A Genetic, Transgenic, and Transcriptomic Analysis of Larval Salivary Gland Physiology in Drosophila melanogaster

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A GENETIC, TRANSGENIC, AND TRANSCRIPTOMIC ANALYSIS

OF LARVAL SALIVARY GLAND PHYSIOLOGY

IN DROSOPHILA MELANOGASTER

by

Elana A. Paladino

Bachelor of Arts
University of California, Los Angeles
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A dissertation submitted in partial fulfillment
of the requirements for the

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Elana A. Paladino

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ABSTRACT

A Genetic, Transgenic, and Transcriptomic Analysis of Larval Salivary Gland Physiology in Drosophila melanogaster

by

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Dr. Andrew J. Andres, Examination Committee Chair
Associate Professor of Biological Sciences
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Cholesterol is the precursor to a unique class of lipophilic signaling molecules called steroid hormones that initiate the development of sexual characteristics, reactions to stress, and maintenance of metabolism, among many other functions. Although much progress has been made in understanding the function of these signaling hormones, we do not fully understand how a single steroid can cause many distinct, tissue-specific responses. Drosophila melanogaster is an effective model for understanding steroid hormone action because of its simplicity. The steroid molting hormone 20-hydroxyecdysone (hereafter, 20E) is the primary active steroid in Drosophila and mediates not only larval molts and the emergence of a sexually mature adult, but also is an effector of many other tissue-specific actions from the embryonic to larval stages and into adulthood. An effective approach for studying hormone signaling is to use molecular genetics in which genes (that have roles in 20E-signaling pathways) are overexpressed or silenced in a specific tissue at a defined time during development. Even more, Drosophila is a tractable model organism with many tools that are widely available, and large-scale genetic screens are easily executed. We chose to focus on the salivary gland of larval Drosophila as a primary model for 20E signaling in that several
specific, measurable responses to the steroid occur at different times during development.

Using this model system, my dissertation research was centered on two major aims:

1) identify genes in the 20E-regulated process of glue secretion in the larval salivary gland; and 2) test the hypothesis that an ATP-Binding Cassette (ABC) transporter, E23, participates in a negative feedback loop that acts directly on the hormone 20E.

For the first part of my dissertation, I mapped and characterized mutations that block 20E-regulated processes in the salivary gland of larval Drosophila. These mutations were generated in an EMS-mutagenesis screen for blockages in the steroid-regulated process of salivary-gland glycoprotein (glue) secretion and expulsion. I mapped four homozygous recessive, loss-of-function mutations that block the expulsion of the glue protein, labeled with a GFP reporter. For each mutation, I generated a list of approximately ten candidate genes after mapping each mutation with a deficiency kit. I refined the mapping as more deficiencies became available, and I used complementation testing with Drosophila lines that had single-gene disruptions to narrow down the candidates even further. Finally, I sequenced mutant DNA in order to find the molecular nature of the mutations and confirm the genes with the suspected mutations. In doing so, I mapped two mutations to single loci on the third chromosome.

For the second part of my dissertation, I characterized a 20E-activated gene named E23, which encodes an ABC transporter. In humans, cholesterol secretion is mediated by a subset of ABC transporters that are similar to E23. Whole-body cholesterol homeostasis relies in part on the efflux of cholesterol and cholesterol derivatives from cellular compartments. Because ABC transporters are crucial in maintaining the metabolism and homeostasis of cholesterol and lipids derived from cholesterol, mutations
in ABC transporters result in severe heritable diseases such as Tangier disease, Dubin-Johnson syndrome, sitosterolemia, and adrenoleukodystrophy. Of the more than 50 ABC transporter genes in *Drosophila melanogaster*, *E23* is the only known gene to be activated by 20E (mediated by the ecdysone receptor). We tested the hypothesis that E23 is part of a negative feedback loop that acts directly on the hormone 20E. We overexpressed *E23* in specific tissues (including the larval salivary gland) that respond to 20E, at a defined time during development. We found that *E23* overexpression phenocopies the loss of the ecdysone receptor, but this can be overcome by exposing tissues to high levels of 20E *in vivo*. Using Illumina RNA-seq on the larval salivary gland, we also found that *E23* overexpression has widespread consequences on gene expression, including the expression of temporally-specific 20E targets. These results support our hypothesis that E23 has a negative regulatory role on 20E signaling through modulating the levels of 20E. This work is novel in that it suggests a previously unknown mechanism for effectively controlling the hormone exposure of target tissues, which may contribute to the diverse responses to a single hormone in flies, and furthermore, it serves as a springboard for future experiments to test the mechanism and specificity of E23 for the steroid hormone 20E.
ACKNOWLEDGEMENTS

This work would not have been possible without the advice, support, and involvement of a talented group of people. Foremost, I owe an extreme debt of gratitude to Dr. Andres (and Chris Andres) and my doctoral committee of past and present: Dr. Jeff Shen, Dr. Frank van Breukelen, Dr. Deborah Hoshizaki, Dr. Ernesto Abel-Santos, and Dr. Laurel Raftery. It was with their encouragement that I saw my doctorate through to its completion and through their superior educational abilities that I became a better scientist.

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molecular biology. Even more, Ben encouraged me to believe in myself, and he filled my life with laughter. He is the reason I persevered after my father died, and he will always hold a special place in my heart.

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DEDICATION

This work is dedicated to my dad

Bernard Milton Paladino

(March 7, 1943 - January 13, 2007)
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CHAPTER 1
THE SALIVARY GLAND:
A MODEL FOR STEROID HORMONE SIGNALING

1.1: Drosophila is a Model for Steroid Hormone Signaling

Drosophila melanogaster is a powerful genetic model organism that has been used in biological research for over a century. Its long history has allowed scientists to develop an arsenal of genetic and molecular tools that, in combination with genomic and bioinformatic approaches, allow an investigator to answer virtually any biological question (see Arias, 2008 for a historical summary).

Drosophila continues to prove its value to the progress of scientific knowledge. Its strength as a model organism is obvious when examining the information we have gained from such endeavors as the Nobel-prize winning research that laid the blueprint for embryonic development not only in Drosophila but in animals including humans (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984; Lewis, 1986), and from the studies of basic cell signaling pathways that are strikingly similar to other eukaryotes (Rubin et al., 2000; Reiter et al., 2001; Bier, 2005). For example, some of the most significant findings on the molecular basis for hereditary human colorectal cancer originated from studies of the wingless/wnt-signaling pathway in Drosophila (Kinzler and Vogelstein, 1996; Clevers and Nusse, 2012).

Furthermore, it is not mere coincidence that Drosophila is the workhorse of genetic research, for it was the pioneering work of Thomas Hunt Morgan and his students, Alfred Sturtevant, Calvin Bridges, and Hermann Muller, that led to the finding that the hereditary material (i.e., genes) are contained on chromosomes (Bridges, 1916a, 1916b;
Sturtevant et al., 1919); and even further, that chromosomes could be physically mutated by X-rays (Muller, 1927), so that the resulting mutant phenotypes could be described and located to specific loci in a forward genetic approach (Lindsley et al., 1972).

Of critical importance to the work contained herein is the over 80 year history of the contributions of *Drosophila* to genome science, specifically as a model for steroid-induced global changes in gene expression within a specific tissue (see Rubin and Lewis, 2000). In effect, the cellular biology of *Drosophila* facilitated large studies of steroid hormones and gene expression many decades prior to the completion of *Drosophila’s* genome sequence. This was possible because some larval tissues such as the salivary gland contain large polyploid cells with endoreplicated chromosomes that are a thousand times thicker than the interphase chromosomes of diploid cells (Lee et al., 2009). As a result, changes in gene expression can be visualized cytologically as puffs (reviewed in Zhimulev et al., 2004). Even today the physical and cytogenetic mapping of alleles benefit from the polytene maps constructed by Bridges in the 1930s (for example, see Bridges and Bridges, 1938).

Perhaps most importantly, exposing the polytene chromosomes *in vitro* and *in vivo* to steroid hormones induces the sequential puffing of distinct yet reproducible regions of the chromosomes. The chromosomal puffs were hypothesized to be areas of transcription due to the action of the steroid hormone 20-hydroxyecdysone (20E) (Ashburner, 1972, 1973, 1974; Ashburner et al., 1974; Ashburner and Richards, 1976; Richards, 1982). Hence, in the 1960s and 1970s, the Genomic Era was born where sizeable studies of gene expression cascades led to the characterization of the puffing regions, such that a large number of the individual genes within those regions could be placed into a 20E-initiated
genetic circuit (Ashburner et al., 1974). Moreover, the complete genome sequence of *D. melanogaster* was finished in 2000, and it was the first of the complex organisms to be sequenced, opening the door for genome-scale discovery of the battery of genes in the steroid-regulated circuit (Adams et al., 2000).

### 1.2: The Insect Steroid Hormone 20-Hydroxyecdysone

The steroid hormone 20-hydroxyecdysone (20E) is the endocrine signal that was critical for the polytene puffing studies of the mid-twentieth century. It is a 27-carbon molecule derived from dietary cholesterol, and it is the primary active steroid in *Drosophila melanogaster* (Lafont and Dauphin-Villemant, 2011). Ingested cholesterol is enzymatically altered by a series of cytochrome P450s (encoded by the *Halloween*-suite of genes including *phantom*, *disembodied*, and *shadow*) in the prothoracic gland (which is part of an organ called the ring gland) of larval *Drosophila* to form the 20E-precursor α-ecdysone (Chavez et al., 2000; Warren et al., 2002, 2004).

The precursor α-ecdysone is further hydroxylated to form 20E by another cytochrome P450 (the product of the *shade* gene) in tissues that are peripheral to the ring gland such as the fat body, Malpighian tubules, and midgut (Petryk et al., 2003) (Figure 1.1A). Because they are very water soluble due to their numerous hydroxyl groups, ecdysteroids are thought to be transferred to target tissues without specific carrier molecules in the hemolymph (Gilbert and Chino, 1974) even though the possibility exists for a yet-to-be discovered hemolymph-binding protein or lipoprotein particle.

After α-ecdysone is converted to 20E and 20E enters its target cell possibly through passive diffusion (Gilbert and Chino, 1974), it must also enter the nucleus, where a
nuclear hormone receptor is located (Koelle et al., 1991; Yao et al., 1993; Gauhar et al., 2009). The functional ecdysone receptor is a heterodimer of two proteins, EcR (ecdysone receptor) (Koelle et al., 1991) and USP (ultraspiracle) (Shea et al., 1990; Oro et al., 1992). When bound at ecdysone response elements (EcREs) of some target genes, the EcR/USP heterodimer is associated with corepressors such as SMRTER (Tsai et al., 1999), and as such, EcR/USP has a repressive role in the absence of 20E. Once 20E binds, corepressors dissociate and coactivators are recruited (Gates et al., 2004), changing the conformation of the heterodimer and resulting in high levels of hormone receptor activation of the target gene (Yao et al., 1993) (Figure 1.1C-D).

Interestingly, concerning the molecular mechanism of 20E-induced gene regulation, more recent evidence suggests that another nuclear hormone receptor, E75A, associates with the corepressor SMRTER and competes for EcR/USP binding sites on the DNA (Johnston et al., 2011). Thus, E75A would have the proposed repressive function and not necessarily the unliganded EcR/USP receptor. Surprisingly, new evidence also suggests that EcR and USP are localized in the cytoplasm when 20E levels are lower (Johnston et al., 2011), which contradicts that accepted paradigm that EcR/USP is perpetually nuclear localized even in the absence of hormone (Koelle et al., 1991).

Much is known about 20E hormone signaling at the molecular level, but the genetic pathways are not linear or completely understood. It is known that 20E-initiated gene expression is required throughout the life cycle of Drosophila as it mediates important developmental transitions such as molting at the end of each larval instar and metamorphosis into adulthood (Garen et al., 1977; Riddiford, 1993; Riddiford et al., 2000; Schwedes and Carney, 2012). However, 20E is not ubiquitously present at specific
basal levels/titers; instead, it floods the animal in waves of *de novo* synthesized hormone (Chino et al., 1974; King et al., 1974; Dauphin-Villemant et al., 1995), corresponding to major developmental changes such as molting to the subsequent instar or transitioning to a pupa from a larval state (Riddiford, 1993) (Figure 1.2A).

Nevertheless, the fluctuating titers of 20E result from several metabolic factors that include not only the rate of *de novo* synthesis of α-ecdysone, but also the rate of conversion of α-ecdysone into the metabolically active 20E, and the inactivation and/or excretion of 20E (Koolman and Karlson, 1985). The Malpighian tubules, fat body, and midgut are the primary tissues of steroid metabolism, and the steroids are eventually excreted by way of the gut or Malpighian tubules (Koolman and Karlson, 1985; Lafont and Koolman, 1985). The fact that the steroid hormone is so precisely regulated suggests that its levels are critical for inducing properly timed developmental and physiological events.
Figure 1.1: The structure of 20E and the canonical mechanism of 20E signaling via EcR/USP. (A) Dietary cholesterol is converted to α-ecdysone in the prothoracic gland of larval Drosophila. (B) α-ecdysone moves through the hemolymph and is converted to 20E in peripheral tissues such as the fat body. (C) In the absence of 20E in target tissues, EcR/USP is believed to act as a repressor and is bound by corepressors. (D) When threshold levels of 20E are present, the ecdysone receptor is de-repressed via the recruitment of coactivators, resulting in high levels of gene activation.
Figure 1.2: The global and tissue-specific roles of 20E during development. (A) *Drosophila* grows through ecdysis and molting, that is, the shedding and rebuilding of its cuticular structures at times that correlate with systemic increases in 20E. There are two larva-to-larva molts, followed by a larva-to-pupa molt (pupariation) and a pupa-to-adult molt. Ecdysteroid titers are redrawn from Riddiford, 1993. (B) 20E causes tissue specific physiological changes, which have been well studied at metamorphosis, including the cell death of larval tissues, the growth and differentiation of adult precursor tissues called imaginal discs, and the reorganization of larval tissues such as the dissociation of the fat body. Although not depicted, 20E causes many other tissue-specific physiological events, occurring at many different stages during development including both embryonic and adult, in addition to the larval stages.
1.3: Decoding the Spatially and Temporally Specific Responses to 20E

Besides the global function of the steroid hormone in molting, 20E causes precisely timed, tissue specific changes prior to and during metamorphosis that can be categorized into three major groups: 1) the programmed cell death of larval tissues; 2) the differentiation of adult structures from precursor organs called imaginal discs; and 3) the reorganization of larval tissues during metamorphosis (reviewed in Riddiford, 1993) (Figure 1.2B). A major question that prompted the studies in this dissertation is: “How can a single steroid cause so many spatially and temporally specific physiological events?”

