 20E synthesis, including *molting defective* (*mld*), *βftz-f1*, *giant* and *without children* (*woc*) (Neubueser et al., 2005; Parvy et al., 2005; Schwartz et al., 1984; Wismar et al., 2000). *Mld* codes for a nuclear zinc finger protein and null mutations of this gene cause a failure to undergo the first larval molt (Neubueser et al., 2005). Intriguingly, in *mld* mutant larvae, the expression of *spok* , a larvally expressed gene encoding a key component of the black box step, that is important for 20E synthesis during the larval stage, is absent, which is likely responsible for the molting defects (Ono et al., 2006). However, it remains to be determined whether *mld* directly regulates *spok* transcription.

The gene *βftz-f1* is the *Drosophila* homolog of the vertebrate steroidogenic factor 1 (SF1) and is required for embryogenesis, larval molts and metamorphosis (Yamada et al., 2000). SF1 functions to promote the transcription of the genes directly or indirectly involved in steroidogenesis (Parker et al., 2002; Val et al., 2003). Mutant clones of *βftz-f1* in the PG show reduced levels of the Dib and Phm proteins (Parvy et al., 2005), suggesting that at least some of the defects observed in *βftz-f1* mutants are due to decreased expression of 20E biosynthetic enzymes.

Woc is a zinc-finger transcription factor (Wismar et al., 2000), which is thought to control the expression of 7,8-dehydrogenase, as inferred from the biochemical finding that the conversion from cholesterol to 7dC is blocked in *woc* mutant PG (Warren et al., 2001). With the recent identification of a putative 7,8-dehydrogenase Nvd, it would be interesting to test if the expression of *nvd* is indeed modulated by Woc.

Giant encodes a b-ZIP transcription factor that is featured by a basic domain and

a leucine-zipper domain for DNA binding (Capovilla et al., 1992). *Giant* is a gap gene that is involved in early embryonic patterning (Eldon and Pirrotta, 1991). Mutations in *giant* cause ecdysteroid deficiency that gives rise to delayed metamorphosis and a giant larva phenotype (Schwartz et al., 1984). Recently, it was found that *giant* may directly regulate E production by determining the fate of PTTH neurons (Ghosh et al., 2010). However, the mechanism responsible for *giant*'s function in specifying PTTH neuron fate remains to be investigated.

4.2. Extracellular signaling molecules involved in regulating 20E biosynthesis

4.2.1. Prothoracicotropic hormone (PTTH)

PTTH, a brain-derived small peptide that plays a central role in insect metamorphosis, has been proposed to be the primary factor that promotes 20E synthesis from the PG. Understanding how PTTH modulates 20E synthesis is critical to deciphering timing mechanisms.

PTTH identification

Nine decades ago, Kopec used classic ligation experiments on gypsy moth larva to nicely demonstrate the involvement of the brain in insect metamorphosis (Kopec, 1922). His work not only predicted the existence of a brain-derived diffusible factor required for metamorphosis but also laid out the foundation for modern neuroendocrinology. Despite the early prediction, this factor, now known as prothoracicotropic hormone (PTTH), was not purified until twenty years ago when it was

obtained from the silkworm *Bombyx mori* (Kawakami 1990). The mature *Bombyx* PTTH is composed of two identical monomers, each of which is a proteolytic cleavage product from a precursor protein (Kawakami et al., 1990). The amino acid sequence of the monomer contains seven cysteine residues which contribute to one intermolecular disulfide bond and three intramolecular disulfide bonds (Ishibashi et al., 1994). After successful cloning from *Bombyx*, *ptth* was subsequently identified and isolated from a variety of other Lepidoptera species including *Manduca sexta* (Gilbert et al., 2002; Shionoya et al., 2003) and quite recently from *Drosophila melanogaster* (McBrayer et al., 2007). Interestingly, while the relative positions of the seven cysteine residues are invariably conserved, the amino acid sequences of the PTTH molecules from different insect species do not show significant similarities (McBrayer et al., 2007; Shionoya et al., 2003). Despite the low conservation of the amino acid sequences of PTTHs, it has been demonstrated that *Bombyx* PTTH at high concentrations is able to induce ecdysteroidogenesis in the PG of *Rhodnius prolixus*, a bloodsucking bug from the Hemiptera order (Steel and Vafopoulou, 1997). These observations suggest that PTTHs likely have evolved from a common ancestor protein and may employ similar downstream mechanisms to trigger ecdysteroidogenesis.

PTTH synthesis and release

In Lepidoptera such as *Bombyx* and *Manduca*, PTTH is synthesized by a pair of neurosecretory cells in each brain hemisphere (Mizoguchi et al., 1990; O'Brien et al., 1988). These four neurons terminate their axons onto a neurohemal organ named corpus allatum (CA). Therefore PTTH produced from these neurons is first stored in the

CA. It is then released into the hemolymph in response to unknown signals and delivered systematically through the hemolymph to the PG. In *Drosophila*, the PTTH producing neurons extend their axons from the brain and terminate directly onto the PG portion of the ring gland so that PTTH is targeted to the PG without being circulated in the hemolymph (McBrayer et al., 2007). By using Northern blot to measure the transcriptional profile of *ptth*, McBrayer et al. (2007) found that the synthesis of *ptth* mRNA in third instar *Drosophila* larvae exhibits a cyclic pattern (McBrayer et al., 2007). Interestingly, this cyclic pattern of *ptth* transcription approximately coincides with the temporal profile of the so-called “commitment peaks”, which are small E peaks occurring at the early and mid third instar stage before the appearance of the large E titer at start of metamorphosis (Warren et al., 2006). Commitment peaks are believed to ensure that the larvae undergo a pupal molt (metamorphosis) instead of a larval molt (Riddiford 1995). Additionally, a dramatic up-regulation of the *ptth* transcript proceeds the formation of the large ecdysteroid titer prior to metamorphosis (McBrayer et al., 2007). Similar findings regarding the pattern as well as the function of the discrete PTTH titers during the last larval instar stage have also been reported from *Lepidoptera* studies, strongly suggesting that PTTH release likely triggers the production of E from PG (Bollenbacher and Gilbert, 1980; Dai et al., 1995).

While the mechanisms controlling the periodic synthesis of PTTH remain elusive, the release of PTTH has been linked to two factors: critical weight and the photoperiod. Critical weight is a point beyond which starvation can no longer affect the timing of metamorphosis (Mirth and Riddiford, 2007). In *Manduca*, the attainment of critical weight is thought to trigger a rapid down-regulation of juvenile hormone (JH) by enhancing the activity of juvenile hormone esterase (JHE) (Mirth and Riddiford, 2007; Nijhout, 2003).

The function of JH in most insects is to antagonize metamorphosis by inhibiting PTTH release (Rountree and Bollenbacher, 1986). The administration of JH delays the onset of metamorphosis while removal of the corpus allatum (CA), the gland responsible for JH synthesis, causes early pupation (Nijhout and Williams, 1974). The clearance of the JH by JHE alleviates the inhibitory effect on PTTH secretion, which subsequently induces the synthesis of E to initiate metamorphosis. In *Drosophila*, however, genetic ablation of the CA does not shorten the developmental time required to reach metamorphosis (Riddiford et al., 2010). Therefore, the mechanisms of how achieving critical weight leads to PTTH release in *Drosophila* remain elusive. The second factor that impacts PTTH release is photoperiod. Once the inhibition by JH in removed, PTTH secretion is allowed to occur during a brief window of time known as the 'photoperiodic gate' (Truman, 1972; Truman and Riddiford, 1974). This photoperiodic gate only opens during specific times of the day. If the gate (Truman and Riddiford, 1974) is closed, PTTH release is postponed until the next opening of the gate.

The PTTH signal transduction pathway

Although it has been well stablished that PTTH stimulates ecdysteroid synthesis in the PG, the detailed molecular mechanisms of this event remain largely elusive. Work in *Manduca* has revealed the involvement of second messenger cAMP and Ca^{2+} in the early steps of the PTTH signaling pathway. A rapid production of cAMP in the PG was observed after PTTH stimulation (Smith et al., 1984). The increase in the cAMP level is dependent on the elevation of the intracellular Ca^{2+} concentration from both the external and internal Ca^{2+} sources (Smith and Gilbert, 1989) because deprivation of Ca^{2+} from

the PG causes failure to generate cAMP upon PTTH stimulation (Smith et al., 1985). This finding indicates the existence of a Ca^{2+} activated adenylate cyclase, which is indeed expressed in *Manduca* PG (Meller et al., 1988). Downstream of the adenylate cyclase are probably cAMP-dependent protein kinases (PKAs) based on the observations that a PKA-inhibiting cAMP analog can block ecdysteroid synthesis in the PG (Smith et al., 1986). However no direct evidence for an absolute requirement of PKAs in ecdysteroidogenesis thus far has been established. Other important players in the PTTH pathway are ERK and ribosomal protein S6 whose phosphorylation appears to be critical for PTTH-induced ecdysteroidogenesis (Rybczynski et al., 2001; Rybczynski and Gilbert, 2003; Song and Gilbert, 1994; Song and Gilbert, 1997).

The involvement of cAMP in PTTH-stimulated ecdysteroid synthesis suggested that a G-protein coupled receptor (GPCR) pathway was likely activated by PTTH. On the other hand, the necessity of ERK phosphorylation/activation for the steroidogenic effect of PTTH indicated a MAP kinase pathway downstream of PTTH. The identification of the PTTH receptor, therefore, was key to revealing the true nature of the PTTH pathway. Despite intensive searches, the identity of the PTTH receptor remained unknown for decades. Fortunately, by taking the advantage of molecular genetic tools in *Drosophila*, Rewitz et al. (2009b) were able to demonstrate that Torso, a receptor tyrosine kinase, is the PTTH receptor (Rewitz et al., 2009b). Knockdown of *torso* specifically in the PG results in a delay of metamorphosis, reminiscent of the *ptth* neuron ablation phenotype. They also found that effectors downstream of Torso are likely to be Ras, Raf and ERK, because knockdown of these factors in the PG gives rise to a similar developmental delay phenotype. Intriguingly, expression of a constitutively active Ras (Ras^{V12}) in the PG accelerates the onset of metamorphosis while a dominant-negative Raf (Raf^{K497M}) delays

entry to the pupa stage (Caldwell et al., 2005), lending further support to the idea that the PTTH pathway is mediated by Ras, Raf and ERK. Furthermore, *Bombyx* Torso is found to be predominantly expressed in the PG and is able to activate ERK phosphorylation *in vitro* in response to PTTH addition (Rewitz et al., 2009b), suggesting that the utilization of Torso as the PTTH receptor is conserved among insect species. Although it seems difficult to reconcile the involvement of cAMP in ecdysteroidogenesis if the PTTH signal is transmitted via a MAP kinase pathway, an intriguing finding in mammals that a cAMP-dependent guanine nucleotide exchange factor (GEF) can activate small G proteins (Kawasaki et al., 1998) suggests that the generation of cAMP upon PTTH stimulation may trigger the activation of a cAMP-dependent GEF which then activates Ras, a small G protein. Alternatively, it is possible that PTTH may activate both GPCR and MAP kinase pathways, through an unidentified GPCR.

PTTH loss-of-function phenotype

The steroidogenic effect of PTTH on the PG was exclusively based on *in vitro* studies using isolated PGs. Thus, whether PTTH was solely responsible for triggering metamorphosis *in vivo* remained largely elusive. This issue was recently investigated in *Drosophila* by genetically ablating the PTTH-neurons (McBrayer et al., 2007). Surprisingly, despite a prolonged larval development, PTTH-neuron ablated larvae were still capable of initiating metamorphosis. The prolonged larval stage was due to the low ecdysteroid titer resulting from reduced transcriptional expression of E biosynthetic enzymes. Strikingly, the 20E titer did eventually rise to trigger metamorphosis, although transcription of the biosynthetic enzymes remained low. This observation indicates that

PTTH determines the timing but is not the sole mechanism responsible for generating the rise in the ecdysteroid titer at metamorphosis. Therefore, there must be other mechanisms that can stimulate E synthesis in the PG of the PTTH neuron-ablated larvae. A potential compensation mechanism is insulin/insulin-like growth factor (IGF) signaling initiated by insulin-like peptides in insects that can influence the timing of development.

4.2.2. Insulin-like peptides: DILPs and Bombyxin

In many animal species including humans, nutritional status has been intimately linked to developmental timing (Wyshak and Frisch, 1982; Baker, 1985). One important mechanism that underlies the association between nutrition and development is the highly conserved insulin/IGF signaling pathway (for reviews, see Mirth and Riddiford, 2007; Nijhout, 2003). Insulin/IGF signaling is activated by the binding of an insulin family ligand to an insulin receptor (InR). This binding activates phosphatidylinositide 3-kinase (PI3K) which converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). This conversion is antagonized by a phosphatase known as PTEN which catalyzes the reverse reaction. The formation of PIP3 on the membrane recruits protein kinase B (PKB), also known as Akt, which is subsequently phosphorylated and activated. The active Akt then triggers a number of downstream metabolic events such as increased glucose uptake and storage by phosphorylating target proteins. Additionally, Akt phosphorylates the transcriptional repressor FOXO which, when phosphorylated, is sequestered in the cytosol, thereby alleviating the transcriptional repression on the genes involved in cell growth. Akt can also inactivate tumor sclerosis complex 1 and 2 (TSC1 and 2), the negative regulators of

TOR (target of rapamycin) signaling.

Drosophila has homologues of all the essential components of the insulin/IGF pathway, including seven insulin-like peptides (DILPs), a unique insulin receptor (InR), PI3K, Akt, and PTEN (Brogiolo et al., 2001; Goberdhan et al., 1999; Leevers et al., 1996; Verdu et al., 1999; Weinkove et al., 1999). The seven DILPs display specific temporal and spatial expression patterns. At the embryonic stage, the only detectable *dilps* are 2, 4 and 7 which are expressed in the presumptive mesoderm and midgut rudiment (Brogiolo et al., 2001). During the larval stage, strong expression of *dilps* 2, 3 and 5 is observed in a cluster of seven neurosecretory cells known as the insulin-producing cells (IPCs) located in the anteromedial region of each brain hemisphere (Brogiolo et al., 2001) and their expression is controlled by the availability of nutrients (Ikeya et al., 2002). In contrast, *dilp7* exhibits a segmented expression pattern in the ventral nerve cord (Brogiolo et al., 2001), while *dilp6* does not show prominent expression until late third-instar stage when its transcription in fat bodies is dramatically induced by the rise of the 20E titer (Okamoto et al., 2009; Slaidina et al., 2009). The expression levels of *dilp1* and *dilp3* peak at the pupa stage (Slaidina et al., 2009), however, the exact physiological functions of these DILPs at the pupa stage are unclear.

Several lines of evidence support a significant role of insulin/IGF signaling in regulating pubertal timing in mammals. Firstly, the levels of circulating IGF-1 in many mammalian species including humans show significant increases prior to pubertal onset (Copeland et al., 1982; Handelsman et al., 1987; Juul et al., 1994; Roberts et al., 1990), suggesting that IGF-1 may function to relay the information of somatic growth to developmental timing control mechanism. When added to the median eminence portion of the juvenile female rat brain *in vitro*, IGF-1 is able to trigger the release and/or

synthesis of LH releasing hormone (Hiney et al., 1996). Furthermore, injecting low levels of IGF-1 into the intraventricular space of immature and prepubertal female rats stimulated the release and/or synthesis of LH and advanced the initiation of puberty (Hiney et al., 1996). The stimulatory effect of IGF-1 on LH is likely an indirect result of enhanced expression of kisspeptins, the peptide products of the KiSS-1 gene, which are essential for LH release and pubertal onset (Hiney et al., 2009).

Likewise, insulin/IGF signaling plays a central role in developmental timing in *Drosophila*. Larvae with ablated IPCs exhibit a 7-day delay in the onset of metamorphosis (Rulifson et al., 2002). In addition, a similar developmental delay phenotype is observed in *InR* loss-of-function mutants (Brogiolo et al., 2001), strongly suggesting that insulin/IGF signaling can modify the duration of development. This notion is further enhanced by gain-of-function studies in which the effect of global up-regulation of insulin/IGF signaling was examined. It was found that blocking the PKA pathway in IPCs results in increased systemic insulin/IGF signaling, which, in turn, advances metamorphosis timing (Walkiewicz and Stern, 2009). Remarkably, manipulating the activity of insulin/IGF signaling in the PG is sufficient to cause the same influence on the timing of metamorphosis as observed with global up-regulation of the insulin/IGF signaling activity (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). This strongly suggests that PG is the tissue that relays the nutritional status to developmental timing. Interestingly, the mRNA levels of *phm* and *dib* are significantly reduced upon inactivating the insulin/IGF signaling pathway in the PG (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). These results indicate that insulin/IGF signaling in the PG affects the onset of metamorphosis by promoting the expression of 20E biosynthetic enzymes.

In line with the ecdysteroidogenic role of DILPs in *Drosophila*, the insulin-like peptide bombyxin, originally isolated from the heads of adult *Bombyx* (Nagasawa et al., 1984; Nagasawa et al., 1986), also induces E synthesis by the PG. Surprisingly, bombyxin can stimulate ecdysteroid synthesis by the PG of a related silkworm species *Samia cynthia ricini* but has no effect on the *Bombyx* PGs (Kiriishi et al., 1992). There are more than 30 *bombyxin*-related genes with certain sequence variations present in the *Bombyx* genome (Kondo et al., 1996; Tsuzuki et al., 1997; Yoshida et al., 1997; Yoshida et al., 1998), which possibly results from gene duplication events. So, it is likely that other bombyxin-related peptides may have stronger ecdysteroidogenic effect on *Bombyx*'s own PGs. A recent study that used bovine insulin explicitly showed that insulin can stimulate E synthesis by the PG of the silkworm *Bombyx* both *in vitro* and *in vivo* (Gu et al., 2009). In the same study, the researchers also found that blocking the activity of PI3K suppresses the ecdysteroidogenic effect of bovine insulin, suggesting that this effect is mediated by the insulin signaling pathway (Gu et al., 2009). Evidence supporting a role of insulin-like peptides in E synthesis can also be found in the bloodsucking bug *Rhodnius prolixus*. Direct administration of the anti-insulin antibodies into the *Rhodnius* larvae resulted in a failure to undergo molting (Sevala et al., 1992), indicating that insulin signaling is required for E synthesis.

Based on the observations that insulin signaling promotes E biosynthesis in multiple insect species, it appears that insulin signaling may be responsible for integrating the nutritional inputs to developmental programs that time the onset of metamorphosis.

4.2.3. TOR signaling pathway

In addition to insulin/IGF signaling, target of rapamycin (TOR) signaling also relays nutritional information about an organism's growth and development. TOR is a serine/threonine kinase and is conserved from single cell organisms like yeast to complex multicellular organisms such as mammals (for reviews, see Dann and Thomas, 2006; Wullschleger et al., 2006). By associating with different factors, TOR forms two types of protein complexes, TORC1 and TORC2 that regulate distinct cellular events. TORC1 plays a crucial role in sensing environmental signals such as nutrients particularly amino acids, stress and oxygen, and coordinating cellular processes in response to these signals. TORC2, on the other hand, is involved in regulating the organization of intracellular actin architecture. Under favorable growth conditions, TORC1 is activated and promotes protein translation by phosphorylating the initiation factor 4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6K). Upon phosphorylation, 4E-BP can no longer bind to eIF4E, which then becomes available to form a complex with eIF4G to initiate translation. Phosphorylated and thus activated S6K can phosphorylate ribosomal protein S6, which is believed to increase general translation of mRNAs that contain a 5' tract of oligopyrimidine (TOP). The majority of the TOP mRNAs code for ribosomal proteins and elongation factors whose increased production enhances the capacity of cell's translation machinery. The activation of the TOR pathway is in part induced by insulin pathway via Akt. Akt can phosphorylate and hence prevent the formation of tuberous sclerosis complex 1 and 2 (TSC1/2), which inhibits the activity of Ras homolog enriched in the brain (Rheb), a positive regulator of TOR. Therefore, Akt activation allows TOR to be activated (for reviews, see Inoki et al., 2002; Inoki et al., 2003; Saucedo et al., 2003; Taniguchi et al., 2006; Zhang et al., 2003).

Additionally, TOR signaling can be activated by amino acids present in the environment, although the precise mechanism for how the TOR pathway senses amino acids is not well understood in mammals (Dann and Thomas, 2006).

As in mammals, TOR signaling in *Drosophila* is essential for coupling nutrient availability to growth controls. Mutations in *dTOR* phenocopy both loss-of-function mutations in the insulin/IGF pathway and amino acid deprivation (Oldham et al., 2000; Zhang et al., 2000). Intriguingly, an amino acid transporter encoded by *slimfast* (*slif*) has been proposed as the amino acid sensor of TOR signaling. Knockdown of *slif* in the fat body, the insect equivalent to vertebrate liver and adipose tissue, causes severe systemic growth retardation that can be rescued by TOR signaling activation (Colombani et al., 2003). It is of interest to note that this global growth defect is also accompanied by a developmental delay (Colombani et al., 2003), suggesting that TOR signaling is involved in determining developmental timing. Furthermore, a recent study has demonstrated that inactivation of TOR signaling in the PG leads to a developmental delay that can be reversed by 20E feeding (Layalle et al., 2008). This observation implies that limited nutrient availability inactivates TOR signaling in the PG, which subsequently lengthens development by delaying the synthesis of 20E. Interestingly, the larvae with blocked TOR signaling in the PG exhibit a delayed induction of *phm* and *dib* (Layalle et al., 2008), suggesting that the effect of TOR signaling on 20E synthesis is via regulating the expression of 20E biosynthetic enzymes.

4.2.4. Other neuropeptides that affect ecdysteroidogenesis

Since the discovery of PTTH, the first brain-derived peptide identified that elicits

ecdysteroidogenesis by the PG, a number of additional neuropeptides have been isolated and characterized from various insect species. Those that stimulate 20E production are known as prothoracicotropic factors, while those that inhibit are called prothoracicostatic factors.

4.2.4.1. Prothoracicotropic factors

Small PTTH

In *Manduca*, in addition to the 'big' PTTH, or true PTTH, there also exists a 'small' PTTH whose molecular weight is around 7 kDa. Interestingly, this small PTTH possesses a stronger ecdysteroidogenesis-stimulating effect on the larval PG than the pupal PG, suggesting a stage-specific function of this molecule (Bollenbacher et al., 1984). The small PTTH may also employ the same or a highly similar signal transduction pathway as true PTTH to activate E synthesis by the PG. This hypothesis is based on the observation that the small PTTH increases both intracellular Ca^{2+} concentration and the Ca^{2+}-dependent cAMP generation (Hayes et al., 1995; Watson et al., 1993) similar to true PTTH. Because crude extracts instead of pure or recombinant small PTTH were used in these assays and because the same second messengers are utilized by the small PTTH, it was suspected that the small PTTH might be an artifact from proteolytic cleavage of true PTTH (Gilbert et al., 2002). To address this concern, it is critical to determine the sequence of the small PTTH and whether it is produced under physiological conditions in *Manduca* as well as other insect species.

FXPRL-amide peptides

FXPRL-amide peptides, named after their common FXPRL-amide C terminus, comprise a large family of peptides that play diverse roles in insect physiology such as pheromone synthesis and developmental diapause (Altstein, 2004; Homma et al., 2006; Homma et al., 2006). The receptors of two large *Bombyx* FXPRL-amide peptides, the diapause hormone (DH) and (Homma et al., 2006; Hull et al., 2004) the pheromone biosynthesis activating neuropeptide (PBAN) have been identified as G-protein coupled receptors (GPCR) (Homma et al., 2006; Hull et al., 2004). Intriguingly, analysis on the expression pattern of the GPCR of DH reveals that in addition to the pheromone gland, the receptor is also detected in the PG in the final larval instar stage (Watanabe et al., 2007), indicating a possible involvement of DH in E synthesis. Indeed, incubating the PG with DH elevates the intracellular concentrations of Ca^{2+} and cAMP and promotes ecdysteroidogenesis, although to a lesser degree than PTTH (Watanabe et al., 2007). This ecdysteroidogenic effect of DH has also been demonstrated in the bollworm *Helicoverpa armigera* (Zhang et al., 2004), suggesting that the function of FXPRL-amide peptides in molting and metamorphosis may be conserved among insect species.

4.2.4.2. Prothoracicostatic factors

Prothoracicostatic peptide (PTSP)

In contrast to prothoracicotropic factors, prothoracicostatic factors are inhibitors of PG's activity in producing E. The first prothoracicostatic factor PTSP, a nonapeptide, was isolated from the larval brains of *Bombyx* (Hua et al., 1999). *In vitro* analysis showed that PTSP is able to repress both basal and PTTH-stimulated ecdysteroid

biosynthesis by the PG (Hua et al., 1999). Direct injection of PTSP lowers the hemolymph ecdysteroid titer and subsequently delays the onset of metamorphosis, confirming its inhibitory role in ecdysteroidogenesis *in vivo* (Liu et al., 2004). It is of great interest to note that PTSP shows sequence homology to the highly conserved N-terminal part of galanins, a family of vertebrate neuropeptide that have been found to exert inhibitory action on certain hormone-secreting cells (Tempel and Leibowitz, 1990). Nonetheless, given the extremely high effective concentrations of PTSP required to achieve the inhibitory effect on PG's activity in synthesizing E (Dedos et al., 2001), it remains uncertain if PTSP plays a role in controlling E synthesis under normal physiological conditions.

FMRFamide-related peptides (FaRPs)

A second prothoracicostatic factor (EDVVHSFLRFamide) isolated from pupal brains of *Bombyx mori* was named Bommo-myosuppressin (Bommo-MS) based on a conserved structure to an insect myosuppressin (Yamanaka et al., 2005). In fact, Bommo-MS as well as other insect myosuppressins belongs to the FMRFamide-related peptide family (FaRPs). FaRP family members, which share the C terminal RF amide but unique N terminus, are involved in the regulation of multiple aspects of insect physiology and behavior (Nichols, 2003) but never ecdysteroidogenesis. Surprisingly, Bommo-MS can reduce both the basal and PTTH-stimulated E synthesis by *Bombyx* PGs via down-regulating the cAMP level in a dose-dependent manner (Yamanaka et al., 2005). Unlike PTSP, the amount of Bommo-MS required to achieve the inhibitory effect is within the appropriate physiological range, implying that it likely plays a physiological role *in vivo*. In

the same study, the researchers also cloned the receptor of Bommo-MS (Bommo-MSR), a GPCR that is primarily expressed in the PG but can also be found in the gut and Malpighian tubules (Yamanaka et al., 2005). The temporal expression pattern of Bommo-MS in the brain shows that this neuropeptide is induced during the first half of the last larval instar stage when the ecdysteroidogenic activity of the PG is low (Yamanaka et al., 2005), indicating that Bommo-MS may function to keep the PG inactive during this period. An identical neuropeptide to Bommo-MS has been isolated from *Manduca* (Lu et al., 2002), however whether it possess the same prothoracicostatic activity remains to be tested.

Following the identification of Bommo-MS, four more FaRPs, encoded by the same *Bombyx* FMRFamide gene (*BRFa*) have been purified that have negative effect on E synthesis (Yamanaka et al., 2006). These four neuropeptides, like Bommo-MS, utilize Bommo-MSR as their receptor (Yamanaka et al., 2006). Interestingly, the neurons expressing these four FaRPs directly innervate the PG, suggesting that the neuropeptides are directly delivered onto the PG to activate Bommo-MSR without circulating in the hemolymph. Consistent with the prothoracicostatic function of these four neuropeptides, the neurons that express them show strong firing activities when the hemolymph ecdysteroid titer is low (Yamanaka et al., 2006). One mechanism responsible for their prothoracicostatic effect is by transcriptional suppression of the *Halloween* gene *phm* (Yamanaka et al., 2007). This finding highlights the significance of direct neuronal input in modulating the expression of the E biosynthetic enzymes.

4.2.4.3. Factors of non-neuron origin that affect ecdysteroidogenesis

While most of the factors that exert regulatory functions on PG's activity in synthesizing ecdysteroids are derived from the brain, evidence for the involvement of factors of non-neuron origins in ecdysteroidogenesis by the PG has been emerging.

Gut peptides

Two decades ago, Gelman et al. (1991) found a large amount of prothoracicotropic factors present in the hindgut extracts of the European corn borer *Ostrinia nubilalis* and the gypsy moth *L. dispar*, showing for the first time that in addition to the CNS, the gut can serve as a major source of secreted peptides (Gelman et al., 1991). The prothoracicotropic activity of these factors was demonstrated by their ability to induce a pupal molt of the gypsy moth larvae, possibly via the second messenger cAMP (Gelman et al., 1993). It was later revealed that the hindgut of the last instar *Manduca* larvae also contains similar prothoracicotropic factors (Gelman and Beckage 1995). Nevertheless, efforts have failed to reveal the true identify of these factors except that they likely are low molecular weight molecules (0.5-1.5 kDa). Therefore, more characterizations are required to fully determine their identity as well as molecular actions.

PG autocrine factors

Using isolated *Bombyx* PGs, it was recently discovered that the PG-conditioned medium has the potential to trigger ecdysteroidogenesis *in vitro*, suggesting the existence of a

secreted factor by the PG itself (Gu, 2006; Gu, 2007). Direct injection of this putative autocrine factor into the final instar *Bombyx* larvae causes a dramatic increase in DNA synthesis that normally occurs prior to acute E production as well as the hemolymph ecdysteroid titer, demonstrating the ability of this factor to induce E synthesis *in vivo* (Gu, 2006; Gu, 2007). Interestingly, this putative factor can also promote DNA synthesis by isolated corpus allatum (Gu, 2006), indicating that it may act as a universal tropic factor. Although the structure of this factor remains to be determined, it is known that it is heat stable and has an estimated molecular weight of 1 kDa to 3 kDa. Similar findings on the existence of autocrine factors from PG have been reported in other insect species (Vandersmissen et al., 2007), indicating that stimulation of the ecdysteroidogenic activity of the PG by autocrine factors is likely a general mechanism in regulating ecdysteroid synthesis.

In addition to the secreted peptide factors mentioned above, the ecdysteroidogenic event in the PG is also modulated by a protein modification mechanism, the SUMO modification.

4.2.5. SUMO modification

SUMO (small ubiquitin-related modifier), a highly conserved protein found throughout the eukaryotic kingdom, can be covalently attached to target proteins in a reversible manner. The attachment is catalyzed by three enzymes: the E1-activating enzyme, the E2-conjugating enzyme and the E3 ligase (for review, see Geiss-Friedlander and Melchior, 2007). E1 functions to activate the SUMO protein and catalyzes the conjugation between SUMO and the E2 enzyme. Subsequently, with the

help of an E3 ligase, E2 transfers SUMO to the target protein via an isopeptide bond formed between the glycine residue of SUMO and a lysine residue of the target protein. This covalent modification by SUMO has a wide range of biological effects such as modulating protein-protein/protein-DNA interactions, changing protein subcellular localizations and altering enzymatic activities (David et al., 2002; Wilkinson and Henley, 2010). Therefore, depending on the function of the target protein, the SUMO pathway has critical roles in a variety of cellular processes such as gene transcription, DNA repair, cell cycle regulation and signal transduction (Andreou and Tavernarakis, 2009; Gill, 2005; Lee and Bachant, 2009; Poukka et al., 2000; Verger et al., 2003).

Recently, the SUMO pathway has been linked to insect metamorphosis. Knockdown of *smt3*, the *Drosophila* SUMO gene, specifically in the PG was found to disrupt the generation of the 20E titer, thereby blocking the initiation of metamorphosis (Talamillo et al., 2008). Further analysis revealed that the expression of several 20E biosynthetic enzymes including Dib and Sad was severely affected by down-regulation of the SUMO pathway, which might be the underlying reason for the lack of 20E synthesis (Talamillo et al., 2008). Consistently, PG specific knockdown of *lesswright* (*lwr*), the gene encoding the *Drosophila* E2 conjugating enzyme, also gives rise to the same developmental arrest phenotype at the onset of metamorphosis (personal observation). It would be interesting to investigate whether the E biosynthetic enzymes are directly or indirectly modulated by the SUMO pathway.

While a number of peptide hormones, secreted factors and signaling pathways have been found to regulate E biosynthesis in the PG, additional players that are involved in E biosynthesis are being identified. Here, we report the involvement of TGF-β signaling in ecdysteroidogenesis in the PG.

4.2.6. TGF-β signaling pathway

TGF-β signaling plays pivotal roles in multiple aspects of development including cell proliferation, differentiation and morphogenesis (Derynck and Feng, 1997). TGF-β superfamily ligands are secretory cytokines that can be divided into three major branches: TGF-βs, Activins and Bone Morphogenetic Proteins (BMPs) based on the sequence similarities. Although functionally distinct, these TGF-β superfamily ligands share remarkably similar downstream signaling mechanisms (for review, see Massague, 1998). The canonical signaling pathway is initiated by the binding of a ligand to a type I/type II heterodimeric receptor complex that becomes activated and phosphorylates downstream effectors known as R-Smads. The phosphorylated R-Smads subsequently associate with Co-Smads, forming a R-Smad/Co-Smad complex that functions as a transcription factor to regulate gene expression. Each TGF-β superfamily ligand can activate a specific set of R-Smads, giving rise to the specificity of the signaling pathways (for reviews, Feng and Derynck, 2005; Derynck and Feng, 1997). For example, the R-Smads involved in TGF-β/Activin signaling include Smad2 and Smad3, whereas BMPs signal though Smad1, Smad5 and Smad8. In both cases, Smad4 serves as the Co-Smad (Lagna et al., 1996).

In *Drosophila*, both the TGF-β/Activin and BMP branches of the signaling pathways are well conserved and characterized (for review, see Parker et al., 2004). There are total 4 Activin ligands: *Drosophila* Activin (Actβ), Dawdle (Daw), Myoglianin (Myo) and Maverick (Mav). The BMP ligand family is composed of Decapentaplegic (Dpp), Glass Bottom Boat (Gbb) and Screw. While Activin family ligands bind to the type I receptor Baboon (Babo), BMP ligands activate the type I receptor Thick Vein (Tkv) and

Saxophone (Sax). These type I receptors complex with the type II receptors Punt (Put) and Wishful thinking (Wit) to phosphorylate R-Smads. There are two R-Smads present in the *Drosophila* genome, dSmad2 (the fly homolog of Smad2/3), which mediates Activin signaling (Brummel et al., 1999) and Mad (the fly homolog of R-Smads1/5/8) that transmits BMP signaling (Shimmi et al., 2005a; Shimmi et al., 2005b). The co-Smad in *Drosophila* is known as Medea, which associates with either dSmad2 or Mad, forming a trimeric Smad complex (Wisotzkey et al., 1998).