Principally, three overlapping levels of control must be considered as to how a systemic signal such as 20E can have temporal and tissue-specific functions. The first is that the differing titers of 20E throughout development cause tissue-specific gene activation depending on the nature of the hormone response element (EcRE) (for example, see Andres and Cherbas, 1992, 1994). Different genes have EcREs that vary in structure and function, and they are predicted to have higher or lower affinities for the ecdysone receptor (Champlin and Truman, 1998; Li and White, 2003). Thus, individual genes may be more or less responsive to the hormone depending on the structure of the EcRE and on the amount of hormone to which the tissues are exposed.

Second, the constellation of transcriptional regulators in a given tissue at a given time determines the specific response. This hypothesis has been supported by research demonstrating differing expression profiles and functions for the three isoforms of EcR (Robinow et al., 1993; Talbot et al., 1993; Truman et al., 1994; White et al., 1997; Cherbas et al., 2003). It has also been supported by research in which temporally-
specific 20E responses require the EcR component of the receptor, but USP was either not required or had a repressive role (Ghbeish and McKeown, 2002; Costantino et al., 2008). In addition, any factor that regulates the ecdysone receptor could play a role. For example, the tissue-specific expression of coactivators and corepressors dictate the activity of the ecdysone receptor (Dressel et al., 1999; Tsai et al., 1999; Bai et al., 2000; Francis et al., 2010). Furthermore, a tissue-specific E3 ubiquitin ligase (encoded by Ariadne1) was demonstrated to bind to and ubiquitylate specific isoforms of EcR, which would lead to unique EcR isoform localization due to differing levels of proteasomal degradation (Gradilla et al., 2011).

An offshoot of the idea that control is achieved by the specific constellation of transcriptional regulators present in a cell is called the Ashburner model. This model defines the global response to the hormone at the molecular level and will be discussed further in Section 1.5. Briefly, the model states that a battery of genes is activated by 20E in a hierarchy, with the earlier gene products dictating what later genes will be activated. Thus, the specific expression of earlier genes in the hierarchy could lead to tissue-specific responses (Ashburner et al., 1974). For example, the early gene Broad Complex (BR-C), which encodes a complex of zinc-finger transcription factors, has several isoforms that have unique expression patterns and functions (von Kalm et al., 1994).

Finally, the third consideration is the length of time a tissue is exposed to the hormone. Thus, even though the hormone is a systemic signal, tissue-specific factors may deactivate the hormone resulting in varying exposure. To the extent of what is currently known about 20E catabolism, a critical inactivating enzyme is Cyp18a1, and it has very high levels of expression in 20E target tissues such as the salivary glands,
epidermis, fat body, and midgut early in the third larval instar (Guittard et al., 2011). It is believed to play a major catabolic role during metamorphosis when the pupa exists in a system devoid of food intake or waste removal (Guittard et al., 2011). However, Cyp18a1 does not appear to have a critical role prior to metamorphosis because the animal survives to pupariation in Cyp18a1 loss-of-function animals (Guittard et al., 2011). Yet, 20E is metabolized at earlier times in development as indicated by the intermittent low whole-animal titers (Warren et al., 2006).

For this work, we are interested in the connection between the global aspects of the Ashburner model and the idea that the intracellular levels of 20E are specifically regulated to bring about different responses. Interestingly, the ATP-binding cassette (ABC) transporter named E23 (encoded by Early gene at 23/E23) is an early gene in Ashburner’s model, and has been proposed to negatively regulate 20E through lowering the effective concentration of 20E within cells (Hock et al., 2000; Thummel, 2002; Thummel and Chory, 2002). It is an intriguing intersection linking Ashburner’s model to tissue-specific hormone regulation. Further, E23 is a focus of this dissertation and will be discussed in Sections 1.6-1.8.

1.4: The Larval Salivary Gland—20E-Mediated Glue Synthesis and Secretion

In order to explore temporally and spatially-specific gene activation by 20E, we have developed a robust model system using the larval salivary gland of Drosophila (Biyasheva et al., 2001; Costantino et al., 2008). This is ideal to study the temporally distinct changes brought about by 20E signaling because of several unique responses to the steroid that are separated in time but require 20E to occur. We have centered our
studies on two of these physiological events that are specific to the salivary gland and are triggered by 20E in the third instar (but separated by approximately 14-18 hours). First is the production of a glycoprotein glue mixture during the middle of the third instar, and second (occurring ~18 hours later) is the secretion of the glue into the lumen of the salivary gland where it can then be expelled out of the salivary gland and through the mouth of a late larva. The expectorated glue allows a prepupa to adhere to a surface during metamorphosis (Korge, 1977).

The development of transgenic stocks carrying fluorescently-tagged glue proteins (under the control of their endogenous regulatory sequences) has facilitated the study of glue synthesis and secretion (Costantino et al., 2008) because with this tool, both glue production and expulsion can be visualized with a low-power fluorescent microscope. Using both confocal and fluorescent microscopy, we characterized the progression of wild-type glue production and secretion with the fluorescently-tagged glue protein (glueRED). Becoming familiar with the normal progression of these processes is necessary for interpreting data in the chapters that follow (see Figure 1.3 for a complete description).

Different titers of 20E trigger each of the responses (Figure 1.4A). A small pulse of 20E in the mid-third instar results in the production of the glycoprotein glue mixture; whereas, the large pre-metamorphic pulse of 20E causes the exocytosis of glue (Biyasheva et al., 2001; Costantino et al., 2008). Hence, these two distinct salivary-gland specific functions of 20E can be used to understand how a single hormone causes a variety of responses. What is notable about both processes is that they are mediated by early gene products. Specific isoforms of the transcription factor BR-C have been
demonstrated as a requirement for glue gene expression and secretion, as shown through loss-of-function and rescue experiments with individual isoforms encoded by BR-C (Biyasheva et al., 2001; Costantino et al., 2008).

In addition to glue secretion, the pre-metamorphic pulse of 20E initiates a complex hierarchy of gene activity involving the activation of the early and late genes, which are so named because they form temporally specific puffs on the giant polytene chromosomes in the salivary gland nuclei. The puffing activity has helped researchers to build testable models of the 20E-triggered cascade of gene activation that results in tissue specific responses. Understanding the genetic cascade is also critical to understanding the specific factors involved in glue synthesis and secretion. Furthermore, using the fluorescently-tagged glue protein (glueGRN), we sought to characterize additional genes involved in the steroid-regulated circuit that triggers secretion; hence, we conducted a loss-of-function screen for secretion mutants (see Chapter 2), leading to the identification of novel regulators of secretion, that may be unique effectors of the 20E-signaling cascade in the salivary gland during this temporally specific physiological event.
Figure 1.3: Cellular images of the production and secretion of salivary gland glue. On the left, the larval or prepupal stage of development is given. On the right, an illustration of the salivary gland in the context of the larva or prepupa and the region that was imaged is provided. In the confocal images, glue protein is labeled with a DsRed tag and the nuclei with Green Fluorescent Protein (GFP). (A) Prior to glue production, the salivary gland is less than 500 µm long. (B) Both the nuclei and the gland continue to grow as glue is produced during the middle of the third larval instar. Typically the distal half of the gland is full of glue by the midpoint of the third instar, as shown in the illustration on the right. (C) The gland reaches over 1 mm in length as it fills with glue during the last part of the third instar. In addition, the glue-filled vesicles (0.5-2 µm) become larger granules (2-6 µm) as more glue is packaged. (D) Just prior to the white prepupal (WPP) stage, the cells secrete the contents of the granules into the lumen of the gland. (E) At WPP, through its mouth, the animal expectorates the glue, which covers the ventral surface of the prepupal case adhering it to a solid surface, as illustrated on the right. Residual glue remains in the gland. (F) Ten hours after pupariation, the gland is undergoing cell death. Any glue remaining inside the salivary gland will be absorbed as the tissue is histolyzed. In all cellular images and illustrations, anterior is to the right. The scale bar in (A) is 20 µm. The illustrations are not to scale. Confocal images are the same magnification (630x with 1.5x optical zoom).
Figure 1.4: Responses to 20E and the 20E hierarchy in the salivary gland. (A) Glue production, glue secretion, and salivary gland histolysis are induced by increases in 20E at three different time periods of development. The titers are redrawn from Warren et al., 2006. (B) The pre-metamorphic response to 20E has been studied at the molecular level in the salivary glands due to the puffing cascade visible on polytene chromosomes. The inset box summarizes the Ashburner model of 20E-activated gene expression, which proposes EcR/USP bound by 20E has two primary effects: 1) the direct induction of the early genes; and 2) the repression of the late genes. Two to four hours after early gene induction, the proteins encoded by the early genes are predicted to repress their own activation and also induce and amplify a larger set of late genes that carry out the necessary physiological process at a given time. The 20E-cascade products are color-coded and correspond to the colors in the inset box.
1.5: 20E-Regulated Genes in the Puffing Hierarchy in the Salivary Gland

Understanding the exquisiteness of gene regulation in complex organisms begins by dissecting genetic pathways in model systems. As previously noted, *Drosophila melanogaster* has been a powerful model for understanding hormone-regulated gene cascades as early as the 1950s because the steroid hormone 20E causes polytene chromosomes in the salivary gland to form visible puffs in locations where genes are being expressed, which allows for the ease of direct observation of gene expression (Becker, 1959; Korge, 1975).

As such, the larval salivary gland is an excellent tool because the puffing patterns have allowed researchers to build models linking the steroid hormone to a specific cascade of gene expression that triggers a distinct physiological event. With modern techniques, we can compare the polytene puffing cascade in the pre-metamorphic salivary gland to steroid-triggered gene expression in other tissues and at other times in the salivary gland to define the distinctions among 20E-regulated events (see Chapters 4 and 5).

The Ashburner model was built upon the puffing activity observed in pre-metamorphic salivary gland polytene chromosomes. It states that the ecdysone receptor directly induces the genes in early puffs while repressing the genes in late puffs. The *early* gene products then activate the genes in late puffs while causing their own repression through a negative feedback loop (Ashburner et al., 1974) (Figure 1.4B).

There are actually four described groups of puffs based on their timing (intermolt, early, early-late, and late) (Ashburner et al., 1974). Intermolt puffs are active before the pre-metamorphic pulse, and contain the *glue* genes such as *Sgs1*, *Sgs3*, *Sgs4*, *Sgs5*, *Sgs7*,...
and Sgs8 (reviewed in Lehmann, 1996). Gene activation is highly dependent on the concentration of 20E (Ashburner, 1973), and the intermolt puffs regress as the pre-metamorphic titer increases and drives the early gene puffs. Early puffs respond to 20E within minutes of exposure even in the presence of protein synthesis inhibitors (Ashburner, 1972, 1974). Five main genes have been emphasized in the study of the early puffs: BR-C, E74, E75, E63-1, and E23 (reviewed in Andres and Thummel, 1992 and Thummel, 2002).

In support of the Ashburner model, BR-C, E74, and E75 encode transcription factors that regulate downstream puffs and genes. BR-C encodes four zinc finger transcription factor isoforms (Spokony, 2007), and the BR-C early puff has been demonstrated to control tissue-specific responses to 20E (von Kalm et al., 1994). In addition, the expression levels of the early genes vary among different tissues, which may contribute to the tissue specificity of these responses (Huet et al., 1993). E74 has two isoforms controlled by two alternate promoters with different affinities for the ecdysone receptor, supporting the concentration dependence of gene activation (Ashburner, 1973). In addition E74 directly activates late genes, supporting its role as an early gene in the Ashburner model (Burtis et al., 1990; Fletcher et al., 1995; Urness and Thummel, 1995). The nuclear hormone receptor E75 (with three isoforms, E75A, B, and C) localizes to both early and late puffs, indicating that it may be regulating genes at these puffs (Segraves and Hogness, 1990; Hill et al., 1993). E75A also participates in the negative feedback loop proposed in the Ashburner model by repressing BR-C (Dubrovsky et al., 2004).

However, E63-1 and E23 are unlike the other early genes in that they do not encode
transcriptional regulators. \textit{E63-1} encodes a Ca\textsuperscript{2+}-binding protein that is involved in salivary gland glue secretion (see Chapter 4; Andres and Thummel, 1995; Biyasheva et al., 2001), and \textit{E23} encodes an ABC transporter (Hock et al., 2000) and will be discussed further in Section 1.6.

The early-late puffs arise when the early puffs are at their maximal size and include genes such as \textit{E78, DHR3}, and \textit{DHR39}, all of which encode nuclear hormone receptors (Stone and Thummel, 1993). However, the difference in the timing of mRNA expression between the \textit{early} and \textit{early-late} genes was refined by RT-PCR of individual salivary glands and found to be too subtle to distinguish as separate groups even though we will still treat them as such in this dissertation (Huet et al., 1995). The \textit{early-late} genes may regulate downstream genes in the puffing cascade similar to the \textit{early} genes. For example, the \textit{early-late} gene \textit{DHR3} is required as early as the embryonic stage (Carney et al., 1997), and is also required for metamorphosis because it represses the stage-specific puff encoding another nuclear hormone receptor \textit{\beta ftz-F1} (White et al., 1997).

The late puffs are the last to be induced and require prior protein synthesis (Ashburner, 1974), suggesting a role for the \textit{early} gene products in activating the genes in the late puffs (Ashburner et al., 1974). Although there are many late puffs identified (>100), only a few of the genes have been characterized including those at the 71E puff (Restifo and Guild, 1986; Wright et al., 1996), 62E puff (Keegan et al., 2001), 63E puff (Stowers et al., 2000), and 82F puff (Stowers et al., 1999). The genes in the 71E puff form a salivary gland-specific, coordinately-expressed cluster of small peptides, and they are believed to be involved in immunity defense during metamorphosis (Wright et al., 1996). The 71\textit{E} gene cluster is also useful for studies looking at the disruption of \textit{late}
gene expression by mutations in earlier gene products (Urness and Thummel, 1995).

Finally, there are puffs that arise during a developmentally defined period of time, containing genes such as \( \beta ftz-F1 \) and \( E93 \). Both gene products are required for metamorphic processes including the histolysis of the salivary gland (Lee and Baehrecke, 2001). \( \beta ftz-F1 \) is activated when 20E levels are low, and encodes a nuclear hormone receptor that is necessary for \( E93 \) expression and for progression through metamorphosis (Lavorgna et al., 1993; Broadus et al., 1999). \( E93 \) is required to initiate programmed cell death pathways (Lee and Baehrecke, 2001; Lee et al., 2002).

In summary, in many aspects the Ashburner model has been supported in molecular and genetic studies, but at the same time, the model has been refined as more 20E-regulated genes have been characterized and as technological innovations have become available. For example, not all early puff products control the expression of late puffs (e.g., \( E63-1 \)), and not all 20E-regulated primary response genes are found within puffs (see Andres and Thummel, 1992). Furthermore, other models have come to light. Most notably, a repressive role for the unliganded ecdysteroid receptor has been demonstrated in more recent studies of imaginal discs and the developing eye (Schubiger and Truman, 2000; Ghbeish and McKeown, 2002; Schubiger et al., 2005). Thus, in some circumstances, 20E-induced gene expression may be caused by an active de-repression.