Despite the enormous number of developmental pathways that TGF-β signaling regulates, its involvement in modulating developmental timing has not been investigated. It is interesting to note, however, that in *C. elegans* TGF-β signaling appears to regulate the levels of Daf-9, a cytochrome P450 protein that carries out the final step in synthesis of the steroid derivative dafachronic acid (Motola et al., 2006). Dafachronic acid levels in turn control entry into an alternative protective developmental pathway known as dauer formation which helps the organism survive times of stress such as poor nutrient conditions.

My thesis project therefore examines whether TGF-β signaling plays a role in regulating metamorphic timing in *Drosophila*. I found that knockdown of various Activin signaling components in the PG leads to a developmental arrest phenotype at the final larval instar stage. This defect was due to decreased expression of several 20E biosynthetic enzymes, which affected the 20E titer required for metamorphosis. The reduced expression of the 20E biosynthetic enzymes is likely a consequence of impaired PTTH and insulin/IGF pathways. This study has demonstrated for the first time that Activin signaling is essential for initiating metamorphosis. The findings from this study has also revealed an intricate interplay between the signaling pathways that influence

metamorphic timing, thereby providing telling insights into how multiple inputs from different signaling pathways are integrated to set the developmental clock. Given that TGF-β signaling controls similar biological and physiological processes in both flies and humans, it may be that the highly conserved Activin pathway controls pubertal timing in humans as well.

Chapter 2:

Activin Signaling Modulates the Competence of the Prothoracic Gland during *Drosophila* Metamorphosis

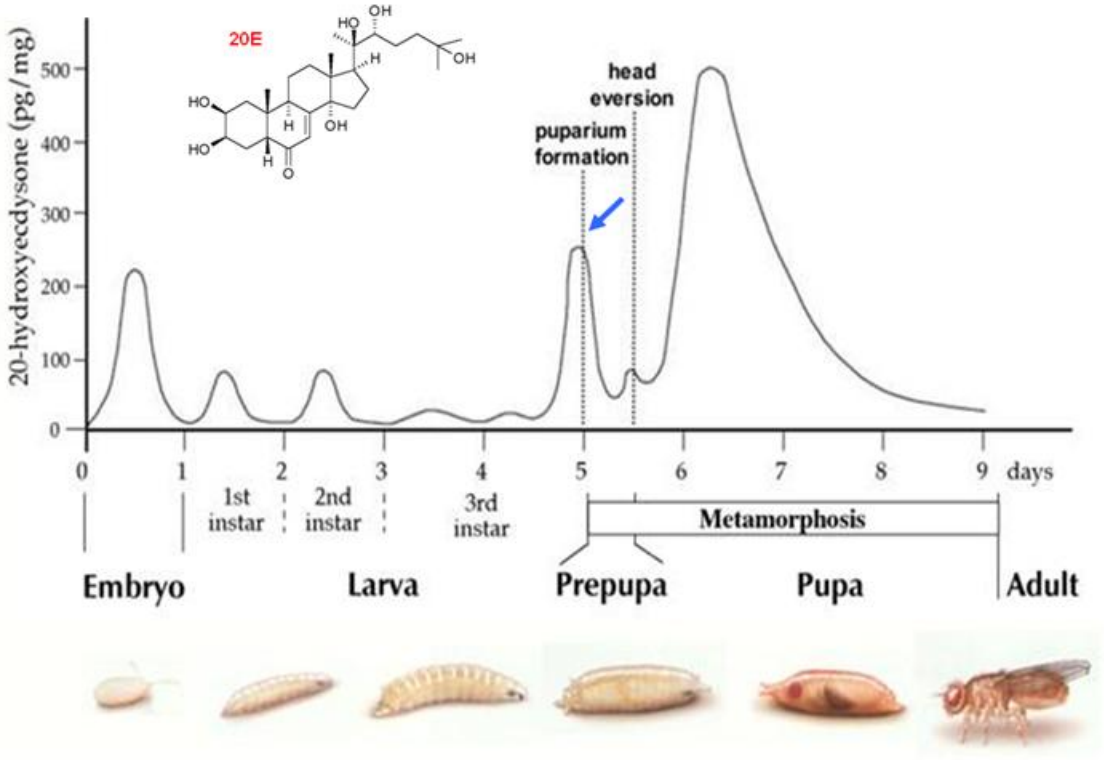
Introduction

In many multi-cellular organisms, developmental transitions are necessary for proper progression from the juvenile stage to adulthood. In humans, for instance, puberty initiates a series of changes such as growth of the body, development of secondary sex characters and maturation of reproductive organs that culminate in a mature adult. In the kingdom of insects, which comprises the most diverse and abundant species on earth, the animals also go through major developmental changes, some of the most dramatic of which are seen holometabolous insects that undergo complete metamorphosis of. During metamorphosis, insect larvae stop feeding, enter into the pupal stage and completely remodel their body plan to produce sexually mature adults. Although puberty and insect metamorphosis appear to be considerably different, both processes rely on steroid hormones that can systemically coordinate gene expression and ultimately cause physiological changes in the whole animal. While human sex steroid hormones are essential for puberty (Ebling and Cronin, 2000; Mauras et al., 1996; Sisk and Foster, 2004), insect steroid hormones, particularly ecdysone (E) and its active metabolite 20-hydroxyecdysone (20E), play pivotal roles in modulating metamorphosis (Gilbert et al., 1997; Gilbert et al., 2002) (Figure 2-1 A).

During larval and pupal stages, the prothoracic gland (PG) is the major endocrine organ in which biosynthesis of E takes place (Figure 2-1B). The production of E in the

Figure 2-1:

A


20E
20-hydroxyecdysone (pg/mg)
500
400
300
200
100
0
head eversion
puparium formation
0
1
2
3
4
5
6
7
8
9
days
1st instar
2nd instar
3rd instar
Metamorphosis
Embryo
Larva
Prepupa
Pupa
Adult

B

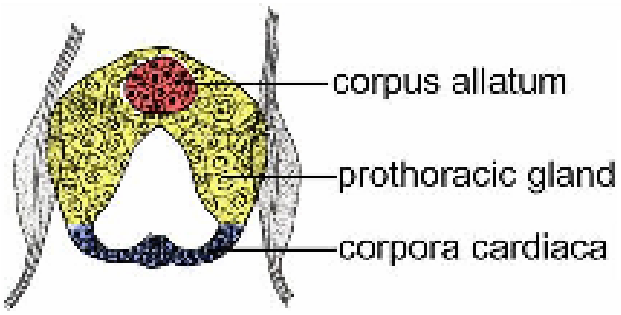

corpus allatum
prothoracic gland
corpora cardiaca

Figure 2- 1. The temporal profile of 20E during *Drosophila* development.

(A). The graph shows the developmental profile of the whole body 20E levels in *Drosophila*. The post-embryonic development of *Drosophila* includes 3 larval instar stages, the pupal stage and the adult stage. The 20E pulses are believed to trigger major developmental transitions including two larval molts and the pupal molt, also known as metamorphosis (arrow). The 20E peak required for metamorphosis is much larger in magnitude than those required for the larval molts. High levels of 20E are also needed for embryonic development as well as pupal development. (Modified from Thummel C.S. 2001 Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. Dev. Cell. 4,453-65).

(B). The anatomy of the *Drosophila* prothoracic gland. The ring gland is composed of three endocrine glands, the prothoracic gland (yellow), the corpus allatum (red) and the corpora cardiacum (blue). Modified from Morphology of the ring gland. (2010). FlyMove. Retrieved August 19, 2010, from http://flymove.uni-muenster.de/Organogenesis/Ringgland/OrgRingpage.html

PG requires the action of the enzymes encoded by the Halloween gene family. With the exception of *neverland* (*nvd*) and *shroud* (*sro*), all the Halloween genes identified so far including *spook* (*spo*), *spookier* (*spok*), *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*) and *shade* (*shd*) code for cytochrome P450 proteins that sequentially convert cholesterol to 20E (Gilbert et al., 2002; Gilbert, 2004). The Nvd protein is a Reiske-type dehydrogenase that is thought to aid in the conversion of cholesterol to 7-dehydrocholesterol (7dC) (Yoshiyama et al., 2006). *Spo* and *spok* are functionally equivalent paralogs that only differ in their temporal expression patterns (Ono et al., 2006). Both appear to act at the so-called Black Box step, perhaps in conjunction with a dehydrogenase coded for by *sro* (Niwa et al., 2010), to convert 7dC to the 3β,5β [H] ketodiol. Phm, Dib and Sad sequentially catalyze the last three steps of E synthesis by adding hydroxyl groups to various positions on this modified cholesterol derivative (Warren et al., 2002; Warren et al., 2004). Once made and released from the PG, E is rapidly converted by Shd to 20E in the peripheral tissues such as the gut, epidermis and fat bodies (Petryk et al., 2003). In *Drosophila* and other holometabolous insects, a large increase in the titer of E/20E generated at the end of the third-instar stage triggers metamorphosis by inducing the expression of the downstream target genes that coordinate and regulate metamorphic events (Andres and Thummel, 1992; Baehrecke, 1996).

Because entry into the pupal stage arrests feeding, the final body size is influenced by the timing of the E/20E peak that sets the duration of the feeding interval. Thus, elucidating the mechanisms that modulate and time the production of the E/20E titer at the end of the third instar stage is crucial to understanding several aspects of insect development. In the past several decades, a great deal of work has centered on understanding the role that various neuropeptides play in modulating the ecdysone

response (for review, see Marchal et al., 2010). Foremost among these is prothoracicotropic hormone (PTTH), a brain-derived factor that functions as a positive regulator of E biosynthesis in the PG (Gilbert et al., 1988). PTTH stimulation of the PG cells initiates a signal transduction pathway that leads to the production and secretion of a surge of the steroid hormone E (Gilbert et al., 1997; Gilbert et al., 2002). Recently, the *Drosophila* PTTH receptor has been identified as Torso (Rewitz et al., 2009b), a receptor tyrosine kinase that activates the Ras/Raf/MEK/ERK pathway during embryonic terminal development in response to Trunk, a distal relative of PTTH (Li, 2005; Perrimon et al., 1995). Knockdown of Ras, Raf or ERK in the PG gives rise to a developmental delay phenotype similar to that caused by PTTH-neuron ablation, suggesting that the same downstream MAPK pathway components are employed to transduce PTTH-Torso signaling in the PG (Rewitz et al., 2009b). Consistent with this view, over-activation of Ras and Raf in the PG leads to accelerated larval development due to increased E production (Caldwell et al., 2005). One potential mechanism for how the PTTH pathway promotes E biosynthesis is through the regulation of the transcriptional expression of E biosynthetic enzymes. Genetic ablation of the PTTH neurons results in marked reduction in the steady state mRNA levels of several E biosynthetic enzymes just prior to metamorphosis when compared to wild type larvae (McBrayer et al., 2007). The positive effect of PTTH signaling on E biosynthetic enzyme transcription is likely to contribute to the increased upregulation of the E titer prior to metamorphosis (Gilbert, 2004; Parvy et al., 2005).

In addition to PTTH signaling, insulin/IGF (insulin growth factor) signaling (IIS), a highly conserved signaling pathway from flies to humans, has been implicated in controlling E synthesis. In *Drosophila*, there are seven insulin-like peptides (DILPs) that serve as the ligands to activate a single insulin-like receptor (InR) (Mirth and Riddiford,

2007; Wu and Brown, 2006). The most intensively studied DILPs, DILP2, 3 and 5, are expressed in two clusters of seven neurosecretory cells known as insulin-producing cells (IPCs) located symmetrically on the two brain hemispheres (Ikeya et al., 2002). Genetic ablation of the IPCs or partial loss-of-function of InR causes a development delay (Brogiolo et al., 2001; Rulifson et al., 2002), a phenotype reminiscent to low ecdysteroid mutants (Garen et al., 1977; Wismar et al., 2000). PG specific expression of a dominant negative PI3K, a kinase downstream of InR or PTEN, a phosphatase that antagonizes PI3K (Goberdhan et al., 1999), results in low ecdysteroid titer (Mirth et al., 2005). In contrast, PG-specific expression of a wild type PI3K shortens the larval stages, resulting in smaller flies (Colombani et al., 2005; Mirth et al., 2005), presumably due to increased E levels. These observations suggest that IIS signaling influences metamorphosis timing by acting on the PG to affect E synthesis.

Coupled with IIS signaling is the Target of Rapamycin (TOR) pathway that coordinates development according to the nutritional status. The activation of TOR signaling is regulated by IIS signaling through the action of Akt (Taniguchi et al., 2006) as well as the availability of amino acids (Dann and Thomas, 2006). Interestingly, genetic manipulations that reduce TOR activity in the PG prolonged the larval stage by delaying the formation of the E titer required for metamorphosis, indicating that developmental timing is also intimately linked to nutritional information via TOR signaling (Layalle et al., 2008).

Here, we report that TGF-β signaling plays a role in regulating E synthesis. We found that knockdown of various Activin pathway components but not BMP signaling transducers in the PG causes developmental arrest at the last larval stage. The arrest is due to the absence of the ecdysteroid 20E peak required for eliciting pupariation. The defect in 20E production is caused in part by reduced expression of the E biosynthetic

enzymes Nvd, Dib and Spok. Finally, we present evidence showing that Activin signaling likely affects the competence of the PG to respond to metamorphic signals provided by PTTH and IIS signaling.

Results

Activin-like ligands are expressed in the ring gland and neurosecretory cells

In an effort to analyze the dynamics and tissue specific expression patterns of the *Drosophila* Activin family members throughout development, we fused candidate regulatory sequences of *Activin-β* (*Actβ*), *dawdle* (*daw*) and *myoglianin* (*myo*) with Gal4 and used these constructs to drive the expression of a UAS-nuclear-GFP transgene. During the third instar stage, we noticed distinct expression of *daw*-Gal4>nucGFP in the prothoracic gland (PG) portion of the ring gland (Figure 2-2A) and this expression was confirmed by *in situ* hybridization (Figure 2-2B). In the case of *myo* we observed expression in a large number of surface glial cells (Figure 2-2C). *Actβ in situ* and GFP reporter expression is seen in a cluster of lateral anterior brain cells that are in the approximate position of the insulin producing cells (IPCs) as well as a significant number of additional cells in the central nervous system (CNS) including the optic lobes, mushroom bodies and the ventral ganglion (Figure 2-2A and B), consistent with previous reports (Gesualdi and Haerry, 2007; Zhu et al., 2008). To determine if some of these CNS cells correspond to neurosecretory cells, we doubled stained with DIMM, a nuclear bHLH transcription factor whose expression is restricted to neurosecretory cells including IPCs (Hewes et al., 2006; Park et al., 2008). Indeed, many *Actβ*-positive cells including the IPCs on the brain lobes and various cells in the ventral ganglion are DIMM-positive (Figure 2-3). Becasue Daw, Myo and Actβ are all expressed in the

Figure 2-2:

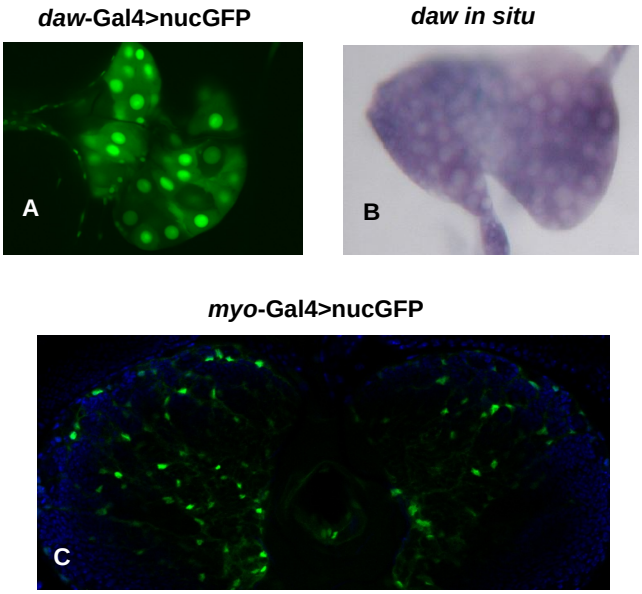


Figure 2-2. The expression pattern of the TGF-β superfamily ligands Daw and Myo.

(A). A nuclear GFP protein accumulates in the nuclei of the PG cells of *daw*-Gal4>UAS-GFP larvae.

(B). The *daw* transcript is detected in the PG by *in situ* hybridization.

(C). The expression of a nuclear GFP protein in a large number of surface glial cells in the CNS of the *myo*-Gal4>UAS-GFP larvae. The blue channel is the DAPI staining.

Figure 2-3:

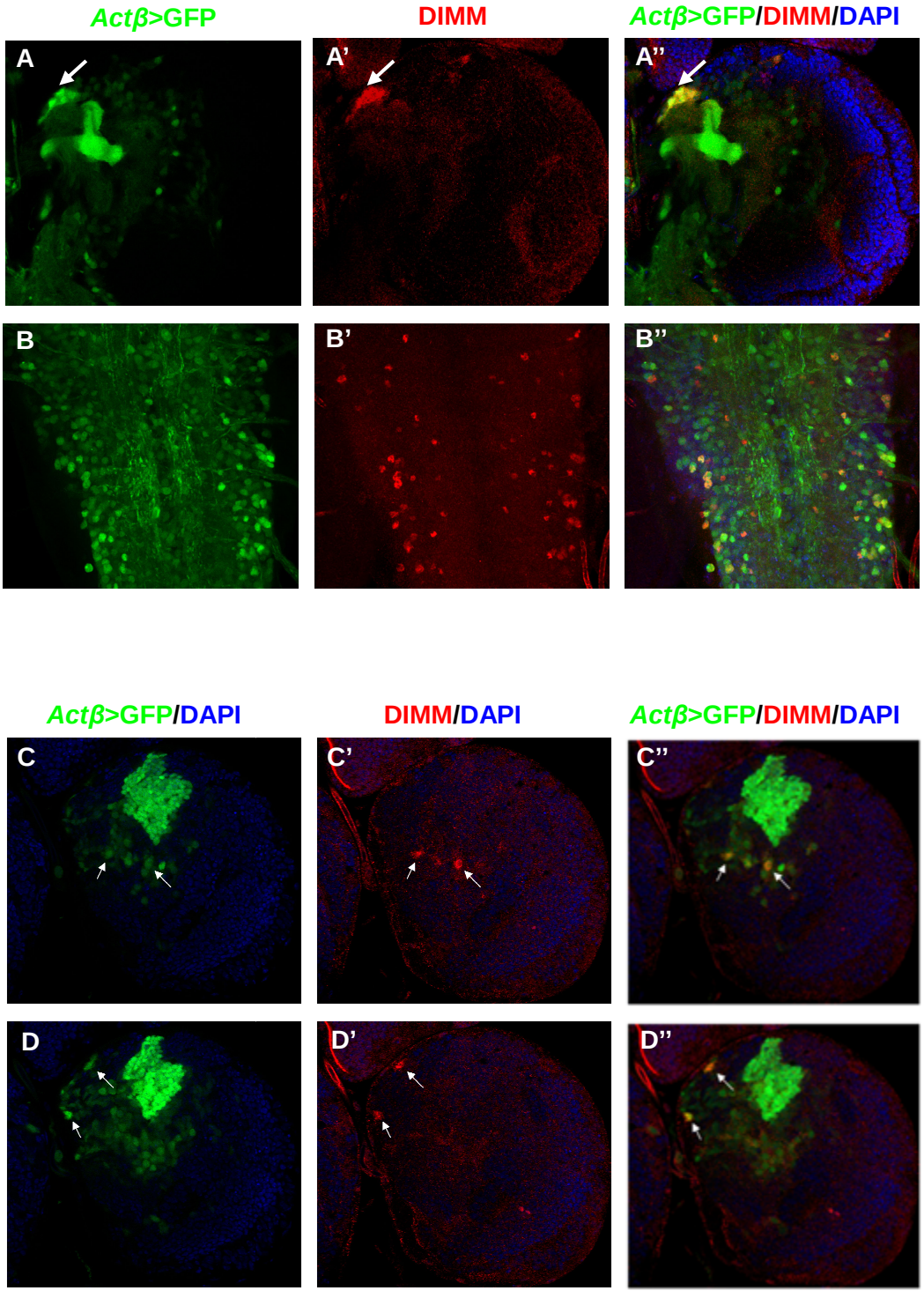

Actβ>GFP
DIMM
Actβ>GFP/DIMM/DAPI
A
A'
A''
B
B'
B''
Actβ>GFP/DAPI
DIMM/DAPI
Actβ>GFP/DIMM/DAPI
C
C'
C''
D
D'
D''

Figure 2-3. Actβ is expressed in neurosecretory cells in the CNS.

(A). DIMM and GFP co-localize in a cluster of cells that are in approximate position to the insulin-producing cell (IPC) cluster in the brain (big arrow) of the *Actβ*-Gal4>UAS-GFP animals.

(B). Many cells in ventral ganglion of the *Actβ*-Gal4>UAS-GFP animals are positive for DIMM.

(C and D). There are several DIMM positive cells in the brain hemisphere that are expressing *Actβ*.

neuroendocrine tissue and because expression of *daw* or *myo* in any endocrine tissues rescues the *daw* or *myo* mutants respectively (data not shown), it seems likely that they circulate in the hemolymph and may provide systemic regulatory signals.

Loss and gain of Activin signaling in PG affect developmental timing

The observation that several Activin-type ligands are expressed in the ring gland and neurosecretory cells led us to examine whether perturbation of this pathway specifically in the ring gland produces any developmental abnormalities. To this end, we used Akh (CC), Phm (PG) and Aug21 (CA) Gal4 drivers to specially overexpress or knockdown various Activin signaling components in different portions of the ring gland. Overexpression of a constitutively activate form of Babo (Babo*), the type I receptor for Activin-type ligands (Brummel et al., 1999) or knockdown of dSmad2, the primary transcriptional transducer of *Drosophila* Activin signaling (Brummel et al., 1999) in either the CA or the CC produced no obvious developmental phenotypes (data not shown). In contrast, overexpression of Babo* or Actβ in the PG resulted in larvae that started wandering while still in a relatively small size (Figure 2-4A). The mouth hooks of these small larvae exhibited second instar characteristics (Figure 2-4B), indicating that they failed to progress to the third instar stage. Intriguingly, after wandering for about two days, these second instar larvae form stage precocious puparia (Figure 2-4C). However, the puparia fail to survive to pharate adults, perhaps because they do not achieve a minimal viable weight (Figure 2-4D). Growth analysis of these second instar precocious larvae showed that they suffered from a delayed development and took approximately 6.5 days after egg laying to reach 50% pupariation compared to the typical 5-day period in wild type larvae (Figure 2-4E).

Although these gain-of-function experiments demonstrate that excess Activin

Figure 2-4:

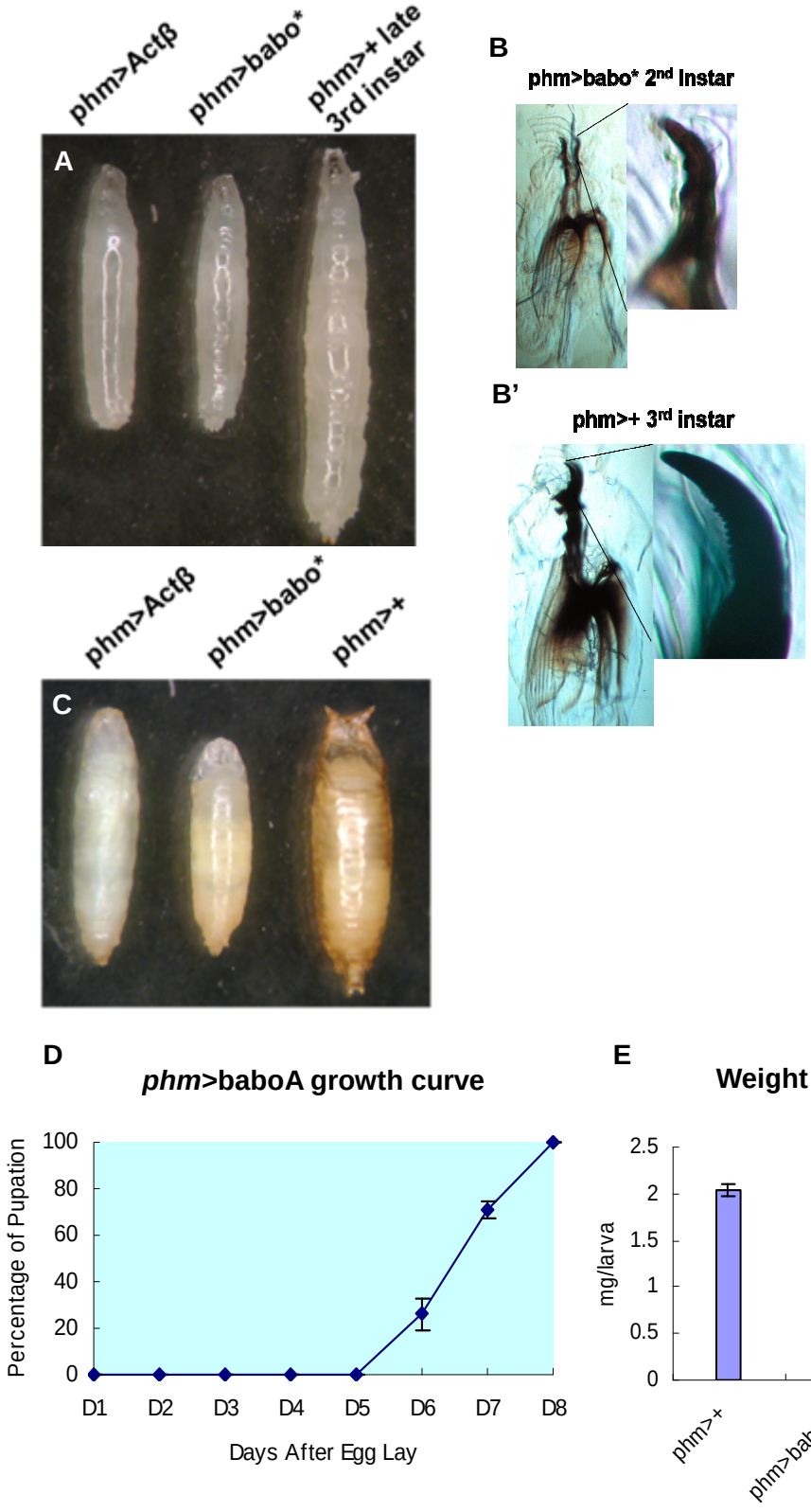

phm>Actβ
phm>babo*
phm>+ late 3rd instar
A
B
phm>babo* 2nd Instar
B'
phm>+ 3rd instar
phm>Actβ
phm>babo*
phm>+
C
D
phm>baboA growth curve
Percentage of Pupation
100
80
60
40
20
0
D1
D2
D3
D4
D5
D6
D7
D8
Days After Egg Lay
E
Weight
mg/larva
2.5
2
1.5
1
0.5
0
phm>+
phm>baboA

Figure 2-4. Over-activation of Activin signaling in the PG leads to precocious pupariation.

(A). Ectopic expression of the ligand Actβ (left) or a constitutively active receptor Babo* (middle) in the PG results in small wandering larvae as compared to a 3rd instar control wandering larva (right).

(B). The mouth hook of the small wandering larvae in (A, Left or Middle) exhibits 2nd instar characteristics while (B') the mouth hook of the 3rd instar control larva in (A, right) shows 3rd instar characteristics, such as the serrated teeth structure.

(C). The small wandering larvae in (A, Left and Middle) formed stage precocious pupae (Left and Middle), which failed to survive to the pharate adult stage. A normal pupa formed from a 3rd instar control larva is shown on the right that finally emerged as an adult fly.

(D). The growth curve of *phm*>babo* larvae showed that these larvae take approximately 6.5 days after egg lay to reach 50% precocious pupation.

(E). The *phm*>babo* second-instar wandering larvae is roughly half the weight of the control third-instar wandering larvae.

signaling on the PG can alter developmental progression through larval stages, we sought to determine if Activin signaling is required in the PG for normal development. Accordingly, we employed RNAi to specifically knockdown the *dSmad2* expression in the PG using a variety of PG expressed Gal4 drivers (Figure 2-5, A and B). Since dSmad2 is the sole downstream mediator of canonical Activin signaling, its knockdown should remove contributions of all Activin-type ligands including Actβ, Daw, Myo and perhaps Maverick, a TGF-β ligand whose precise subfamily classification is not clear from either signaling or phylogenetic studies (Nguyen et al., 2000). Interestingly, larvae in which *dSmad2* is knocked down in the PG (thereafter referred to as *dSmad2* RNAi larvae) failed to initiate metamorphosis and arrested at the third instar stage for more than 2 weeks, during which time they kept feeding and grew to a very large size (Figure 2-5C). To eliminate the possible off-target effects of the RNAi technology, we used several UAS-*dSmad2* RNAi constructs that target different regions of the *dSmad2* gene. All of these constructs individually gave rise to the same non-pupariating phenotype (Table 2-1). Expression of a UAS-Flag-*dSmad2* transgene in the PG reversed the developmental arrest caused by *dSmad2* RNAi, further demonstrating the specificity of *dSmad2* RNAi (data not shown). To show that the non-pupariating phenotype is specific for the loss of Activin signaling rather than other unrelated cellular functions of the *dSmad2* gene, we knocked down the receptor Babo which normally phosphorylates and activates dSmad2 as well as Punt, a type II receptor and Medea, a transcriptional cofactor for dSmad2. Again, using several RNAi lines we observed the non-pupariating phenotype (Figure 2-5, C and D; Table 2-1). In contrast, knockdown of Mad, the transcriptional transducer of the BMP pathway in the PG cells showed no effect on developmental timing even though these RNAi lines were capable of generating strong BMP loss-of-function phenotypes

Figure 2-5:

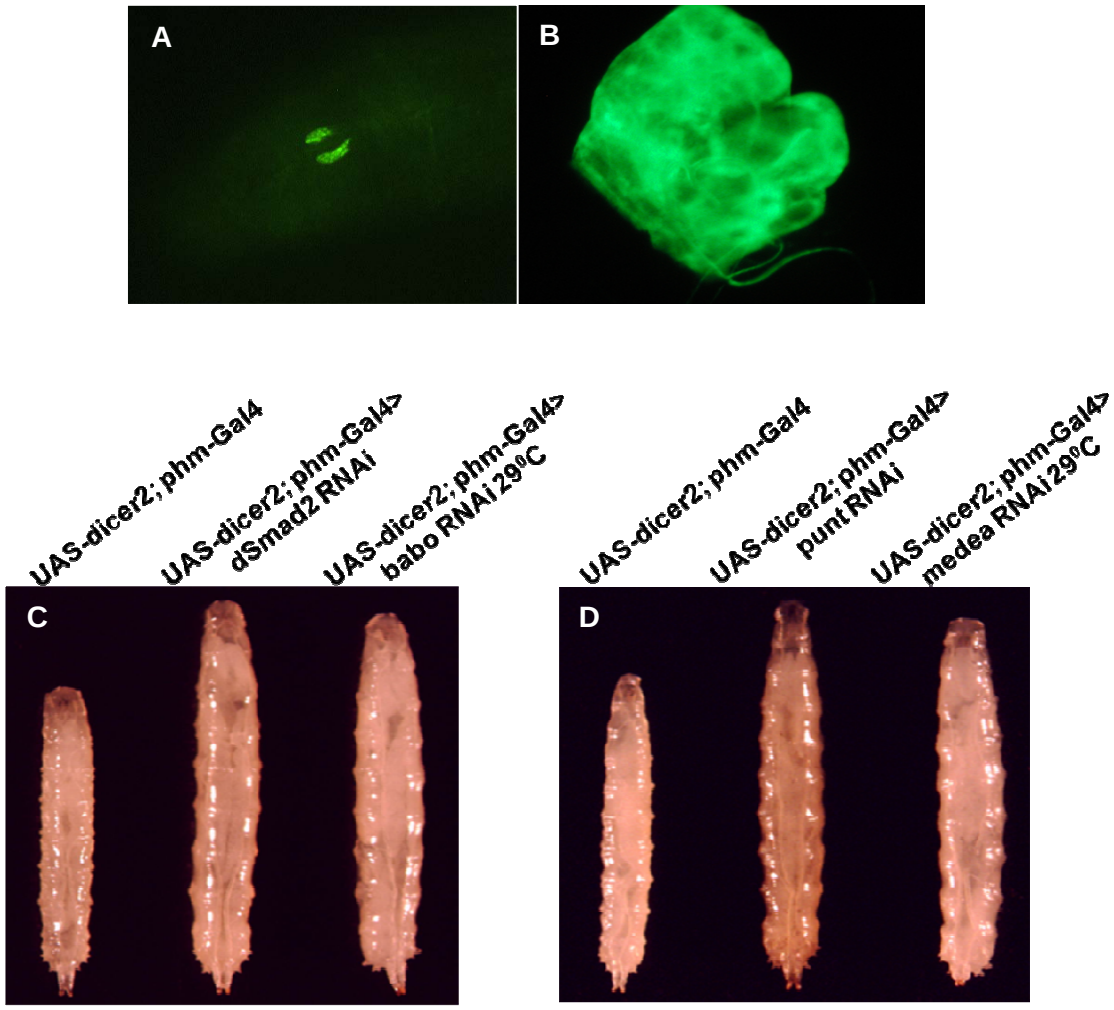

A
B
UAS-dicer2; phm-Gal4
UAS-dicer2; phm-Gal4> dSmad2 RNAi
UAS-dicer2; phm-Gal4> babo RNAi 29°C
C
UAS-dicer2; phm-Gal4
UAS-dicer2; phm-Gal4> punt RNAi
UAS-dicer2; phm-Gal4> medea RNAi 29°C
D

Figure 2-5. Loss of Activin signaling in the PG causes defects in initiating metamorphosis.

(A and B). A UAS-*cd8-gfp* transgene is specifically expressed in the PG when driven by *phm*-Gal4 in the whole larvae view (A) and in an isolated PG (B).

(C). PG specific knockdown of dSmad2, the primary transcription transducer of Activin signaling and Babo, the type I receptor that activates dSmad2, leads to developmental arrest in the third instar stage. These larvae failed to initiate metamorphosis and remained in the third instar stage for at least two weeks. The third instar stage normally lasts for two days. Due to the increased duration of the larval growth period, these larvae are larger than the control.