For our studies, knowledge of the temporally defined gene cascade has allowed us to test gene candidates for their involvement in the signaling pathway via loss-of-function and gain-of-function experiments. I will introduce the gene \( E23 \) that we examined for its role in the cascade in the next section.
1.6: *E23* is an *Early* Gene in the 20E Hierarchy

Using *in situ* hybridization with cosmid probes to polytene chromosomes, the gene *E23* was found within the 23E early puff, which is located on the left of arm of chromosome two (Hock et al., 2000). Notably, *E23* is induced very early (within 5 minutes) in the genetic hierarchy of cultured polytene chromosomes exposed to 20E (Ashburner, 1972). Furthermore, *E23* is only transcribed in the presence of 20E as a primary response gene (Hock et al., 2000). Thus, in the presence of cyclohexamide, a protein synthesis inhibitor, *E23* is still transcribed (Hock et al., 2000). Correspondingly, *E23* mRNA levels are elevated when whole-animal 20E levels are high, which was discovered by using an *E23*-specific probe in a developmental northern blot of all pre-adult stages of development (Hock et al., 2000).

*E23* developmental expression has several unique features to other *early* genes. First, *E23* transcripts are maternally deposited, as indicated by elevated levels of *E23* mRNA in adult females and not males, and in 0–2 hour embryos (Hock et al., 2000). Second, late embryonic expression is not observed for *E23* (but it is for *E74* and *E75*) (Hock et al., 2000). Third, *E23* transcripts remain abundant for a longer time (up to 10 hours) after pupariation before the prepupal 20E pulse (Hock et al., 2000). Fourth, *E23* is one of the most highly induced genes in the ovaries during the process of oogenesis in adult females (Chintapelli et al., 2007; Graveley et al., 2011). These unique features of E23 may provide some clues to its pleiotropic function.

While most *early* genes in the Ashburner model were predicted to be transcription factors that can induce genes that are turned on later in the cascade, *E23* does not encode a transcription factor. Instead, *E23* encodes a member of the ATP-binding cassette
(ABC) transporter family as evidenced by amino acid conservation with other ABC transporters (Hock et al., 2000). It is not known why an ABC transporter would be induced so early in the genetic cascade or what the function of the transporter is, but some indications come from characterized ABC transporters in other systems. In the next section, a general overview of ABC transporters is provided to provide a context for the role of E23 in metamorphosis.

1.7: ATP-Binding Cassette Transporters

ABC proteins belong within the general category of “cellular transporters.” Common to all cellular transporters are the alpha helices in their transmembrane domains and their function to import or export substances from the cytoplasm/extracellular space or within organelles. Furthermore, transporters can be classified into two broad categories: passive or active (Figure 1.5A). Passive transporters use differential ion gradients to drive the movement of a substrate; whereas, active transporters either use the hydrolysis of ATP (primary active transport) or an electrochemical gradient (secondary active transport) to drive the movement of a substrate. ABC transporters belong to the primary active transport category, along with P-ATPases (such as the Na\(^+\)/K\(^+\) pump) and V-ATPases (such as the H\(^+\) ATPase used in cellular respiration) (Holland, 2003; Dahl et al., 2004).

Interestingly, there is a difference in the function of ABC transporters in bacteria and eukaryotes in general. Most bacterial ABCs pump in essential compounds like sugar, vitamins, and minerals that cannot enter by simple diffusion. Bacterial transporters also use a periplasmic binding protein to assist in the transport process (Holland, 2003; Cui and Davidson, 2011; Klein and Lewinson, 2011). Whereas, eukaryotic ABCs mostly
move substances out from the cytoplasm to the extracellular space or into an intracellular compartment like the endoplasmic reticulum, mitochondrion, or peroxisome; and they do not require an accessory protein (Holland, 2003). However, there are ABC transporters that are dedicated to moving hydrophobic substances into cells as a metabolic process (Albrecht and Viturro, 2007). Yet, the majority of eukaryotic ABC transporters move hydrophobic substances out of cells for transport to other organs or secretion from the body.

The eukaryotic family of ABC transporters is characterized by the presence of an ATP-binding cassette region (AKA nucleotide binding domain), which hydrolyzes ATP to support energy-dependent substrate exportation from the cytoplasm to the extracellular space. Full-length transporters contain two mirror-image halves that are separated by a linker region; whereas, half-transporters, e.g. E23, function as homo- or heterodimers and may be localized to the plasma membrane. Full ABC transporters have two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs); whereas, half transporters only have one of each, so they must pair with another half transporter to be functional. Each TMD is comprised of 6 to 11 membrane-spanning alpha helices and provides the substrate specificity of the transporter. The NBD is located in the cytoplasm and is used to transfer energy to transport a substrate (Holland, 2003; Hollenstein et al., 2007; Vasiliiou et al., 2009).

ABC transporters are further classified into subcategories (A-G) based on three main criteria: 1) amino acid sequence homology in their NBDs and TMDs; 2) similarity in gene structure (half versus full-length transporters); and 3) the order of their domains (Table 1.1). Within the NBD, a Walker A and Walker B consensus sequence is found in
all ABC transporters along with a conserved signature ABC motif (Dean et al., 2001; Holland, 2003). Class G of ABC proteins (ABCG) form “reverse” half-transporters with the NBD located at the N-terminus (N-NBD-TMD-C), and they are known to export lipophilic substances such as cholesterol and bile (Dean et al., 2001; Hazard and Patel, 2007). E23 belongs to this class of transporters (Dean et al., 2001).

The mechanism for ABC transporters begins with the substrate binding to the transporter. Thus, the specificity of the transporter for its substrate may be contained within the amino acid sequence, but it may also be the higher order structure that determines this specificity. Exporters, as would be the case with most eukaryotic ABC transporters, recruit substrates directly from the cytoplasm or in some cases from within the plasma membrane. Next, the requirement for ATP-triggered movement of substrates involves first the recruitment and binding of ATP to the NBD of the protein, which is facilitated by the binding of the substrate to the transporter. Hydrolysis of ATP at the two ATP cassettes leads to a conformational change in the TMD that is referred to as the power stroke. Because the power stroke and ATP hydrolysis occur on only on one side of the plasma membrane, it is thought that substrate movement is unidirectional. Finally, the transporter returns to its initial relaxed state and ADP and inorganic phosphate dissociate (van der Does and Tampe, 2004).
Figure 1.5: Classification, gene structure, and conserved domains of E23. (A) ABC transporters are primary active transporters, requiring ATP hydrolysis for movement of substrates. (B) The gene structure of E23 is shown. Boxes represent exons. Colored boxes represent mRNA in the open reading frame. The location in the mRNA containing the sequence coding for the nucleotide binding domain (NBD) and transmembrane domain (TMD) is also depicted. (C) The E23 polypeptide contains the Walker A, Walker B, and ABC Signature motif within its NBD, an indication that it is an ABC transporter.
TABLE 1.1

Classification of ABC transporters in humans and *Drosophila*

<table>
<thead>
<tr>
<th>ABC Class</th>
<th>Domain Organization</th>
<th>Full/ Half</th>
<th>Examples from <em>Drosophila</em></th>
<th>Examples from Humans – Substrate/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>¹N-TMD-NBD-TMD-NBD-C</td>
<td>Full</td>
<td>CG5944 – unknown</td>
<td>ABCA1 – Lipid trafficking</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ABCA4 – Vitamin A (Eye)</td>
</tr>
<tr>
<td></td>
<td>N-TMD-NBD-C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>¹N-TMD-NBD-TMD-NBD-C</td>
<td>Both</td>
<td>Mdr49 – methotrexate²</td>
<td>MDR – Chemo-drugs</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Blood/Brain Barrier)</td>
</tr>
<tr>
<td></td>
<td>N-TMD-NBD-C</td>
<td></td>
<td></td>
<td>TAP – Peptides (ER)</td>
</tr>
<tr>
<td>C</td>
<td>N-TMD-NBD-TMD-NBD-C</td>
<td>Full</td>
<td>Sur – sulfonylurea receptor³</td>
<td>MRP – Drug conjugates to organic ions</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td>⁴CFTR Channel – Chloride ions</td>
</tr>
<tr>
<td>D</td>
<td>N-TMD-NBD-C</td>
<td>Half</td>
<td>CG2316 – non-steroidal ecdysteroid agonist⁵</td>
<td>ALD – Long chain fatty acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Peroxisome)</td>
</tr>
<tr>
<td>E/F</td>
<td>N-NBD-C</td>
<td>N/A</td>
<td>CG1703 – unknown</td>
<td>OABP – oligo-adenylate</td>
</tr>
<tr>
<td></td>
<td>N-NBD-NBD-C</td>
<td></td>
<td></td>
<td>(viral immunity)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ABCF1 – non-transport function (translation regulation)</td>
</tr>
<tr>
<td>G</td>
<td>N-NBD-TMD-C</td>
<td>Half</td>
<td>white, scarlet, brown – eye pigment precursors⁶</td>
<td>ABCG1 – Cholesterol regulation</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ATET – tracheal⁷</td>
<td>ABCG2 – Drug resistance</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E23 – 20E⁸</td>
<td>ABCG5/8 – Sterol transport</td>
</tr>
</tbody>
</table>

The domain organizations and functions of ABC transporters define their seven classes (A-G) according to the HUGO Gene Nomenclature Committee (www.genenames.org). Classes E and F are combined into one group defined by the lack of a TMD and involvement in non-transport processes. Information presented is summarized from *ABC Proteins From Bacteria to Man* (Holland, 2003) and Dean et al., 2001.

¹Some full transporters may have N-NBD-TMD-NBD-TMD-C domain organization.
²Chahine and O’Donnell, 2009
³Nasonkin et al., 1999
⁴The CFTR protein is a channel, not requiring ATP hydrolysis for transport.
⁵Mosallanejad et al., 2010
⁶Ewart et al., 1994
⁷Kuwana et al., 1996
⁸Hock et al., 2000 and *this work* support this role.
1.8: The ABC Transporter E23

Previous work has shown that eukaryotic ABC transporters can function as exporters and play important roles in regulating steroid hormones and sterol homeostasis. In yeast, for example, the well characterized PDR5 and SNQ2 transporters export dexamethasone (Kralli et al., 1995; Mahé et al., 1996). PDR5 is also capable of extruding non-steroidal ecdysone agonists (Hu et al., 2001; Retnakaran et al., 2001). Similarly, the mammalian P-glycoprotein (P-gp) has been shown to transport cortisol, aldosterone, and dexamethasone (Bourgeois et al., 1993; Gruol et al., 1994). In light of this evidence, we propose that E23 is exporting 20E.

The most critical support for this hypothesis is that E23 is capable of acting as a negative regulator of 20E responses (Hock et al., 2000). Specifically in the study, the ectopic expression of E23 (driven by a heat shock promoter) resulted in the loss of transcriptional activation of 20E gene targets (E74, E75, and a EcRE-lacZ reporter) when tissues were cultured in the presence of 20E (Hock et al., 2000).

Furthermore, both the NBD and TMD of E23 share amino acid sequence similarity with half transporters of the ABCG class (Dean et al., 2001) (Figure 1.5B, C). These transporters mostly function to transport lipophilic substances in humans (Tarr et al., 2009). Specifically, E23 shares high amino acid sequence similarity with yeast ADP1, Drosophila ATET, Drosophila white, human ABCG1/murine ABC8/human white, and human ABCG2/MXR/ABCP1/BCRP (Hock et al., 2000). Perhaps the most studied of the ABCG class in Drosophila, the white gene is well known for its mutant phenotype in the adult eye first noticed by Thomas Hunt Morgan in the early 1900s. The white protein forms heterodimers with other half transporters (encoded by brown and scarlet) to allow
pigment precursors such as guanine or tryptophan to enter the pigment-producing granules of the compound eye (Ewart et al., 1994; Thomas and Wassarman, 1999).

Again, half transporters must form a homo- or heterodimer in order to function, with each half containing only one NBD and one TMD. It is not known whether E23 has a heterodimer partner. Interestingly, ATET encodes an ABCG transporter expressed mainly in the trachea during all larval stages, but its substrate and binding partner are not known (Kuwana et al., 1996). If E23 forms a heterodimer with another ABCG protein such as ATET, it would add a layer of complexity to the ability of E23 to transport its specific substrate.

Returning to the major question as to how spatially and temporally specific responses occur when there is a single, systemic signal, we propose that E23 has a distinctive role by extruding 20E from target cells (Figure 1.6). We hypothesize that the negative regulation of 20E signaling via E23 occurs at different magnitudes, depending how much E23 protein is present in a particular tissue at a given time. Although other mechanisms of tissue-specific gene expression are still at play, E23 would work in concert with these to trigger a fine-tuned response to the hormone. This hypothesis is an intriguing expansion to Ashburner’s original idea and provides a novel way of attenuating the responses to the hormone throughout the life cycle of Drosophila.
Figure 1.6: A model of the negative autoregulatory loop initiated by E23. As the result of the hormone 20E binding to the nuclear hormone receptor (EcR/USP), the gene *E23* is transcribed. Subsequently, the E23 ABC transporter negatively regulates 20E signaling directly by actively extruding the hormone. Lastly, by negatively regulating the intracellular levels of 20E, the expression of *E23* would in turn be attenuated.
1.9: Summary

The fundamental research question of this work is: How are the temporally and spatially distinct responses to the steroid hormone 20E implemented at the molecular level? We use the salivary gland as a model to explore the activity of the hormone because there are several temporally distinct responses involving glue production, glue secretion, and gland histolysis and because we have developed an accurate fluorescent glue reporter. The focus of our work is on the pre-metamorphic response to 20E during the larval and prepupal stages of Drosophila development.

We have taken several approaches to answer our research question. In Chapters 2, 4, and 5, we identify molecules involved in the 20E-regulated pre-metamorphic responses in the salivary gland. Specifically, in Chapter 2, we conduct a genetic screen for secretion defects, and in Chapters 4 and 5, we implement a transcriptome analysis of the salivary gland using RNA-seq. In Chapter 3, we take a candidate-based gene approach and study the role of E23 in the 20E genetic circuit. Our results demonstrate that E23 acts as a powerful negative regulator of 20E, which may help to define the spatially and temporally specific responses to the hormone. Furthermore, our results from Chapters 2, 4, and 5 support the idea that many tissue-specific, 20E-regulated primary response genes function not as activators of downstream genes in the 20E-initiated battery but instead as direct effectors of specific physiological responses.
CHAPTER 2

MAPPING MUTATIONS THAT BLOCK STEROID-REGULATED SECRETION IN THE LARVAL SALIVARY GLAND

2.1: Introduction

Steroid hormones play a critical role in vertebrate development and homeostasis by binding to cognate nuclear receptors, which primarily function as ligand-activated transcription factors (King-Jones and Thummel, 2005). These hormones also maintain the physiology of the tissues with which they interact. For example, it has been previously demonstrated that steroid signaling can invoke exocytosis, or the regulated fusion of vesicles with the plasma membrane. The importance of regulated exocytosis cannot be understated, as it is necessary for immune function and hormone secretion among many other functions in mammals.