(D). PG specific knockdown of Punt, a type II receptor as well as Medea, the co-Smad that can form a functional transcription complex with dSmad2, gives rise to a similar developmental arrest phenotype.

Table 2-1. The RNAi constructs that lead to developmental arrest when expressed in the PG.

dSmad2 RNAi constructs	*punt* RNAi constructs	*babo* RNAi constructs
#14609 (Vienna Stock Center) Target sequence: cDNA (nucleotide 652-1049)	#848 and #37279 (Vienna Stock Center) Target sequence: cDNA (nucleotide 1076-1247)	#853 and #3825 (Vienna Stock Center) Target sequence: cDNA (nucleotide 1771-1942)
#105687 (Vienna Stock Center) Target sequence: 3' UTR	#26756 (Bloomington Stock Center) Target sequence: cDNA (nucleotide 785-1236)	#41B3 (The O'Connor Lab) Target sequence: cDNA (nucleotide 1123-1640)
#3A2 (The O'Connor Lab) Target sequence: cDNA (nucleotide 713-1136)		

when expressed in other tissues (data not shown). In addition, simultaneous knockdown of both dSmad2 and Mad in the PG did not enhance the dSmad2 knockdown (data not shown). The above data suggest that Activin signaling in the PG mediated by Babo, Punt, dSmad2 and Medea is essential for proper developmental timing and initiation of metamorphosis.

Loss of Activin signaling leads to low 20E due to E enzyme downregulation

Next we explored the underlying mechanism(s) of how Activin signaling affects metamorphosis. Since TGF-β signaling regulates cell fate during development (Larsson and Karlsson, 2005; Mondal et al., 2004; ten Dijke et al., 2003), we first examined whether Activin signaling is required for specifying PG cell fate. Normal expression of Phm and Sad in the *dSmad2* RNAi PG indicates that loss of Activin signaling did not interfere with the specification of the PG cells (Figure 2-6, E and E'). Also, both the morphology and the cell number of the *dSmad2* RNAi PG appear to be normal compared to the control (Figure 2-6A and A'). Although we did not observe any major morphological defects in the PG, we noticed that the *dSmad2* RNAi larvae have smaller brains and imaginal discs (Figure 2-6A, B and C), despite the fact that the larvae are abnormally large. These phenotypes have been reported in *without children* mutants which have abnormally low ecdysteroid levels (Wismar et al., 2000). Consistently, the B1 isoform of the ecdysone receptor (EcR-B1), whose expression in the CNS normally occurs in the late third instar larvae and peaks at the white pupa (WP) stage when the 20E titer reaches its maximum, is largely diminished from the brain of the *dSmad2* RNAi larvae (Figure 2-6D). Because these phenotypes indicated a possible low ecdysteroid defect, we examined whether *dSmad2* RNAi larvae exhibit a decreased 20E titer. Indeed,

Figure 2-6:

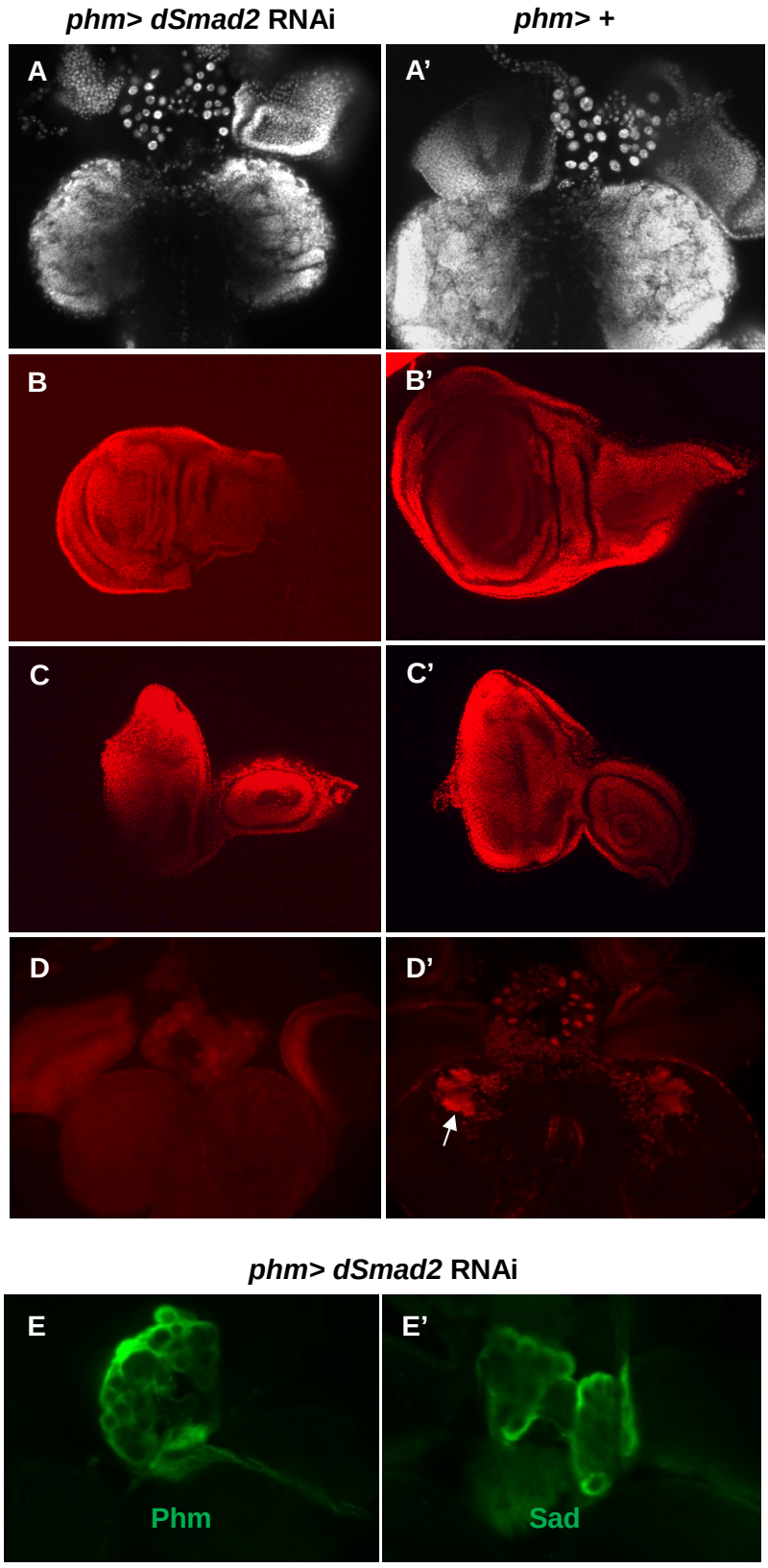


Figure 2-6. Loss of Activin signaling in the PG affects the size of brain and imaginal discs.

(A and A'). The brain of *dSmad2* RNAi larvae exhibits reduced size as compared to the control. Note that loss of Activin signaling does not affect the development of the PG since there are no obvious abnormalities in the morphology and the cell number of the PG of the *dSmad2* RNAi larvae as compared to the control *phm*>+ animals.

(B and B'). The leg imaginal discs of *dSmad2* RNAi larvae are smaller than the control.

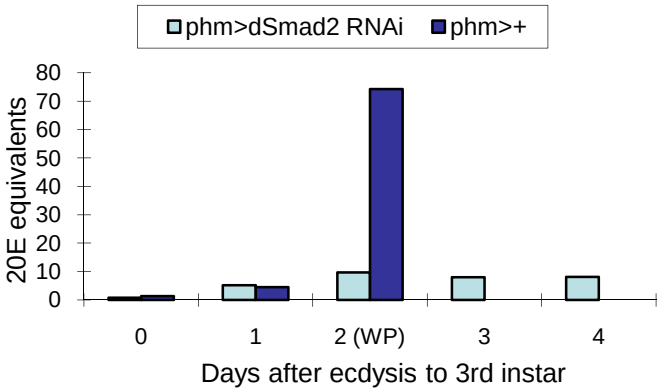
(C and C'). The wing imaginal discs of *dSmad2* RNAi larvae are smaller than the control.

(D and D'). The expression of EcR-B1 is completely absent in the CNS of the dSmad2 RNAi larvae (D). Note that the mushroom body of a wild type brain (arrow in D') has strong expression of EcR-B1.

(E and E'). The expression of the Phm and Sad protein in the PG is detected in the *dSmad2* RNAi larvae.

Figure 2-7:

A


phm>dSmad2 RNAi
phm>+
20E equivalents
80
70
60
50
40
30
20
10
0
0
1
2 (WP)
3
4
Days after ecdysis to 3rd instar

B


+20E

Figure 2-7. Loss of Activin signaling in the PG affects the 20E titer required for metamorphosis.

(A). Whereas there is a large 20E titer detected in the control larvae prior to metamorphosis [also known as the white pupa (WP) stage], the level of 20E fails to pulse at the onset of metamorphosis in the *dSmad2* RNAi larvae and remains at the basal level during the prolonged third instar stage.

(B). Exogenous 20E feeding can rescue the 10 days old *dSmad2* RNAi third instar larvae to the pupa stage. While some rescued larvae formed normal-looking pupae (right), some pupae display abnormalities such as the lack of anterior spiracles (left) and uneven distribution of the pigment on the pupa case, suggesting that the larvae are gradually losing competence to develop into pupae during the larval arrest. None of the rescued pupae emerged as adults because a high amount of 20E is indispensible for pupa development and 20E feeding ceases during the pupal stage.

an analysis on 20E levels revealed that *dSmad2* RNAi larvae do not generate a 20E peak during the prolonged third instar stage. In control larvae that normally undergo pupariation ~48 hrs after ecdysis into the third instar stage, there is a sharp rise in total ecdysteroid content (Figure 2-7A). In contrast, *dSmad2* RNAi larvae failed to mount an ecdysone surge even after 4 days in the third instar stage (Figure 2-7A). Feeding *dSmad2* RNAi larvae 20E, however, rescued their ability to undergo metamorphosis, confirming that 20E deficits underlie the failure to elicit metamorphosis (Figure 2-7B). Based on these observations, we conclude that the Activin signaling pathway mediated by dSmad2 modulates E metabolism in the PG.

One possible explanation for impaired 20E production in *dSmad2* RNAi larvae is that the E biosynthetic enzymes are not properly expressed in the PG. To test this idea, we used immunostaining to examine whether E biosynthetic enzymes are expressed in the PGs of *dSmad2* RNAi larvae. Intriguingly, we found that staining for Spok and Dib, two P450 enzymes required in the PG for the synthesis of E is reduced in *dSmad2* RNAi larvae (Figure 2-8A and A'). Curiously, an occasional individual PG cell (Figure 2-8A) still exhibits significant staining likely caused by cell heterogeneity in *phm*>Gal4 expression.

To more quantitatively demonstrate this down-regulation, we performed Western blot analysis on brain-ring gland complexes (BRGCs) to measure the levels of the Dib and Spok protein (Figure 2-8B and B'). Densitometry showed that BRGCs from *dSmad2* RNAi larvae have extremely low levels of Dib ranging from a 5-fold reduction at the beginning of the third instar to an 11-fold loss at the onset of metamorphosis as compared to the control larvae in the same the stage (Figure 2-8C). Spok also showed significant reduction in *dSmad2* RNAi larvae (Figure 2-8C'). The levels of Dib and Spok decreased further in the prolonged third instar stage, possibly resulting from gradual degeneration of the PG (Figure 2-9). Conversely, Phm and Sad protein levels were

Figure 2-8:

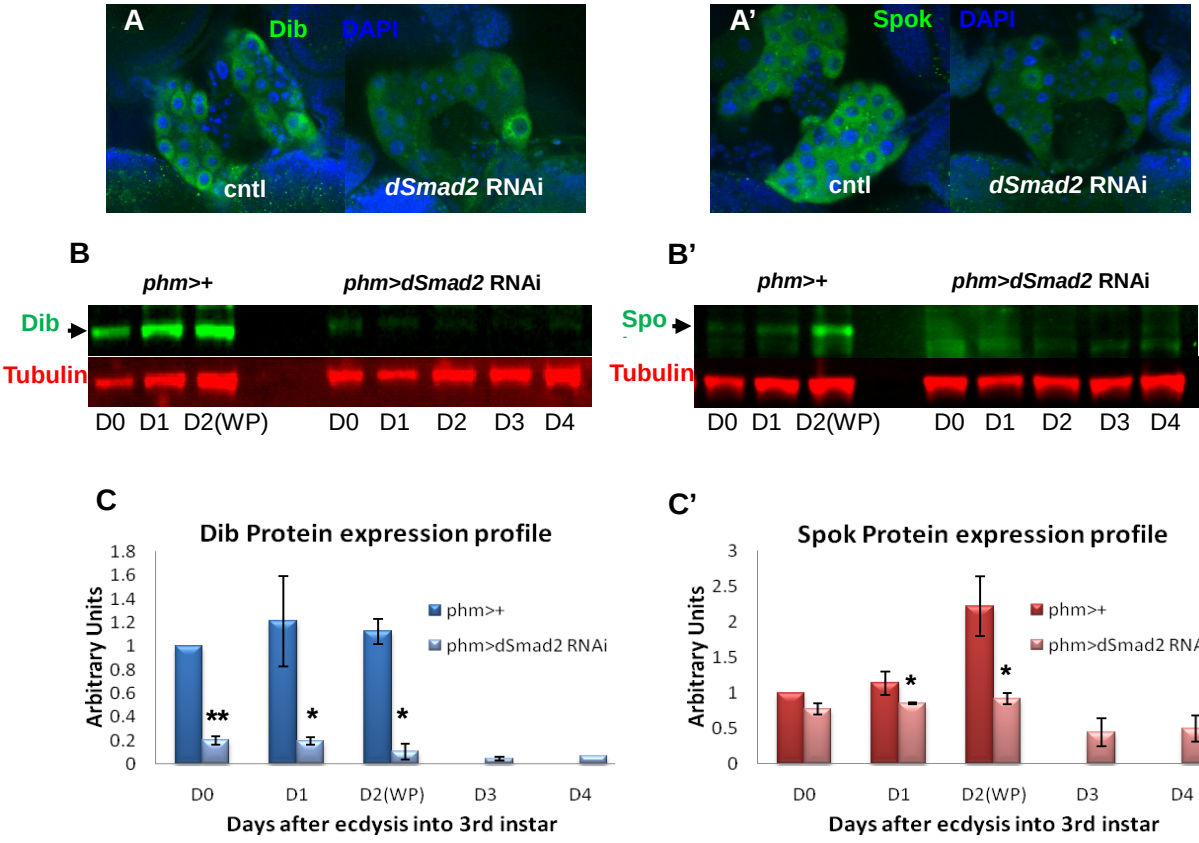

A
Dib
DAPI
cntl
dSmad2 RNAi
A'
Spok
DAPI
cntl
dSmad2 RNAi
B
phm>+
phm>dSmad2 RNAi
Dib
Tubulin
D0 D1 D2(WP) D0 D1 D2 D3 D4
B'
phm>+
phm>dSmad2 RNAi
Spo
Tubulin
D0 D1 D2(WP) D0 D1 D2 D3 D4
C
Dib Protein expression profile
Arbitrary Units
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
phm>+
phm>dSmad2 RNAi
**
*
*
D0 D1 D2(WP) D3 D4
Days after ecdysis into 3rd instar
C'
Spok Protein expression profile
Arbitrary Units
3
2.5
2
1.5
1
0.5
0
phm>+
phm>dSmad2 RNAi
*
*
D0 D1 D2(WP) D3 D4
Days after ecdysis into 3rd instar

Figure 2-8. Activin signaling regulates E biosynthetic enzymes in the PG.

(A and A'). The expression of Dib (A) and Spok (A') is down-regulated in the PG of *dSmad2* RNAi larvae as shown by immunostaining.

(B). The Western blot analysis demonstrates that *dSmad2* RNAi causes a significant reduction at the Dib protein level at the 3rd instar stage. The PG-brain complexes were dissected and subjected to immunoblotting. Total 4 PG-brain complexes were loaded on each lane. Tubulin was used as a loading control.

(C). The quantitative analysis of the Western blot results in (B). The Dib protein level in each lane is normalized to the tubulin level. The normalized Dib level in the control larvae at D0 is set as 1. Three independently collected biologically equivalent samples were used to obtain the statistic information. Error bars represent SD. The Student T-Test was used to calculate the statistical significance. *P<0.05, **P<0.005.

Figure 2-9:

***phm>dSmad2* RNAi larvae 3rd instar stage**

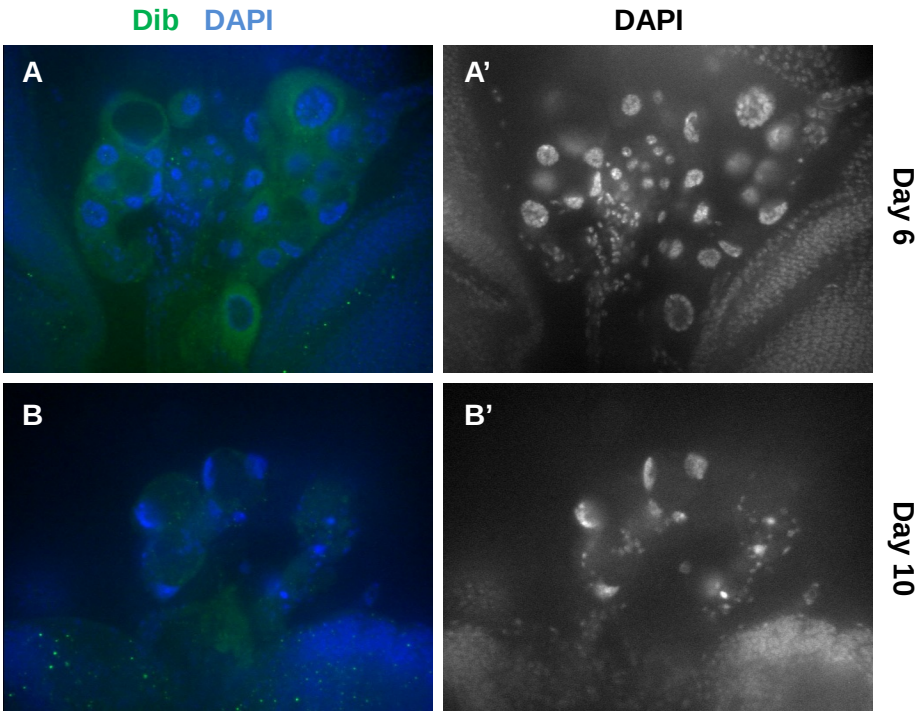


Figure 2-9. The PG degenerates in the prolonged third instar stage.

(A). The expression of Dib in the PG is extremely low in 6 days old *dSmad2* RNAi larvae as shown by immunostaining. In contrast to the normal round-looking nuclei, some of the PG nuclei are long and appear condensed.

(B). The PG of a 10 days old *dSmad2* RNAi larvae has undergone extensive cell death with only a few abnormal nuclei visible at this stage. The Dib level is undetectable.

Figure 2-10:

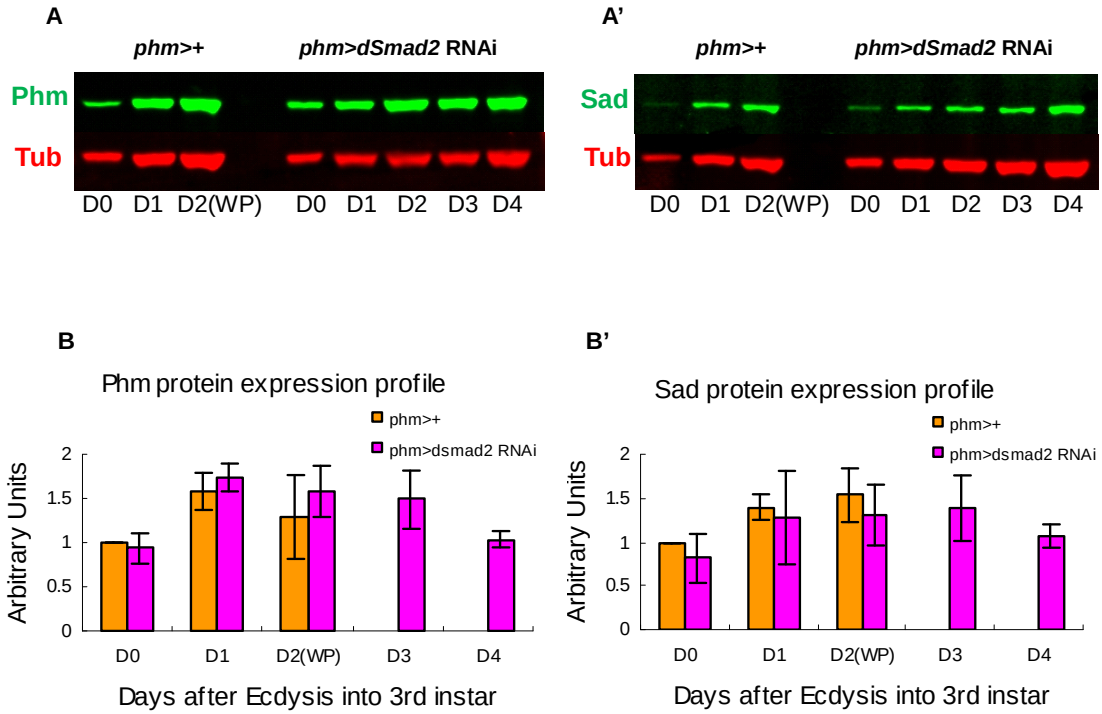

A
phm>+
phm>dSmad2 RNAi
Phm
Tub
D0 D1 D2(WP) D0 D1 D2 D3 D4
A'
phm>+
phm>dSmad2 RNAi
Sad
Tub
D0 D1 D2(WP) D0 D1 D2 D3 D4
B
Phm protein expression profile
phm>+
phm>dsmad2 RNAi
Arbitrary Units
2
1.5
1
0.5
0
D0 D1 D2(WP) D3 D4
Days after Ecdysis into 3rd instar
B'
Sad protein expression profile
phm>+
phm>dsmad2 RNAi
Arbitrary Units
2
1.5
1
0.5
0
D0 D1 D2(WP) D3 D4
Days after Ecdysis into 3rd instar

Figure 2-10. The protein expression levels of Phm and Sad in *dSmad2* RNAi larvae show no significant difference from the controls.

(A and B). The Western blot analysis of the protein levels of Phm (A) and Sad (B) in the control *phm*-Gal4>+ larvae and *phm*-Gal4>*dSmad2* RNAi larvae. For the control larvae, three time points were selected for protein level analysis, D0 (immediately after the L2-to-L3 molt), D1 (1 day after the L2-to-L3 molt) and D2 (2 days after the L2-to-L3 molt) which is also known as the white pupa stage (WP) in the wild type. Because *dSmad2* RNAi larvae could not initiate metamorphosis, D3 and D4 were included for their prolonged 3rd instar stage.

(A' and B'). The quantitative analysis of the Western blot results. The Phm protein level in each lane was normalized to the tubulin level. The normalized Dib level in the control larvae at D0 was set as 1. The Student T-Test was used to calculate the statistical significance. No significant differences were found at D1, D1 and D2 time points between the *dSmad2* RNAi larvae and the controls. The levels of Phm and Sad in *dSmad2* RNAi larvae persisted in D3 and showed a declining trend at D4.

Figure 2-11:

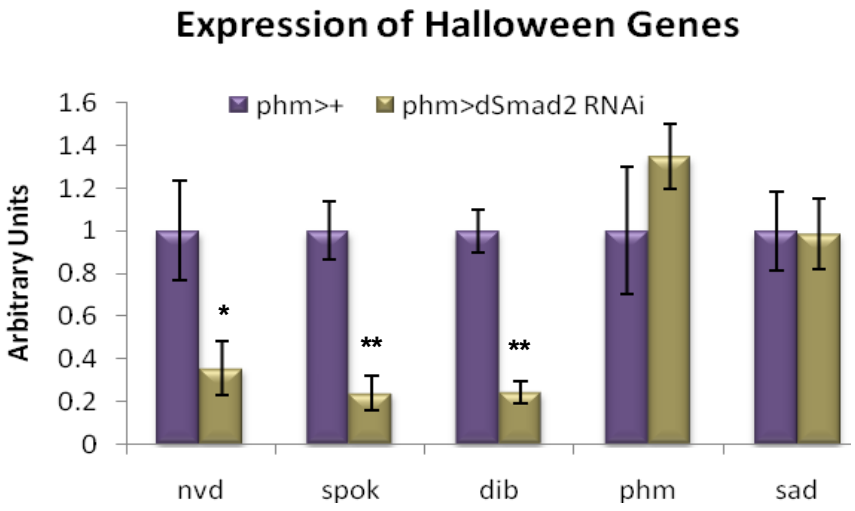

Expression of Halloween Genes
phm>+
phm>dSmad2 RNAi
Arbitrary Units
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
nvd
spok
dib
phm
sad

Figure 2-11. Loss of Activin signaling disrupts the transcription of the Halloween genes.

The qPCR analysis on the transcript levels of the genes involved in E biosynthesis shows that *nvd*, *spok* and *dib* have significant reductions at their transcript levels in *dSmad2* RNAi larvae, whereas the expression of *phm* and *sad* is normal. While *nvd* has a fourfold decrease, *spok* and *dib* show an approximately five fold reduction. The gene expression levels were normalized to the mRNA levels of the ribosomal protein rpL23 in the same samples. Three independently collected biologically equivalent samples were used to obtain the statistic information. Error bars represent SD. The Student T-Test was used to calculate the statistical significance. *P<0.05, **P<0.005.

Table 2-2. The primers used for qPCR experiments.

Gene	Forward	Reverse
nvd	5’-GGAAGCGTTGCTGACGACTGTG-3’	5’-TAAAGCCGTCCACTTCCTGCGA-3’
dib	5’-TGCCCTCAATCCCTATCTGGTC-3’	5’-ACAGGGTCTTCACACCCATCTC-3’
spok	5’-GCCGAGCTAAATTTCTCCGCTT-3’	5’-TATCTCTTGGGCACACTCGCTG-3’
phm	5’-GGATTTCTTTCGGCGCGATGTG-3’	5’-TGCCTCAGTATCGAAAAGCCGT-3’
sad	5’-CCGCATTCAGCAGTCAGTGG-3’	5’-ACCTGCCGTGTACAAGGAGAG-3’
rpL23	5’-GACAACACCGGAGCCAAGAACC-3’	5’-GTTTGCGCTGCCGAATAACCAC-3’

unaffected in *dSmad2* RNAi larvae (Figure 2-10).

Since TGF-β signaling regulates gene transcription, we sought to ask whether down-regulation of Dib and Spok at the protein level is a consequence of reduced transcription. To address this question, we performed quantitative RT-PCR analysis on the RNA samples isolated from BPGCs in staged *dSmad2* RNAi larvae and the control larvae. The qPCR result demonstrated that, indeed, the steady-state levels of *dib*, *spok* and *nvd* transcripts were significantly decreased (Figure 2-11). Consistent with the protein expression data, the transcript levels of *phm* and *sad* were not altered by *dSmad2* RNAi (Figure 2-11). The above experiments identified the E biosynthetic genes *dib*, *spok* and *nvd* as being potential regulatory targets of Activin signaling in the PG and suggested that the reduced expression of these genes likely contributed to the *dSmad2* RNAi phenotype.

Loss of Spok expression may account for the developmental arrest

The identification of multiple genes involved in the E biosynthetic pathway including *spok*, *dib* and *nvd* as potential targets of Activin signaling prompted us to determine whether there is a key target or whether the combination of all or some of these genes, when down-regulated, contributes to the low ecdysteroid production in *dSmad2* RNAi larvae. Ectopic expression of a UAS-*nvd* transgene (data not shown) or a UAS-*dib* transgene (Figure 2-12) in the PG of *dSmad2* RNAi larvae could not rescue the defect in initiating metamorphosis. Due to the unavailability of a UAS-*spok* transgene, we attempted to express *spo* which demonstrates functional equivalence to *spok* (Ono et al., 2006). Intriguingly, with *spo* expression in the PG, the *dSmad2* RNAi larvae successfully initiated metamorphosis and developed into viable adults. Additionally,

Figure 2-12:

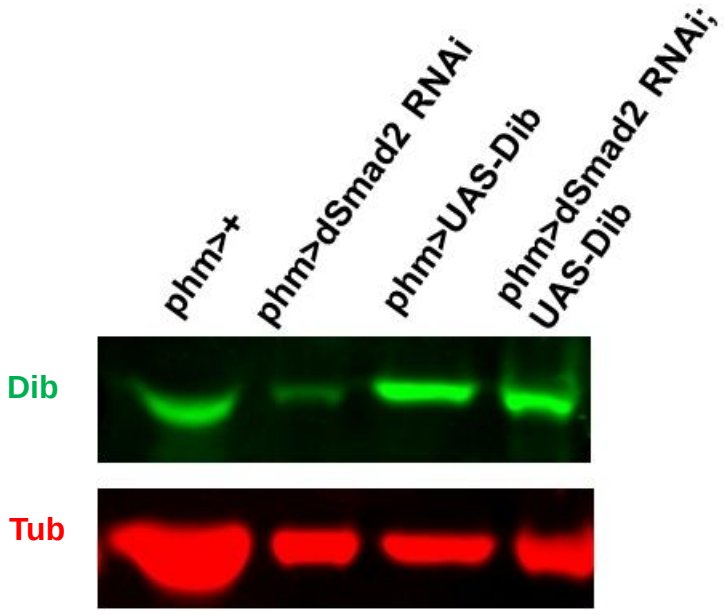

phm>+
phm>dSmad2 RNAi
phm>UAS-Dib
phm>dSmad2 RNAi; UAS-Dib
Dib
Tub

Figure 2-12. Expression of Dib fails to rescue *dSmad2* RNAi larvae.

Ectopic expression of a UAS-dib transgene in the PG of *dSmad2* RNAi larvae restored the wild type expression level of Dib, as shown in the green channel of the Western blot. However, the *phm> dSmad2* RNAi; UAS-*dib* animals are still unable to initiate metamorphosis (data not shown). Tubulin is used as the loading control.

Figure 2-13:

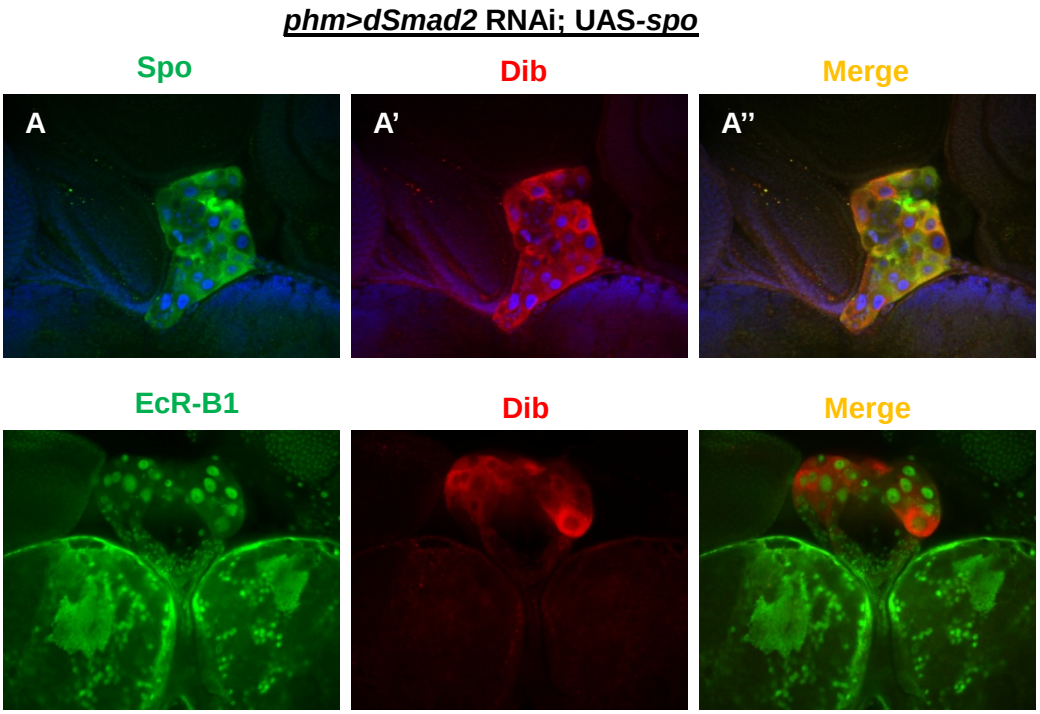


Figure 2-13. Spok is likely a key target of Activin signaling in the PG.

(A). Expression of Spo, which is functionally equivalent to Spok, in the PG of the *dSmad2* RNAi larvae fully rescued the *dSmad2* RNAi larvae to the adult stage. The Dib expression, which is reduced in *dSmad2* RNAi larvae, is increased upon *spo* expression. (B). The expression of EcR-B1 is detected in both PG cells and the CNS of the late wandering stage of the *phm>dSmad2* RNAi; UAS-*spo* animals.

intense EcR-B1 expression was detected in the brain of the *phm*>UAS-*spo*; UAS-*dSmad2* RNAi larvae (Figure 2-13B), indicating that Spo expression rescued the formation of the 20E titer. Of particular interest was the observation that the level of Dib was significantly up-regulated by Spo expression (Figure 2-13), which implied that Dib expression relies on Spok. Since Spok is an upstream enzyme in the 20E biosynthetic pathway, this result indicated that the induction or stabilization of downstream enzymes such as Dib may depend upon the synthesis of some upstream reaction intermediates.