The regulation of exocytosis has been demonstrated to act through both genomic and non-genomic mechanisms. For example, in human spermatozoa, progesterone exposure leads to an influx of $\text{Ca}^{2+}$ that is so rapid it cannot occur through a transcriptional mechanism; the result of this rapid signaling is the exocytosis of predominantly proteolytic enzymes into the oocyte zona pellucida during the process of fertilization (Baldi et al., 2009; Strünker et al., 2011). However, longer exposure to steroid hormones such as glucocorticoids, thyroid hormone, 1,25-Dehydroxyvitamin D$_3$ (calcitriol), estradiol, and progesterone have been implicated in a transcriptional response resulting in exocytosis that is required for immunity and homeostasis in mammals (Krey and Kamel, 1990; Ortmann et al., 1995; Thomas et al., 1996; Bonaterra et al., 1998; Shalita-Chesner et al., 1998).
In both genomic and non-genomic mechanisms, nonetheless, fluctuations in intracellular Ca\(^{2+}\) levels have a distinct role in modulating secretion (Thomas et al., 1996; Ortmann et al., 1998). Furthermore, steroid-hormone signaling has been shown to regulate intracellular Ca\(^{2+}\) levels (Tornquist and Tashjian, 1989; Ritchie, 1993; Thomas et al., 1996). However, the mechanism that connects hormone signaling to Ca\(^{2+}\) increases and ultimately secretion is not completely described.

The salivary gland of *Drosophila melanogaster* is a powerful model to study steroid-regulated exocytosis because it produces and secretes a glycoprotein glue mixture, and this process has been demonstrated to function through a steroid-regulated genomic mechanism requiring Ca\(^{2+}\) influxes (Biyasheva et al., 2001). In *Drosophila*, a single steroid hormone 20-hydroxyecdysone (20E) has many roles both on a global scale (such as molting) and in individual tissues (such as tissue histolysis) (reviewed in Riddiford, 1993). The larval salivary gland responds to 20E in at least three different ways as it is exposed to the hormone at three distinct times during development including: 1) the synthesis of a glycoprotein glue mixture during the mid-third instar (Korge, 1977; reviewed in Lehmann, 1996); 2) the secretion of the glue 2-4 hours (hrs) before puparium formation (Zhimulev and Kolesnikov, 1975; Biyasheva et al., 2001); and 3) the autophagic cell death of the gland 14-16 hrs after puparium formation (APF) (reviewed in Andrew and Myat, 2005; Yin and Thummel, 2005)

For our study, a distinction must be made between glue secretion and expulsion. Glue secretion is the 20E-regulated process of delivery and exocytosis of glue granules to the lumen of the gland prior to the prepupal stage of development (Zhimulev and Kolesnikov, 1975; Biyasheva et al., 2001). In contrast, glue expulsion is the process in
which a prepupa expectorates this glycoprotein glue mixture from the lumen of the salivary gland, through the ducts of the gland, and out through the mouth (Fraenkel, 1952; Fraenkel and Brookes, 1953). It results in the animal being covered by a sticky glue mixture. For expulsion to occur, it is necessary for secretion to precede it.

The process of glue expulsion is correlated with a series of whole-cuticle peristaltic contractions, during which the glue moves into the duct of the salivary gland and out through the mouth. The programmed contractions last up to several minutes, and after they cease, the animal quickly becomes stationary in preparation for pupation, which occurs approximately 12 hrs later. It has been postulated that the purpose of expulsion is to allow a larva that is about to go through metamorphosis to adhere itself beneath a surface where it would be hidden from predation (Fraenkel, 1952; Fraenkel and Brookes, 1953). In summary, glue secretion involves regulated granule exocytosis; whereas, glue expulsion is more similar to salivation, which in other systems, has been shown to involve both exocytosis and the rapid movement of electrolytes and water through channels in the cell membrane (for a review of salivation in vertebrates, see Melvin et al., 2005). However, salivary gland glue expulsion has not yet been characterized molecularly in Drosophila.

Although 20E is necessary for secretion, it is not known whether there is a non-genomic response resulting in secretion and expulsion. In order to characterize both genomic and non-genomic mechanisms of secretion and expulsion, we conducted a trial F_2-genetic screen for recessive mutations on the third chromosome (~40% of the genome). We used Ethyl Methanesulfonate (EMS) to induce mutations, and we isolated strains that failed to expel the glue that is produced in the larval salivary gland. In order
to efficiently screen for secretion and expulsion mutants, we included a GFP-tagged glue transgene under the control of its endogenous regulatory sequences in the mutagenized strain. Thus, we could visualize whether the glue was expelled using a low-resolution fluorescent microscope.

We characterized four mutants on the third chromosome that produced but failed to expel glue, and we used a deficiency kit to cytologically and molecularly map the mutations. We further refined our mapping strategy to isolate the genes containing two of the four mutations. Using higher resolution microscopy, we found that several of the mutants underwent a disordered form of secretion; while, the expulsion of the glue remained completely blocked. This study revealed that the enzyme responsible for the biosynthesis of acetylcholine (Choline acetyltransferase) and \textit{Lgr3} encoding a G-Protein coupled receptor (GPCR) potentially have an important role in controlling the process of secretion and expulsion. Furthermore, given what is known about how acetylcholine and GPCRs work in other processes, it is plausible that the genomic response to the steroid hormone coalesces with other signaling pathways to trigger the downstream cellular responses responsible for regulated secretory events.

\textbf{2.2: Materials and Methods}

\textbf{2.2.a: \textit{Drosophila} Stocks and Culture}

Crosses were performed at ambient room temperature on a standard-recipe cornmeal molasses medium. The third chromosome deficiency kit was obtained from the Bloomington \textit{Drosophila} Stock Center (BDSC, Bloomington, IN, USA) (Parks et al., 2004; Cook et al., 2012). Lethal P-element insertions on the third chromosome were
obtained from the BDSC. The third chromosome P-element collection consists of lethal P-element insertions characterized by the Berkeley *Drosophila* Genome Project (Bellen et al., 2011). The RNAi lines were obtained from the Harvard collection at the BDSC. The following lines were used in the study: 1) *glueGRN-3* = *[w*]<sup>1118</sup>; *P{w+, Sgs3-GFP}3* (Vaskova et al., 2000); 2) *{sgGal4, glueGRN} = *[w*]<sup>1118</sup>; *P{w+, sgGal4}; P{w+, Sgs3-GFP}3*); 3) *{glueGal4, glueGRN} = *[w*]<sup>1118</sup>; *P{w+, glueGal4}; P{w+; Sgs3-GFP}]* (Vaskova et al., 2000); 4) *UAS-EcR-DNF645A* (Cherbas et al., 2003); 5) *y w; Actin5C-Gal4/Cyo* (FBst0004414); 6) *DTS4, TM8/TM6C, Tb, Sb* (FBst0002632). In addition, all stocks listed in Tables 2.3-2.6 were obtained from the BDSC or the *Drosophila* Genetic Resource Center (DGRC, Kyoto, Japan), as noted in the table. Stocks with *glueGal4* or *sgGal4* combined with *glueGRN* were generated by standard genetic crosses.

### 2.2.b: EMS Screen for Glue Production, Secretion, and Expulsion

To isolate mutations that affect glue secretion or expulsion, we carried out an F2-loss-of-function screen of the third chromosome. Newly eclosed *glueGRN-3* males (aged for 3-5 days post eclosion) were starved for 12 hrs, and etherized to increase dehydration. They were then fed 25 mM EMS (Sigma, St. Louis, MO, USA) in 10% sucrose on a 1MM filter paper disc (Whatman plc, Kent, ME, UK) for 9 hrs. Males were allowed to recover on regular food overnight before mating with virgin females of the genotype *w; DTS4/ TM6C, Tb Sb*. Individual F<sub>1</sub> males (lacking the *Stubble* and *Tubby* markers) were crossed to 3–5 *DTS4/ TM6C, Tb Sb* virgin females. Approximately 1,600 individual F<sub>1</sub> males were crossed. Each F<sub>2</sub> stock was raised at 29°C to selectively kill off any progeny
carrying the chromosome bearing \emph{DTS4}. Thus, only mutated \emph{glueGRN/TM6C, Tb Sb} offspring survived, and they were sibling mated to produce a stock. These lines were assigned a unique letter and number and scored for glue defects. Absence of the dominant pupal marker \emph{Tubby} was used to identify homozygous mutant pupae (see Figure 2.1 for the crossing scheme). Thus, F\textsubscript{2} stocks carrying a mutation on the third chromosome were scored for glue defects, and those that displayed a mutant phenotype were preserved for mapping and characterization.

2.2.c: Screening for Larval Salivary Glands that Fail to Produce, Secrete, or Expel Glue

Salivary glands were visualized by salivary-gland specific GFP expression in whole animals using a low-resolution fluorescent microscope (see Microscopy and Imaging). Lines were scored for glue defects by observing GFP in third instar larvae and prepupae through the wall of a culture vial. If no GFP were visible, the animal was classified as \emph{glueless}. If GFP were visible but was not expelled, the animal was classified as \emph{drymouth}. If GFP were visible and was only partially expelled, the animal was classified as \emph{drooler}. See Figure 2.2 for phenotypes.

2.2.d: Deficiency Mapping and Complementation Analysis

We used both cytologically and molecularly-defined deficiencies on the third chromosome from the BDSC to map each mutation because the design of the screen with third chromosome balancers and \emph{DTS4} only allowed for the recovery of third chromosome mutations. All deficiencies initially were re-balanced over \emph{TM6C} with the dominant pupal marker \emph{Tubby} to facilitate identifying mutant-over-deficiency offspring.
in the F₁ during mapping. For the mapping, we crossed males from each deficiency to virgins from each of the four drymouth mutations. Because we isolated mutants on the third chromosome, we crossed each of the mutants to approximately 180 deficiencies, covering 97.5% and 98.9% of the left and right arms of the third chromosome, respectively (Cook et al., 2012). We examined the F₁ for secretion and expulsion defects, and if present, we marked that deficiency as the location of the mutation. We defined smaller regions as per overlapping deficiencies that failed to complement the mutation. Once each mutation was mapped to a cytological or molecular region of the third chromosome, we carried out complementation analysis with known mutations and P-element disruptions in each region. We crossed ~20 virgins from each mutant line to ~10 males and examined the F₁ for noncomplementation (blocked glue expulsion and/ lethality).

2.2.e: Using RNAi Against Gene Candidates to Phenocopy the Mutations

We crossed eight available UAS-RNAi lines (against the gene candidates) from BDSC to salivary gland drivers (glueGal4 and sgGal4) containing the glueGRN glue reporter. We analyzed the F₁ for secretion and expulsion defects that resulted in blockages in glue expulsion, as in the mutant lines. We also crossed the RNAi lines to the Actin5C-Gal4 (ubiquitous) driver, and we looked for lethality as a preliminary indication that the gene candidate should be looked at further in the case of a lethal mutant. The Actin5C driver did not include a GFP-glue reporter.
2.2.f: Microscopy and Imaging

Whole animals were imaged using a Leica FL III fluorescent stereomicroscope with a filter cube for GFP (Leica Microsystems Inc., Buffalo Grove, IL, USA). Low-resolution images were visualized with a 10x ocular and a Plan APO 1x objective, using between 2.6 and 4.0 zoom. Images were taken and processed using Spot Insight QE Model #4.2 digital camera and Spot Insight software (McBain Systems, Simi Valley, CA, USA).

For high-resolution images, salivary glands were dissected at the appropriate stages in *Drosophila*-PBS (DPBS, see Appendix A), and mounted on 25 x 75-mm glass slides (VWR, Radnor, PA, USA) with 1MM cellulose filter paper spacers (Whatman) and standard 18-mm² coverslips (VWR) with a thickness between 0.17 and 0.25 mm. Samples were imaged using an LSM 510 Axioplan Confocal Microscope (Carl Zeiss Inc., Oberkochen, Baden-Württemberg, Germany), with a water-immersion objective (Zeiss, W Plan-Apochromat 63x/NA:0.9). LSM 510 image-analysis software (Zeiss) and PowerPoint (Microsoft Co., Redmond, WA, USA) were used for image post-processing. For several confocal images, a second confocal microscope (Nikon Instruments Inc., Melville, NY, USA) equipped with a water-immersion objective was used (Nikon Instruments, Plan Apo VC 60x/NA:1.2; Cover glass 0.13-0.19), and NIS image software (Nikon) and PowerPoint software (Microsoft) were used for image processing and analysis.

2.2.g: DNA Isolation and PCR

To purify DNA from the mutants and parental strain *glueGRN*, we used the Wizard Genomic DNA Isolation Kit (Promega, Fitchburg, WI, USA). We designed PCR primers
to each of the exons of the candidate genes using the open source software ExonPrimer (http://ihg.gsf.de/ihg/ExonPrimer.html). The primer sequences are found in Figure 2.8, and the primers were obtained from IDT (Integrated DNA Technologies Inc., Coralville, IA, USA). We performed PCR using standard protocols with GoTaq Green Master Mix (Promega). PCR products were verified on 2% agarose gels. Dyes and other contaminants were removed from PCR products using Qiagen Quick PCR Purification Kit (Qiagen Sciences LLC, Louisville, KY, USA). We sequenced the ~300 bp PCR products using terminator sequencing with an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Finally, we aligned the mutant sequences to the parental and published exon sequences using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov). Sequences with potential mutations were verified in at least three replicate sequencing reactions using the products of three separate PCR experiments.
**Figure 2.1**

**Figure 2.1:** EMS screen of the third chromosome to isolate secretion and expulsion mutants. Step 1: (F0) glueGRN-3 males fed 25 mM EMS are mated to balanced females carrying the Dominant temperature sensitive (DTS4) allele. Only chromosomes mutagenized in the male germline are transmitted to the F1. Step 2: (F1) Individual male progeny (with a possible mutation, marked by the asterisk) are mated to balanced DTS4 females, and the F2 progeny are raised at 29°C to kill all DTS-bearing larvae and to isolate and amplify the mutated third chromosome. Step 3: (F2) Lines with amplified mutant chromosomes are sibling mated to establish homozygous mutants for screening. Step 4: Finally, homozygous mutants are screened for recessive glue defects on the third chromosome. Note that mutations on the other chromosomes (X, 2, or 4) would not be selected and retained in this chromosome-specific screen. The Tubby offspring served as a heterozygous control in the screen.
2.3: Results

2.3.a: An EMS Screen for Salivary Gland Glue Secretion and Expulsion Mutants

We conducted a pilot screen in which we mutagenized adult males expressing a GFP-tagged glue protein (glueGRN), and used a dominant temperature sensitive (DTS4) mutation balanced over TM6C to select only for mutant third chromosomes (Figure 2.1). We established F2 stocks carrying one mutation on the third chromosome balanced over TM6C, Tb, Sb, and we screened for glue defects by observing the GFP-tagged Sgs3 protein through the culture vial in the homozygous mutants, identified by the absence of the Tubby marker. We isolated three categories of mutants: 1) those that are unable to produce glue (glueless); 2) those that produce glue but only partially expel the glue (drooler); and 3) those that produce but completely fail to expel glue (drymouth) (Figure 2.2 A-D). In total, we isolated 27 mutants that either failed to produce or expel the glueGRN protein. Two of the mutations were allelic; while, the other 25 mapped to different genes as distinguished by inter se complementation (Table 2.1).

As a first step in characterizing the isolated mutants, we quantified the larvae in each mutant line that exhibited a glue production or expulsion phenotype (Figure 2.2E). Because we were interested in the process of steroid-regulated secretion for this study, we set apart four drymouth mutants defined by the absence of glue expulsion in greater than 90% of the population (Figure 2.2E). Because by inter se complementation analysis the drymouth mutants represent four different genes, we have used standardized Drosophila nomenclature to rename them DmA<sup>k46</sup>, DmB<sup>k67</sup>, DmC<sup>d7</sup>, DmD<sup>o57</sup>. The superscript represents the single alleles of each gene that we isolated. We also determined that DmB<sup>k67</sup>, DmC<sup>d7</sup>, and DmD<sup>o57</sup> ultimately arrest in the prepupal stage (Table 2.1), an
indication that the genes containing the mutations are pleiotropic and cause defects that prevent major developmental processes and metamorphosis into adulthood. Whereas, $DmA^{k46}$ is homozygous viable, which is an indication that the mutation is located in a gene that is required for the process of glue secretion or expulsion, but it might not have a broader developmental role unless the mutation is a weak hypomorph.
Figure 2.2: Mutant expulsion phenotypes recovered in the screen. For illustration purposes, a pupa with (A) wild-type expulsion is compared to (B) a drooler mutant, (C) a drymouth mutant, and (D) a glueless mutant. Images are overexposed in (A-D), so that the cuticles of the pupae are visible. (E) The chart represents the phenotypes of each mutant line as a percentage of animals expressing that phenotype. The four mutant strains with over 90% of prepupae having blocked glue expulsion were characterized and mapped.
TABLE 2.1
Phenotypes of mutants recovered in the screen for glue secretion and expulsion defects

<table>
<thead>
<tr>
<th>Mutant</th>
<th>% Express glueGRN</th>
<th>% Expel glueGRN</th>
<th>Phenotype</th>
<th>Lethal Phase</th>
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<td>parental glueGRN-3</td>
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<td>100</td>
<td>Parental/Control</td>
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</tr>
<tr>
<td>B4</td>
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<td>NA</td>
<td>Glueless</td>
<td>Prepupal</td>
</tr>
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<td>Glueless</td>
<td>Prepupal</td>
</tr>
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<td>Prepupal</td>
</tr>
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<td>Glueless</td>
<td>Prepupal</td>
</tr>
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<td>Glueless</td>
<td>Prepupal</td>
</tr>
<tr>
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<td>*0</td>
<td>Drooler</td>
<td>Prepupal</td>
</tr>
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<td>Prepupal</td>
</tr>
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</tr>
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</tr>
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<td>Prepupal</td>
</tr>
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<td>*0</td>
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</tr>
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</tr>
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<td>*0</td>
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</tr>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>2</td>
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<td>Prepupal</td>
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</table>

Mutant alleles uncovered in the screen for glue production, secretion, and expulsion defects are listed. The percentage of mutant larvae expressing and expelling GFP-tagged glue is given along with the parental (glueGRN-3) strain on chromosome 3. Mutants were grouped into categories based on the level of glue production and expulsion with Glueless not producing glue, Drooler producing and partially expelling glue, and Drymouth producing glue but having blocked glue expulsion. Inter se complementation analysis revealed that E30 and G45 are allelic. The phase of lethality is approximate as some mutants are lethal in multiple stages.

*Animals partially expel glue and are not fully blocked.
2.3.b: Cellular Characterization of the Mutants

Next we examined the morphology of the salivary gland in the four expulsion mutants, and we determined whether they also had defective secretion using higher resolution microscopy. With low-resolution microscopy, we observed that three of the four drymouth mutants (DmA\(^{k46}\), DmB\(^{k67}\), and DmC\(^{k7}\)) had a complete block in glue expulsion; whereas, approximately 2% of DmD\(^{k57}\) expelled glue normally. We compared the phenotype of the parental glueGRN-3 strain to the mutants. The whole-animal phenotypes of DmA\(^{k46}\) and DmB\(^{k67}\) are shown to illustrate expulsion defects (Figure 2.3). The homozygous viable mutant DmA\(^{k46}\) undergoes typical pupal development (Figure 2.3D-F) as compared to wildtype (Figure 2.3A-C), but its salivary gland is bloated with glue that is not expelled (2.3D, E). By 12 hrs APF, the glue is located centrally in the salivary gland, and partially moves into the duct (Figure 2.3F). Because pupae are enclosed within a pupal case, glue expulsion is not possible during the prepupal stage even though glue is seen in the duct. The salivary gland undergoes histolysis at the proper time in DmA\(^{k46}\) mutants, approximately 12-14 hrs APF.

In contrast to DmA\(^{k46}\), many DmB\(^{k67}\) mutants arrest in the larval stage, and those that survive to pupariation have several striking features. First, the block in glue expulsion is evident from white puparium formation until approximately 12 hrs APF when the animal ceases development and expires (Figure 2.3J-L). Glue is not visible in the ducts of the gland even though glue is centrally located during the late prepupal stage (Figure 2.3L). Second, melanotic tumors are visible in many of the DmB\(^{k67}\) third instar larvae, and many of these grow in size as the animal enters the prepupal stage and eventually dies (Figure 2.3J’ and L’).
In order to define the secretion phenotype of each of the four mutations, we used confocal imaging to characterize the status of the glue at the cellular level. We hypothesized that the mutants would not secrete glue because secretion occurs prior to glue expulsion; and thus, glue must be secreted into the lumen of the salivary gland before it can be expelled through the mouth of a prepupa. We characterized wild-type secretion with the \textit{glueGRN-3} parental line for comparison with the mutants. Wild-type secretion is an orderly process that begins with the maturation of glue-filled vesicles (0.5-2 \, \mu m) into larger granules (2-5 \, \mu m) (Figure 2.4A-C). The granules fuse with the apical membrane of the salivary gland cells, emptying their contents into the lumen of the gland (Figure 2.4D, E). After secretion, expulsion follows; however, residual glue granules are always detected in the cells of white prepupae that have already expelled most of their contents (Figure 2.4F).

As a negative control for secretion, we crossed a \textit{UAS-EcR-DN} line to a salivary gland driver \textit{glueGal4}, also containing \textit{glueGRN}. In these larvae, 20E signaling is perturbed because the mutant receptor cannot be activated by 20E (Cherbas et al., 2003). As a result, granules mature, but they are not secreted (Figure 2.4G-J). All four mutants have defects in secretion, but they do not have a complete block in secretion like \textit{UAS-EcR-DN}. For example, the vesicles of \textit{DmA}\textsuperscript{k46} do not fully mature; yet, glue is secreted in some cases (Figure 2.4M). Nevertheless, an abundance of unsecreted glue remains in many cells, adding to the expulsion blockage (Figure 2.4N). Although \textit{DmB}\textsuperscript{k67} does not have a defect in granule maturation (Figure 2.4O, P), glue secretion appears to be dysregulated, as the cells burst open during a stage when all the glue should have already been secreted (Figure 2.4R). The mutant \textit{DmC}\textsuperscript{j7} has both a granule maturation problem
and a secretion block (Figure 2.4T, U). At puparium formation, glue enters the lumen in
\textit{DmC}^{67} \textit{mutants} (Figure 2.4V).

Finally, the \textit{DmD}^{657} \textit{mutant} has an asynchronous vesicle maturation phenotype with
adjacent cells forming smaller vesicles and larger granules (Figure 2.4X). Glue is not
secreted at the proper time in \textit{DmD}^{657} \textit{mutants}, and the granules become oversized
(Figure 2.4Y). Like \textit{DmB}^{667}, the cells of \textit{DmD}^{657} appear to burst open, spilling glue into
the lumen by white puparium formation (Figure 2.4Z). In summary, if salivary gland
secretion, strictly speaking, is the regulated fusion of glue granules with the apical
membrane and the movement of the glue into the lumen at a defined time during
development, all four \textit{drymouth} mutants are secretion defective. However, there is not an
absence of glue in the lumen in some cases; instead, the process is simply dysregulated,
occurring at the wrong time in a disordered manner. Nevertheless, the cellular
phenotypes are consistent with apical membrane dysfunctions in the cases where the cells
appear to burst or leak their contents into the lumen.
Figure 2.3: *DmAk46* and *DmBk67* mutant glue expulsion phenotypes. (A-C) In wild-type animals, glue is expelled to the outside of the animal during puparium formation. (B) Residual glue remains in the salivary gland but is absorbed quickly during salivary gland histolysis. In contrast, (D-F) the *DmAk46* mutant and (G-I) the *DmAk46* mutant-over-the-deficiency (BL-24909) do not expel glue during puparium formation. (F) In the *DmAk46* mutant by 12 hrs APF, the glue appears as a thin strip, located in the lumen and duct of the salivary gland, similarly to *DmAk46* over the deficiency (I). Although glue is in the duct, it is never expelled because the mouthparts are no longer exposed externally. (J-L) In addition, the mutant *DmBk67* does not expel glue before or after puparium formation. (L) The glue appears in the central portion of the gland around 12 hrs APF, but it cannot be expelled at that time. (J' and L') Insets show melanotic tumors in similarly staged animals. Images in (C) and (I) are overexposed in order to visualize the weak GFP signal. White arrowheads point to glue that has been expelled to the outside of the animal. Yellow arrowheads point to glue that is internally located within the salivary gland or salivary gland duct. Scale bars: 500 µm.
Figure 2.4

Figure 2.4: The cellular phenotypes of the drymouth (Dm) mutants. Confocal images of wildtype, glueGal4/UAS-EcR-DN, and mutants were taken at 630x and 1.5 zoom. (A-C) In wild-type animals, glue is produced and packaged into vesicles (0.5-2 µm) during the mid-third instar of larval development. (D) The glue-filled vesicles mature into secretory granules (2-5 µm) as more glue is synthesized. (E) Glue secretion occurs when the granules fuse with the apical membrane of the salivary gland, and glue is released into the lumen. The cells become flat and rectangular after the majority of the glue has been secreted. (F) By white puparium formation, only residual glue remains in the salivary gland. (G-J) As a negative control, glueGal4/UAS-EcR-DN fully blocks 20E-regulated secretion when driven in the mid-third instar salivary gland. (K, O, S, W) All four drymouth mutants produce glue, but the vesicles of (L) DmA^{k46} and (T) DmC^{k7} do not mature. (X) DmD^{o57} undergoes an asynchronous vesicle maturation with adjacent cells having smaller vesicles. Glue is secreted into the lumen in (M) DmA^{k46} and (Q) DmB^{k67} mutants, but it is not secreted in (U) DmC^{k7} and (Y) DmD^{o57}. By white puparium formation, glue secretion is abnormal in all mutants with some containing oversized granules (R, V, Z) that appear to burst within the cytoplasm (N, R, Z). In many cases, cells appear to lyse so that glue empties into the lumen (R, Z) in contrast to the orderly secretion of glue in wildtype (D, E). Mutants: DmA^{k46} (K-N); DmB^{k67} (O-R); DmC^{k7} (S-V); DmD^{o57} (W-Z). In images where the lumen is not visible, white arrowheads point in the direction that glue secretion would follow. L: lumen. Scale bar (W): 25 µm.
2.3.c: Mapping of Third-Chromosome Mutations Using Cytologically and Molecularly Defined Deficiencies

Our next step was to find the location of each mutation in the drymouth lines. We mapped all four mutants, and the key deficiencies uncovering the mutant regions along with their cytolocations are listed in Table 2.2. We mapped \textit{DmD}\textsuperscript{657} to the left arm of the third chromosome, and the other three mutants (\textit{DmA}\textsuperscript{k46}, \textit{DmB}\textsuperscript{k67}, and \textit{DmC}\textsuperscript{47}) to the right arm. \textit{DmC}\textsuperscript{47} mapped to two separate but adjacent regions on chromosome 3R. The molecular coordinates flanking each mutation are shown in Figure 2.6.

In order to discover whether the mutants were nulls or hypomorphs, we characterized the severity of the mutant-over-deficiency phenotypes. For \textit{DmD}\textsuperscript{657} and \textit{DmC}\textsuperscript{47}, we quantified the glue expulsion phenotypes of two overlapping deficiencies crossed to the mutants; for \textit{DmB}\textsuperscript{k67} we quantified the stage of lethality with each deficiency; and for \textit{DmA}\textsuperscript{k46}, we compared the mutant-over-deficiency expulsion phenotype to the homozygous mutant. We found that \textit{DmC}\textsuperscript{47} crossed to the deficiencies had a less severe expulsion phenotype, with some larvae fully expelling glue (Figure 2.5A). Nevertheless, there was 100% lethality with these deficiencies in the 89A-89B region crossed to \textit{DmC}\textsuperscript{47} (Figure 2.5B). The weaker expulsion phenotype may be due to \textit{DmC}\textsuperscript{47} mapping to two separate regions. The \textit{DmD}\textsuperscript{657} mutant crossed to the overlapping deficiencies in the 66B-66C region also contained a small number of progeny that fully expelled glue (Figure 2.5C). Nevertheless, the \textit{DmD}\textsuperscript{657} homozygote fully expels glue 2% of the time as well.

We also characterized the expulsion phenotype of the \textit{DmA}\textsuperscript{k46} mutant over the only deficiency that failed to complement it and found the phenotype to be identical to the \textit{DmA}\textsuperscript{k46} homozygotes (Figure 2.3G-I). In addition, \textit{DmB}\textsuperscript{k67} failed to complement 10
deficiencies, and the phenotypes were variable ranging from more severe with an earlier lethal phase to less severe with the animals surviving as pupae (Table 2.3). In summary, it is likely that \( DmB^{k67} \) is a hypomorph; whereas, \( DmA^{k46} \) appears to be a null because its phenotype is identical to the mutant over the deficiency. Finally, it is not clear whether \( DmC^{c7} \) and \( DmD^{o57} \) are hypomorphs because the mutant-over-deficiency phenotypes were less severe than the homozygous mutants alone.

In addition, we noticed that two deficiencies when crossed to \( DmB^{k67} \) resulted in partial lethality, and these deficiencies overlapped only in cytolocation 92A11 (3R: 15467758..15468450), outside of the 91C region where we originally mapped the mutation. We explored this further by quantifying the robustness of lethality when \( DmB^{k67} \) was crossed to each of the deficiencies and by defining the stages of lethality (Table 2.3). \( DmB^{k67} \) had a full lethal phenotype that recapitulated the \( DmB^{k67} \) homozygous phenotype when crossed to deficiencies in the 91C region; whereas, \( DmB^{k67} \) only had partial lethality when crossed to deficiencies in the 92A11 region, which molecular coordinates uncover only one gene \( CG16718 \), encoding a putative calcium-dependent chloride channel (Lake et al., 2004).