PTTH signaling is impaired by blocking Activin Signaling in the PG

In Lepidoptera and *Drosophila*, the rise of the E/20E titer at the onset of metamorphosis is thought to be triggered in part by the neuropeptide PTTH (Ishizaki and Suzuki, 1994; McBrayer et al., 2007; Smith and Gilbert, 1989). Therefore, we investigated the possibility that loss of Activin signaling might indirectly affect E production by modifying PTTH signaling. To test this hypothesis, we first examined whether the ligand PTTH is expressed normally. We found that the *ptth* transcript is properly expressed in a pair of neurons on each brain hemisphere in *dSmad2* RNAi larvae (Figure 2-14A). Next, we sought to test whether the downstream components of the PTTH pathway are affected by *dSmad2* RNAi. Recently, Rewitz et al. (2009) demonstrated that Torso, a receptor tyrosine kinase, is the PTTH receptor and that it signals through the Ras-Raf-Erk cascade (Rewitz et al., 2009b). Thus, we examined the expression of *torso* in *dSmad2* RNAi larvae. Intriguingly, the level of the *torso* mRNA was markedly reduced in *dSmad2* RNAi larvae as demonstrated by both *in situ* hybridization and qPCR analysis (Figure 2-14B and C). Furthermore, phosphorylated Erk (pErk), an indicator of PTTH signal activation, also showed strong down-regulation (Figure 2-14D and D'), consistent with a blockage of the PTTH pathway at the receptor level by

Figure 2-14:

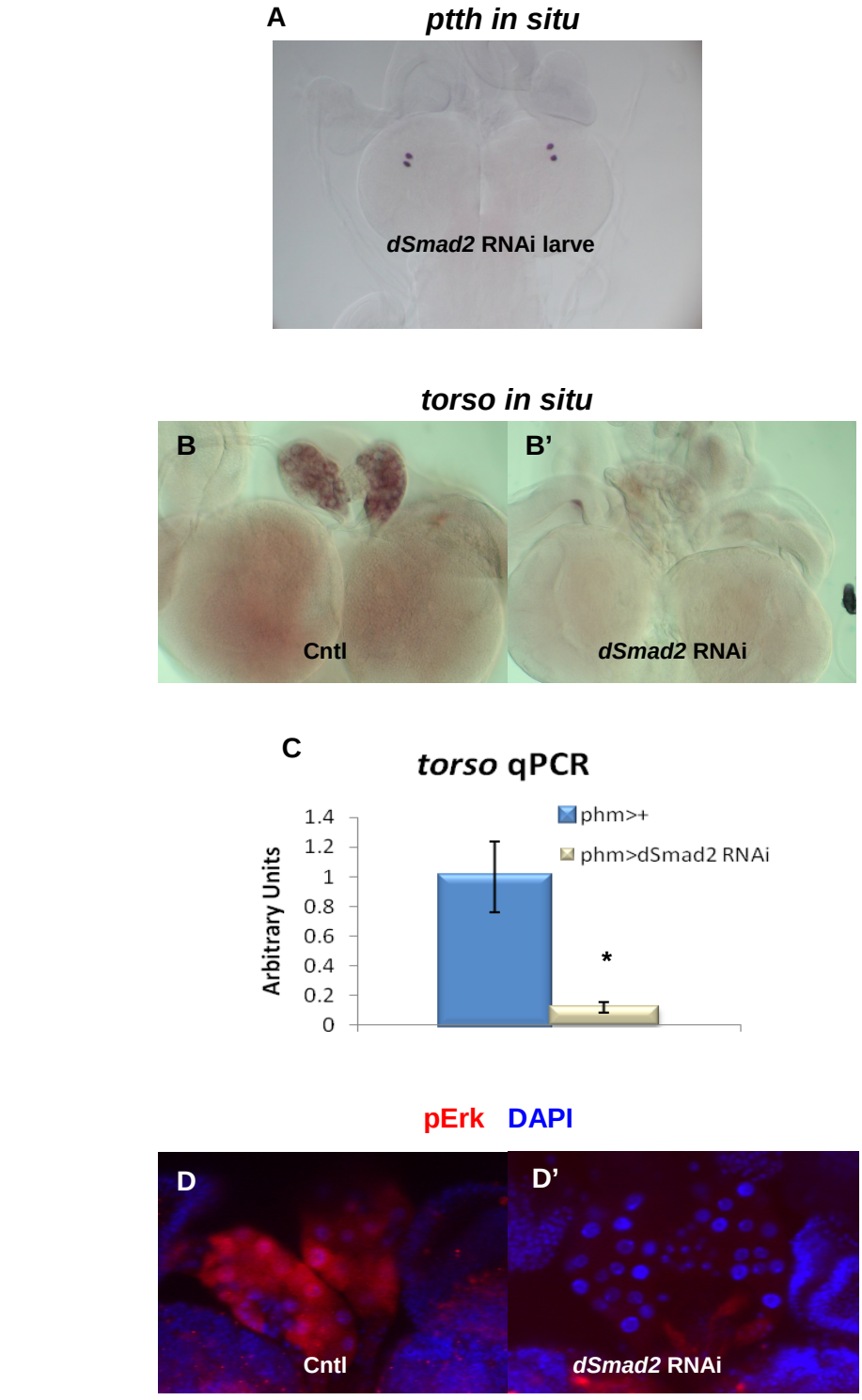

A
ptth in situ
dSmad2 RNAi larve
torso in situ
B
Cntl
B'
dSmad2 RNAi
C
torso qPCR
Arbitrary Units
1.4
1.2
1
0.8
0.6
0.4
0.2
0
phm>+
phm>dSmad2 RNAi
*
pErk DAPI
D
Cntl
D'
dSmad2 RNAi

Figure 2-14. Activin signaling affects PTTH signaling by modulating the expression of the PTTH receptor Torso.

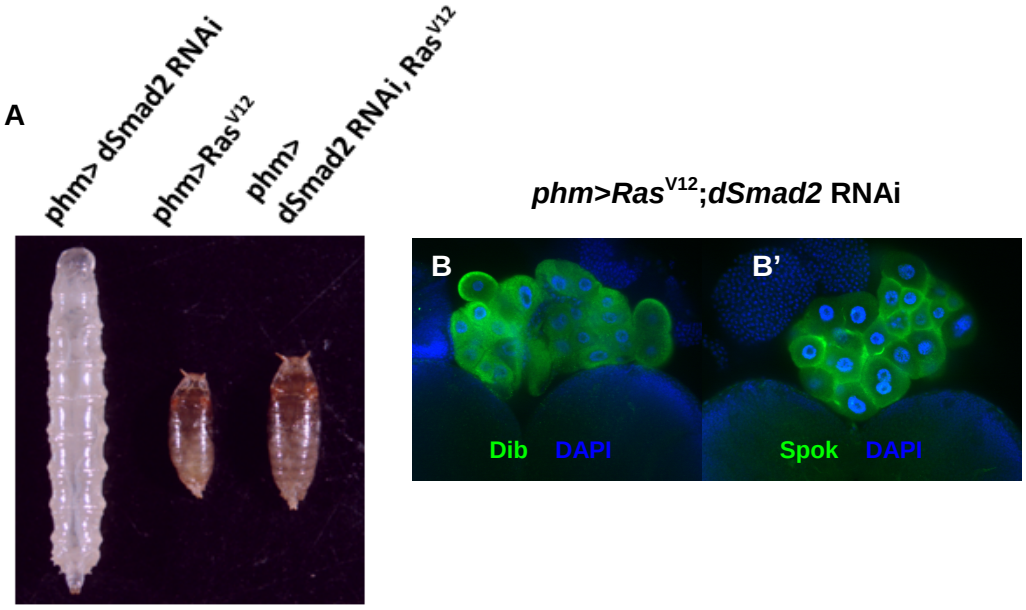
(A). The *ptth* transcript is detected in the PTTH neurons in *dSmad2* RNAi larvae by *in situ* hybridization.

(B and B'). *In situ* hybridization reveals that the *torso* transcript is highly down-regulated in *dSmad2* RNAi larvae.

(C). The qPCR result shows a significant down-regulation of the *torso* transcript in *dSmad2* RNAi larvae. Three independently collected biologically equivalent samples were used to obtain the statistic information. The mRNA levels of the ribosomal protein rpL23 in the same samples were used for normalization. Error bars represent SD. The Student T-Test was used to calculate the statistical significance. *P<0.05.

(D and D'). The immunohistochemical analysis reveals that *dSmad2* RNAi larvae have a remarkable reduction of pErk staining in the PG. pErk staining is shown in red and DAPI is shown in blue. All the images were taken under the same exposure time.

Figure 2-15:


A
phm> dSmad2 RNAi
phm>Ras^{V12}
phm> dSmad2 RNAi, Ras^{V12}
phm>Ras^{V12};dSmad2 RNAi
B
Dib DAPI
B'
Spok DAPI

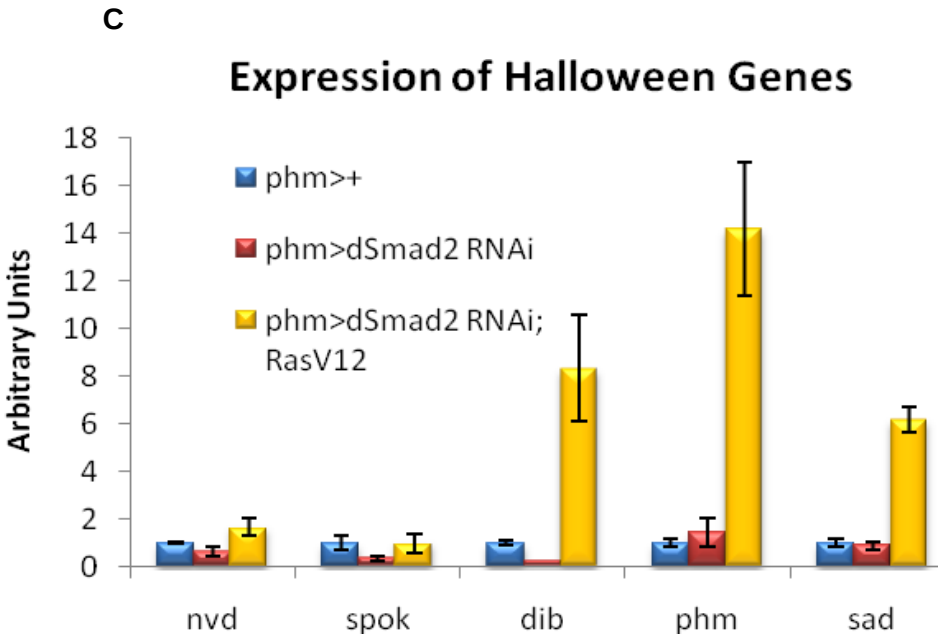

C
Expression of Halloween Genes
Arbitrary Units
18
16
14
12
10
8
6
4
2
0
phm>+
phm>dSmad2 RNAi
phm>dSmad2 RNAi; RasV12
nvd
spok
dib
phm
sad

Figure 2-15. Activation of PTTH signaling rescues *dSmad2* RNAi phenotype by elevating the transcription of the Halloween genes.

(A). Expression of a constitutively active form of Ras (Ras^{V12}) in the PG rescues the *dSmad2* RNAi larvae to the pharate stage.

(B and B'). Expression of Ras^{V12} increases the expression of Dib and Spok in the PG of *dSmad2* RNAi larvae as shown by immunostaining.

(C). Expression of Ras^{V12} elevates the transcript levels of the E biosynthetic enzymes *nvd*, *spok*, and *dib* whose expression is down-regulated in *dSmad2* RNAi larvae. The mRNA levels of phm and sad are also up-regulated with Ras^{V12} expression. The gene expression levels were normalized to the mRNA levels of the ribosomal protein rpL23 in the same samples. Three independently collected biologically equivalent samples were used to obtain the statistic information. Error bars represent SD. The Student T-Test was used to calculate the statistical significance. For *nvd*, *spok*, *dib*, *phm* and *sad*, the upregulation at their transcript levels upon Ras^{V12} expression is statistically significant compared to their levels in *dSmad2* RNAi larvae.

dSmad2 RNAi.

If the loss of PTTH signaling was responsible for the lack of E in *dSmad2* RNAi larvae, we rationalized that reactivating the PTTH pathway might partially restore E biosynthetic enzyme expression and rescue the pupariation defect. To reactivate PTTH signaling, we expressed a constitutively active form of Ras (Ras^{V12}) in the PG. Ras is an essential component in PTTH signaling that acts downstream of Torso (Rewitz et al., 2009b). Expression of Ras^{V12} alone in the PG accelerated larval development and gave rise to small third instar pupae, similar to a previous report (Caldwell et al., 2005) (Figure 2-15A). When Ras^{V12} was expressed in the PG of *dSmad2* RNAi larvae (*phm*-Gal4> *dSmad2* RNAi; Ras^{V12}), the larvae successfully initiated metamorphosis (Figure 2-15A). We reasoned that this rescue was likely a consequence of restored expression of E biosynthetic enzymes, in particular, Spok. At the protein level, Spok and Dib, which are barely detectable in *dSmad2* RNAi larvae, are strongly expressed in the PG of *phm*-Gal4> *dSmad2* RNAi; UAS-Ras^{V12} larvae (Figure 2-15B and B'). This increase at the protein level is probably a result of increased transcription because the level of the *nvd*, *spok* and *dib* mRNA increased to almost normal or even more than the wild type levels upon Ras^{V12} expression (Figure 2-15C). Surprisingly, transcription of *phm* and *sad*, which are not affected by *dSmad2* RNAi also exhibit significant up-regulation (Figure 2-15C), indicating that PTTH signaling likely regulated the expression of *phm* and *sad* as suggested previously (McBryer 2007). The expression of *molting defective* (*mld*), which encodes a transcription factor required for *spok* expression (Neubueser et al., 2005; Ono et al., 2006), is not influenced by Ras^{V12} expression (data not shown), suggesting that the up-regulation on mRNA levels is not seen on all PG expressed genes when the PTTH signal pathway is hyper activated. Overall, these results indicate that loss of Activin signaling in the PG impairs the expression of the PTTH receptor Torso and thus

indirectly reduces E levels by rendering PG cells incompetent to respond to PTTH stimulation.

IIS signaling is disturbed by loss of Activin signaling in the PG

While loss of *torso* expression in *dSmad2* RNAi larvae is consistent with the observed developmental timing defect, it is important to note that ablation of PTTH producing neurons or knockdown of *torso* expression in the PGs only delayed development during the third instar stage (McBrayer et al., 2007; Rewitz et al., 2009b) and did not arrest it as did knockdown of Activin signaling components. We previously speculated that a second prothoracicotropic signal exists that is responsible for producing an eventual rise in the E titer in PTTH signal negative larvae that ultimately enables them to undergo metamorphosis (McBrayer et al., 2007). The more severe developmental arrest phenotype exhibited by *dSmad2* RNAi larvae compared to the developmental delay seen in PTTH ablated larvae could be accounted for by a loss of competence to respond to the alternative prothoracicotropic signal. While the molecular identity of that signal remains unknown, we speculated that insulin-like factors might be good candidates since genetic manipulations of IIS signaling in the PG have revealed that IIS signaling has a positive impact on 20E titers (Colombani et al., 2005; Mirth et al., 2005). We first sought to determine whether IIS signaling in the PG is disturbed by loss of Activin signaling. To address this issue, we utilized a pleckstrin homology domain-GFP (PH-GFP) fusion protein, which served as an indicator of IIS signaling activation (Britton et al., 2002). While the PH-GFP protein was clearly localized on the membrane of the wild type PG cells, its membrane localization was severely disrupted in the *dSmad2* RNAi PG (Figure 2-16A), indicating that IIS signaling was likely down-regulated by blocking Activin signaling. Analysis of the mRNA levels of the IIS signaling components

revealed that whereas *Pi3K* and *Akt* showed mild but significant reduction, *InR* exhibited a remarkable decrease in the BRGC of the *dSmad2* RNAi larvae as compared to the wild type (Figure 2-16B). Although down-regulation in both the PG and the brain could account for the observed transcriptional reduction of *InR*, expressing *InR* or its downstream mediator *Akt* specifically in the PG rescued the ability of the *dSmad2* RNAi larvae to undergo the larval-to-pupal transition (Figure 2-16C). These results strongly suggest that loss of InR in the PG contributes to the negative effect of *dSmad2* RNAi on 20E production. Conversely, blocking Activin signaling in the PG did not affect the transcriptional expression of *TOR* and *S6k*, two key TOR signaling transducers (Figure 2-16B). This finding indicates that loss of Activin signaling in the PG does not affect TOR signaling, consistent with the genetic evidence that further up-regulating TOR signaling in the PG by expressing Rheb, a positive regulator of TOR signaling (Inoki et al., 2003; Saucedo et al., 2003; Stocker et al., 2003), was unable to modify the non-pupariating phenotype of the *dSmad2* RNAi larvae (data not shown).

To determine if the rescue by up-regulating IIS signaling was a result of enhanced expression of the E biosynthetic enzymes, we analyzed the mRNA levels of *nvd*, *dib* and *spok* and the protein levels of Dib and Spok. Although up-regulation of IIS signaling had no effect on the steady state mRNA levels of these E enzymes (Figure 2-17A), there is a considerable elevation of the amount of the Dib and Spok protein upon IIS signaling stimulation in *dSmad2* RNAi larvae (Figure 2-17B, B' C and C'). Altogether, the above results suggest that insulin signaling may be compromised in *dSmad2* RNAi larvae and that its primary effect is at the post-transcriptional level.

Figure 2-16:

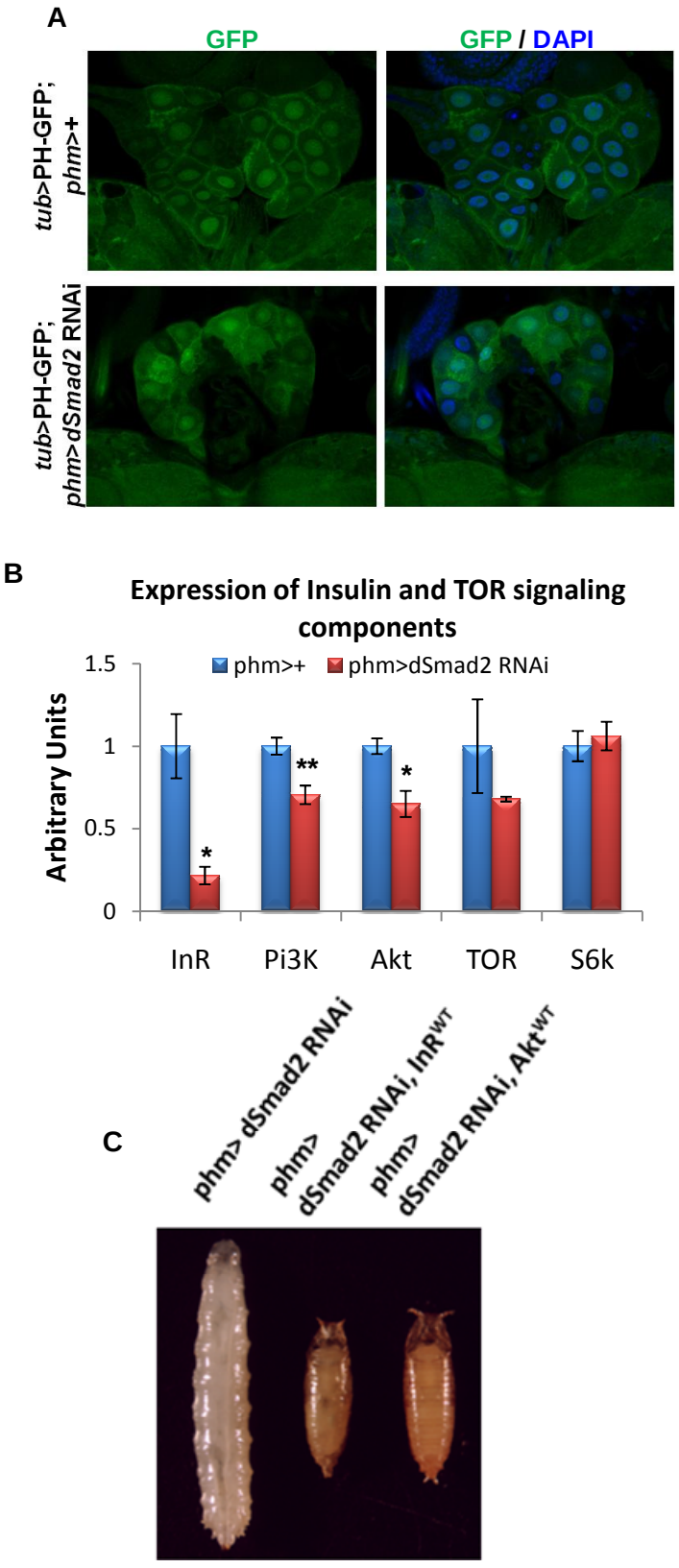

A
GFP
GFP / DAPI
tub>PH-GFP; phm>+
tub>PH-GFP; phm>dSmad2 RNAi
B
Expression of Insulin and TOR signaling components
phm>+
phm>dSmad2 RNAi
Arbitrary Units
1.5
1
0.5
0
*
**
*
InR
Pi3K
Akt
TOR
S6k
C
phm> dSmad2 RNAi
phm> dSmad2 RNAi, InR^WT
phm> dSmad2 RNAi, Akt^WT

Figure 2-16. Insulin signaling is disrupted by loss of Activin signaling in the PG.

(A). Compared to the control (tub>PH-GFP; phm>+), the membrane localization of the PH-GFP fusion protein on the PG cells is severely disrupted in the *dSmad2* RNAi PG (*tub*>PH-GFP; *phm>dSmad2* RNAi). Instead, the PH-GFP protein is largely localized in the cytosol of the *dSmad2* RNAi PG cells.

(B). The expression levels of *InR*, *Pi3K* and *Akt* are significantly reduced in the BPGC of the *dSmad2* RNAi larvae with InR having the most pronouncing decrease (five-fold). The transcript levels of TOR and S6k are not changed by *dSmad2* RNAi. The gene expression levels were normalized to the mRNA levels of the ribosomal protein rpL23 in the same samples. Three independently collected biologically equivalent samples were used to obtain the statistic information. Error bars represent SD. The Student T-Test was used to calculate the statistical significance. * $P<0.5$, ** $P<0.05$.

(C). Expression of either a wild type InR or Akt transgene in the PG rescues the *dSmad2* RNAi larvae to the adult stage.

The model

Based on the finding that Activin signaling is essential for the proper expression of the PTTH receptor Torso and the insulin pathway receptor InR, we propose that the role of Activin signaling is to confer competence to the PG so that it can respond to the metamorphic signals such as PTTH and DIPLs (Figure 2-18). While PTTH signaling which is transduced by Ras/Raf/Erk likely plays a role in controlling transcriptional expression, IIS signaling appears to regulate translation of the E biosynthetic proteins, particularly the possibly rate-limiting enzyme, Spok.

Discussion

What is the ligand of the Activin pathway?

Here we present evidence showing that the canonical Activin signaling pathway plays a critical role in triggering metamorphosis by regulating the expression of E biosynthetic enzymes. While loss-of-function data clearly demonstrate that Babo, Punt, dSmad2 and Medea function as the downstream mediators of the Activin pathway, the identity of the ligand remains uncertain. Because only Actβ overexpression leads to a precocious phenotype, we propose that Actβ, which is produced and released from the *Actβ*-expressing neurons into the hemolymph, could act in an endocrine fashion to initiate Activin signaling in the PG. However, the *Actβ* single null mutants show no defects in initiating metamorphosis while *Actβ*;*daw* double null mutants cannot undergo larval-to-pupal transition (unpublished results). These observations suggest that Actβ and Daw may function redundantly to modulate metamorphosis. What is puzzling to us is that unlike *Actβ*, *daw* overexpression in the PG fails to produce the precocious pupariation phenotype (data not shown). We speculated that the amount of the

Figure 2-17:

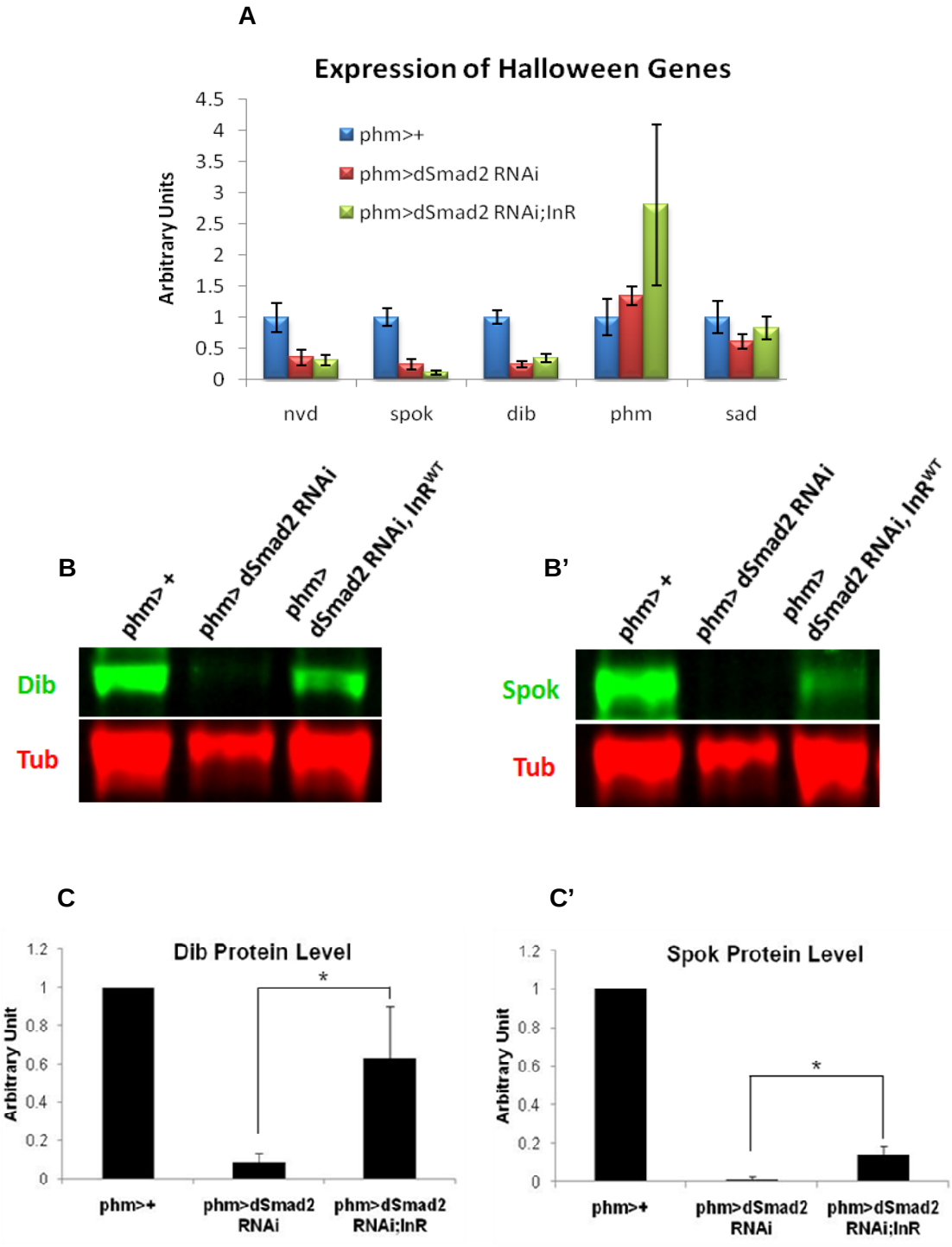

A
Expression of Halloween Genes
Arbitrary Units
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
phm>+
phm>dSmad2 RNAi
phm>dSmad2 RNAi;InR
nvd
spok
dib
phm
sad
B
phm> +
phm> dSmad2 RNAi
phm> dSmad2 RNAi, InR^WT
Dib
Tub
B'
phm> +
phm> dSmad2 RNAi
phm> dSmad2 RNAi, InR^WT
Spok
Tub
C
Dib Protein Level
Arbitrary Unit
1.2
1
0.8
0.6
0.4
0.2
0
*
phm>+
phm>dSmad2 RNAi
phm>dSmad2 RNAi;InR
C'
Spok Protein Level
Arbitrary Unit
1.2
1
0.8
0.6
0.4
0.2
0
*
phm>+
phm>dSmad2 RNAi
phm>dSmad2 RNAi;InR

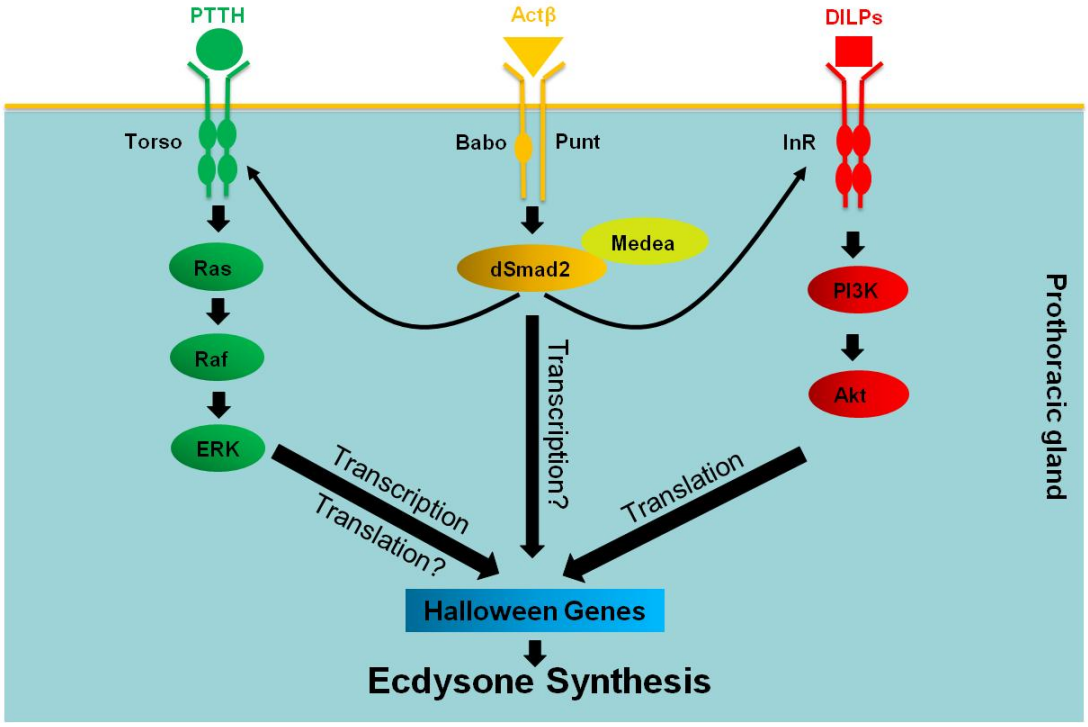
Figure 2-17. Insulin signaling enhances the translation of the Halloween genes.

(A). Expression of InR in *dSmad2* RNAi larvae does not increase the transcription of the E biosynthetic enzymes including *nvd*, *spok*, *dib*, *phm* and *sad*. The gene expression levels were normalized to the mRNA levels of the ribosomal protein rpL23 in the same samples. Three independently collected biologically equivalent samples were used to obtain the statistic information. Error bars represent the SD. The Student T-Test was used to calculate the statistical significance. For the transcript levels of *nvd*, *spok*, *dib*, *phm* and *sad*, there is no statistical significant difference between the *phm>dSmad2* RNAi larvae and *phm>dSmad2 RNAi; InR* larvae.

(B and B'). Expression of a wild type InR increases the protein levels of Dib and Spok in *dSmad2* RNAi larvae.

(C and C'). The graphs represent the quantitation of the Western blot results in B and B'. Expression of InR leads to a 7-fold increase of the Dib protein level and a 15-fold increase of the Spok protein level. The statistical analysis is performed on three independent biological samples for each genotype. Error bars represent the SD. The Student T-Test was used to calculate the statistical significance. * $P<0.05$.

Figure 2-18:


PTTH
Actβ
DILPs
Torso
Babo
Punt
InR
Medea
dSmad2
Ras
Raf
ERK
PI3K
Akt
Transcription?
Transcription
Translation?
Translation
Halloween Genes
Ecdysone Synthesis
Prothoracic gland

2-18. Activin signaling confers PG's competence to respond to PTTH and insulin signaling.

The PG of Drosophila is subject to the regulation by multiple pathways including the PTTH pathway, insulin pathway and Activin pathway. Activin signaling in the PG is initiated by the binding Actβ and possibly Daw to the Babo and Punt receptor complex and transduced by dSmad2 and Medea. The function of this Activin pathway is to time the onset of metamorphosis by modulating the formation of the ecdysteroid titer. While Activin signaling may directly regulate the transcription of Halloween genes that code for E biosynthetic enzymes, this signaling pathway likely controls E synthesis via regulating the expression of Torso and InR, the receptors for PTTH and DILPs respectively. PTTH signaling is involved in promoting the transcriptional expression of the Halloween genes, although it may also stimulate translation of these genes. Insulin signaling, on the other hand, positively influences the translation, but not the transcription, of the Halloween genes. Loss of either PTTH or insulin signaling only delays metamorphosis due to their redundant function on the expression of the E biosynthetic enzymes. Blocking Activin signaling which disrupts PG's ability to receive both PTTH and insulin signaling completely prevents the activation of the Halloween genes and therefore leads to developmental arrest.

endogenous Daw has exceeded the capacity of its receptor and therefore further increase in the level of Daw by over-expression causes no effect. Indeed, co-expression of Daw and Babo(c), the receptor isoform through which Daw signals (Jensen et al., 2009), in the PG leads to precocious pupariation (data not shown), suggesting that the receptor is a limiting factor for generating the precocious phenotype. Additionally, we investigated whether the other two Activin ligands, Myo and Mav, are involved in regulating metamorphosis. Expressing *myo* and *mav* in the PG does not cause precocious development (data not shown). This indicates that Myo and Mav may not participate in activating the Activin signaling pathway in the PG, although we cannot exclude the possibility that their receptors are limiting as well.

Is Activin signaling required for larval molts?

In addition to the larva-to-pupal molt, the larval-to-larval transitions also require discrete titers of E. Does Activin signaling play a role in larval molts by regulating E synthesis? In an effort to answer this question, we closely monitored the developmental profile of the *dSmad2* RNAi larvae. We found that approximately 10%-15% of the larvae die at the L2-to-L3 transition with molting defects (Figure 2-19), implying that Activin signaling is necessary for generating the E titers during the L2-to-L3 larval molt. However, we did not notice any significant development timing difference between the *dSmad2* RNAi larvae and the control at the L1 and L2 stages, suggesting that Activin signaling is not required for basal E production.

Figure 2-19:

L2 to L3 transition

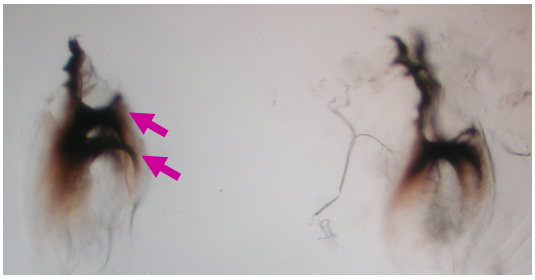


Figure 2-19. The *dSmad2* RNAi larvae have molting defects.