We crossed deficiencies from the primary region to which \( DmB^{k67} \) mapped (91C) to the two deficiencies that overlap at 92A11 to see whether lethality would also result. We did this to verify that there was not a second mutation in \( DmB^{k67} \) at 92A11. When we crossed the deficiencies together, the trans-allelic combination exhibited low-level lethality in the pupal stage and prior. This result suggests that \( DmB^{k67} \) may interact with the deficiencies in the 92A11 cytolocation, and that it does not contain a second mutation.
### TABLE 2.2

Deficiency mapping results for DmA\(^{k46}\), DmB\(^{k67}\), DmC\(^{7}\), and DmD\(^{p57}\) glue expulsion mutants

<table>
<thead>
<tr>
<th>Mutant ID</th>
<th>Estimated map position</th>
<th>Cytological deficiencies tested in map region</th>
<th>Molecularly-defined deficiencies tested in map region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Deficiency</td>
<td>Stock no.</td>
</tr>
<tr>
<td>DmA(^{k46})</td>
<td>3R: 96E6-96E9</td>
<td>Df(3R)mbc-R1</td>
<td>(BL-2585)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)96B</td>
<td>(BL-4531)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)Espl3</td>
<td>(BL-5601)</td>
</tr>
<tr>
<td>DmB(^{k67})</td>
<td>3R: 91C-91D</td>
<td>Df(3R)Cha7</td>
<td>(BL-3011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)Cha1a</td>
<td>(BL-5599)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)BX5</td>
<td>(BL-3119)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)07280</td>
<td>(BL-2409)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)Dl-M2</td>
<td>(BL-5597)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)Cha9</td>
<td>(BL-5600)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)DI-KX23</td>
<td>(BL-2411)</td>
</tr>
<tr>
<td>DmC(^{7})</td>
<td>3R: 89A-89B and 89D-89E</td>
<td>Df(3R)sbd105</td>
<td>(BL-756)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)Po2</td>
<td>(BL-3524)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)c(3)G-2</td>
<td>(BL-3527)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)sbd45</td>
<td>(BL-3678)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)P115</td>
<td>(BL-1467)</td>
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<tr>
<td></td>
<td></td>
<td>Df(3R)Spf</td>
<td>(BL-4906)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)P10</td>
<td>(BL-3483)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3R)Exel6112</td>
<td>(BL-7737)</td>
</tr>
<tr>
<td>DmD(^{p57})</td>
<td>3L: 66B-66C</td>
<td>Df(3L)RM5-2</td>
<td>(BL-4502)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3L)ZP1</td>
<td>(BL-5877)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3L)jpl-NR</td>
<td>(BL-1520)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3L)66C-G28</td>
<td>(BL-1541)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3L)66C-165</td>
<td>(BL-209)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Df(3L)66C-ex83</td>
<td>(BL-28804)</td>
</tr>
</tbody>
</table>

Four glue expulsion mutations were mapped using the 3\(^{rd}\) chromosome deficiency kit provided by the Bloomington Drosophila Stock Center. Only the deficiencies in the mapped region are listed, and those that fail to complement the mutations are underlined. The estimated map position is given. For the precisely mapped molecular coordinates of each mutation, refer to Figure 2.6.
Figure 2.5: The glue expulsion phenotypes of \textit{Dmc}^{c7} and \textit{DmD}^{57} mutants. (A) Cytological deficiencies crossed to \textit{Dmc}^{c7} were scored for glue expulsion, and although 100% of \textit{Dmc}^{c7} homozygotes do not expel glue, \textit{Dmc}^{c7} over deficiencies resulted in less severe or wild-type phenotypes in 10% of the prepupae. (B) However, lethality was 100%, which is identical to \textit{Dmc}^{c7} homozygotes. (C) \textit{DmD}^{57} over deficiencies displayed a similar phenotype to \textit{DmD}^{57} homozygotes with less than 10% expelling glue normally.
**TABLE 2.3**

Phenotypes and lethal phases of *DmB*\(^{k67}\) crossed to deficiencies

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Stock no.</th>
<th>Cyto-location</th>
<th>Percent Tb+ lethal</th>
<th>Lethality Summary</th>
<th>Notes on Lethal Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>DmB</em>(^{k67})</td>
<td>N/A</td>
<td>N/A</td>
<td>100% (n=45)</td>
<td>Full lethal</td>
<td>Pupal lethal and prior</td>
</tr>
<tr>
<td>Df(3R)BSC509</td>
<td>BL-25013</td>
<td>91A3-91D5</td>
<td>95.8% (n=48)</td>
<td>Full Lethal</td>
<td>Pharate lethal and prior</td>
</tr>
<tr>
<td>Df(3R)ED2</td>
<td>BL-6962</td>
<td>91A5-91F1</td>
<td>100.0% (n=38)</td>
<td>Full Lethal</td>
<td>Pupal lethal and prior</td>
</tr>
<tr>
<td>Df(3R)Exel6180</td>
<td>BL-7659</td>
<td>91B5-91C5</td>
<td>100.0% (n=15)</td>
<td>Full Lethal</td>
<td>Pupal lethal and prior</td>
</tr>
<tr>
<td>Df(3R)BSC742</td>
<td>BL-26840</td>
<td>91B8-91F1</td>
<td>100.0% (n=13)</td>
<td>Full Lethal</td>
<td>Pharate lethal and prior</td>
</tr>
<tr>
<td><em>Df(3R)Di-KX23</em></td>
<td>BL-2411</td>
<td>91C7/D1-92A5/8</td>
<td>35.3% (n=51)</td>
<td>Full Lethal</td>
<td>Pupal lethal and prior</td>
</tr>
<tr>
<td><em>Df(3R)ED5938</em></td>
<td>BL-24139</td>
<td>91D4-92A11</td>
<td>26.1% (n=153)</td>
<td>Partial Lethal</td>
<td>Pharate lethal and prior</td>
</tr>
<tr>
<td><em>Df(3R)ED6025</em></td>
<td>BL-8964</td>
<td>92A11-92E6</td>
<td>45.0% (n=140)</td>
<td>Partial Lethal</td>
<td>Pharate lethal and prior</td>
</tr>
</tbody>
</table>

Additional complementation testing was performed for deficiencies crossed to *DmB*\(^{k67}\) in order to quantify and estimate the stage of lethality as compared to the *DmB*\(^{k67}\) homozygote. Further mapping of *DmB*\(^{k67}\) revealed that there is partial lethality when crossed to deficiencies covering the 92A11 region.

*Balanced over TM3, so only 50% (at most) of the Tb+ are the non-balanced genotype of interest*
Figure 2.6: Gene candidates that are found in the regions where the mutants map. 
(A) There are 7 gene candidates and 2 microRNAs in the 42 kb region where \( DmAk46 \) maps.  (B) \( DmBk67 \) maps to a 69 kb region containing 15 gene candidates.  The mapped regions for \( DmCl7 \) (C) and \( DmDo57 \) (D) are also shown.  The molecular coordinates of each region are listed above the image.  The gray arrowheads represent each gene, pointing to indicate the DNA strand (forward or reverse) on which the gene is located. Images were generated using “GBrowse” on flybase.org (Crosby et al., 2007).
2.3.d: Using RNAi to Phenocopy the Mutations

As a strategy to map the mutations to individual genes, we used an RNAi approach against eight of the gene candidates in an attempt to replicate the block in glue expulsion and pupal lethality (if applicable). All the gene candidates we tested secreted and expelled glue normally (Table 2.4). Although some of the crosses resulted in lethality, for those that were viable, we could not exclude the possibility that the UAS-RNAi was not working efficiently. We expect that our RNAi strategy will be improved by combining the glueGRN glue reporter with the ubiquitous Act5C-Gal4 driver because the original loss-of-function mutations were not limited to the salivary glands; thus, it may be the loss of a signal originating outside of the salivary gland that is causing the glue blockage. Alternately, none of the eight candidates we tested may be the gene that is mutated in our loss-of-function lines. The RNAi strategy will be expanded to test gene candidates in future studies using a larger set of UAS-RNAi reagents.

2.3.e: Complementation Analysis with P-element Disruptions and Classic Mutants

As a second strategy to precisely map the mutations to genes, we crossed the mutants to P-element disruption lines and classical mutants that were available for the gene candidates. For DmA\textsuperscript{646}, seven genes are found within the 96E region (Figure 2.6A), and all have viable P-element disruption lines available to test for complementation analysis. For DmB\textsuperscript{667}, 15 genes are located within the 91C region (Figure 2.6B), but only two genes had available P-element disruptions or chemically-induced mutations. Within the 66B-66C region where DmD\textsuperscript{657} maps, there are 12 genes with five P-element disruption lines available from Bloomington and one available from Kyoto (Figure 2.6C). Finally,
there are a total of 18 genes, spanning the two regions where \( DmC^{67} \) maps (Figure 2.6D), with nine available P-element disruption lines.

We tested all available P-element disruption lines and mutants for complementation with the four mutants. The \( DmC^{67} \) mutant was potentially the result of two point mutations in two separate genes, and it did not fail to complement any of the P-element disruption lines. In addition, none of the available lethals failed to complement \( DmD^{657} \) (Table 2.5). An EMS-induced, recessive lethal mutation in the gene \( ChA \) (Choline Acetyltransferase) failed to complement \( DmB^{667} \) (Table 2.5), and a P-element disruption of the gene \( Lgr3 \) crossed to \( DmA^{k46} \) had a partial block in expulsion in approximately one-third of the progeny (Table 2.6). Therefore, the mutation in \( DmB^{667} \) was potentially located in the \( ChA \) gene because the heteroallelic combination of the EMS mutation and \( DmB^{667} \) was 100% lethal. \( DmA^{k46} \) was possibly located in \( Lgr3 \), a GPCR involved in signal transduction.

2.3.f: Molecular Characterization of the Mutations

In order to define the molecular nature of the mutations in the mapped lines, we sequenced the gene candidates that failed to complement \( DmA^{k46} \) and \( DmB^{k67} \). Using PCR, we amplified each of the exons of \( ChA \) and \( Lgr3 \) in the mutant lines and control parental line \( glueGRN-3 \) (Figure 2.8). We also sequenced the exons from the gene in the 92A11 region (\( CG16718 \)) that \( DmB^{667} \) partially failed to complement in the deficiency mapping analysis. We have not located the specific mutations in the exons of any of the gene candidates, so we expanded our sequencing effort to other genes in the 96E region where \( DmA^{k46} \) maps. We analyzed the sequences of two microRNAs (\( miR92a \) and
miR92b) and the transcription factor jigr1, but we were unable to locate a specific mutation in the coding sequences of these candidates. Thus, we have genetic evidence that two of the mutations map to specific genes (ChA and Lgr3, Figure 2.8) on the third chromosome, but we will broaden our efforts to include an analysis of transcripts from these alleles in the mutant background and an analysis of other gene candidates for DmA^{kd6} in future sequencing endeavors so that we are able to uncover the precise location of the mutations in the DNA.
### TABLE 2.4

Testing UAS-RNAi lines against gene candidates for \(DmA^{k46}\) and \(DmB^{k67}\) in the salivary gland

<table>
<thead>
<tr>
<th>Mutant ID</th>
<th>Available UAS-RNAi Stock no.</th>
<th>Lethality with Act5C-Gal4</th>
<th>Glue expulsion with glueGal4</th>
<th>Glue expulsion with sgGal4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(DmA^{k46}) jigr1</td>
<td>BL-31921 viable</td>
<td>Full Expulsion (97.3%, n=284)</td>
<td>Full Expulsion (99.1%, n=322)</td>
<td></td>
</tr>
<tr>
<td>RASSF8</td>
<td>BL-28323 viable</td>
<td>Full Expulsion (97.8%, n=133)</td>
<td>Full Expulsion (100%, n=123)</td>
<td></td>
</tr>
<tr>
<td>Lgr3</td>
<td>BL-28789 partial lethal</td>
<td>Full Expulsion (95.7%, n=112)</td>
<td>Full Expulsion (96.7%, n=352)</td>
<td></td>
</tr>
<tr>
<td>(DmB^{k67}) Mekk1</td>
<td>BL-35402 lethal</td>
<td>Full Expulsion (96.6%, n=144)</td>
<td>Full Expulsion (99.4%, n=171)</td>
<td></td>
</tr>
<tr>
<td>CG7708</td>
<td>BL-28613 partial lethal</td>
<td>Full Expulsion (99.7%, n=344)</td>
<td>Full Expulsion (99.0%, n=196)</td>
<td></td>
</tr>
<tr>
<td>VACht</td>
<td>BL-27684 lethal</td>
<td>Full Expulsion (100%, n=151)</td>
<td>Full Expulsion (98.1%, n=208)</td>
<td></td>
</tr>
<tr>
<td>Gos28</td>
<td>BL-34724 lethal</td>
<td>Full Expulsion (100%, n=215)</td>
<td>Full Expulsion (98.4%, n=240)</td>
<td></td>
</tr>
<tr>
<td>CG31224</td>
<td>BL-33969 lethal</td>
<td>Full Expulsion (99.5%, n=198)</td>
<td>Full Expulsion (100%, n=148)</td>
<td></td>
</tr>
</tbody>
</table>

UAS-RNAi lines against \(DmA^{k46}\) and \(DmB^{k67}\)-gene candidates were crossed to salivary gland drivers glueGal4 and sgGal4 and assayed for glue expulsion. All candidates expelled glue properly when knocked down in the salivary gland by RNA interference. The lethality of the offspring is indicated when the UAS-RNAi is driven ubiquitously with Actin5C-Gal4 and was determined by scoring for the presence or absence of dominant markers in adults.
TABLE 2.5

Lethal P-Element disruptions and mutants tested with *DmB*\(^{67}\) and *DmD*\(^{57}\)

<table>
<thead>
<tr>
<th>Mutant ID</th>
<th>Gene candidate</th>
<th>Stock no. of lethal</th>
<th>Complementation analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>DmB</em>(^{67})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG31230</td>
<td>N/A</td>
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<td>CG7702</td>
<td>N/A</td>
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</tr>
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<td>CG7705</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
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<td>N/A</td>
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</tr>
<tr>
<td>CG7708</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muc91C</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CG14300</td>
<td>N/A</td>
<td></td>
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</tr>
<tr>
<td>CG34282/3</td>
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<td>VACHT</td>
<td>N/A</td>
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<tr>
<td>ChA</td>
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<td>Complement</td>
<td></td>
</tr>
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<td>BL-15153</td>
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</tr>
<tr>
<td></td>
<td>BL-32009</td>
<td>Fail (100%)</td>
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</tr>
<tr>
<td>CG7714</td>
<td>N/A</td>
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<tr>
<td>CG7715</td>
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<td>N/A</td>
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</tr>
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<td>Mekk1</td>
<td>KY-105282</td>
<td>Complement</td>
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<td>DmB(^{67})</td>
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<tr>
<td>Ubc12</td>
<td>BL-16178</td>
<td>Complement</td>
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<td>CG13679</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG32364</td>
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<td></td>
</tr>
<tr>
<td>CG8006</td>
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<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td></td>
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<tr>
<td>Dscam4</td>
<td>BL-17117</td>
<td>Complement</td>
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</tbody>
</table>