10% to 15% of the *dSmad2* RNAi larvae fail to survive the L2-to-L3 transition. These deceased larvae had double mouth hooks, indicating that they could not undergo proper molting.

Activin pathway components are not SUMOylated

It was of particular interest to note that the specific knockdown of SUMO, a small protein modifier that covalently attaches to and thus regulates the activity of its target proteins, in the PG causes a developmental arrest at the third instar stage (Talamillo et al., 2008). The arrested larvae are deficient in ecdysteroids and display greatly reduced Dib levels in the PG cells (Talamillo et al., 2008). These strikingly similar phenotypes to *dSmad2* RNAi larvae led us to seek whether SUMOylation is indispensible for the activity of the Activin pathway components. We were particularly interested in Medea because Medea is SUMOylated *in vitro* (Miles et al., 2008). By using the S2 cell culture system, however, we were unable to detect any SUMOylated forms of Medea as well as dSmad2 under various signaling conditions (data not shown). While different experimental settings and distinct *in vitro* systems may account for the difference in detecting Medea SUMOylation, we speculate that Medea SUMOylation, if it does happen in the PG *in vivo*, does not affect 20E biosynthesis. This speculation is based on the finding that Medea SUMOylation only modestly inhibits TGF-β signaling (Miles et al., 2008), which is in contrast to a strong positive effect of SUMOylation on 20E production (Talamillo et al., 2008). Since we did not observe any Activin signaling components being SUMOylated, it is likely that the 20E biosynthetic enzymes and/or various transcriptional factors that are involved in 20E generation are the direct targets of SUMO modification.

Spok is likely a key target of Activin signaling

A biosynthetic pathway commonly contains a few key reaction steps that are catalyzed by rate-limiting enzymes. These enzymes are subject to stringent regulation to modulate the overall rate of the entire pathway. The rescue of the *dSmad2* RNAi phenotype by Spo expression but not Dib or Nvd suggests that Spo/Spok may serve as

a critical control point in the 20E biosynthetic pathway. In Lepidoptera, Spo has been proposed to be a rate-limiting enzyme based on the accumulation of 7dC, the precursor for the black box step in which Spo/Spok is involved (Grieneisen et al., 1991; Lafont et al., 2005; Sakurai et al., 1986). Intriguingly, studies in *Manduca* have revealed that both the abundance and phosphorylation of Spo are rapidly and significantly increased upon PTTH stimulation, suggesting that the activity of this possible rate-limiting enzyme is modulated through its expression and also by posttranslational modification (Rewitz et al., 2009a). Although the phosphorylation sites of *Manduca* Spo are not conserved in *Drosophila*, it is likely that *Drosophila* Spo has other phosphorylation sites or is also strictly regulated at the transcription and/or translation levels. While we found that *spok* transcript levels are drastically lowered, *mld*, the only known transcription factor that controls *spok* expression (Ono et al., 2006), is not affected by *dSmad2* RNAi (data not shown). This indicates that Activin signaling may either directly or via other signaling pathways such as PTTH signaling regulate *spok* transcription in order to set the pace for 20E production.

Roles of PTTH and IIS signaling in developmental timing

The timing of developmental transitions is generally modulated by both intrinsic genetic programs and external environment cues. In insects, accumulating evidence has revealed that PTTH signaling serves as an intrinsic clock to determine the onset of metamorphosis (McBrayer et al., 2007) while IIS signaling incorporates environmental conditions to modify the timing of this major event (Colombani et al., 2005; Mirth et al., 2005; Rulifson et al., 2002; Walkiewicz and Stern, 2009). How these two signaling pathways are integrated at the molecular level, however, is poorly understood. We found that while ectopic expression of Ras^{V12} in the PG of *dSmad2* RNAi larvae increases the

mRNA levels of the E enzymes, expression of InR elevates their protein levels. These findings strongly suggest that PTTH signaling and IIS signaling affect transcription and translation of the E biosynthetic enzymes respectively in the PG and provide a mechanism for how developmental timing can be modulated by these two pathways. This simple yet elegant mechanism has several advantages. First of all, it allows fine adjustments on developmental timing based on the external conditions. In the presence of rich nutrients, the resulting high levels of IIS signaling stimulate the translation of E enzymes, leading to early entry into the pupal stage. On the other hand, when food is scarce, the stimulatory effect on the translation of the E biosynthetic enzymes by IIS signaling is remarkably diminished, resulting in a prolonged larval stage. This allows the larvae to continue to feed until IIS signaling reaches a certain threshold to trigger translational expression of E biosynthetic enzymes, which subsequently elicits an E titer necessary for metamorphosis to occur. Consequently, the larvae can store enough nutrients and energy to survive the metamorphic event. The second advantage is that inactivation of one pathway can be compensated by up-regulation of the other. Since PTTH and IIS signaling stimulate the transcriptional and translational expression of E enzymes respectively, loss of the transcriptional stimulation as in the case of PTTH deficient larvae can be compensated by a translational up-regulation upon IIS signaling over-activating and *vice versa*. Our observation that up-regulation of either the PTTH or IIS pathway rescues the non-pupariating defect of the *dSmad2* RNAi larvae lends support to this hypothesis. This hypothesis also provides an explanation for the ability of the PTTH neuron-ablated larvae to undergo metamorphosis after a prolonged larval period (McBrayer et al., 2007). Presumably, it is the IIS signaling pathway that eventually provokes translation of the E biosynthetic enzymes in PTTH neuron-ablated larvae. Of course, future analyses are needed to fully test this idea.

DILP6 could be the ligand that activates the IIS signaling pathway in the PG. DILP6 is not expressed until late 3rd instar stage when larvae cease feeding (Okamoto et al., 2009; Sakurai et al., 1986; Slaidina et al., 2009), which coincides with the timing of metamorphosis. However, the *dilp6* mutants do not show any developmental delays, implying that other DILPs are compensating for the loss of DILP6 in initiating metamorphosis (Slaidina et al., 2009). It is also possible that DILP 3 and DILP5, whose expression is coordinated by nutritional status (Ikeya et al., 2002) are responsible for the activation of IIS signaling in the PG. In this way, the inputs from the environment will be integrated to adjust the timing of metamorphosis.

Activin signaling confers tissue competence

Competence factors have been long been known to play a central role in modulating cellular responses to hormonal signals (Broadus et al., 1999). One generally recognized mechanism through which a factor gives competence to a cell or a tissue is by regulating the expression of the receptor for a particular signal (Shi et al., 1996). One fundamental goal in the competence field is to identify competence factors and understand their molecular actions. Our observation that Activin signaling in the PG regulates the expression of the PTTH receptor Torso and the IIS receptor InR strongly suggests that Activin signaling functions as a competence factor by modulating the PG's ability to respond to PTTH and DILPs. We speculate that the reason for evolving such a competence factor is twofold. First of all, because DILPs are systemic signals, it is essential to develop a mechanism that allows the PG to interpret the systemic signals in a stage-specific manner. Uncontrolled expression of InR would interfere with the timing when the PG can respond to DILPs and thus affect the normal duration of development.

Our observation that genetic manipulation of Activin signaling, which presumably affects the temporal expression of InR, results in either precocious (gain-of-function) or delayed metamorphosis (loss-of-function) highlights the importance of a tissue's timely response to hormonal signals. Secondly, the brain-derived competence factor Actβ may provide a protective mechanism against premature metamorphosis. Since the ligand Actβ is secreted by the neurons in the CNS, the level of Actβ may function as an indicator for the developmental status of the brain. Depending on the hemolymph level of Actβ as reflected by the strength of Activin signaling in the PG, the PG could monitor the readiness of the larval brain to undergo metamorphic transformation.

Whether the regulation of the *torso* and *InR* expression by Activin signaling is direct or indirect remains an open question. Given that dSmad2/Medea functions as a transcription factor, Activin signaling is likely to be directly involved in the transcription of these receptors. Future experiments that aim to identify Activin responsive elements in the promoters of *torso* and *InR* could clarify whether Activin signaling is having a direct effect.

In the mammalian ovary systems, Activin induces the expression of the receptor for follicle stimulating hormone (FSH) in rat granulosa cells (Hasegawa et al., 1988; Xiao et al., 1992), contributing to the stage-specific response of the developing follicles to the FSH stimulation (Knight and Glister, 2001). In this respect, the function of Activin as a competence factor seems to be evolutionarily conserved from insects to mammals. Moreover, the effect of Activin on the FSH receptor is at the transcription level (Nakamura et al., 1993), consistent with our speculation that *Drosophila* Activin signaling may directly regulate transcription of the receptors Torso and InR.

The role of Activin signaling in developmental timing is conserved

Besides *Drosophila*, TGF-β/Activin signaling is also involved in developmental timing controls in other species. For instance, the nematode worm *C. elegans* employs the TGF-β pathway to make a nutritional dependent decision on whether to continue the normal development program into a mature adult or to enter a developmentally arrested stage known as a dauer. The decision depends on nutritional as well as other indicators of environmental conditions (for a review, see Fielenbach and Antebi, 2008). The TGF-β pathway ligand encoded by *daf-7* is expressed in the chemosensory neurons of the amphid sensillum and its expression is modulated by environmental cues sensed by these neurons. When the conditions favor development, Daf-7 is turned on in the amphid neurons and suppresses the formation of dauer. Conversely, when the environment is not suitable for normal development, DAF-7 becomes down-regulated, allowing the entry to the dauer stage. Interestingly, *daf-9*, which encodes a cytochrome P450 protein necessary for production of dafachronic acid, an ecdysone-like steroid hormone, acts downstream of the DAF-7 TGF-β pathway (Gerisch et al., 2001; Jia et al., 2002). Thus, like *Drosophila*, TGF-β signaling in *C. elegans* also exerts development control by regulating cytochrome P450 expression.

In humans, the timing of the pubertal onset is modulated primarily by the hypothalamus-pituitary-gonad axis of the neuroendocrine system (Sisk and Foster, 2004). One key component of this axis is the ability of the reproductive organs to produce sex steroid hormones. Failure to synthesize sufficient amounts of sex steroids severely hinders the normal onset of puberty as manifested in disorders such as Klinefelter syndrome and Turner syndrome (Kesler, 2007; Smyth and Bremner, 1998). Although the importance of sex steroid hormones is paramount, their regulation is not well understood. Several lines of evidence from multiple species have demonstrated the

involvement of Activin signaling in steroid hormone synthesis in mammalian ovaries. For example, Activin was found to enhance the activity of CYP 450 aromatase and stimulate estradiol production (Hutchinson et al., 1987; Miro et al., 1991; Miro and Hillier, 1992). Our observation that *Drosophila* Activin signaling is also required for steroid hormone synthesis by regulating the expression of cytochrome P450 proteins strongly suggests that the function of this ancient signaling pathway in developmental transitions is conserved. Therefore, studies on how Drosophila Activin signaling modulates developmental timing may have significant relevance to our understanding of how human puberty is initiated.

In addition to Activin signaling, IGF signaling, which is highly conserved from flies to humans, also modulates puberty timing (Hiney et al., 1996). Mammalian insulin-like growth factors (IGF), which are homologous to *Drosophila* DILPs in particular to DILP6 (Okamoto et al., 2009), have been shown to play critical roles in steroidogenesis during ovarian follicular development and maturation (Silva et al., 2009). However, whether the actions of IGFs are dependent on Activin signaling has not been investigated. Our findings that *Drosophila* insulin signaling is modulated by Activin signaling indicate that these two signaling pathways may also interact with each other in mammals and therefore provide insights into the mechanisms that control the actions of IGFs in human ovary.

Tissue-specific knockdown has a more severe metamorphosis phenotype than null mutants

In *Drosophila*, *dSmad2* is an essential gene and involved in multiple aspects of development such as neuroblast proliferation (Zhu et al., 2008) and adult neuron differentiation (Zheng et al., 2006). While the majority of the *dSmad2* null mutants

display a high degree of larval lethality, a small percentage of them successfully undergo metamorphosis although they display lethality in a much later pharate adult stage (Figure 2-20A). This observation is in direct contrast to the non-pupariating phenotype of the *dSmad2* RNAi larvae. Moreover, the *dSmad2* null mutants that survive to the wandering stage have proper expression of the Dib protein in the PG (Figure 2-20B), suggesting that they do not suffer from the low ecdysteroid defect as *dSmad2* RNAi larvae. Similarly, *babo* null mutants are also able to initiate metamorphosis and express Dib in the PG (Figure 2-20B'), further suggesting that PG specific loss of Activin signaling components results in more severe developmental timing defects than null mutants. To rule out the possibility that this phenomenon is an artifact of RNAi, we used *daughterless*-Gal4, a ubiquitous driver to knockdown *dSmad2*. Unlike *phm*-Gal4>*dSmad2* RNAi animals, the *daughterless*-Gal4>*dSmad2* RNAi larvae reach relatively normal size at the end of their third instar stage and a small percentage of them are able to initiate metamorphosis. These phenotypes are similar to *dSmad2* nulls, indicating that the effect of RNAi is specific. Since the *dSmad2* RNAi larvae differ from the *dSmad2* null larvae by the presence of dSmad2 in peripheral tissues, we propose two models, namely the inductive model and repressive model that could provide possible explanations to the apparent discrepancy in terms of the ability to initiate metamorphosis (Figure 2-21). In the inductive model, the biosynthesis of E is under the positive influence of both dSmad2 in the PG and an inducing signal X(i) from periphery. DSmad2 in the peripheral tissues functions as a repressor that keeps X(i) inactive. In the wild type larvae, although X(i) is repressed, the presence of dSmad2 in the PG is sufficient to maintain normal E biosynthesis. In *dSmad2* RNAi larvae, the loss of dSmad2 in the PG affects E biosynthesis and therefore the larvae fail to initiate metamorphosis due to the lack of E. In *dSmad2* null larvae, the absence of dSmad2

Figure 2-20:

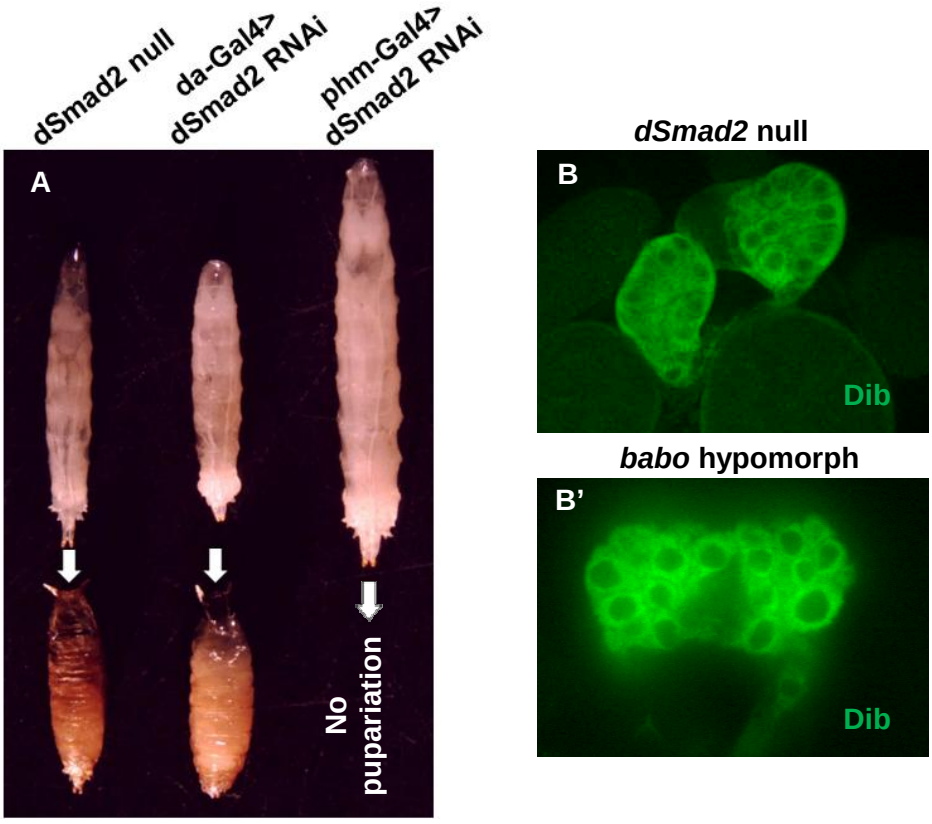

dSmad2 null
da-Gal4> dSmad2 RNAi
phm-Gal4> dSmad2 RNAi
A
No pupariation
dSmad2 null
B
Dib
babo hypomorph
B'
Dib

Figure 2-20. The *dSmad2* RNAi larvae have more severe developmental phenotype than *dSmad2* null mutants.

(A). In contrast to the non-pupariating phenotype of dSmad2 RNAi larvae, *dSmad2* null mutants are able to initiate metamorphosis despite high larval lethality. The *dSmad2* null mutants exhibit lethality at the pupa stage. Ubiquitous knockdown of *dSmad2* using *daughterless*-Gal4 (*da*-Gal4) gives rise to a similar phenotype to *dSmad2* null mutants. Compare to the large size of the *dSmad2* RNAi larvae, the *dSmad2* null and *da*-Gal4>*dSmad2* RNAi larvae have relatively normal size at the wandering stage.

(B). The Dib protein is strongly expressed in the PG of *dSmad2* null and babo hypomorphic mutants.

from periphery leads to the disinhibition of X(i), which in turn acts on the PG to promote E synthesis. Thus, *dSmad2* null mutants produce normal amounts of E for metamorphosis initiation. In the repressive model, the E biosynthesis is negatively modulated by a repressive signal X(r) from periphery. This repressor is induced by dSmad2 present in the peripheral tissues. The function of dSmad2 in the PG is to suppress the repressive effect of X(r) on E biosynthesis and thus ensure the proper production of E from the PG in the wild type situation. In *dSmad2* RNAi larvae, the loss of dSmad2 in the PG relieves the suppression on the inhibitory effect by X(r) and subsequently E biosynthesis is repressed. In *dSmad2* null larvae, the additional loss of dSmad2 in the periphery causes down-regulation of X(r). Consequently, E synthesis is resumed in the absence of the repressor X(r) and allows the larvae to undergo metamorphosis.

Because IGFs in mammals can trigger early onset of puberty (Hiney et al., 1996), the *Drosophila* insulin-like peptides are good candidates for the inducer. Among the seven insulin-like peptides in *Drosophila*, DILP6, which exhibits the closest structural resemblance to vertebrate IGFs and which shows a drastic increase in gene expression before the start of metamorphosis (Okamoto et al., 2009; Slaidina et al., 2009; Slaidina et al., 2009), may function as a potent inducer. Consistent with the inductive model, we observed that the amount of the *dilp6* mRNA of *dSmad2* null larvae is comparable to that of the wild type at the white pupa stage, whereas the transcript level of *dilp6* of *dSmad2* RNAi larvae fails to rise (Figure 2-22). To further test if DILP6 could act as an inducer, we ectopically expressed *dilp6* in the fat bodies of the *dSmad2* RNAi larvae using *cg*-Gal4, a fat body driver. Nonetheless, the *cg*-Gal4; *phm*-Gal4>*dSmad2* RNAi; UAS-*dilp6* animals still arrest in the third instar larval stage. Perhaps the expression level of *dilp6* by *cg*-Gal4 is not sufficient to elicit metamorphosis, especially considering the InR level

Figure 2-21:

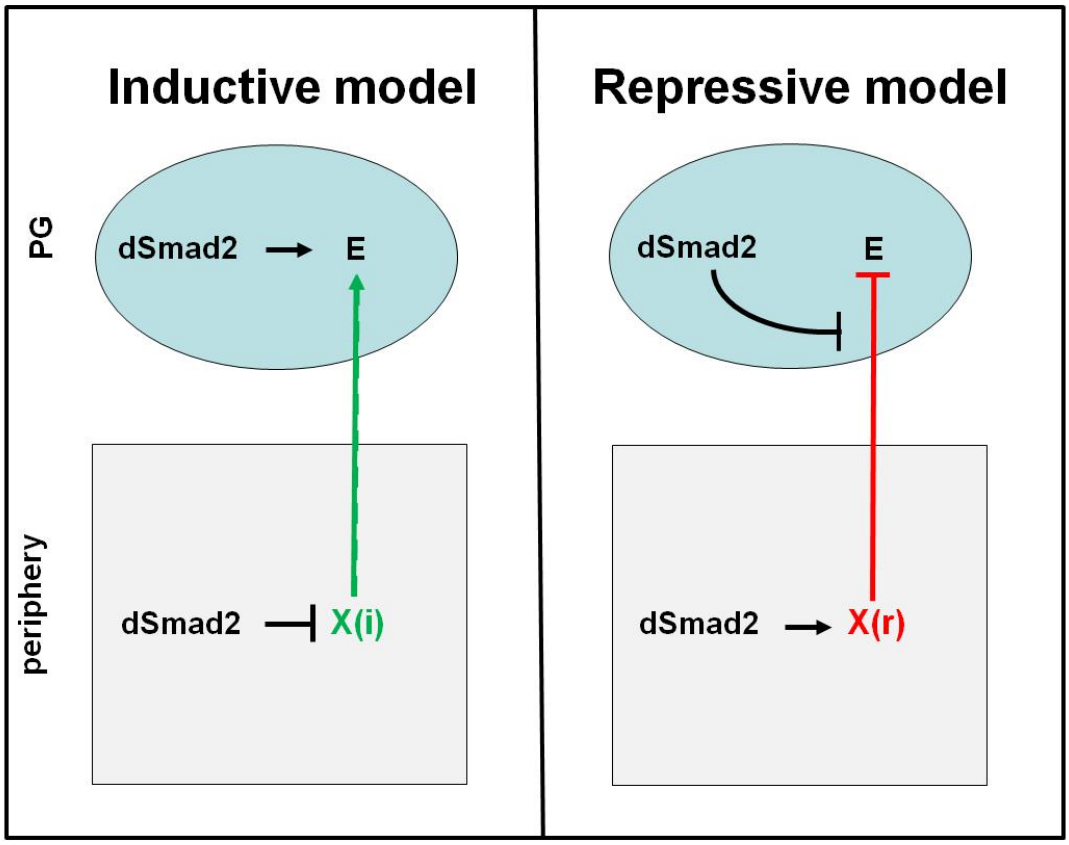

Inductive model
Repressive model
PG
periphery
dSmad2
E
dSmad2
X(i)
dSmad2
E
dSmad2
X(r)

Figure 2-21. The inductive and repressive models.

The models that explain the dissimilar metamorphosis phenotypes observed in dSmad2 RNA i larvae and dSmad2 null mutants. Refer to the text for details.

is drastically reduced in *dSmad2* RNAi larvae.Alternatively, DILP6 may not be able to stimulate metamorphosis. Given that *dilp6* is inducible by 20E (Okamoto et al., 2009; Slaidina et al., 2009) and the 20E titer attains its maximum level at the white pupa stage, the wild type level of *dilp6* in the *dSmad2* nulls is likely a consequence instead of a cause of the initiation of metamorphosis.

In summary, the results from PG specific knockdown of *dSmad2* has uncovered an important role of Activin signaling in ecdysteroidogenesis which otherwise would be masked by the compensatory mechanisms induced in the null mutants.

Future directions

To further test the inductive/repressive models, it is key to identify the inducer/repressor. Although DILP6 is unable to induce metamorphosis in *dSmad2* RNAi larvae, it is possible that the expression of *dilp6* under the Gal4 drivers we used did not reach an effective level to trigger metamorphosis. Alternatively, other signaling molecules are responsible for initiating metamorphosis in *dSmad2* null mutants. In *Manduca*, for instance, in addition to PTTH, a peptide known as the small PTTH exerts stimulatory effect on E synthesis (Marchal et al., 2010). Therefore, it is likely that *Drosophila* may contain other peptides that function like the small PTTH in *Manduca*.

For the repressive model, one good candidate for the repressor is juvenile hormone (JH) whose inhibitory effect on E production has been well characterized (Nijhout and Williams, 1974; Rountree and Bollenbacher, 1986). It would be interesting to measure the JH titer in the *dSmad2* RNAi larvae. If JH contributes to the low ecdysteroid titer, we anticipate seeing an elevated JH level in *dSmad2* RNAi larvae. Static factors from the imaginal discs also have the potential to act as the repressor.

Figure 2-22:

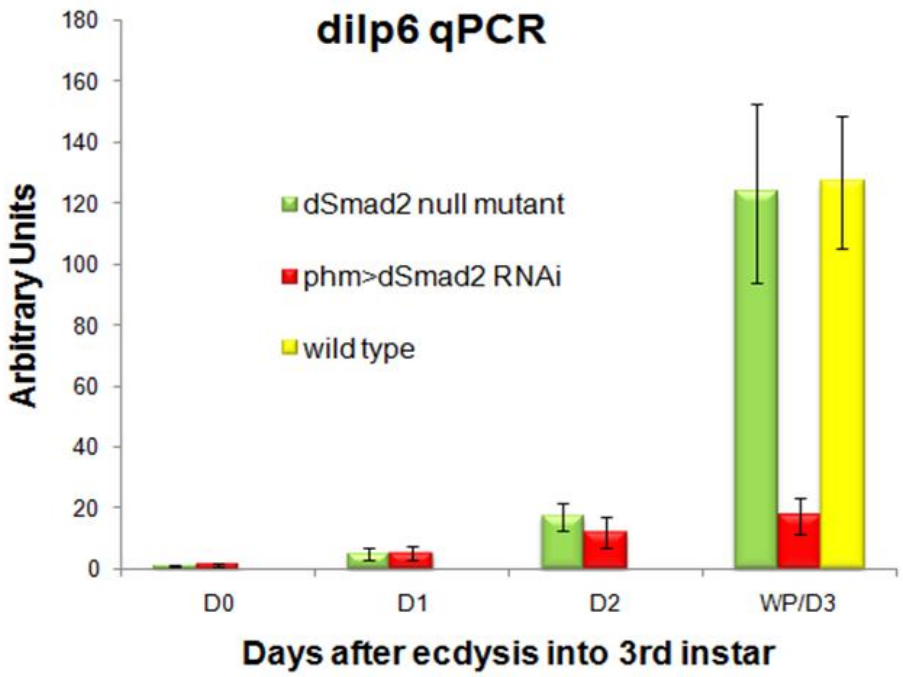

dilp6 qPCR
Arbitrary Units
180
160
140
120
100
80
60
40
20
0
dSmad2 null mutant
phm>dSmad2 RNAi
wild type
D0
D1
D2
WP/D3
Days after ecdysis into 3rd instar

Figure 2-22. The expression of dilp6 in *dSmad2* null and *dSmad2* RNAi larvae.

The temporal expression profile of *dilp6* during the third larval instar stage was analyzed by qPCR. In both the *dSmad2* null and *dSmad2* RNAi larvae, the expression of *dilp6* is gradually increasing from D0, the beginning of the third instar stage to D2, two days after ecdysis into the third instar stage. During this period, the levels of dilp6 in the *dSmad2* null and *dSmad2* RNAi larvae are comparable. Due to delayed larval development of dSmad2 null larvae, they enter the white pupa (WP) stage at D3. At this stage, dSmad2 null larvae show a significantly higher *dilp6* mRNA level than the *dSmad2* RNAi larvae. The level of *dilp6* of the *dSmad2* null larvae at the WP stage is similar to that of control larvae at the WP stage. At each time point, total mRNA was isolated from 5 larvae per genotype. For statistical analysis, three independent biological samples were collected. The ribosomal PL23 gene is used as the reference gene.

Damaged imaginal discs delay the onset of metamorphosis (Halme et al., 2010; Hussey et al., 1927; Simpson et al., 1980). Therefore, certain factors induced by the damaged discs relay information to the developmental control mechanism so that the repair of the discs can be coordinated with the developmental program. Similarly, the retinoic acid (RA) pathway promotes developmental arrest upon disc damage (Halme et al., 2010). To examine if RA is involved in developmental arrest resulting from *dSmad2* RNAi in the PG, we will analyze whether mutations such as *ninaB*, *santa maria*, *Formaldehyde dehydrogenase (Fdh)* and *Aldh* that are involved in RA synthesis (Halme et al., 2010) can suppress the non-pupariating phenotype. Additionally, several neuropeptides collectively known as the prothoracicostatic factors have been discovered in *Bombyx* that possess inhibitory effects on ecdysteroidogenesis in the PG (Yamanaka et al., 2005; Yamanaka et al., 2006). *Drosophila* may also express similar neuropeptides that function as potent repressors to antagonize E synthesis.

Another key issue regarding the model is the precise identity of the peripheral tissue(s) where the action of dSmad2 leads to suppressed E synthesis. One strategy to address this question is to perform a screen that utilizes individual tissue specific Gal4 drivers in combination with the PG Gal4 driver (*phm*-Gal4) to knockdown *dSmad2*. Presumably, knockdown of *dSmad2* in both the PG and this yet-to-be-identified peripheral tissue will produce similar phenotype, i.e. being able to undergo metamorphosis, as the *dSmad2* null mutants.

Intriguingly, when we searched the literature for similar examples, we noticed that a gene known as *slimfast* (*slif*) that encodes an amino acid transporter, when knocked down in fat bodies, displays more severe growth defects than *slif* nulls (Colombani et al., 2003). So, this novel finding that null mutations result in more severe phenotypes than tissue-specific knockdowns is not specific to *dSmad2*. Further investigation is required to

determine whether this finding reflects a general phenomenon and what the evolutionary significance associated with it may be.

Experimental Procedures

Drosophila Strains and husbandry

All the fly stocks and crosses were maintained on standard cornmeal food at 25℃. The fly strains used include *daw*-Gal4 (Zhu et al., 2008), *actβ*-Gal4 (Ting et al., 2007), UAS-babo*(Brummel et al., 1999) and *phm*-Gal4 (Ono et al., 2006), UAS-Flag-dSmad2 (Ting et al., 2007). UAS-*dSmad2* RNAi (#14609 and #105687), UAS-*babo* RNAi (#3825 and #853), UAS-*punt* RNAi (#848 and #37279) and UAS-*medea* RNAi (#19688 and #19689) were obtained from the Vienna *Drosophila* RNAi center. UAS-nuGFP (#4775), UAS-Akt (#8191), UAS-InR (#8262) were obtained from the Bloomington *Drosophila* Stock Center. UAS-Daw and UAS-Actβ were generated in a y,w^{1118} background by using standard methods. UAS-Ras^{V12} and *tub*-PH-GFP are kind gifts from Dr.Neufeld.

Staging Larvae

Females were allowed to lay eggs on apple juice agar plates for 6 hrs. Larvae were synchronized at the L2-to-L3 transition. The newly molted third instar larvae were then collected, transferred into vials containing standard cornmeal food and allowed to develop to the desired time points. The larvae were reared inside an insulated, moist chamber at 25℃–28℃ under a 12 hr light/dark cycle.

20E Feeding

The 20E feeding experiment was performed as described (McBrayer et al., 2007).

Whole-Body Ecdysteroid Titer Determination

50 Larvae per genotype per time point were collected and frozen at -80^{o}C. The following procedures for ecdysteroid extraction and determination by radioimmunoassay can be found in Warren et al., (2006).

In Situ Hybridization

The procedures for *in situ* hybridization and for generating the labeled probes were followed according to Chavez et al. (2000). cDNAs were used to generate both the anti-sense and sense probes.

Immunofluorescence

Brian-Ring Gland complexes were dissected in pre-chilled PBS, fixed in 3.7% formaldehyde in PBS for 20 min at room temperature and were washed extensively in PBS with 0.1% Triton X-100 (PBT). The primary antibodies used in this study include rabbit anti-PHM, 1:1000 (Parvy et al., 2005), rabbit anti-DIB, 1:1000 (Parvy et al., 2005), rabbit anti-Sad, 1:1000 (raised against the peptide sequence CIRVQEDQRRPHDEA), guinea pig anti-Spo/Spok, 1:100 (raised against the peptide sequence CDWSQLQQKRRNLARRH), and mouse monoclonal anti-MAPK activated, 1:10,000 (Sigma, M8159). Guinea pig anti-DIMM antibody, 1:500 is a kind gift from P. Taghert. Fluorescent conjugated secondary antibodies (ALEXA FLUOR®) were purchased from Invitrogen and were used at a 1:500 dilution. DNA was stained by DAPI (1 µg/mL) in PBT for 5 min (Sigma). All primary and secondary antibodies were diluted in PBT. Primary antibody incubation was carried out at 4℃ ove rnight and secondary antibody

incubation was performed at room temperature for 2 hrs. DNA was stained by DAPI. Images were acquired using a Zeiss LSM710 laser scanning confocal microscope.

qRT-PCR

For each sample, 10 ring gland-brain complexes were dissected and stored in TRIzol® Reagent (Invitrogen). Total RNA was isolated by using RNeasy mini kit and RNase-Free DNase Set (QIAGEN). Reverse transcription was carried out by using ThermoScript RT PCR System (Invitrogen). qRT-PCR was performed on Roche LightCycler® 480 System (Roche) using LightCycler® DNA Master SYBR Green I (Roche). The amount of *Drosophila* ribosomal protein L23 (rpL23) was used to normalize the transcript level of the genes examined in the same sample. Three independent biological samples for each genotype at each specific time point were collected to reduce variation. The qRT-PCR primers used are listed in Table S1.

Western blotting

Western blotting was carried out according to standard protocols. The primary antibodies include mouse anti-α-tubulin, 1:5,000 (Sigma, # T9026), rabbit anti-PHM, 1:1000 (Parvy et al., 2005), rabbit anti-DIB, 1:1000 (Parvy et al., 2005), rabbit anti-Sad, 1:1000 (raised against the peptide sequence CIRVQEDQRRPHDEA), guinea pig anti-Spok, 1:5,000 (raised against the peptide sequence that matches the Spok protein (FBgn0086917) from amino acid 356 to 485). The secondary antibodies used are IRDye 700 and 800, 1:10,000 and blots were scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Chapter 3: Dual Function of *Drosophila* Sno: an antagonizer of BMP signaling but a mediator of Activin signaling

Introduction

Ski was originally identified as the oncogenic component of the avian Sloan-Kettering virus which has the ability to transform chick embryo fibroblasts (Li et al., 1986). This *v-ski* oncogene arises from a deletion event of a nuclear protein encoded by *c-ski* and only contains the N-terminus of the c-Ski protein. Another important member of the *ski* proto-oncogene family is *sno* (*ski*-related *no*vel gene), which was discovered by probing a human cDNA library with the *v-ski* probe (Nagase et al., 1993). The *ski/sno* proto-oncogene family is highly conserved and can be found in a variety of species including humans (Nagase et al., 1993; Pearson-White, 1993; Pearson-White and Crittenden, 1997), mouse (Namciu et al., 1995), chicken (Stavnezer et al., 1986), zebra fish (Kaufman et al., 2000) and *Xenopus* (Sleeman and Laskey, 1993).