Complementation testing was performed with EMS mutants and P-element disruptions crossed to *DmB*\(^{67}\) and *DmD*\(^{57}\). *DmB*\(^{67}\) failed to complement a classical EMS-induced allele of the gene ChA. Whereas, *DmD*\(^{57}\) complemented all of the available lines. KY-Kyoto DGRC; BL-Bloomington BDSC.
TABLE 2.6

P-Element disruption lines tested with the viable \( \text{Dm}^{A^{kd}} \) mutant

<table>
<thead>
<tr>
<th>Mutant ID</th>
<th>Gene candidate</th>
<th>Stock no.</th>
<th>Complementation analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Dm}^{A^{kd}} )</td>
<td>( \text{jigr}1 )</td>
<td>BL-16746</td>
<td>Complement</td>
</tr>
<tr>
<td>( \text{tankyrase} )</td>
<td>BL-17132</td>
<td>Complement</td>
<td></td>
</tr>
<tr>
<td>( RASSF8 )</td>
<td>BL-31807</td>
<td>Complement</td>
<td></td>
</tr>
<tr>
<td>( \text{Lgr3} )</td>
<td>BL-25253</td>
<td>Fail (29.7%, n=172)</td>
<td></td>
</tr>
<tr>
<td>( \text{CG4730} )</td>
<td>BL-22128</td>
<td>Complement</td>
<td></td>
</tr>
<tr>
<td>( \text{CG5039} )</td>
<td>BL-17935</td>
<td>Complement</td>
<td></td>
</tr>
<tr>
<td>( \text{CG4743} )</td>
<td>BL-19089</td>
<td>Complement</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BL-18615</td>
<td>Complement</td>
<td></td>
</tr>
</tbody>
</table>

Complementation analysis was performed by evaluating F\(_1\)-salivary gland glue expulsion. P-element disruption line BL-25253 crossed to \( \text{Dm}^{A^{kd}} \) resulted in a third of the progeny partially blocked. There was only one fully blocked larva. BL-Bloomington BDSC.
Figure 2.7

A

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutation</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>DmA- Lgr3&lt;sup&gt;k46&lt;/sup&gt;</td>
<td>not located</td>
<td>Lgr3</td>
<td>G Protein-Coupled Receptor; Signal Transduction</td>
</tr>
<tr>
<td>DmB- ChA&lt;sup&gt;k67&lt;/sup&gt;</td>
<td>not located</td>
<td>Choline Acetyltransferase (ChA)</td>
<td>Synthesis of Acetylcholine from Enzyme Acetyl-CoA and Choline</td>
</tr>
</tbody>
</table>

B

![ChA-PA diagram](image)

α: (aa 83-485) CoA-dependent Aeryltransferase domain

C

![Lgr3-PA diagram](image)

α: (aa 7-29) transmembrane helix
β: (aa 31-70) Low-density lipoprotein receptor class A
γ: (aa 204-226) Leucine rich repeat
δ: (aa 447-704) GPCR Rhodopsin-like superfamily

Figure 2.7: Identification of DmA<sup>k46</sup> and DmB<sup>k67</sup> mutations. (A) DmA<sup>k46</sup> was identified as an allele of the ChA gene and DmA<sup>k46</sup> as an allele of Lgr3. (B) A schematic of the ChA protein is illustrated along with the location of the CoA-dependent acyltransferase domain in gray. (C) A schematic of the Lgr3 protein is shown with the GPCR sequence and other domains in gray. The exon boundaries corresponding to the amino acid locations are represented by vertical lines, and the numbers represent amino acid positions in the proteins in (B) and (C).
2.4: Discussion

2.4.a: A Genetic Screen for Novel Regulators of Salivary Gland Glue Secretion and Expulsion

The use of a GFP-tagged salivary glue protein allowed us to rapidly screen for secretion and expulsion mutants and resulted in the discovery of two new genes potentially involved in these processes. \( DmB^{667} \) mapped to \( ChA \), which encodes the enzyme choline acetyltransferase, involved in the production of acetylcholine (ACh) from Enzyme CoA and choline. Because ACh is known to play a role in muscle contraction by altering the permeability plasma membranes and allowing for the influx of ions (Wessler et al., 1998) we expect that the mutation in \( ChA \) prevents the biosynthesis of acetylcholine and results in a block in the contraction cycle of muscle tissue that must occur in order for glue to be expelled.

Intriguingly, the \( ChA \) mutation interacted with deficiencies in a nearby region containing the gene \( CG16718 \), encoding a calcium-activated chloride channel (CaCC). These channels are expressed (in part) in the Cajal cells of mice that control smooth muscle contraction (Huang et al., 2009c). Hence, knockout mice for CaCC have diminished gastric smooth muscle contraction. One possibility is that the products of \( CG16718 \) and \( ChA \) are involved in the same pathway that ultimately leads to the muscle contractions necessary for glue expulsion. It is also plausible that there is an interaction between the two genes because the other EMS mutant specific to \( ChA \) (BL-32009) also results in partial lethality when crossed to the deficiencies in the non-overlapping region.

Furthermore, the best candidate for the \( DmA^{k46} \) mutant is \( Lgr3 \) because the P-element disruption line partially failed to complement \( DmA^{k46} \) in one-third of the progeny.
However, a confounding variable is that the P-element line has a GFP reporter, making the progeny difficult to score for expulsion defects. Nevertheless, if $DmA^{k46}$ maps to the $Lgr3$ encoding a GPCR, the mutation may be preventing the increase in $\text{Ca}^{2+}$ that must precede salivary gland glue exocytosis because increasing intracellular $\text{Ca}^{2+}$ levels is a known function for GPCRs (Brody and Cravchik, 2000). Although the ligand for Lgr3 is not currently known, it is predicted to be similar to the LH/FSH/TSH-class of hormones. It has been posited that this class of hormones may serve as antidiuretics in *Drosophila* (Sellami et al., 2011). It is not clear exactly what role the GPCR may have in glue expulsion and secretion, but because of the numerous roles of GPCRs in signal transduction, further characterization will be necessary.

2.4.b: The Genomic Effect of Secretion is Linked to a Probable Non-Genomic Effect of Expulsion

Secretion is known to be regulated by the EcR/USP heterodimer through 20E signaling in the salivary gland (Biyasheva et al., 2001). Moreover, perturbations in the 20E pathway in the salivary gland block glue from ever being secreted into the lumen. However, the mutations we have characterized are clearly blocking expulsion of glue but are not completely blocking secretion. Although secretion is abnormal in these mutants, glue eventually enters the lumen. This may be due to the fact that the mutations are not in genes downstream of the ecdysteroid response. Nevertheless, the expression of septal Choline Acetyltransferase in murine models is the result of retinoid activation of the $\alpha$-retinoic acid receptor (Pedersen et al., 1995), and $Lgr3$ was found in a gain-of-function screen for regulators of steroid hormone-controlled neuroendocrine remodeling (Zhao et
al., 2008). Thus, it is possible that ChA and Lgr3 expression is linked to 20E signaling in *Drosophila*. On the other hand, acetylcholine and GPCRs have such diverse roles during development that the block in expulsion could be the result of a non-genomic mechanism being perturbed in a separate signaling pathway. It is not known whether 20E triggers non-genomic effects from the results of our trial screen.

Thus, these mutants are potentially not directly linked to the genomic effects of 20E signaling, but instead they may be involved in another signal transduction pathway that must also be intact for secretion and expulsion to be complete. GPCRs such as Lgr3 are notorious for activating a cascade of secondary messengers, triggering Ca$^{2+}$ release, a necessary precursor to secretion (Brody and Cravchik, 2000). In addition, acetylcholine has many non-genomic excitatory and inhibitory effects on muscle (Wessler et al., 1998), which might be required for secretion or expulsion. Alternately, the lack in expulsion could be a secondary effect of another physiological event outside the salivary gland being perturbed. Future screens will be on a larger scale and address this issue by isolating *bona fide* secretion mutants, having an identical phenotype to *glueGal4/UAS-EcR-DN* animals with perturbed 20E signaling in the salivary gland.

2.4.c: The Broader Roles of the Genes Uncovered in the Screen

With delicate care, a larva can survive into adulthood without its salivary gland. Therefore, it is intriguing that three of the four mutations that block the expulsion of glue are also lethal. This speaks to them having a pleiotropic role in development, as would be expected. Whereas, the fourth mutation *DmA*kr, which is viable, may have a true role in the salivary gland as a mediator of glue secretion. Future experiments will include
other functional tests of the mutants to see what other processes are disrupted. In addition, future experiments will examine whether the genes $ChA$ and $Lgr3$ are directly regulated by 20E or 20E effectors.

In sum, the screen was successful. It should be noted that the screen was not saturating because we only screened the third chromosome (40% of the genome), and we uncovered just two mutants that were alleles of the same gene. Had we saturated the third chromosome with mutations, we would have found many more inter se complementation groups, with at least two alleles of each gene. Thus, it will be very informative once we conduct the screen on a larger scale with full saturation coverage of all four chromosomes. We expect that we will find even more genes that are members of pathways that can be synergistic with steroid hormone signaling but are not traditionally known to be directly activated by steroid hormones.
**Figure 2.8**

A *ChA-RA* (gene: 26.82 Kb; transcript: 3,991 bp)

*B* *Lgr3-RA* (gene: 11.41 Kb; transcript: 3,007 bp)

*C* *jigr1-RA* (gene: 12.42 Kb; transcript: 1,852 bp)

<table>
<thead>
<tr>
<th>Exon Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Exon Size (bp)</th>
<th>Product Size (bp)</th>
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<td>jigr1_Exon3_2</td>
<td>GGATCATACAGCTGTTGAC</td>
<td>CGCATAGATGAGACAGAC</td>
<td>(687)</td>
<td>370</td>
</tr>
</tbody>
</table>

**Figure 2.8:** PCR primers for the gene candidates for *DmA* and *DmB*. The exons are represented by red boxes and the untranslated regions are represented by unfilled boxes for (A) the *DmA* candidate gene *ChA* and (B) the *DmB* candidates *Lgr3* and *jigr1*. Images in (A) and (B) were generated by Ensembl.org (Flicek et al., 2012). The PCR primer sequences and predicted product sizes are indicated in (C).
CHAPTER 3
ECTOPIC E23 OVEREXPRESSION BLOCKS ECDYSONE SIGNALING IN LARVAL TISSUES

3.1: Introduction

Steroid hormones initiate complex regulatory circuits by binding to cognate nuclear-hormone receptors and activating genes that are necessary for homeostasis, metabolism, and cellular differentiation among many other functions (reviewed in King-Jones and Thummel, 2005). *Drosophila melanogaster* is a powerful model for steroid hormone signaling because invertebrate nuclear hormone receptors mechanistically are very similar to those of vertebrates (Mangelsdorf et al., 1995; King-Jones and Thummel, 2005). The steroid hormone 20-hydroxyecdysone (20E) initiates a cascade of gene activation that has been examined extensively in the larval salivary gland due to the presence of giant polytenized chromosomes, which have facilitated tissue-specific genomic studies prior to the era of sequenced genomes (Ashburner, 1972, 1973, 1974; Ashburner and Richards, 1976; Andres et al., 1993).

Steroid hormone-mediated gene expression flows in three visible waves in the salivary gland, characterized as “early”, “early-late”, and “late” puffs (Ashburner, 1973; Ashburner and Richards, 1976). These findings were revolutionary in that a complex regulatory circuit was reduced to a simplified model in which the proteins encoded by a small set of early genes were capable of shutting down their own expression while allowing for the much larger group of late genes to be turned on (Ashburner et al., 1974). The late genes were subdivided into two groups, with the early-late genes requiring the activated ecdysone-receptor complex for their expression, and the late-late genes
requiring the withdrawal of the activated-ecdysone-receptor complex for activation (reviewed in Andres and Thummel, 1992; Thummel, 2002). Ultimately, this gene cascade is activated in 20E-responsive tissues resulting in diverse physiological effects, and essentially controlling insect development.

Some of the early genes studied in this model encoded proteins that were not transcriptional activators or repressors, as one might expect (Thummel, 2002). One such gene is E23, named for its early (“E”) expression in the cascade and its chromosomal location (location 23 on chromosome 2). E23 encodes an ATP-Binding Cassette (ABC) transporter that shares protein sequence homology with the product of the white gene (Hock et al., 2000) and is a member of a class of ABC transporters that must form dimers to function (Dean et al., 2001). In the 20E cascade, E23 may fulfill the role of a negative regulator because ectopic expression of E23 results in decreased expression of the 20E-induced genes E74 and E75 (Hock et al., 2000).

We sought to test the hypothesis that E23 is capable of negatively regulating 20E signaling; and thus, E23 would be able to block responses to 20E in any tissue. Because 20E-regulated physiological responses have been well-characterized in many tissues (reviewed in Riddiford, 1993; Riddiford et al., 2000), our strategy was to overexpress the E23 transporter in these tissues using the UAS/Gal4 system (Brand and Perrimon, 1993) and compare the results to phenotypes that are manifested when a dominant negative form of the ecdysone receptor (EcR) is able to bind ligand but is unable to activate downstream genes (Cherbas et al., 2003).

For many of our experiments, the larval salivary gland of Drosophila is our focus. The larval salivary gland provides an elegant model system to study hormone signaling.
because there are three easily observable responses to 20E that have been carefully timed and characterized; thus, blocking ecdysone signaling in this tissue has very predictable results. The first response to 20E is the synthesis of a glycoprotein glue mixture. Synthesis occurs during the middle of the third larval instar at approximately 24 hours (hrs) before puparium formation (BPF) in response to an elevated hormone titer (Zhimulev and Kolesnikov, 1975; Warren et al., 2006). The second response is the secretion of this glue mixture into the lumen of the salivary gland. Secretion occurs approximately 4 hrs BPF during the late third instar during the pre-metamorphic pulse of 20E (Zhimulev and Kolesnikov, 1975; Biyasheva et al., 2001; Warren et al., 2006). In addition, this glue is expelled from the lumen through the mouth of the larva; it then drips down the front of the larval cuticle, subsequently allowing the animal to stick to a solid surface. Finally, the third major response at the end of the prepupal period is the histolysis of the salivary gland. The cell death of the salivary gland, which has characteristics of both autophagy and programmed cell death, is complete by about 14-16 hrs after puparium formation (APF) (Lee and Baehrecke, 2001; reviewed in Yin and Thummel, 2005).