The transforming property of Ski has been mapped to the N-terminal region of the protein (Zheng et al., 1997). Sno, which shares a high sequence homology with Ski over a large region on the N-terminus, when expressed at high levels, is also capable of transforming chicken embryo fibroblasts (Boyer et al., 1993). The ability of Ski/Sno to transform cells is attributed to their antagonizing roles on the signal transduction activated by TGF-β, a ubiquitous cytokine that has growth inhibitory effects on epithelial, endothelial and lymphoid cells (Bachman and Park, 2005; Zhu et al., 2005). The inhibitory effect of the Ski/Sno proteins on TGF-β signaling arise from their ability to bind directly to the Smad2, Smad3 and Smad4 proteins (Liu et al., 2001; Luo, 2004). This binding is believed to disrupt the proper formation of active Smad complex and displace

the transcriptional co-activator p300/CBP (Akiyoshi et al., 1999; Wu et al., 2002), thereby prohibiting Smad proteins from activating the target genes. Additionally, Ski/Sno can recruit a number of transcription repressors such as N-CoR, histone deacetylase and mSin3 to the promoter regions of Smad target genes so that the genes that are involved in repressing growth and proliferation are silenced (Akiyoshi et al., 1999; Luo et al., 1999; Sun et al., 1999a). Indeed, in many cell types, over-expression of Ski/Sno severely compromises the activation of TGF-β target genes and therefore relieves the cell cycle arrest induced by TGF-β (Stroschein et al., 1999; Wang et al., 2000). Consistent with the oncogenic property of the Ski/Sno proteins, high levels of Ski/Sno are detected in a number of human tumor cell lines obtained from melanoma, carcinomas of esophagus and breast (Fumagalli et al., 1993; Imoto et al., 2001; Zhang et al., 2003). Interestingly, the expression of Ski/Sno is regulated by TGF-β signaling. Addition of TGF-β to cells results in rapid degradation of Ski/Sno via the ubiquitin-proteasome pathway (Stroschein et al., 1999; Sun et al., 1999b). However, prolonged treatment of TGF-β on the other hand leads to an elevation in the amount of the Sno protein (Stroschein et al., 1999). It was thus proposed that the initial down-regulation of Ski/Sno permits the transcriptional activation of TGF-β target genes whereas the gradual up-regulation of these proteins serves as a negative feedback mechanism that eventually turns off TGF-β signaling (Stroschein et al., 1999). According to this model, the high levels of Ski/Sno in tumor cells saturated the capacity of the protein degradation machinery so that excessive Ski/Sno renders the tumor cells resistance to the growth inhibitory effect of TGF-β signaling (Liu et al., 2001)(see Figure 3-1).

While the oncogenic property of over-expressed Ski/Sno has been well documented, the physiological functions of the endogenous Ski and Sno in relation to

Figure 3-1:

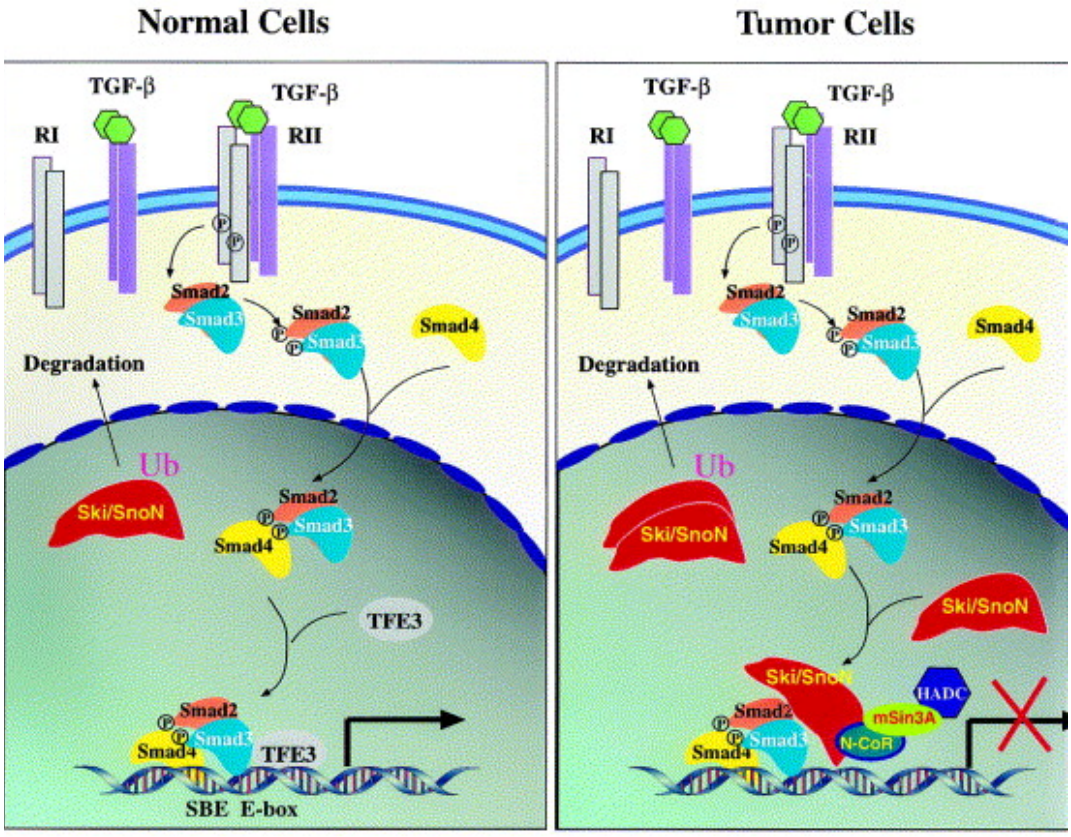

Normal Cells
Tumor Cells
TGF-β
TGF-β
RI
RII
Smad2
Smad3
Smad4
Degradation
Ub
Ski/SnoN
TFE3
SBE
E-box
HADC
mSin3A
N-CoR

Figure 3-1. The mechanisms by which Ski/Sno antagonizes TGF-β signaling and contributes to tumorous growth.

In normal cells, TGF- β signaling is initiated by the binding of the ligand to a type I/type II tetrameric receptor complex. The signal is then transmitted by Smad2, Smad3 and Smad4, which associate to form an active transcription complex. This complex interacts with other transcription activators such as TEF3, binds to Smad Binding Elements (SBE) located in the promoter regions of the genes, and activates the transcription of these genes that are involved in growth arrest. The endogenous Ski/Sno is degraded via the ubiquitin/proteosome pathway. In tumor cells, Ski/Sno is over-expressed and cannot be completely degraded by the ubiquitin/proteosome machinery. The accumulated Ski/Sno binds to and thus disrupts the formation of an active Smad2/3/4 complex. In addition, Ski/Sno recruits transcription repressors such as N-CoR, mSin3A and HADC to the TGF-β responsive genes and suppress their transcription. As a result, cells escape from the growth inhibitory effect of TGF signaling and undergo uncontrolled growth. (Adapted from Liu X., Sun Y., Weinberg R.A. and Lodish H.F. 2001. Ski/Sno and TGF-beta signaling. Cytokine Growth Factor Rev. 12:1-8.)

TGF-β signaling remain obscure. While the endogenous *ski* is ubiquitously expressed ata low level in the adult mice (Nomura et al., 1989), *ski* is mainly detected in the neural tube, neural crest cells and skeletal muscles during embryonic development (Lyons et al., 1994; Namciu et al., 1995). Consistent with this expression pattern, the ski null mice display defects in neural tube closure, craniofacial abnormalities and decreased muscle mass (Berk et al., 1997; Colmenares et al., 2002). Similar craniofacial phenotypes have also been observed in mice over-expressing a BMP signaling target gene Msx2 (Winograd et al., 1997). Given that Ski can inhibit BMP signaling by binding and thus inactivating the Smad complex responsible for transmitting BMP signaling (Wang et al., 2000), the craniofacial defects in *ski* null mice are likely a consequence of up-regulated BMP signaling. However, whether the abnormalities in neural tube formation and skeletal muscle development are related to Ski's antagonistic role in TGF-β signaling is unclear. Similar to *ski*, the expression of the endogenous *sno* gene in adult mice is relatively low (Pearson-White and Crittenden, 1997). Knockdown of *sno* by RNAi has demonstrated that *sno* can mediate TGF-β signaling in a cell type-specific manner (Sarker et al., 2005). The function of the endogenous *sno* was also addressed by constructing *sno* knockout mice. One *sno* knockout mouse line uncovered an essential function of *sno* during embryonic development: *sno* null mice died at an early embryonic stage with defects in blastocyst formation (Shinagawa et al., 2000). Intriguingly, the same study also showed that the $sno^{+/-}$ heterozygote mice had a higher incidence of tumor formation, indicating a tumor suppressor role of the endogenous *sno* gene (Shinagawa et al., 2000). Based on the above observations, endogenous *sno* may mediate TGF-β signaling rather than antagonizing TGF-β signaling. Nonetheless, the mechanism by which *sno* can serve this dual role remains completely unknown. Paradoxically, another independently generated

sno null mouse line was viable but had T cell activation defects (Pearson-White and McDuffie, 2003). Therefore, no definite conclusion can be drawn on the role of endogenous *sno* in vertebrates.

The nematode *C. elegans* also has a Ski/Sno homolog encoded by *daf-5*, which plays a critical role in determining the entry into the dauer stage (Fielenbach and Antebi, 2008). DAF-5 is able to bind to DAF-3, a Smad protein in the TGF-β pathway. The domain of DAF-5 that mediates DAF-5/DAF-3 interaction is highly conserved which is responsible for the Ski/Smad interaction in vertebrates (da Graca et al., 2004). Intriguingly, genetic analysis revealed that DAF-5 may function as a co-factor for DAF-3 based on similar loss-of-function phenotypes (Thomas et al., 1993). However, DAF-3 is an unconventional Smad because its activity, instead of being activated, is inhibited by TGF-β signaling (Fielenbach and Antebi, 2008). This is contrast to the conventional TGF-β pathway in vertebrates where the activities of Smads are activated by signaling (Feng and Derynck, 2005). Therefore, *C. elegans* is not an ideal system to address the function of Ski/Sno regarding to TGF-β signaling. *Drosophila*, on the other hand, which contains one *sno* homolog and where TGF-β pathways are highly conserved, provides a simple and convenient system that allows us to investigate the relation between endogenous *sno* and TGF-β signaling. In this study, we found that *Drosophila* Sno can promote Activin/Babo signaling while inhibiting Dpp signaling by shunting Medea from Mad to dSma2 (Takaesu et al., 2006).

Results

Because this work (Takaesu et al., 2006) is a collaborative effort, I marked the parts accomplished by me as “my work included in the paper”. For the data that are not

Figure 3-2:

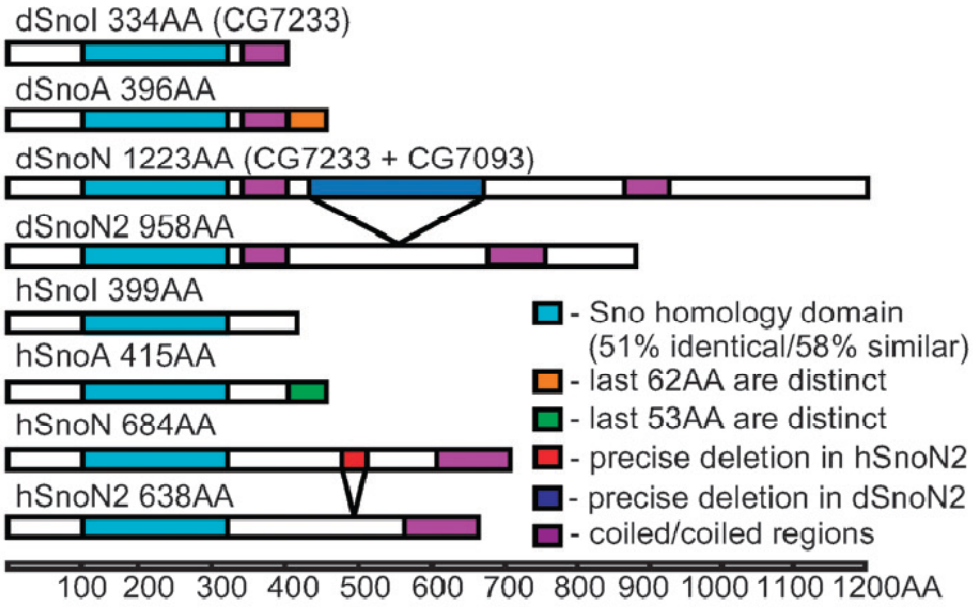

dSnoI 334AA (CG7233)
dSnoA 396AA
dSnoN 1223AA (CG7233 + CG7093)
dSnoN2 958AA
hSnoI 399AA
hSnoA 415AA
hSnoN 684AA
hSnoN2 638AA
- Sno homology domain (51% identical/58% similar)
- last 62AA are distinct
- last 53AA are distinct
- precise deletion in hSnoN2
- precise deletion in dSnoN2
- coiled/coiled regions
100 200 300 400 500 600 700 800 900 1000 1100 1200AA

Figure 3-2. *Drosophila* Sno protein has four isoforms that correspond to the four human isoforms.

The four dSno protein isoforms identified are dSnoI, dSnoA, dSnoN and dSnoN2 with dSnoN being the longest isoform. dSnoI and dSnoA are encoded by *CG7233* while dSnoN and dSnoN2 contain regions that are encoded by *CG7093* in addition to *CG7233*. All dSno isoforms possess the Sno homology domain at the N terminus and the coiled-coil domains at the C terminus. (Adapted from Takaesu et al., 2006. dSno facilitates baboon signaling in the *Drosophila* brain by switching the affinity of Medea away from Mad and toward dSmad2. Genetics. 174:1299-313.)

included in the paper, I annotated those as “unpublished results”.

dSno identification and null allele generation

The *Drosophila* database listed CG7233 as the *Drosophila* homologue of the *sno* gene and annotated it as *snoN*. By probing the *Drosophila* cDNA libraries with CG7233, a total of 12 different cDNAs were isolated which encode 4 protein isoforms (figure 3-2) that correspond to the four human Sno isoforms namely SnoN, SnoN2, SnoA and SnoI with SnoN as the longest isoform (Pearson-White and Crittenden, 1997; Pearson-White and McDuffie, 2003). The *CG7233* gene, which was annotated as *snoN* actually encodes the shortest isoform *dSnoI*. The longest isoform *dSnoN* is encoded by both *CG7233* and a downstream gene *CG7093*. All four *Drosophila* Sno (dSno) isoforms contain the Sno homology domain on the N terminus which is 51% identical to the human Sno homology domain. At the C terminus, dSno isoforms have coiled-coil motifs similar to their human homologues.

In order to study the role of the endogenous sno gene, it was essential to generate *dSno* null mutations. One way to generate null alleles in *Drosophila* is by imprecise P element excision. In this method, a P element that localizes near the gene of interest is excised by expressing a transposase, the enzyme catalyzing this excision event. At a certain frequency, the excision of the P element also carries away a genomic region that contains the gene of interest, resulting in the generation of a null allele. By searching the fly database, a single P element insertion close to CG7233 was found in a fly line bearing the *l(2)sh1402* allele which is homozygous lethal at the pupa stage. Because the P element is also close to a neighboring gene *CG7231*, it was possible that the lethality of *l(2)sh1402* might be due to loss of *CG7231* function instead of *dSno*. To eliminate this possibility, a rescue experiment was performed in which a UAS-*dSno*

(*CG7233*) transgene was expressed by a heat-shock Gal4 driver in the *l(2)sh1402* homozygous animals. Expression of *dSno* indeed rescued the lethality of the *l(2)sh1402* homozygous animals to the adult stage, demonstrating that the P element insertion in the *l(2)sh1402* disrupts the *dSno* gene. This allele was thus a *dSno* null or strong hypomorphic allele and is designated as $dSno^{sh1402}$. From $dSno^{sh1402}$, two *dSno* deletion alleles, $dSno^{EX17B}$ and $dSno^{EX4B}$, were generated using the imprecise P element excision method. They were considered as *dSno* mutant alleles based on their inability to complement the $dSno^{sh1402}$ alleles. Animals with the genotype $dSno^{sh1402}/dSno^{EX4B}$ displayed lethality at larval-to-pupal transition, which is earlier than the $dSno^{sh1402}/dSno^{EX17B}$ heterozygous animals, suggesting that $dSno^{sh1402}/dSno^{EX4B}$ represents the strongest hypomorphic combination. So, we used the $dSno^{sh1402}/dSno^{EX4B}$ heterozygous animals as the most severely affected *dSno* mutant.

dSno mutants show a similar brain phenotype to Activin signaling mutants (my work included in the paper by Takaesu et al., 2006)

Analysis on the expression pattern of *dSno* revealed strong *dSno* expression in the CNS at both the embryonic stage and third instar larval stage (Figure 3-3). This expression pattern intrigued us to examine the CNS of *dSno* mutants for any abnormalities. We found that *dSno* mutant animals have a small brain, which is reminiscent of strong *babo* and *dSmad2* mutant brain phenotypes. Babo and dSmad2 are *Drosophila* Activin signaling transducers (Brummel et al., 1999). The *dSmad2* and *babo* mutants have severe defects at the optic lobes where the axons of the photoreceptors innervate the lamina and medulla (Zhu et al., 2008) (Figure 3-4). Normally, the photoreceptors R1-R6 extend axons to the lamina, whereas R7 and R8 project their axons to the medulla where they form a lattice-like network (Figure 3-4). In

Figure 3-3:

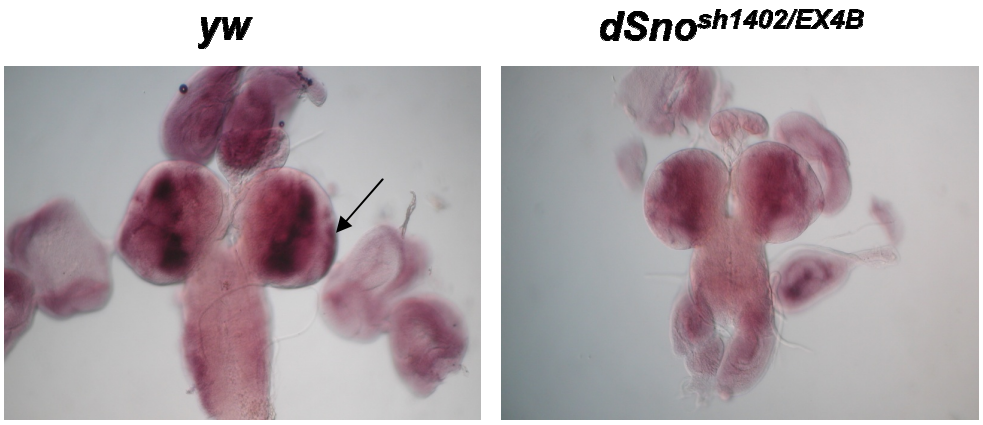


Figure 3-3. The *dSno* transcript is strongly expressed in the larval CNS.

In situ hybridization reveals strong *dSno* expression in the brain of a third instar wild type larva *(yw*). Note the *dSno* mRNA is enriched in the optic lobes where the photoreceptors innervate the brain (arrow). The expression of *dSno* is dramatically reduced in a *dSno* hypomorphic mutant CNS ($dSno^{sh1402/EX4B}$). The presence of low amounts of the *dSno* transcript is likely because the P element inserted in the 5' region of the *dSno* gene in the $dSno^{sh1402}$ allele interferes with, but does not completely eliminate, *dSno* transcription. Data are not shown for the negative staining of the sense probe.

Figure 3-4:

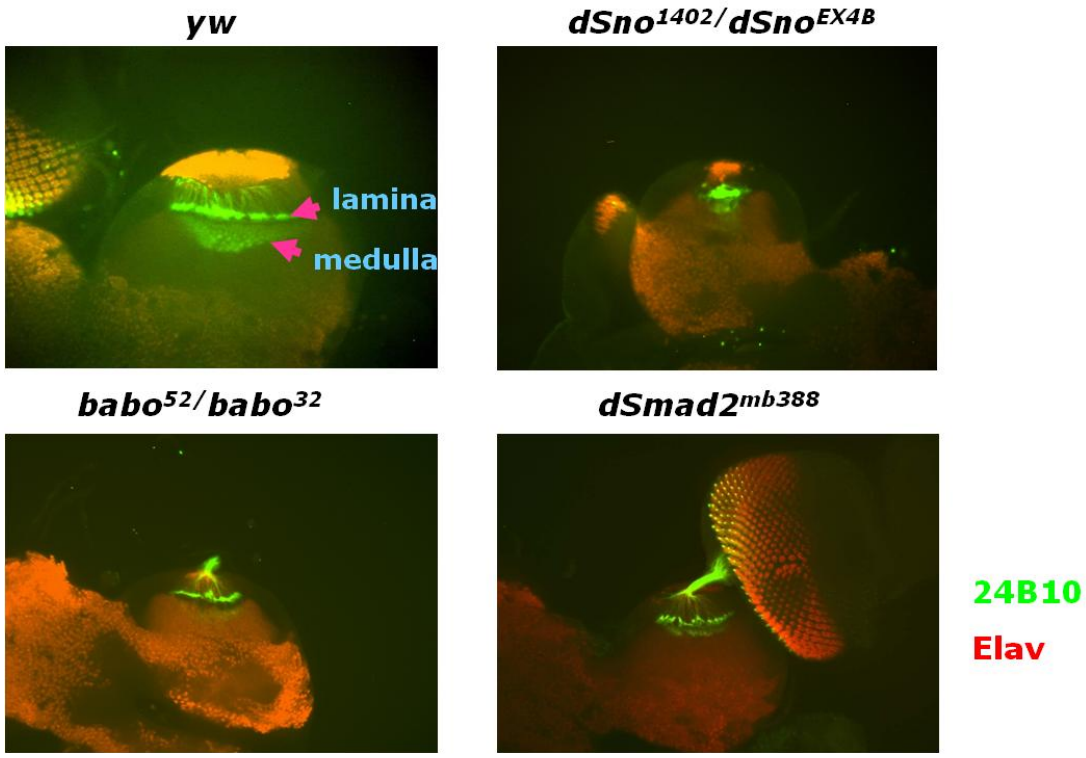

yw
dSno1402/dSnoEX4B
lamina
medulla
babo52/babo32
dSmad2mb388
24B10
Elav

Figure 3-4. The *dSno*, *babo* and *dSmad2* mutants have defects in optic lobes.

Optic lobes stained with anti-24B10 (green) and Elav (red) antibodies. In the wild type, note the large lamina cap, lamina plexus (arrowhead) and R7–R8 lattice area in the medulla (arrow). In all mutants, these areas are much reduced.

Figure 3-5:

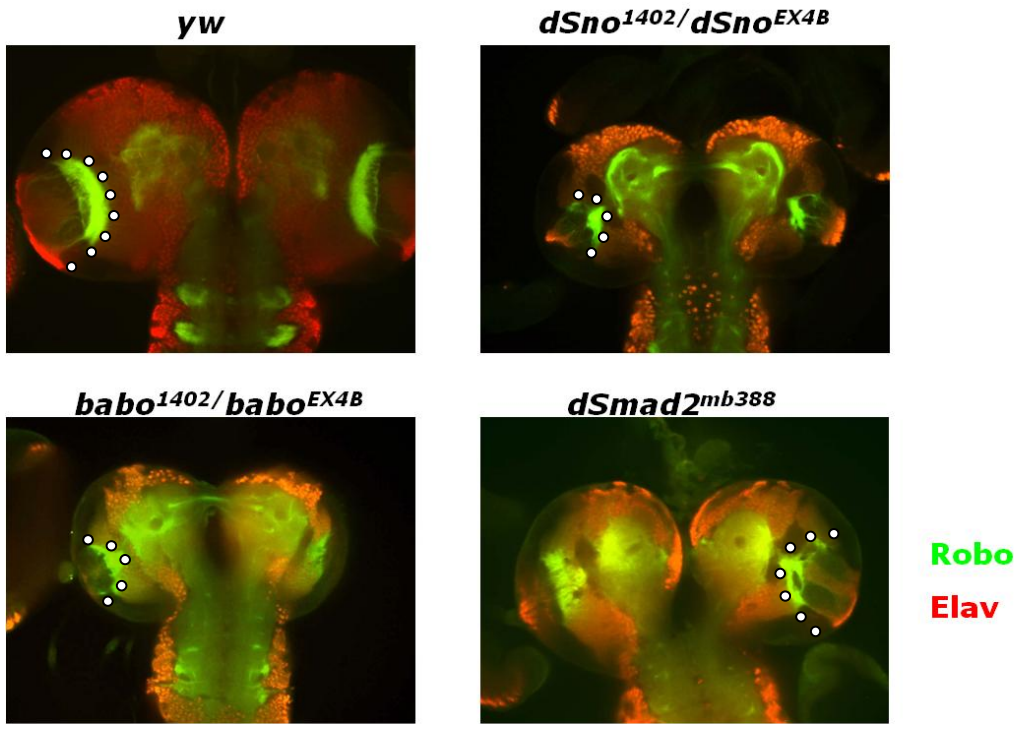

yw
dSno1402/dSnoEX4B
babo1402/baboEX4B
dSmad2mb388
Robo
Elav

Figure 3-5. The *dSno*, *babo* and *dSmad2* mutants have reduced neuropil.

Larval brains stained with anti-Robo (green) and Elav (red). The white dots outline Robo staining in the medulla neuropil. All mutants have the reduced neuropil which is also altered in its morphology.

Figure 3-6:

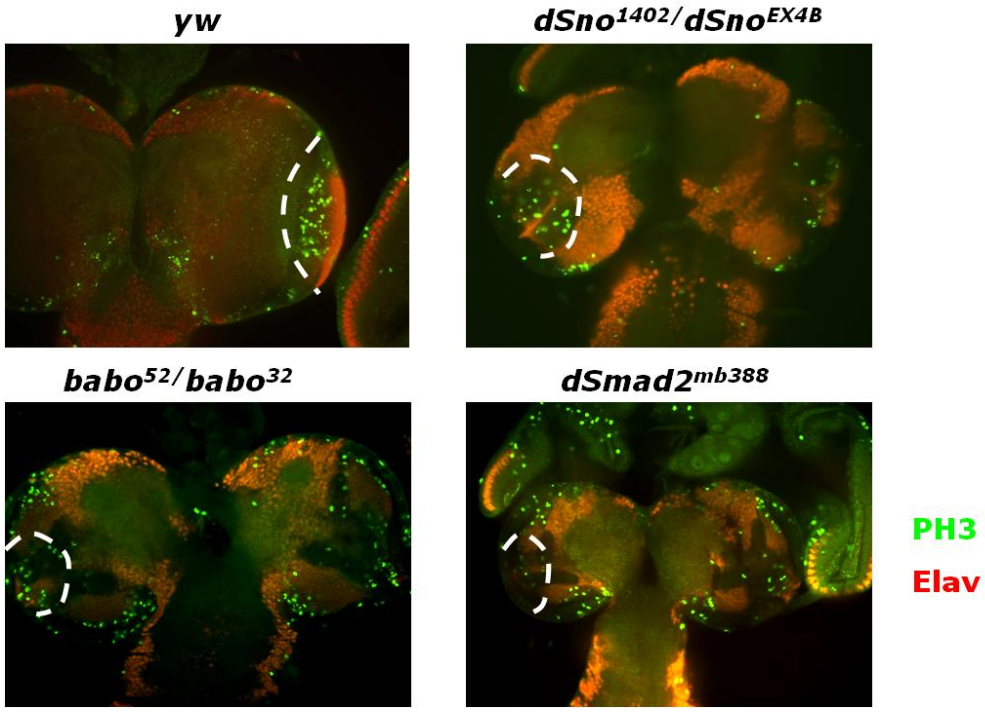


Figure 3-6. The *dSno*, *babo* and *dSmad2* mutants have reduced cell proliferation in optic lobes.

Optic lobes stained with anti-phospho-histone H3 (green) and Elav (red). The white dashes outline the inner proliferation zone. All mutants show reduced numbers of cells in M phase in the inner proliferation zone.

Figure 3-7:

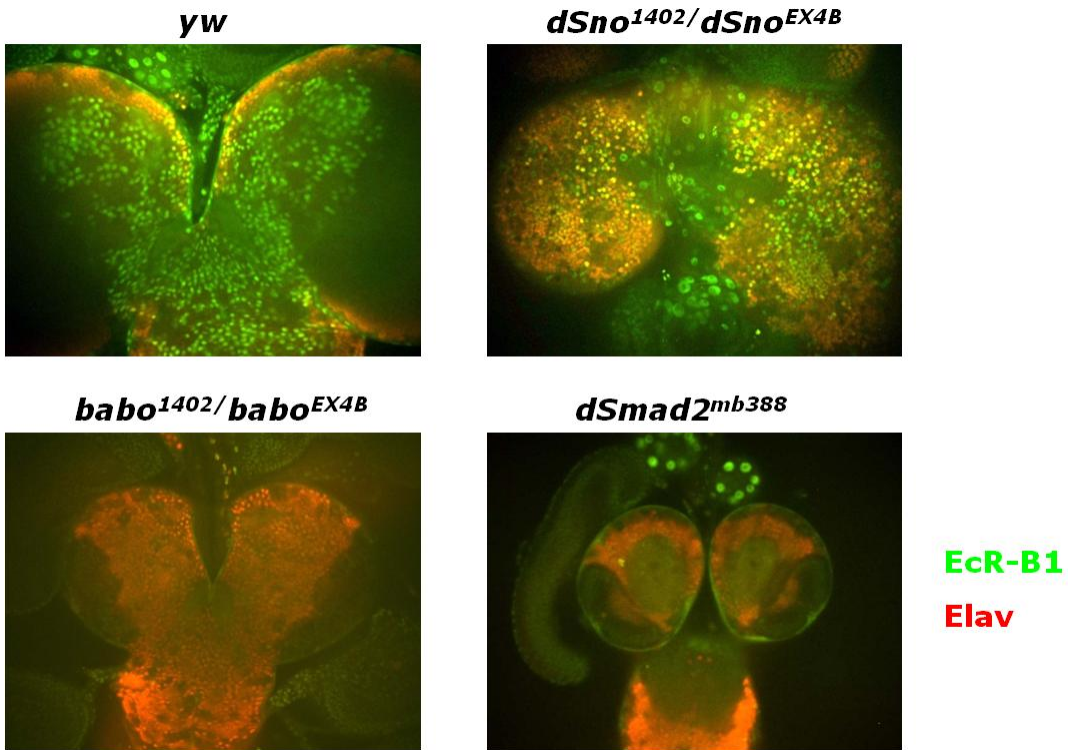

yw
dSno1402/dSnoEX4B
babo1402/baboEX4B
dSmad2mb388
EcR-B1
Elav

Figure 3-7. The *dSno* mutants show no defects in EcR-1B expression in CNS.

Brain lobes stained with anti-EcR-1B (green) and Elav (red). Both *babo* and *dSmad2* mutants have greatly reduced EcR-1B expression on CNS while *dSno* mutants do not.

Table 3-1. Comparison of the brain phenotypes of *sno* mutants with Activin signaling mutants.

	Brain size	Lamina and medulla	Cell proliferation	EcR-1B expression
***babo* and *dSmad2* mutants**	**reduced**	**collapsed**	**defective**	**undetectable**
***sno* mutants**	**reduced**	**collapsed**	**defective**	**normal**

dSmad2 and *babo* mutants, the lamina is markedly reduced in size (Figure 3-4). Also, the axons of the R7 and R8 photoreceptors fail to form the regular lattice network. Additionally, the medulla neuropil shows a significant decrease in size with altered architecture. This defect is likely due to the reduced rate of cell proliferation as revealed by the number of the M-phase cells based on phospho-H3 staining (Brummel et al., 1999) (Figure 3-5 and 3-6). Similarly, *dSno* mutants also have reduced lamina, malformed medullar and smaller medulla neuropil, which probably result from defects in cell proliferation (Figure 3-7). Intriguingly, the expression of EcR-B1, the only known target of Babo signaling which is expressed in the mature neurons (Zheng et al., 2006), is not altered in *dSno* mutants (Figure 3-7). Altogether, these observations (summarized in Table 3-1) suggest that dSno likely mediates the Babo/dSmad2 pathway to maintain proper rate of cell division in the optic lobes but not in the mature neurons.

dSno overexpression antagonizes BMP signaling

Similar to the vertebrate counterparts, we found that high levels of dSno can antagonize BMP signaling (Takaesu et al., 2006). In *Drosophila*, BMP signaling is required for crossvein formation and normal wing development. dSno, when over-expressed in the wing, resulted in flies with small wings, truncated veins and malformed crossveins. These phenotypes closely resemble those of the flies that highly express Mad^{1}, a dominant-negative allele that competes with endogenous Mad for Medea in the wing. This suggests that dSno may inhibit BMP signaling by sequestering Medea from Mad. Consistent with this idea, co-expression of Mad with dSno rescued the phenotypes caused by dSno overexpression.

Figure 3-8:

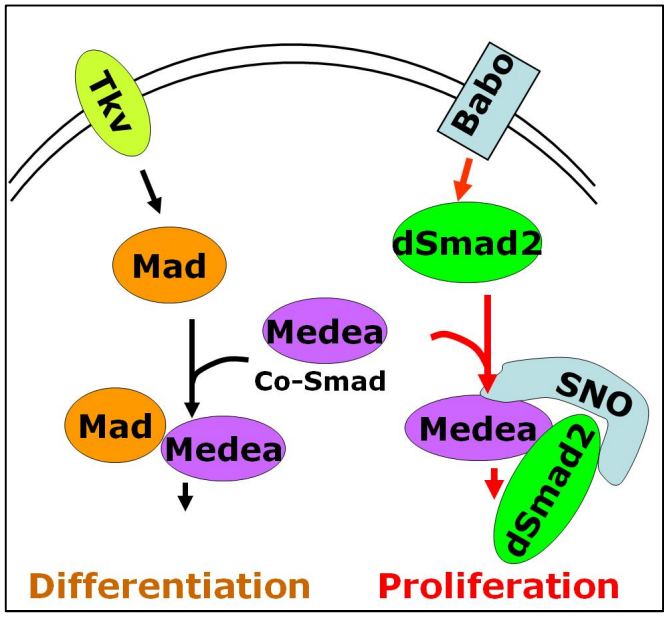

Tkv
Babo
Mad
dSmad2
Medea
Co-Smad
SNO
Mad
Medea
Medea
dSmad2
Differentiation
Proliferation

Figure 3-8.

In optic lobes, BMP signaling mediated by the Mad/Medea complex likely plays a role in cell differentiation whereas Activin signaling possibly regulates cell proliferation through the Smad2/Medea transcription complex. The function of dSno is to promote the formation of the dSmad2/Medea complex by shunting Medea away from the Mad to dSmad2. In the absence of dSno, the normal cell proliferation rate cannot be maintained due to reduced amount of the dSmad2/Medea complex formation, resulting in defects in optic lobes. Thickvein (Tkv) and Baboon (Babo) are the type I receptors that phosphorylate and activate Mad and dSmad2, respectively.

The pathway switch model

Since the BMP and Activin/Babo pathways are similar in basic mechanisms and share Medea as the co-Smad, it is intriguing how dSno could exert opposite effects on these two pathways. Insights were gained into this mystery with biochemical experiments showing that dSno can physically interact with Medea and that this interaction promotes the Medea/dSmad2 complex formation which subsequently decreases the availability of Medea for the Medea/Mad complex (Takaesu et al., 2006). Based on these data, we proposed a pathway switch model for dSno's dual function: dSno shunts Medea from BMP signaling to Babo signaling by facilitating the association between Medea and dSmad2 and reducing the interaction between Medea and Mad (Figure 3-8). Given that Activin signaling via Babo is vital for cell proliferation (Brummel et al., 1999) and that BMP signaling through Tkv (a type I receptor of BMP signaling) is required for cell differentiation (Yoshida et al., 2005), this model explains how neuroblasts can maintain proliferation in the presence of BMP signaling that promotes cell differentiation.

Mapping the break points of dSno null alleles (my work included in the paper by Quijano et al., 2010)

Although genetic complementation tests suggested that *dSno*^{EX17B} and *dSno*^{EX4B} are null or strong *dSno* alleles, it was not clear how large the deletions were in the *dSno*^{EX17B} and *dSno*^{EX4B} alleles. Thus, it was critical to precisely map the break points of these two alleles. To map the break points, I employed the PCR method combined with DNA sequencing (detailed method can be found in the experimental procedure section). Results show that *dSno*^{EX17B} contains an approximate 5 kb deletion that includes the 3' of *CG7228 (pes)*, the whole *CG7231* open r+eading frame (ORF) and possibly the

promoter of *CG7233* (*snoN*). For the *dSno*^{EX4B} allele, 4 gene/ORFs including *CG7227*, *CG7228* (*pes*), *CG7231* and *CG7233* (*snoN*) are deleted and the deletion spans a 21 kb region (Figure 3-9). The function of *CG7227* and *CG7228* seem to be related but neither has been fully characterized. Both of them possibly encode novel scavenger receptors that show homology to the human CD36 superfamily members that are involved in odor detection (Nichols and Vogt, 2008). CG7227 has been implicated in both starvation response in Drosophila head (Fujikawa et al., 2009) and synaptic growth at neuron muscular junction (NMJ) (Haussmann et al., 2008). CG7228 may play a role in a detoxification mechanism (Li et al., 2009). For CG7231, little is known about its function. Based on the mapping data, the *dSno*^{EX4B}/*dSno*^{sh1402} larvae are also heterozygous for *CG7227*, *CG7228* and *CG7231*. Due to the unavailability of the null mutant alleles of *CG7227*, *CG7228* and *CG7231*, it remains to be determined whether loss of one copy of these genes contributes to the lethality of the *dSno*^{EX4B}/*dSno*^{sh1402} animals.

Generating new dSno alleles (unpublished results)

Because the *dSno*^{EX17B} and *dSno*^{EX4B} are large deletion alleles in which several neighboring genes are also deleted, it was possible that reduced expression of the products from these deleted neighboring genes might contribute to the *dSno* lethal phenotype. Therefore, it was essential to generate *dSno* alleles that do not affect other genes. To do this, I carried out EMS mutagenesis, hoping to obtain *dSno* alleles with small lesions such as non-sense and mis-sense point mutations. Out of approximately 2000 flies I screened, one *dSno* allele was recovered based on its inability to complement the *dSno*^{sh1402} allele and was named *dSno*^{3}. However, sequencing the *CG7233 (snoN)* locus, which is included in all the *dSno* isoforms, failed to reveal any mutations. We then considered the possibility that the *dSno* phenotype originated from

Figure 3-9:

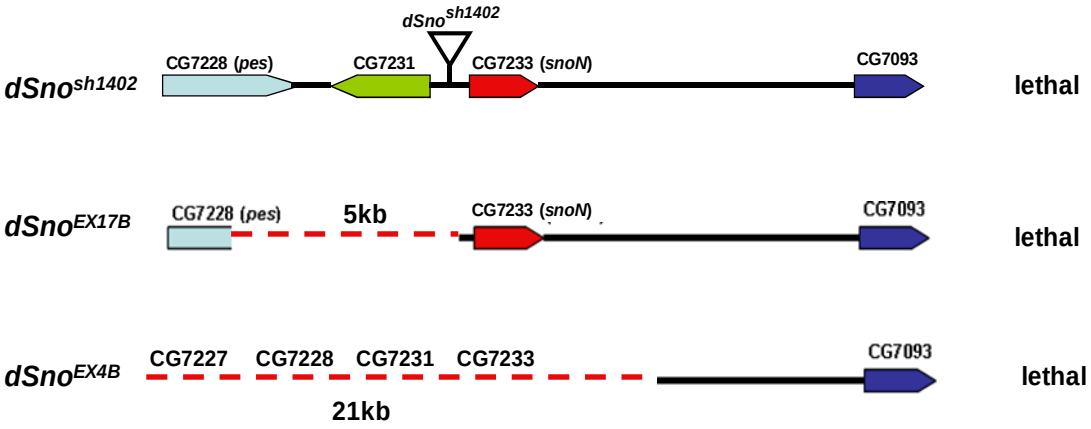

dSno^sh1402
CG7228 (pes)
CG7231
CG7233 (snoN)
CG7093
lethal
dSno^EX17B
CG7228 (pes)
5kb
CG7233 (snoN)
CG7093
lethal
dSno^EX4B
CG7227
CG7228
CG7231
CG7233
21kb
CG7093
lethal

Figure 3-9. Mapping the break points of *dSno* mutant alleles.

The P element on the *dSno*^{sh1402} chromosome is located at the 5' of both the *CG7233* (*snoN*) and *CG7231*. The P element imprecise excision event that generated *dSno*^{EX17B} deletes a 5kb region that includes *CG7231* and the 3' portion of *CG7228 (pes). CG7233* is still present on the *dSno*^{EX17B} chromosome, although its promoter is probably disrupted. In the case of *dSno*^{EX4B}, an approximately 21kb genomic region that contains *CG7227*, *CG7228 (pes), CG7231* and *CG7233* (*snoN*) is deleted.

the *dSno*^{sh1402} allele was a consequence of loss of function of the gene CG7231 that is located adjacent to *CG7233* given that the P element in *dSno*^{sh1402} could also disrupt the promoter of *CG7231* (Figure 3-9). Nonetheless, no mutations were found within the region of *CG7231*.

Discussion

dSno gain-of-function

In vertebrates, the primary mechanism of how high levels of Ski/Sno are able to transform cells is by antagonizing TGF-β signaling (Liu et al., 2001; Luo, 2004). In *Drosophila*, dSno, when overexpressed, is also capable of inhibiting signaling initiated by Dpp a TGF-β superfamily ligand (Barrio et al., 2007; Ramel et al., 2007; Takaesu et al., 2006). This antagonistic action of dSno is due to the inhibitory binding of dSno to, andthus sequestration of, Medea, a cofactor for Mad that transduces the Dpp signaling as supported by both genetic (Barrio et al., 2007) and biochemical studies (Barrio et al., 2007; Takaesu et al., 2006). While high levels of dSno can inhibit the BMP branch of TGF-β signaling, it remains controversial whether dSno can down-regulate the Activin branch of TGF-β signaling in Drosophila. Barrio et al., (2007) reported that dSno can suppress the wing phenotype induced by expressing a constitutively active Babo receptor (Babo*) and interpreted this result as that dSno can also antagonize Activin signaling (Barrio et al., 2007), which is in direct contrast to the role of dSno as an Activin signaling mediator proposed by us (Takaesu et al., 2006). It is important to realize that in addition to phosphorylating dSmad2, Babo can also activate Mad, the BMP signaling transducer (Jensen et al., 2009). Thus, the Babo* overexpressing wing phenotype, which is reminiscent to enhanced BMP signaling, is likely a consequence of Mad

activation. Thus, the inhibitory effect of dSno on Babo* over-expression is perhaps due to a suppression on Mad activity induced by Babo*. Intriguingly, a synergistic effect on the wing phenotype between dSno and dSmad2 observed by the same authors (Barrio et al., 2007) implies that dSno can mediate Activin signaling by augmenting dSmad2 activity, consistent with our observations in the optic lobes (Takaesu et al., 2006).

dSno loss-of-function

The larval and early pupal lethality observed from the dSno mutants derived from $dSno^{sh1402}$, the P-element insertion allele as well as $dSno^{EX17B}$ and $dSno^{EX4B}$, the deletion alleles, demonstrate that *dSno* is an essential gene required for proper development (Takaesu et al., 2006). However, it is important to recognize that other genes in addition to *dSno* are also affected in these alleles and therefore likely contribute to the lethality. Consistent with this notion, several groups have reported that *dSno* loss-of-function mutants are viable (Barrio et al., 2007; Ramel et al., 2007; Shravage et al., 2007). A null *dSno* mutation $dSno^{174}$ generated from an imprecise P element excision event that only deletes CG7233 is homozygous viable, although the viability is significantly reduced (Shravage et al., 2007). Another strong loss-of-function *dSno* allele, $snoN^{GS\text{-}C517T}$, which contains a premature stop codon and thus potentially encodes a truncated protein that is missing the essential domains for Ski/Sno's normal activities, gives rise to homozygous viable flies (Ramel et al., 2007). Additionally, Barrio et al., (2007) generated a *dSno* deficiency fly line designated as *Df(2L)snoN* in which *CG7233 (snoN), CG7231* and part of the *CG7228* are removed (Barrio et al., 2007). Again, *Df(2L)snoN* produces homozygous viable flies (Barrio et al., 2007). Altogether, these results indicate that *dSno* is not an essential gene for development. The lethal phenotype we observed from homo- or heteroallelic combinations of $dSno^{sh1402}$, $dSno^{EX17B}$ and $dSno^{EX4B}$ alleles is likely due to

a lethal mutation present on the original *dSno*^{sh1402} chromosome from which the *dSno*^{EX17B} and *dSno*^{EX4B} alleles were derived.

While it is clear that loss of dSno function does not cause lethality, it remains elusive whether endogenous *dSno* plays any role during development. Interestingly, flies homozygous for *dSno*^{174} and *snoN*$^{GS\text{-}C517T}$, two null mutations, albeit viable display significantly reduced viability and fertility (Barrio et al., 2007; Shravage et al., 2007), suggesting that dSno is required for reproduction and optimal survival. Both of these dSno null flies have normal vein patterning in the adult wing (Barrio et al., 2007; Shravage et al., 2007), suggesting that loss of dSno does not interfere with Dpp signaling, which plays critical roles in the wing vein formation (Strigini and Cohen, 1999). Interestingly, the *dSno* null mutants show patterning defects of the egg shells which are similar to the phenotypes resulting from Dpp signaling over-activation (Shravage et al., 2007). This result indicates that endogenous *dSno* may act as an inhibitor to Dpp signaling during egg shell patterning. Based on the above observations, it appears that, consistent with gain-of-function studies, dSno antagonizes Dpp signaling in a tissue-specific manner.

Experimental procedures

In Situ hybridization

The procedures for *in situ* hybridization and for generating the labeled probes were followed according to Chavez et al. (2000). The *dSno* (*CG7233*) cDNA was used to generate both the anti-sense and sense probes.

Immunohistochemistry

Larval brains together with eye imaginal disks were dissected and fixed in PBS containing 3.7% formaldehyde for 1 hr at room temperature, followed by three washes for 10 min in PBS plus 0.1% Triton X-100 (PBT). Tissue was incubated with primary antibody overnight at 4° followed by four 10-min washes in PBT. Secondary antibodies were incubated for 2 to 4 hr followed by four 10-min washes in PBT. Brains and disks where mounted in 80% glycerol/20% PBT. The rat anti-Elav (7E8A10), mouse anti-chaoptin (24B10), and mouse anti-Robo (13C9) antibodies were obtained from Developmental Studies Hybridoma Bank and used at 1/400, 1/1000, and 1/50 dilutions, respectively. Rabbit antiphosphorylated histone H3 (pSer10, H-0412, Sigma, St. Louis) antibody was used at 1/400 dilution. Secondary anti-mouse rabbit and rat antibodies linked to AlexaFlour 568 or FITC (Molecular Probes, Eugene, OR) were used at 1/300 dilution.

Mapping the break points of *dSno*^{EX17B} and *dSno*^{EX4B}

A series of primer sets, each of which is approximately 1 kb apart were designed across the region from *CG7233* to *CG7224*. The break points of dSno^{EX17B} and dSno^{EX4B} were first roughly determined by the successful amplification of the PCR products upstream and downstream of the deleted regions. Then the forward primer of the upstream primer set and the reverse primer of the downstream primer set were used to amplify the fragment that contains the junction of the break points in each deletion allele. For *dSno*^{EX4B}, the forward primer used is 5' **tagcccctcattttcacagc** 3' and the reverse primer is 5 '**cgccactcgtcgatagatag** 3'. For *dSno*^{EX17B}, the forward primer is 5' **aactggcggagatgcttg** 3' and the reverse primer is 5 '**cagggcttatgacgaatatgg** 3'. The PCR fragments obtained were then cloned into the pCR2.1 TOPO vector (Invitrogen)

and sequenced using the forward and reverse PCR primers that were used to amplify the fragments. The sequences were blasted to find the break points of each deletion allele.

EMS Mutagenesis

100 *yw* males were transferred to empty vials and allowed to dehydrate for 30 min. The dehydrated males were then transferred into vials that contained 25mM EMS solution (10 mM Tris pH7.5 and 1% sucrose) and left overnight. The next morning, the males were put into clean empty vials for 30 min to get rid of the residual EMS on their bodies. Then the males were transferred into vials with ordinary fly food for recovery. The males were allowed to mate with females carrying the second chromosome balancer CyO-GFP. Only the CyO-GFP male progeny was recovered because the *dSno* gene is on the second chromosome. Each individual male was subsequently crossed to virgin females with the genotype $dSno^{sh1402}$/CyO-GFP. Their progeny was screened for larval or pupal lethality which can be reflected by the lack of non-GFP pupae and non-CyO-GFP adults.

Bibliography

Akiyoshi, S., Inoue, H., Hanai, J., Kusanagi, K., Nemoto, N., Miyazono, K., and Kawabata, M. (1999). c-Ski acts as a transcriptional co-repressor in transforming growth factor-beta signaling through interaction with smads. J. Biol. Chem. *274,* 35269-35277.

Altstein, M. (2004). Role of neuropeptides in sex pheromone production in moths. Peptides *25,* 1491-1501.

Andreou, A.M., and Tavernarakis, N. (2009). SUMOylation and cell signalling. Biotechnol. J. *4,* 1740-1752.

Andres, A.J., Fletcher, J.C., Karim, F.D., and Thummel, C.S. (1993). Molecular analysis of the initiation of insect metamorphosis: a comparative study of Drosophila ecdysteroid-regulated transcription. Dev. Biol. *160,* 388-404.

Andres, A.J., and Thummel, C.S. (1992). Hormones, puffs and flies: the molecular control of 0

Ashburner, M. (1974). Sequential gene activation by ecdysone in polytene chromosomes of Drosophila melanogaster. II. The effects of inhibitors of protein synthesis. Dev. Biol. *39,* 141-157.

Bachman, K.E., and Park, B.H. (2005). Duel nature of TGF-beta signaling: tumor suppressor vs. tumor promoter. Curr. Opin. Oncol. *17,* 49-54.

Baehrecke, E.H. (1996). Ecdysone signaling cascade and regulation of Drosophila metamorphosis. Arch. Insect Biochem. Physiol. *33,* 231-244.

Baker, E.R. (1985). Body weight and the initiation of puberty. Clin. Obstet. Gynecol. *28,* 573-579.

Barrio, R., Lopez-Varea, A., Casado, M., and de Celis, J.F. (2007). Characterization of dSnoN and its relationship to Decapentaplegic signaling in Drosophila. Dev. Biol. *306,* 66-81.

Beckstead, R.B., Lam, G., and Thummel, C.S. (2007). Specific transcriptional responses to juvenile hormone and ecdysone in Drosophila. Insect Biochem. Mol. Biol. *37,* 570-578.

Berk, M., Desai, S.Y., Heyman, H.C., and Colmenares, C. (1997). Mice lacking the ski proto-oncogene have defects in neurulation, craniofacial, patterning, and skeletal muscle development. Genes Dev. *11,* 2029-2039.

Bollenbacher, W.E., Katahira, E.J., O'Brien, M., Gilbert, L.I., Thomas, M.K., Agui, N., and Baumhover, A.H. (1984). Insect prothoracicotropic hormone: evidence for two molecular forms. Science *224,* 1243-1245.

Boyer, P.L., Colmenares, C., Stavnezer, E., and Hughes, S.H. (1993). Sequence and biological activity of chicken snoN cDNA clones. Oncogene *8,* 457-466.

Britton, J.S., Lockwood, W.K., Li, L., Cohen, S.M., and Edgar, B.A. (2002). Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. Dev. Cell. *2,* 239-249.

Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., and Woodard, C.T. (1999). The Drosophila beta FTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. Mol. Cell *3,* 143-149.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001). An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Curr. Biol. *11,* 213-221.

Brummel, T., Abdollah, S., Haerry, T.E., Shimell, M.J., Merriam, J., Raftery, L., Wrana, J.L., and O'Connor, M.B. (1999). The Drosophila activin receptor baboon signals through dSmad2 and controls cell proliferation but not patterning during larval development. Genes Dev. *13,* 98-111.

Caldwell, P.E., Walkiewicz, M., and Stern, M. (2005). Ras activity in the Drosophila prothoracic gland regulates body size and developmental rate via ecdysone release. Curr. Biol. *15,* 1785-1795.

Capovilla, M., Eldon, E.D., and Pirrotta, V. (1992). The giant gene of Drosophila encodes a b-ZIP DNA-binding protein that regulates the expression of other segmentation gap genes. Development *114,* 99-112.

Carstea, E.D., Morris, J.A., Coleman, K.G., Loftus, S.K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M.A., Pavan, W.J., Krizman, D.B.*, et al.* (1997). Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. Science *277,* 228-231.

Chavez, V.M., Marques, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., Burr, J., Natzle, J.E., and O'Connor, M.B. (2000). The Drosophila disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. Development *127,* 4115-4126.

Clayton, R.B. (1964). The utilization of sterols by insects. J. Lipid Res. *15,* 3-19.

Colmenares, C., Heilstedt, H.A., Shaffer, L.G., Schwartz, S., Berk, M., Murray, J.C., and Stavnezer, E. (2002). Loss of the SKI proto-oncogene in individuals affected with 1p36 deletion syndrome is predicted by strain-dependent defects in Ski-/- mice. Nat. Genet. *30,* 106-109.

Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carre, C., Noselli, S., and Leopold, P. (2005). Antagonistic actions of ecdysone and insulins determine final size in Drosophila. Science *310,* 667-670.

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., and Leopold, P. (2003). A nutrient sensor mechanism controls Drosophila growth. Cell *114,* 739-749.

Copeland, K.C., Kuehl, T.J., and Castracane, V.D. (1982). Pubertal endocrinology of the baboon: elevated somatomedin-C/insulin-like growth factor I at puberty. J. Clin. Endocrinol. Metab. *55,* 1198-1201.

da Graca, L.S., Zimmerman, K.K., Mitchell, M.C., Kozhan-Gorodetska, M., Sekiewicz, K., Morales, Y., and Patterson, G.I. (2004). DAF-5 is a Ski oncoprotein homolog that functions in a neuronal TGF beta pathway to regulate C. elegans dauer development. Development *131,* 435-446.

Dai, J.D., and Gilbert, L.I. (1991). Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of Drosophila melanogaster: a cytophysiological analysis of the ring gland. Dev. Biol. *144,* 309-326.

Dai, J.L., Mizoguchi, A., Satake, S., Ishizaki, H., and Gilbert, L.I. (1995). Developmental changes in the prothoracicotropic hormone content of the Bombyx mori brain-retrocerebral complex and hemolymph: analysis by immunogold electron microscopy, quantitative image analysis, and time-resolved fluoroimmunoassay. Dev. Biol. *171,* 212-223.

Dann, S.G., and Thomas, G. (2006). The amino acid sensitive TOR pathway from yeast to mammals. FEBS Lett. *580,* 2821-2829.

David, G., Neptune, M.A., and DePinho, R.A. (2002). SUMO-1 modification of histone deacetylase 1 (HDAC1) modulates its biological activities. J. Biol. Chem. *277,* 23658-23663.

Dedos, S.G., Nagata, S., Ito, J., and Takamiya, M. (2001). Action kinetics of a prothoracicostatic peptide from Bombyx mori and its possible signaling pathway. Gen. Comp. Endocrinol. *122,* 98-108.

Derynck, R., and Feng, X.H. (1997). TGF-beta receptor signaling. Biochim. Biophys. Acta *1333,* F105-50.

Ebling, F.J., and Cronin, A.S. (2000). The neurobiology of reproductive development. Neuroreport *11,* R23-33.

Eldon, E. D., and Pirrotta, V., (1991). Interactions of the Drosophila gap gene giant with maternal and zygotic pattern-forming genes. Development. *111,* 367-78.

Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240,* 889-895.

Feng, X.H., and Derynck, R. (2005). Specificity and versatility in tgf-beta signaling through Smads. Annu. Rev. Cell Dev. Biol. *21,* 659-693.

Fielenbach, N., and Antebi, A. (2008). C. elegans dauer formation and the molecular basis of plasticity. Genes Dev. *22,* 2149-2165.

Fujikawa, K., Takahashi, A., Nishimura, A., Itoh, M., Takano-Shimizu, T., and Ozaki, M. (2009). Characteristics of genes up-regulated and down-regulated after 24 h starvation in the head of Drosophila. Gene *446,* 11-17.

Fumagalli, S., Doneda, L., Nomura, N., and Larizza, L. (1993). Expression of the c-ski proto-oncogene in human melanoma cell lines. Melanoma Res. *3,* 23-27

.Garen, A., Kauvar, L., and Lepesant, J.A. (1977). Roles of ecdysone in Drosophila development. Proc. Natl. Acad. Sci. U. S. A. *74,* 5099-5103.

Geiss-Friedlander, R., and Melchior, F. (2007). Concepts in sumoylation: a decade on. Nat. Rev. Mol. Cell Biol. *8,* 947-956.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., and Antebi, A. (2001). A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Dev. Cell. *1,* 841-851.

Gesualdi, S.C., and Haerry, T.E. (2007). Distinct signaling of Drosophila Activin/TGF-beta family members. Fly (Austin) *1,* 212-221.

Ghosh, A., McBrayer, Z. and O'Connor, M.B. (2010). The *Drosophila* gap gene *giant* regulates ecdysone production through specification of the PTTH-producing neurons. Submitted.

Gilbert, L.I. (2004). *Halloween* genes encode P450 enzymes that mediate steroid hormone biosynthesis in Drosophila melanogaster. Mol. Cell. Endocrinol. *215,* 1-10.

Gilbert, L.I., Combest, W.L., Smith, W.A., Meller, V.H., and Rountree, D.B. (1988). Neuropeptides, second messengers and insect molting. Bioessays *8,* 153-157.

Gilbert, L.I., Rybczynski, R., and Warren, J.T. (2002). Control and biochemical nature of the ecdysteroidogenic pathway. Annu. Rev. Entomol. *47,* 883-916.

Gilbert, L.I., Song, Q., and Rybczynski, R. (1997). Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity. Invert. Neurosci. *3,* 205-216.

Gill, G. (2005). Something about SUMO inhibits transcription. Curr. Opin. Genet. Dev. *15,* 536-541.

Goberdhan, D.C., Paricio, N., Goodman, E.C., Mlodzik, M., and Wilson, C. (1999). Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. Genes Dev. *13,* 3244-3258.

Grieneisen, M.L., Warren, J.T., Sakurai, S., and Gilbert, L.I. (1991). A putative route to ecdysteroids: metabolism of cholesterol *in vitro* by mildly disrupted prothoracic glands of *Manduca Sexta*. Insect Biochem. *21,* 41-51.

Gu, S.H. (2007). Autocrine activation of ecdysteroidogenesis in the prothoracic glands of the silkworm, Bombyx mori. J. Insect Physiol. *53,* 538-549.

Gu, S.H. (2006). Autocrine activation of DNA synthesis in prothoracic gland cells of the silkworm, Bombyx mori. J. Insect Physiol. *52,* 136-145.

Gu, S.H., Lin, J.L., Lin, P.L., and Chen, C.H. (2009). Insulin stimulates ecdysteroidogenesis by prothoracic glands in the silkworm, Bombyx mori. Insect Biochem. Mol. Biol. *39,* 171-179.

Halme, A., Cheng, M., and Hariharan, I.K. (2010). Retinoids regulate a developmental checkpoint for tissue regeneration in Drosophila. Curr. Biol. *20,* 458-463.

Handelsman, D.J., Spaliviero, J.A., Scott, C.D., and Baxter, R.C. (1987). Hormonal regulation of the peripubertal surge of insulin-like growth factor-I in the rat. Endocrinology *120,* 491-496.

Hasegawa, Y., Miyamoto, K., Abe, Y., Nakamura, T., Sugino, H., Eto, Y., Shibai, H., and Igarashi, M. (1988). Induction of follicle stimulating hormone receptor by erythroid differentiation factor on rat granulosa cell. Biochem. Biophys. Res. Commun. *156,* 668-674.

Haussmann, I.U., White, K., and Soller, M. (2008). Erect wing regulates synaptic growth in Drosophila by integration of multiple signaling pathways. Genome Biol. *9,* R73.

Hayes, G.C., Muehleisen, D.P., Bollenbacher, W.E., and Watson, R.D. (1995). Stimulation of ecdysteroidogenesis by small prothoracicotropic hormone: role of calcium. Mol. Cell. Endocrinol. *115,* 105-112.

Hewes, R.S., Gu, T., Brewster, J.A., Qu, C., and Zhao, T. (2006). Regulation of secretory protein expression in mature cells by DIMM, a basic helix-loop-helix neuroendocrine differentiation factor. J. Neurosci. *26,* 7860-7869.

Hiney, J.K., Srivastava, V., Nyberg, C.L., Ojeda, S.R., and Dees, W.L. (1996). Insulin-like growth factor I of peripheral origin acts centrally to accelerate the initiation of female puberty. Endocrinology *137,* 3717-3728.

Hiney, J.K., Srivastava, V.K., Pine, M.D., and Les Dees, W. (2009). Insulin-like growth factor-I activates KiSS-1 gene expression in the brain of the prepubertal female rat. Endocrinology *150,* 376-384.

Homma, T., Watanabe, K., Tsurumaru, S., Kataoka, H., Imai, K., Kamba, M., Niimi, T., Yamashita, O., and Yaginuma, T. (2006). G protein-coupled receptor for diapause hormone, an inducer of Bombyx embryonic diapause. Biochem. Biophys. Res. Commun. *344,* 386-393.

Hua, Y.J., Tanaka, Y., Nakamura, K., Sakakibara, M., Nagata, S., and Kataoka, H. (1999). Identification of a prothoracicostatic peptide in the larval brain of the silkworm, Bombyx mori. J. Biol. Chem. *274,* 31169-31173.

Huang, X., Suyama, K., Buchanan, J., Zhu, A.J., and Scott, M.P. (2005). A Drosophila model of the Niemann-Pick type C lysosome storage disease: dnpc1a is required for molting and sterol homeostasis. Development *132,* 5115-5124.

Huang, X., Warren, J.T., Buchanan, J., Gilbert, L.I., and Scott, M.P. (2007). Drosophila Niemann-Pick type C-2 genes control sterol homeostasis and steroid biosynthesis: a model of human neurodegenerative disease. Development *134,* 3733-3742.

Hull, J.J., Ohnishi, A., Moto, K., Kawasaki, Y., Kurata, R., Suzuki, M.G., and Matsumoto, S. (2004). Cloning and characterization of the pheromone biosynthesis activating neuropeptide receptor from the silkmoth, Bombyx mori. Significance of the carboxyl terminus in receptor internalization. J. Biol. Chem. *279,* 51500-51507.

Hussey, R.G., Thompson, W.R., and Calhoun, E.T. (1927). The influence of X-rays on the development of Drosophila larvae. Science *66,* 65-66.

Hutchinson, L.A., Findlay, J.K., de Vos, F.L., and Robertson, D.M. (1987). Effects of bovine inhibin, transforming growth factor-beta and bovine Activin-A on granulosa cell differentiation. Biochem. Biophys. Res. Commun. *146,* 1405-1412.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Curr. Biol. *12,* 1293-1300.

Imoto, I., Pimkhaokham, A., Fukuda, Y., Yang, Z.Q., Shimada, Y., Nomura, N., Hirai, H., Imamura, M., and Inazawa, J. (2001). SNO is a probable target for gene amplification at 3q26 in squamous-cell carcinomas of the esophagus. Biochem. Biophys. Res. Commun. *286,* 559-565.

Inoki, K., Li, Y., Xu, T., and Guan, K.L. (2003). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. *17,* 1829-1834.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. *4,* 648-657.

Ishibashi, J., Kataoka, H., Isogai, A., Kawakami, A., Saegusa, H., Yagi, Y., Mizoguchi, A., Ishizaki, H., and Suzuki, A. (1994). Assignment of disulfide bond location in prothoracicotropic hormone of the silkworm, Bombyx mori: a homodimeric peptide. Biochemistry *33,* 5912-5919.

Ishizaki, H., and Suzuki, A. (1994). The brain secretory peptides that control moulting and metamorphosis of the silkmoth, Bombyx mori. Int. J. Dev. Biol. *38,* 301-310.

Jensen, P.A., Zheng, X., Lee, T., and O'Connor, M.B. (2009). The Drosophila Activin-like ligand Dawdle signals preferentially through one isoform of the Type-I receptor Baboon. Mech. Dev. *126,* 950-957.

Jia, K., Albert, P.S., and Riddle, D.L. (2002). DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. Development *129,* 221-231.

Juul, A., Bang, P., Hertel, N.T., Main, K., Dalgaard, P., Jorgensen, K., Muller, J., Hall, K., and Skakkebaek, N.E. (1994). Serum insulin-like growth factor-I in 1030 healthy children, adolescents, and adults: relation to age, sex, stage of puberty, testicular size, and body mass index. J. Clin. Endocrinol. Metab. *78,* 744-752.

Kaplowitz, P.B., and Oberfield, S.E. (1999). Reexamination of the age limit for defining when puberty is precocious in girls in the United States: implications for evaluation and treatment. Drug and Therapeutics and Executive Committees of the Lawson Wilkins Pediatric Endocrine Society. Pediatrics *104,* 936-941.

Karim, F.D., Guild, G.M., and Thummel, C.S. (1993). The Drosophila Broad-Complex plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. Development *118,* 977-988.

Karim, F.D., Urness, L.D., Thummel, C.S., Klemsz, M.J., McKercher, S.R., Celada, A., Van Beveren, C., Maki, R.A., Gunther, C.V., and Nye, J.A. (1990). The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. Genes Dev. *4,* 1451-1453.

Kataoka, H., Nagasawa, H., Isogai, A., Ishizaki, H., and Suzuki, A. (1991). Prothoracicotropic hormone of the silkworm, *Bombyx mori*: amino acid sequence and dimeric structure. Agric. Biol. Chem. 55,73–86.

Kaufman, C.D., Martinez-Rodriguez, G., and Hackett, P.B.,Jr. (2000). Ectopic expression of c-ski disrupts gastrulation and neural patterning in zebrafish. Mech. Dev. *95,* 147-162.

Kawakami, A., Kataoka, H., Oka, T., Mizoguchi, A., Kimura-Kawakami, M., Adachi, T., Iwami, M., Nagasawa, H., Suzuki, A., and Ishizaki, H. (1990). Molecular cloning of the Bombyx mori prothoracicotropic hormone. Science *247,* 1333-1335.

Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D.E., and Graybiel, A.M. (1998). A family of cAMP-binding proteins that directly activate Rap1. Science *282,* 2275-2279.

Kesler, S.R. (2007). Turner syndrome. Child Adolesc. Psychiatr. Clin. N. Am. *16,* 709-722.

Knight, P.G., and Glister, C. (2001). Potential local regulatory functions of inhibins, activins and follistatin in the ovary. Reproduction *121,* 503-512.

Kondo, H., Ino, M., Suzuki, A., Ishizaki, H., and Iwami, M. (1996). Multiple gene copies for bombyxin, an insulin-related peptide of the silkmoth Bombyx mori: structural signs for gene rearrangement and duplication responsible for generation of multiple molecular forms of bombyxin. J. Mol. Biol. *259,* 926-937.

Kopec, S. (1922). Studies on the necessity of the brain for the inception of insect metamorphosis. Biol. Bull. *42,* 323-342.

Lafont, R., Dauphin-Villemant, C., and Warren, J.T. (2005). Ecdysteroid chemistry and biochemistry. In Comprehensive Molecular Insect Science, Gilbert, L. I., Iatrou, K. and Gill, S. eds., (Oxford: Elsevier) pp. 125-195.

Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996). Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. Nature *383,* 832-836.

Larsson, J., and Karlsson, S. (2005). The role of Smad signaling in hematopoiesis. Oncogene *24,* 5676-5692.

Layalle, S., Arquier, N., and Leopold, P. (2008). The TOR pathway couples nutrition and developmental timing in Drosophila. Dev. Cell. *15,* 568-577.

Lee, M.T., and Bachant, J. (2009). SUMO modification of DNA topoisomerase II: trying to get a CENse of it all. DNA Repair (Amst) *8,* 557-568.

Leevers, S.J., Weinkove, D., MacDougall, L.K., Hafen, E., and Waterfield, M.D. (1996). The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. EMBO J. *15,* 6584-6594.

LeMaire,W.J. (1989). Mechanism of mammalian ovulation. Steroids, 54, 455-469.

Li, W.X. (2005). Functions and mechanisms of receptor tyrosine kinase Torso signaling: lessons from Drosophila embryonic terminal development. Dev. Dyn. *232,* 656-672.

Li, H.M., Sun, L., Mittapalli, O., Muir, W.M., Xie, J., Wu, J., Schemerhorn, B.J., Sun, W., Pittendrigh, B.R., and Murdock, L.L. (2009). Transcriptional signatures in response to

wheat germ agglutinin and starvation in Drosophila melanogaster larval midgut. Insect Mol. Biol. *18,* 21-31.

Li, Y., Turck, C.M., Teumer, J.K., and Stavnezer, E. (1986). Unique sequence, ski, in Sloan-Kettering avian retroviruses with properties of a new cell-derived oncogene. J. Virol. *57,* 1065-1072.

Liu, X., Tanaka, Y., Song, Q., Xu, B., and Hua, Y. (2004). Bombyx mori prothoracicostatic peptide inhibits ecdysteroidogenesis in vivo. Arch. Insect Biochem. Physiol. *56,* 155-161.

Liu, X., Sun, Y., Weinberg, R.A., and Lodish, H.F. (2001). Ski/Sno and TGF-beta signaling. Cytokine Growth Factor Rev. *12,* 1-8.

Lu, D., Lee, K.Y., Horodyski, F.M., and Witten, J.L. (2002). Molecular characterization and cell-specific expression of a Manduca sexta FLRFamide gene. J. Comp. Neurol. *446,* 377-396.

Luo, K. (2004). Ski and SnoN: negative regulators of TGF-beta signaling. Curr. Opin. Genet. Dev. *14,* 65-70.

Luo, K., Stroschein, S.L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999). The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. Genes Dev. *13,* 2196-2206.

Lyons, G.E., Micales, B.K., Herr, M.J., Horrigan, S.K., Namciu, S., Shardy, D., and Stavnezer, E. (1994). Protooncogene c-ski is expressed in both proliferating and postmitotic neuronal populations. Dev. Dyn. *201,* 354-365.

Marchal, E., Vandersmissen, H.P., Badisco, L., Van de Velde, S., Verlinden, H., Iga, M., Van Wielendaele, P., Huybrechts, R., Simonet, G., Smagghe, G., and Vanden Broeck, J. (2010). Control of ecdysteroidogenesis in prothoracic glands of insects: a review. Peptides *31,* 506-519.

Mauras, N., Rogol, A.D., Haymond, M.W., and Veldhuis, J.D. (1996). Sex steroids, growth hormone, insulin-like growth factor-1: neuroendocrine and metabolic regulation in puberty. Horm. Res. *45,* 74-80.

Massague, J. (1998). TGF-beta signal transduction. Annu. Rev. Biochem. *67,* 753-791.

McBrayer, Z., Ono, H., Shimell, M., Parvy, J.P., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-Villemant, C., Gilbert, L.I., and O'Connor, M.B. (2007). Prothoracicotropic hormone regulates developmental timing and body size in Drosophila. Dev. Cell. *13,* 857-871.

Meller, V.H., Combest, W.L., Smith, W.A., and Gilbert, L.I. (1988). A calmodulin-sensitive adenylate cyclase in the prothoracic glands of the tobacco hornworm, Manduca sexta. Mol. Cell. Endocrinol. *59,* 67-76.

Miles, W.O., Jaffray, E., Campbell, S.G., Takeda, S., Bayston, L.J., Basu, S.P., Li, M., Raftery, L.A., Ashe, M.P., Hay, R.T., and Ashe, H.L. (2008). Medea SUMOylation restricts the signaling range of the Dpp morphogen in the Drosophila embryo. Genes Dev. *22,* 2578-2590.

Miro, F., and Hillier, S.G. (1992). Relative effects of activin and inhibin on steroid hormone synthesis in primate granulosa cells. J. Clin. Endocrinol. Metab. *75,* 1556-1561.

Miro, F., Smyth, C.D., and Hillier, S.G. (1991). Development-related effects of recombinant activin on steroid synthesis in rat granulosa cells. Endocrinology *129,* 3388-3394.

Mirth, C., Truman, J.W., and Riddiford, L.M. (2005). The role of the prothoracic gland in determining critical weight for metamorphosis in Drosophila melanogaster. Curr. Biol. *15,* 1796-1807.

Mirth, C.K., and Riddiford, L.M. (2007). Size assessment and growth control: how adult size is determined in insects. Bioessays *29,* 344-355.

Mizoguchi, A., Oka, T., Kataoka, H., Nagasawa, H., and and Suzuki, A. (1990). Immunohistochemical localization of prothoracicotropic hormone-producing cells in the brain of *Bombyx mori*. Dev. Growth Differ. *32,* 591-598.

Mondal, D., Pradhan, L., and LaRussa, V.F. (2004). Signal transduction pathways involved in the lineage-differentiation of NSCs: can the knowledge gained from blood be used in the brain? Cancer Invest. *22,* 925-943.

Moog-Lutz, C., Tomasetto, C., Regnier, C.H., Wendling, C., Lutz, Y., Muller, D., Chenard, M.P., Basset, P., and Rio, M.C. (1997). MLN64 exhibits homology with the steroidogenic acute regulatory protein (STAR) and is over-expressed in human breast carcinomas. Int. J. Cancer *71,* 183-191.

Motola, D.L., Cummins, C.L., Rottiers, V., Sharma, K.K., Li, T., Li, Y., Suino-Powell, K., Xu, H.E., Auchus, R.J., Antebi, A., and Mangelsdorf, D.J. (2006). Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell *124,* 1209-1223.

Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., Ishizaki, H., Mizoguchi, A., Fujiwara, Y., and Suzuki, A. (1984). Amino-terminal amino acid sequence of the silkworm prothoracicotropic hormone: homology with insulin. Science *226,* 1344-1345.

Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., Mizoguchi, A., Fujiwara, Y., Suzuki, A., Takahashi, S.Y., and Ishizaki, H. (1986). Amino acid sequence of a

prothoracicotropic hormone of the silkworm Bombyx mori. Proc. Natl. Acad. Sci. U. S. A. *83,* 5840-5843.

Nagase, T., Nomura, N., and Ishii, S. (1993). Complex formation between proteins encoded by the ski gene family. J. Biol. Chem. *268,* 13710-13716.

Nakagawa, Y., and Henrich, V.C. (2009). Arthropod nuclear receptors and their role in molting. FEBS J. *276,* 6128-6157.

Nakamura, M., Minegishi, T., Hasegawa, Y., Nakamura, K., Igarashi, S., Ito, I., Shinozaki, H., Miyamoto, K., Eto, Y., and Ibuki, Y. (1993). Effect of an activin A on follicle-stimulating hormone (FSH) receptor messenger ribonucleic acid levels and FSH receptor expressions in cultured rat granulosa cells. Endocrinology *133,* 538-544.

Namciu, S., Lyons, G.E., Micales, B.K., Heyman, H.C., Colmenares, C., and Stavnezer, E. (1995). Enhanced expression of mouse c-ski accompanies terminal skeletal muscle differentiation in vivo and in vitro. Dev. Dyn. *204,* 291-300.

Naureckiene, S., Sleat, D.E., Lackland, H., Fensom, A., Vanier, M.T., Wattiaux, R., Jadot, M., and Lobel, P. (2000). Identification of HE1 as the second gene of Niemann-Pick C disease. Science *290,* 2298-2301.

Neubueser, D., Warren, J.T., Gilbert, L.I., and Cohen, S.M. (2005). Molting defective is required for ecdysone biosynthesis. Dev. Biol. *280,* 362-372.

Nguyen, M., Parker, L., and Arora, K. (2000). Identification of maverick, a novel member of the TGF-beta superfamily in Drosophila. Mech. Dev. *95,* 201-206.

Nichols, R. (2003). Signaling pathways and physiological functions of Drosophila melanogaster FMRFamide-related peptides. Annu. Rev. Entomol. *48,* 485-503.

Nichols, Z., and Vogt, R.G. (2008). The SNMP/CD36 gene family in Diptera, Hymenoptera and Coleoptera: Drosophila melanogaster, D. pseudoobscura, Anopheles gambiae, Aedes aegypti, Apis mellifera, and Tribolium castaneum. Insect Biochem. Mol. Biol. *38,* 398-415.

Nijhout, H.F. (2003). The control of body size in insects. Dev. Biol. *261,* 1-9.

Nijhout, H.F., and Williams, C.M. (1974). Control of moulting and metamorphosis in the tobacco hornworm, Manduca sexta (L.): cessation of juvenile hormone secretion as a trigger for pupation. J. Exp. Biol. *61,* 493-501.

Niwa, R., Namiki, T., Ito, K., Shimada-Niwa, Y., Kiuchi, M., Kawaoka, S., Kayukawa, T., Banno, Y., Fujimoto, Y., Shigenobu, S*., et al.* (2010). Non-molting glossy/shroud encodes a short-chain dehydrogenase/reductase that functions in the 'Black Box' of the ecdysteroid biosynthesis pathway. Development *137,* 1991-1999.

Nomura, N., Sasamoto, S., Ishii, S., Date, T., Matsui, M., and Ishizaki, R. (1989). Isolation of human cDNA clones of ski and the ski-related gene, sno. Nucleic Acids Res. *17,* 5489-5500.

Oakley, A.E,, Clifton, D.K., and Steiner, R.A. (2009). Kisspeptin signaling in the brain. Endocr. Rev. *30*, 713-743.

O'Brien, M.A., Katahira, E.J., Flanagan, T.R., Arnold, L.W., Haughton, G., and Bollenbacher, W.E. (1988). A monoclonal antibody to the insect prothoracicotropic hormone. J. Neurosci. *8,* 3247-3257.

Okamoto, N., Yamanaka, N., Yagi, Y., Nishida, Y., Kataoka, H., O'Connor, M.B., and Mizoguchi, A. (2009). A fat body-derived IGF-like peptide regulates postfeeding growth in Drosophila. Dev. Cell. *17,* 885-891.

Oldham, S., Montagne, J., Radimerski, T., Thomas, G., and Hafen, E. (2000). Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. Genes Dev. *14,* 2689-2694.

Omura, T., and Ito, A. (1991). Biosynthesis and intracellular sorting of mitochondrial forms of cytochrome P450. Methods Enzymol. *206,* 75-81.

Ono, H., Rewitz, K.F., Shinoda, T., Itoyama, K., Petryk, A., Rybczynski, R., Jarcho, M., Warren, J.T., Marques, G., Shimell, M.J., Gilbert, L.I., and O'Connor, M.B. (2006). Spook and Spookier code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. Dev. Biol. *298,* 555-570.

Palanker, L., Necakov, A.S., Sampson, H.M., Ni, R., Hu, C., Thummel, C.S., and Krause, H.M. (2006). Dynamic regulation of Drosophila nuclear receptor activity in vivo. Development *133,* 3549-3562.

Park, D., Veenstra, J.A., Park, J.H., and Taghert, P.H. (2008). Mapping peptidergic cells in Drosophila: where DIMM fits in. PLoS One *3,* e1896.

Parker, K.L., Rice, D.A., Lala, D.S., Ikeda, Y., Luo, X., Wong, M., Bakke, M., Zhao, L., Frigeri, C., Hanley, N.A., Stallings, N., and Schimmer, B.P. (2002). Steroidogenic factor 1: an essential mediator of endocrine development. Recent Prog. Horm. Res. *57,* 19-36.

Parker, L., Stathakis, D.G., and Arora, K. (2004). Regulation of BMP and activin signaling in Drosophila. Prog. Mol. Subcell. Biol. *34,* 73-101.

Parvy, J.P., Blais, C., Bernard, F., Warren, J.T., Petryk, A., Gilbert, L.I., O'Connor, M.B., and Dauphin-Villemant, C. (2005). A role for betaFTZ-F1 in regulating ecdysteroid titers during post-embryonic development in Drosophila melanogaster. Dev. Biol. *282,* 84-94.

Pearson-White, S. (1993). SnoI, a novel alternatively spliced isoform of the ski protooncogene homolog, sno. Nucleic Acids Res. *21,* 4632-4638.

Pearson-White, S., and Crittenden, R. (1997). Proto-oncogene Sno expression, alternative isoforms and immediate early serum response. Nucleic Acids Res. *25,* 2930-2937.

Pearson-White, S., and McDuffie, M. (2003). Defective T-cell activation is associated with augmented transforming growth factor Beta sensitivity in mice with mutations in the Sno gene. Mol. Cell. Biol. *23,* 5446-5459.

Perrimon, N., Lu, X., Hou, X.S., Hsu, J.C., Melnick, M.B., Chou, T.B., and Perkins, L.A. (1995). Dissection of the Torso signal transduction pathway in Drosophila. Mol. Reprod. Dev. *42,* 515-522.

Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.P., Li, Y., Dauphin-Villemant, C., and O'Connor, M.B. (2003). Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc. Natl. Acad. Sci. U. S. A. *100,* 13773-13778.

Poukka, H., Karvonen, U., Janne, O.A., and Palvimo, J.J. (2000). Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc. Natl. Acad. Sci. U. S. A. *97,* 14145-14150.

Ramel, M.C., Emery, C.M., Foulger, R., Goberdhan, D.C., van den Heuvel, M., and Wilson, C. (2007). Drosophila SnoN modulates growth and patterning by antagonizing TGF-beta signalling. Mech. Dev. *124,* 304-317.

Rewitz, K.F., Larsen, M.R., Lobner-Olesen, A., Rybczynski, R., O'Connor, M.B., and Gilbert, L.I. (2009a). A phosphoproteomics approach to elucidate neuropeptide signal transduction controlling insect metamorphosis. Insect Biochem. Mol. Biol. *39,* 475-483.

Rewitz, K.F., Yamanaka, N., Gilbert, L.I., and O'Connor, M.B. (2009b). The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis. Science *326,* 1403-1405.

Riddiford, L.M., Truman, J.W., Mirth, C.K., and Shen, Y.C. (2010). A role for juvenile hormone in the prepupal development of Drosophila melanogaster. Development *137,* 1117-1126.

Roberts, C.A., McCutcheon, S.N., Blair, H.T., Gluckman, P.D., and Breier, B.H. (1990). Developmental patterns of plasma insulin-like growth factor-1 concentrations in sheep. Domest. Anim. Endocrinol. *7,* 457-463.

Roth, G.E., Gierl, M.S., Vollborn, L., Meise, M., Lintermann, R., and Korge, G. (2004). The Drosophila gene Start1: a putative cholesterol transporter and key regulator of ecdysteroid synthesis. Proc. Natl. Acad. Sci. U. S. A. *101,* 1601-1606.

Rountree, D.B., and Bollenbacher, W.E. (1986). The release of the prothoracicotropic hormone in the tobacco hornworm, Manduca sexta, is controlled intrinsically by juvenile hormone. J. Exp. Biol. *120,* 41-58.

Rulifson, E.J., Kim, S.K., and Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science *296,* 1118-1120.

Rybczynski, R., Bell, S.C., and Gilbert, L.I. (2001). Activation of an extracellular signal-regulated kinase (ERK) by the insect prothoracicotropic hormone. Mol. Cell. Endocrinol. *184,* 1-11.

Rybczynski, R., and Gilbert, L.I. (2003). Prothoracicotropic hormone stimulated extracellular signal-regulated kinase (ERK) activity: the changing roles of Ca(2+)- and cAMP-dependent mechanisms in the insect prothoracic glands during metamorphosis. Mol. Cell. Endocrinol. *205,* 159-168.

Sakurai, S., Yonemura, N., Fujimoto, Y., Hata, F., and Ikekawa, N. (1986). 7-Dehydrosterols in prothoracic glands of the silkworm, Bombyx mori. Experientia *42,* 1034-1036.

Sarker, K.P., Wilson, S.M., and Bonni, S. (2005). SnoN is a cell type-specific mediator of transforming growth factor-beta responses. J. Biol. Chem. *280,* 13037-13046.

Saucedo, L.J., Gao, X., Chiarelli, D.A., Li, L., Pan, D., and Edgar, B.A. (2003). Rheb promotes cell growth as a component of the insulin/TOR signalling network. Nat. Cell Biol. *5,* 566-571.

Schwartz, M.B., Imberski, R.B., and Kelly, T.J. (1984). Analysis of metamorphosis in Drosophila melanogaster: characterization of giant, an ecdysteroid-deficient mutant. Dev. Biol. *103,* 85-95.

Segraves, W.A., and Hogness, D.S. (1990). The E75 ecdysone-inducible gene responsible for the 75B early puff in Drosophila encodes two new members of the steroid receptor superfamily. Genes Dev. *4,* 204-219.

Sevala, V.M., Sevala, V.L., Loughton, B.G., and Davey, K.G. (1992). Insulin-like immunoreactivity and molting in Rhodnius prolixus. Gen. Comp. Endocrinol. *86,* 231-238.

Shi, Y.B., Wong, J., Puzianowska-Kuznicka, M., and Stolow, M.A. (1996). Tadpole competence and tissue-specific temporal regulation of amphibian metamorphosis: roles of thyroid hormone and its receptors. Bioessays *18,* 391-399.

Shimmi, O., Ralston, A., Blair, S.S., and O'Connor, M.B. (2005a). The crossveinless gene encodes a new member of the Twisted gastrulation family of BMP-binding proteins which, with Short gastrulation, promotes BMP signaling in the crossveins of the Drosophila wing. Dev. Biol. *282,* 70-83.

Shimmi, O., Umulis, D., Othmer, H., and O'Connor, M.B. (2005b). Facilitated transport of a Dpp/Scw heterodimer by Sog/Tsg leads to robust patterning of the Drosophila blastoderm embryo. Cell *120,* 873-886.

Shinagawa, T., Dong, H.D., Xu, M., Maekawa, T., and Ishii, S. (2000). The sno gene, which encodes a component of the histone deacetylase complex, acts as a tumor suppressor in mice. EMBO J. *19,* 2280-2291.

Shionoya, M., Matsubayashi, H., Asahina, M., Kuniyoshi, H., Nagata, S., Riddiford, L.M., and Kataoka, H. (2003). Molecular cloning of the prothoracicotropic hormone from the tobacco hornworm, Manduca sexta. Insect Biochem. Mol. Biol. *33,* 795-801.

Shravage, B.V., Altmann, G., Technau, M., and Roth, S. (2007). The role of Dpp and its inhibitors during eggshell patterning in Drosophila. Development *134,* 2261-2271.

Silva, J.R., Figueiredo, J.R., and van den Hurk, R. (2009). Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. Theriogenology *71,* 1193-1208.

Simpson, P., Berreur, P., and Berreur-Bonnenfant, J. (1980). The initiation of pupariation in Drosophila: dependence on growth of the imaginal discs. J. Embryol. Exp. Morphol. *57,* 155-165.

Sisk, C.L., and Foster, D.L. (2004). The neural basis of puberty and adolescence. Nat. Neurosci. *7,* 1040-1047.

Slaidina, M., Delanoue, R., Gronke, S., Partridge, L., and Leopold, P. (2009). A Drosophila insulin-like peptide promotes growth during nonfeeding states. Dev. Cell. *17,* 874-884.

Sleeman, J.P., and Laskey, R.A. (1993). Xenopus c-ski contains a novel coiled-coil protein domain, and is maternally expressed during development. Oncogene *8,* 67-77.

Smith, W.A., Combest, W.L., and Gilbert, L.I. (1986). Involvement of cyclic AMP-dependent protein kinase in prothoracicotropic hormone-stimulated ecdysone synthesis. Mol. Cell. Endocrinol. *47,* 25-33.

Smith, W.A., and Gilbert, L.I. (1989). Early events in peptide-stimulated ecdysteroid secretion by the prothoracic glands of Manduca sexta. J. Exp. Zool. *252,* 264-270.

Smith, W.A., Gilbert, L.I., and Bollenbacher, W.E. (1985). Calcium-cyclic AMP interactions in prothoracicotropic hormone stimulation of ecdysone synthesis. Mol. Cell. Endocrinol. *39,* 71-78.

Smith, W.A., Gilbert, L.I., and Bollenbacher, W.E. (1984). The role of cyclic AMP in the regulation of ecdysone synthesis. Mol. Cell. Endocrinol. *37,* 285-294.

Smyth, C.M., and Bremner, W.J. (1998). Klinefelter syndrome. Arch. Intern. Med. *158,* 1309-1314.

Song, Q., and Gilbert, L.I. (1997). Molecular cloning, developmental expression, and phosphorylation of ribosomal protein S6 in the endocrine gland responsible for insect molting. J. Biol. Chem. *272,* 4429-4435.

Song, Q., and Gilbert, L.I. (1994). S6 phosphorylation results from prothoracicotropic hormone stimulation of insect prothoracic glands: a role for S6 kinase. Dev. Genet. *15,* 332-338.

Sonis, W.A., Comite, F., Blue, J., Pescovitz, O.H., Rahn, C.W., Hench, K.D., Cutler, G.B.,Jr, Loriaux, D.L., and Klein, R.P. (1985). Behavior problems and social competence in girls with true precocious puberty. J. Pediatr. *106,* 156-160.

Stavnezer, E., Barkas, A.E., Brennan, L.A., Brodeur, D., and Li, Y. (1986). Transforming Sloan-Kettering viruses generated from the cloned v-ski oncogene by in vitro and in vivo recombinations. J. Virol. *57,* 1073-1083.

Steel, C.G., and Vafopoulou, X. (1997). Ecdysteroidogenic action of Bombyx prothoracicotropic hormone and bombyxin on the prothoracic glands of Rhodnius prolixus in vitro. J. Insect Physiol. *43,* 651-656.

Stocco, D.M., and Clark, B.J. (1996). Regulation of the acute production of steroids in steroidogenic cells. Endocr. Rev. *17,* 221-244.

Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. (2003). Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. Nat. Cell Biol. *5,* 559-565.

Strigini, M., and Cohen, S.M. (1999). Formation of morphogen gradients in the Drosophila wing. Semin. Cell Dev. Biol. *10,* 335-344.

Stroschein, S.L., Wang, W., Zhou, S., Zhou, Q., and Luo, K. (1999). Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein. Science *286,* 771-774.

Sun, Y., Liu, X., Eaton, E.N., Lane, W.S., Lodish, H.F., and Weinberg, R.A. (1999a). Interaction of the Ski oncoprotein with Smad3 regulates TGF-beta signaling. Mol. Cell *4,* 499-509.

Sun, Y., Liu, X., Ng-Eaton, E., Lodish, H.F., and Weinberg, R.A. (1999b). SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor beta signaling. Proc. Natl. Acad. Sci. U. S. A. *96,* 12442-12447.

Takaesu, N.T., Hyman-Walsh, C., Ye, Y., Wisotzkey, R.G., Stinchfield, M.J., O'connor, M.B., Wotton, D., and Newfeld, S.J. (2006). dSno facilitates baboon signaling in the

Drosophila brain by switching the affinity of Medea away from Mad and toward dSmad2. Genetics *174,* 1299-1313.

Talamillo, A., Sanchez, J., Cantera, R., Perez, C., Martin, D., Caminero, E., and Barrio, R. (2008). Smt3 is required for Drosophila melanogaster metamorphosis. Development *135,* 1659-1668.

Taniguchi, C.M., Emanuelli, B., and Kahn, C.R. (2006). Critical nodes in signalling pathways: insights into insulin action. Nat. Rev. Mol. Cell Biol. *7,* 85-96.

Tempel, D.L., and Leibowitz, S.F. (1990). Galanin inhibits insulin and corticosterone release after injection into the PVN. Brain Res. *536,* 353-357.

ten Dijke, P., Korchynskyi, O., Valdimarsdottir, G., and Goumans, M.J. (2003). Controlling cell fate by bone morphogenetic protein receptors. Mol. Cell. Endocrinol. *211,* 105-113.

Thomas, J.H., Birnby, D.A., and Vowels, J.J. (1993). Evidence for parallel processing of sensory information controlling dauer formation in Caenorhabditis elegans. Genetics *134,* 1105-1117.

Thummel, C.S. (1996). Flies on steroids--Drosophila metamorphosis and the mechanisms of steroid hormone action. Trends Genet. *12,* 306-310.

Ting, C.Y., Herman, T., Yonekura, S., Gao, S., Wang, J., Serpe, M., O'Connor, M.B., Zipursky, S.L., and Lee, C.H. (2007). Tiling of r7 axons in the Drosophila visual system is mediated both by transduction of an activin signal to the nucleus and by mutual repulsion. Neuron *56,* 793-806.

Truman, J.W., and Riddiford, L.M. (1974). Physiology of insect rhythms. 3. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. J. Exp. Biol. *60,* 371-382.

Tsuzuki, S., Masuta, T., Furuno, M., Sakurai, S., and Iwami, M. (1997). Structure and expression of bombyxin E1 gene: a novel family gene that encodes bombyxin-IV, an insect insulin-related neurosecretory peptide. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. *117,* 409-416.

Val, P., Lefrancois-Martinez, A.M., Veyssiere, G., and Martinez, A. (2003). SF-1 a key player in the development and differentiation of steroidogenic tissues. Nucl. Recept. *1,* 8.

Van den Broek, P.J., Barroso, M., and Lechner, M.C. (1996). Critical amino-terminal segments in insertion of rat liver cytochrome P450 3A1 into the endoplasmic reticulum membrane. Experientia *52,* 851-855.

Vandersmissen, T., De Loof, A., and Gu, S.H. (2007). Both prothoracicotropic hormone and an autocrine factor are involved in control of prothoracic gland ecdysteroidogenesis in Locusta migratoria and Schistocerca gregaria. Peptides *28,* 44-50.

Verdu, J., Buratovich, M.A., Wilder, E.L., and Birnbaum, M.J. (1999). Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. Nat. Cell Biol. *1,* 500-506.

Verger, A., Perdomo, J., and Crossley, M. (2003). Modification with SUMO. A role in transcriptional regulation. EMBO Rep. *4,* 137-142.

Walkiewicz, M.A., and Stern, M. (2009). Increased insulin/insulin growth factor signaling advances the onset of metamorphosis in Drosophila. PLoS One *4,* e5072.

Wang, W., Mariani, F.V., Harland, R.M., and Luo, K. (2000). Ski represses bone morphogenic protein signaling in Xenopus and mammalian cells. Proc. Natl. Acad. Sci. U. S. A. *97,* 14394-14399.

Warren, J.T., Petryk, A., Marques, G., Jarcho, M., Parvy, J.P., Dauphin-Villemant, C., O'Connor, M.B., and Gilbert, L.I. (2002). Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A. *99,* 11043-11048.

Warren, J.T., Petryk, A., Marques, G., Parvy, J.P., Shinoda, T., Itoyama, K., Kobayashi, J., Jarcho, M., Li, Y., O'Connor, M.B., Dauphin-Villemant, C., and Gilbert, L.I. (2004). Phantom encodes the 25-hydroxylase of Drosophila melanogaster and Bombyx mori: a P450 enzyme critical in ecdysone biosynthesis. Insect Biochem. Mol. Biol. *34,* 991-1010.

Warren, J.T., Wismar, J., Subrahmanyam, B., and Gilbert, L.I. (2001). Woc (without children) gene control of ecdysone biosynthesis in Drosophila melanogaster. Mol. Cell. Endocrinol. *181,* 1-14.

Warren, J.T., Yerushalmi, Y., Shimell, M.J., O'Connor, M.B., Restifo, L.L., and Gilbert, L.I. (2006). Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of Drosophila melanogaster: correlations with changes in gene activity. Dev. Dyn. *235,* 315-326.

Watanabe, K., Hull, J.J., Niimi, T., Imai, K., Matsumoto, S., Yaginuma, T., and Kataoka, H. (2007). FXPRL-amide peptides induce ecdysteroidogenesis through a G-protein coupled receptor expressed in the prothoracic gland of Bombyx mori. Mol. Cell. Endocrinol. *273,* 51-58.

Watson, R.D., Yeh, W.E., Muehleisen, D.P., Watson, C.J., and Bollenbacher, W.E. (1993). Stimulation of ecdysteroidogenesis by small prothoracicotropic hormone: role of cyclic AMP. Mol. Cell. Endocrinol. *92,* 221-228.

Weinkove, D., Neufeld, T.P., Twardzik, T., Waterfield, M.D., and Leevers, S.J. (1999). Regulation of imaginal disc cell size, cell number and organ size by Drosophila class I(A) phosphoinositide 3-kinase and its adaptor. Curr. Biol. *9,* 1019-1029.

Wilkinson, K.A., and Henley, J.M. (2010). Mechanisms, regulation and consequences of protein SUMOylation. Biochem. J. *428,* 133-145.

Winograd, J., Reilly, M.P., Roe, R., Lutz, J., Laughner, E., Xu, X., Hu, L., Asakura, T., vander Kolk, C., Strandberg, J.D., and Semenza, G.L. (1997). Perinatal lethality and multiple craniofacial malformations in MSX2 transgenic mice. Hum. Mol. Genet. *6,* 369-379.

Wismar, J., Habtemichael, N., Warren, J.T., Dai, J.D., Gilbert, L.I., and Gateff, E. (2000). The mutation without children(rgl) causes ecdysteroid deficiency in third-instar larvae of Drosophila melanogaster. Dev. Biol. *226,* 1-17.

Wisotzkey, R.G., Mehra, A., Sutherland, D.J., Dobens, L.L., Liu, X., Dohrmann, C., Attisano, L., and Raftery, L.A. (1998). Medea is a Drosophila Smad4 homolog that is differentially required to potentiate DPP responses. Development *125,* 1433-1445.

Wu, Q., and Brown, M.R. (2006). Signaling and function of insulin-like peptides in insects. Annu. Rev. Entomol. *51,* 1-24.

Wu, J.W., Krawitz, A.R., Chai, J., Li, W., Zhang, F., Luo, K., and Shi, Y. (2002). Structural mechanism of Smad4 recognition by the nuclear oncoprotein Ski: insights on Ski-mediated repression of TGF-beta signaling. Cell *111,* 357-367.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell *124,* 471-484.

Xiao, S., Robertson, D.M., and Findlay, J.K. (1992). Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology *131,* 1009-1016.

Yamada, M., Murata, T., Hirose, S., Lavorgna, G., Suzuki, E., and Ueda, H. (2000). Temporally restricted expression of transcription factor betaFTZ-F1: significance for embryogenesis, molting and metamorphosis in Drosophila melanogaster. Development *127,* 5083-5092.

Yamanaka, N., Honda, N., Osato, N., Niwa, R., Mizoguchi, A., and Kataoka, H. (2007). Differential regulation of ecdysteroidogenic P450 gene expression in the silkworm, Bombyx mori. Biosci. Biotechnol. Biochem. *71,* 2808-2814.

Yamanaka, N., Hua, Y.J., Mizoguchi, A., Watanabe, K., Niwa, R., Tanaka, Y., and Kataoka, H. (2005). Identification of a novel prothoracicostatic hormone and its receptor in the silkworm Bombyx mori. J. Biol. Chem. *280,* 14684-14690.

Yamanaka, N., Zitnan, D., Kim, Y.J., Adams, M.E., Hua, Y.J., Suzuki, Y., Suzuki, M., Suzuki, A., Satake, H., Mizoguchi, A.*, et al.* (2006). Regulation of insect steroid hormone biosynthesis by innervating peptidergic neurons. Proc. Natl. Acad. Sci. U. S. A. *103,* 8622-8627.

Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., McKeown, M., Cherbas, P., and Evans, R.M. (1993). Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. Nature *366,* 476-479.

Yoshida, I., Moto, K., Sakurai, S., and Iwami, M. (1998). A novel member of the bombyxin gene family: structure and expression of bombyxin G1 gene, an insulin-related peptide gene of the silkmoth Bombyx mori. Dev. Genes Evol. *208,* 407-410.

Yoshida, I., Tsuzuki, S., Abdel Salam, S.E., Ino, M., Korayem, A.M., Sakurai, S., and Iwami, M. (1997). Bombyxin F1 gene: structure and expression of a new bombyxin family gene that forms a pair with bombyxin B10 gene. Zoolog Sci. *14,* 615-622.

Yoshida, S., Soustelle, L., Giangrande, A., Umetsu, D., Murakami, S., Yasugi, T., Awasaki, T., Ito, K., Sato, M., and Tabata, T. (2005). DPP signaling controls development of the lamina glia required for retinal axon targeting in the visual system of Drosophila. Development *132,* 4587-4598.

Yoshiyama, T., Namiki, T., Mita, K., Kataoka, H., and Niwa, R. (2006). Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. Development *133,* 2565-2574.

Zhang, F., Lundin, M., Ristimaki, A., Heikkila, P., Lundin, J., Isola, J., Joensuu, H., and Laiho, M. (2003). Ski-related novel protein N (SnoN), a negative controller of transforming growth factor-beta signaling, is a prognostic marker in estrogen receptor-positive breast carcinomas. Cancer Res. *63,* 5005-5010.

Zhang, H., Stallock, J.P., Ng, J.C., Reinhard, C., and Neufeld, T.P. (2000). Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes Dev. *14,* 2712-2724.

Zhang, T.Y., Sun, J.S., Zhang, Q.R., Xu, J., Jiang, R.J., and Xu, W.H. (2004). The diapause hormone-pheromone biosynthesis activating neuropeptide gene of Helicoverpa armigera encodes multiple peptides that break, rather than induce, diapause. J. Insect Physiol. *50,* 547-554.

Zhang, Y., Gao, X., Saucedo, L.J., Ru, B., Edgar, B.A., and Pan, D. (2003). Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat. Cell Biol. *5,* 578-581.

Zheng, G., Teumer, J., Colmenares, C., Richmond, C., and Stavnezer, E. (1997). Identification of a core functional and structural domain of the v-Ski oncoprotein responsible for both transformation and myogenesis. Oncogene *15,* 459-471.

Zheng, X., Zugates, C.T., Lu, Z., Shi, L., Bai, J.M., and Lee, T. (2006). Baboon/dSmad2 TGF-beta signaling is required during late larval stage for development of adult-specific neurons. EMBO J. *25,* 615-627.

Zhu, C.C., Boone, J.Q., Jensen, P.A., Hanna, S., Podemski, L., Locke, J., Doe, C.Q., and O'Connor, M.B. (2008). Drosophila Activin- and the Activin-like product Dawdle function redundantly to regulate proliferation in the larval brain. Development *135,* 513-521.

Zhu, Q., Pearson-White, S., and Luo, K. (2005). Requirement for the SnoN oncoprotein in transforming growth factor beta-induced oncogenic transformation of fibroblast cells. Mol. Cell. Biol. *25,* 10731-10744.