Surprisingly, we found that overexpressing UAS-E23 (E23+) or a dominant negative form of EcR (EcR-DN) resulted in identical phenotypes with any larval-tissue Gal4 driver tested but that E23+ did not phenocopy EcR-DN when overexpression was driven in the imaginal discs during larval development. The observation supports other data that 20E signaling via the ecdysone receptor has different roles in larval tissues versus imaginal discs (Talbot et al., 1993; Li and Bender, 2000; Schubiger and Truman, 2000; Davis et al., 2005). Perhaps most importantly, we found that the block in 20E signaling
by overexpression of E23+ could be overcome in vivo by high levels of 20E. These results speak to the mechanism of the E23 protein as a negative regulator of 20E through specific inhibition of the hormone itself. Finally, our findings suggest a novel way that steroid signaling can be attenuated in a tissue-specific fashion during Drosophila development.

3.2: Materials and Methods

3.2.a: Fly Stocks and Maintenance

Drosophila cultures were reared at room temperature (~25°C) on a standard cornmeal medium with light karo syrup and seeded with dry baker’s yeast (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm). The GMR-, dpp-, Ser-, sg-, act5C-, and Act<cd2<Gal4 drivers were obtained from the Bloomington Drosophila Stock Center (BDSC, Bloomington, IN, USA). Eip<sub>657</sub>-Gal4 and UAS-<sup>F645A</sup>EcR (EcR-DN) were gifts from Lucy Cherbas and described in Cherbas et al., 2003. The Lsp2- and Cg drivers (recombined with membrane-localized GFP) were gifts from Deborah Hoshizaki. More information about the drivers can be found in Table 3.1. UAS-E23 (E23+) on chromosome II and III were gifts from Dan Garza and were generated by P-element transformation. All salivary gland drivers were recombined with Sgs3-GFP (glueGRN) or Sgs3-DsRed (glueRED) to mark glue production and secretion (Biyasheva et al., 2001; Costantino et al., 2008). UAS-EcRi and UAS-USPi were gifts from Christophe Antoniewski. Controls consist of the driver crossed to the w<sup>118</sup> parental stock, obtained from the BDSC.
3.2.b: Developmental Staging

Larvae were staged either according to the blue food method as described in Andres and Thummel (1994) or by visualizing landmark stages of salivary gland physiology including glue production in mid-third instar (18-20 hrs before BPF) and glue secretion (~4 hrs BPF). White prepupae (WPP) (0 hrs BPF) were visibly recognizable because of their everted spiracles and untanned cuticles. Mouth hooks and spiracle morphology were also used to developmentally stage larvae during the viability and molting experiments according to the descriptions found in Bodenstein, 1994.

3.2.c: Assaying Glue Production and Secretion in Live Animals and Dissected Glands

For images of live animals we used an endogenously tagged glue protein, glueRED or glueGRN. Activated by the Sgs3 enhancer/promoter region, glueRED has a C-terminal DsRed tag (described in Costantino et al., 2008). Previous experiments have demonstrated that this tagged protein is an accurate reporter for glue synthesis, secretion, and expulsion (Biyasheva et al., 2001; Costantino et al., 2008). We also incorporated a UAS-GFP nuclear or endosomal marker in salivary gland assays to ensure that tissues were visible even in the absence of glue production. We imaged live animals through their cuticles, using a fluorescent stereomicroscope (see below). For high-resolution images of tissues, we selected larvae of the appropriate stages and dissected the glands in DPBS (see Appendix A). We then mounted the tissues on standard 25 x 75-mm microscope slides (VWR, Radnor, PA, USA) with DPBS, 1MM cellulose filter paper spacers (Whatman plc, Kent, ME, UK), and No. 2 coverslips (18 mm², 0.17 to 0.25-mm thickness) (VWR), and we imaged them using confocal microscopy (see below).
3.2.d: Microscopy and Imaging

Low-resolution images of whole animals or dissected tissues were obtained on a fluorescent stereomicroscope containing filter cubes for GFP and DsRed (Leica Microsystems Inc., Buffalo Grove, IL, USA). Images were captured with the Spot Insight QE Model #4.2 digital camera (McBain Systems, Simi Valley, CA, USA) and processed using PowerPoint software (Microsoft Co., Redmond, WA, USA). High-resolution images of dissected salivary glands and other tissues were imaged on an LSM 510 Axioplan confocal microscope equipped with a 63x water immersion lens (NA:0.9) (Carl Zeiss Inc., Oberkochen, Baden-Württemberg, Germany) and post-processed with LSM 510 image-analysis software (Zeiss) and Canvas (ACD Systems, Victoria, British Columbia, Canada) or PowerPoint (Microsoft).

3.2.e: Viability and Statistical Analysis

Approximately 20 females and 10 males of the appropriate genotype were crossed and reared in 25 x 95-mm vials (Genesee Scientific, San Diego, CA, USA) with standard cornmeal medium at ambient room temperature. Parents were moved to new vials every 3 days for 30 days. Larvae were aged and scored for the stage of lethality using developmental staging described previously. The pupa-to-adult percent survival was arcsin square root transformed before performing an ANOVA comparing the adult viability of wildtype, EcR-DN, and E23+ crossed to the driver of interest. A pairwise t-test with Bonferroni correction was used to find significant differences (p<0.05) between genotypes, and significant differences are denoted by different letters. All graphs are presented as the mean percent surviving +/- SEM over three trials. Statistical
computations including the arcsin square root transformations were done in Prism (GraphPad Software Inc., La Jolla, CA, USA) as per *Biostatistical Analysis* (Zar, 2009).

3.2.f: Western Blotting

Approximately 10-20 salivary glands were dissected in DPBS (as described above) to isolate proteins. The glands were pelleted in a microfuge and resuspended in a lysis buffer (see Appendix B) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO, USA) (Laemmli, 1970). Glands were homogenized and boiled for 5 minutes before being stored at 4°C for less than one week. Samples were resolved on 7.5% SDS polyacrylamide gels (see Appendix B). The blots were transferred to Immobilon P membranes (EMD Millipore, Billerica, Massachusetts, USA) as previously described (Vaskova et al., 2000). The following primary antibodies were used: mouse anti-α-Tubulin primary (Sigma) diluted 1:15,000 and anti-E23 (a gift from Dan Garza) diluted 1:20. We used goat anti-mouse-HRP secondary (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:25,000. Protein levels were visualized and quantified using Chemiluminescence ECL(+) Western-blotting detection system (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and a Typhoon 8600 variable mode imager (GE Healthcare).
3.2.g: Generation of Mosaic Salivary Glands using Site-Specific Recombination

(Flp-Out)

The Gal4-Flp-Out recombinase system was used to generate mosaic tissue (Golic and Lindquist, 1989; Struhl and Basler, 1993). Flp was induced by heat shock in flies of the genotype: y w hsFlp, Act<cd2<Gal4; UAS-GFP.nls/+; UAS-E23, glueRED/+.

We generated this line of flies by combining UAS-E23 with UAS-GFP.nls and glueRED. Then we crossed these flies to y w hsFlp; Sp/CyO, Cy; Dr/TM6B, Tb (a gift from Robert Holmgren). From the F1, we picked males with y bodies and Cy wings and that lacked Sp eyes, and we crossed these flies to y w Act<cd2<Gal4 (BDSC). We raised the progeny at room temperature, but in order to induce mosaic tissue with Flippase Recombinase, we heat shocked the larvae in their culture vials in a waterbath at 37°C for 1 hour, and we allowed them to recover overnight. The following day, we selected third instar larvae and WPP that were Tb+ and GFP+ in order to dissect and analyze salivary glands (see Assaying for Glue and Microscopy and Imaging). The intensity of GFP (as a result of the variable excision of FRT sites separating Gal4 coding information from an actin promoter) was used as an indicator of how much E23+ was produced in each salivary gland cell.
<table>
<thead>
<tr>
<th>Driver</th>
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<th>Onset of Expression</th>
<th>References</th>
<th>Stock Information</th>
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<td>Imaginal discs excluding the eye disc</td>
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The **GAL4 drivers** that were crossed to **UAS-E23** and other **UAS-lines** are listed along with their spatial and temporal domains of expression and key references. Stocks available from the Bloomington *Drosophila* Stock Center are notated with BL-.

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3.3: Results

3.3.a: Targeted Overexpression of E23 in 20E-Responsive Larval Tissues Phenocopies the Overexpression of EcR-DN

Processes regulated by 20E have been characterized in tissues such as the larval salivary gland, larval fat body, epidermis, and mushroom body/CNS. We wanted to test the hypothesis that E23 negatively regulates 20E-induced processes in these tissues, so we overexpressed E23 in a variety of tissues using GAL4-specific drivers. If E23 is normally acting as a 20E exporter, we expect that its ectopic expression in any tissue should produce a phenotype similar to that observed when 20E is prevented from properly signaling. For example, it has been demonstrated that blocking receptor function in both larval and imaginal tissues prevents those tissues from responding to in vivo pulses of 20E to either histolyze (larval) or differentiate (imaginal) (Cherbas et al., 2003). Thus, we hypothesize that tissues in which E23 is ectopically overexpressed should behave as if the ecdysone receptor is nonfunctional.

To test this hypothesis, we used the dominant negative line EcR-DN$^{F645A}$, which can bind DNA and dimerize with USP, but it cannot transactivate the expression of target genes, and thus serves as a competitive inhibitor of endogenous EcR (Cherbas et al., 2003). We crossed $E23^+$ or EcR-DN bearing flies to those with a tissue-specific driver in tissues with known ecdysone responses. The drivers used in this study are outlined in Table 3.1, and the results of overexpressing $E23^+$ with these drivers is summarized below.
3.3.a.i: Salivary Gland

We first assayed the pre-metamorphic responses to 20E, which are glue secretion and salivary gland death. We crossed flies carrying the \textit{E23+} transgene to \textit{glueGAL4}, which is expressed during the mid-third instar when glue protein is synthesized (Biyasheva et al., 2001; Cherbas et al., 2003). We visualized the expression and localization of the glue with an endogenously tagged glue protein under its own regulatory control (\textit{glueRED}) (Costantino et al., 2008).

As predicted, the progeny of this cross express glue at the proper time because both \textit{Gal4-} and \textit{glue-}gene expression are initiated at the same time. As development proceeds, glue proteins are normally secreted into the lumen of the gland approximately 4 hrs before pupariation (Biyasheva et al., 2001). However, the larval offspring that were overexpressing \textit{E23} did not secrete glue appropriately, and glue was found in the cells of the salivary gland throughout late larval and pupal development. In addition, \textit{EcR-DN} elicited the same phenotype as \textit{E23+} in which glue secretion was blocked (Figure 3.1).
Figure 3.1: Salivary gland glue is not secreted when $E23^+$ or $EcR-DN$ is ectopically overexpressed prior to pupariation. Each column represents confocal images of salivary gland cells from crosses of the glueGal4 driver to the parental-control ($w^{1118}$) strain (left), negative-control ($EcR-DN$) strain (middle), and experimental ($E23^+$) strain (right). (A-C) glueGal4 is activated during the mid-third instar at the same time as glue is synthesized and packaged into vesicles with a diameter of 1µm. (D-F) In all three groups, the glue granules increase in size (up to 5 µm in these images) as more glue vesicles fuse and mature in the early-late third instar. (G) During the late third instar, glue granules are secreted into the lumen of the gland only in the parental-control group. In the late third instar, (H) glueGal4>$EcR-DN$ and (I) glueGal4>$E23^+$ do not secrete glue granules. (K, L) The block in glue secretion continues through the WPP stage and (N, O) the prepupal period. (J) In control animals from the parental cross, residual glue remains in the WPP salivary gland. (M) The glue deteriorates rapidly as the gland begins to undergo cell death in the prepupal period in the $w^{1118}$ control. The glue is labeled with an endogenous DsRed tag (glueRED), and the vesicle membranes are labeled via a vesicle snare-GFP. Sgs3: glueRED; synB: UAS-Synaptobrevin-GFP (a vesicle membrane marker); L: lumen. The scale bar shown in (A) is 20 µm. All images were taken at the same magnification (630x with 1.5x optical zoom).
Next we looked at the cell death of the salivary gland, another pre-metamorphic event triggered by 20E signaling. Again, we overexpressed \(E23^+\) using the \(\text{glueGal4}\) driver, and we assayed the cell death of the salivary gland. Because no prepupal salivary-gland driver has been generated, we used the same \(\text{glueGal4}\) driver, but we took advantage of the fact that GAL4 protein is stable and persists in the prepupal period. To perform the assay, we used a transgenic line bearing the \(\text{glueGal4}\) driver and a GFP- or DsRed-tagged glue protein (\(\text{glueGRN}\) or \(\text{glueRED}\)) to monitor the status of the gland through the cuticle and pupal case. In controls, glue had been synthesized during the mid-third instar, and had been secreted and expelled by pupariation, as indicated by the fluorescent ring on the pupal case (Figure 3.2A). Cell death proceeded as normal in controls with the salivary gland degrading by 16 hrs APF; by this time, no fluorescent-tagged glue was visible, so pupae were dissected to verify the degradation of the gland. Adult controls had no fluorescence or larval salivary gland (Figure 3.2D).

In contrast to controls, the larval salivary glands of animals with \(E23^+\) overexpressed persisted beyond 14-16 hrs APF (Figure 3.2B), identical to \(\text{EcR-DN}\) phenotypes (Figure 3.2C). Adults eclosed with intact larval salivary glands that were compressed into the head, thorax, or abdomen (Figure 3.2E, F, I). Adults are viable in spite of the persistence of the larval salivary gland, which can be dissected and imaged using confocal microscopy. One striking feature is the oversized nuclei of the cells of the persistent salivary glands that appear to be more polytenized compared to the nuclei of larval controls (Figure 3.2G, H). Likewise, the salivary gland persists into adulthood in \(\text{EcR-DN}\) lines crossed to \(\text{glueGal4}\). Therefore, both pre-metamorphic responses to 20E in the salivary gland are blocked when \(E23^+\) or \(\text{EcR-DN}\) is overexpressed.
Figure 3.2: Salivary glands do not undergo cell death when *E23*+ or *EcR-DN* is ectopically overexpressed. Pupae in (A–C) were imaged after head eversion which occurs after the salivary gland is normally degraded. Adults are imaged in (D, E, F, and I). The parental controls (*glueGRN*) in (A) and (D) underwent normal salivary gland death. The animals with *E23*+ overexpressed maintained a larval salivary gland during the pupal stage (B) and into adulthood, where it was located in the thorax (E), head (F) or abdomen (I). The same phenotype is observed when *EcR-DN* is expressed in the same way (C). Insets show salivary glands dissected from the pictured pupa or adult. (G, H) Confocal images of the cells of salivary glands from *glueGal4* animals crossed to (G, control) *w*1118 or (H) *E23*+. (G) In a late third instar salivary gland, the nucleus is on average 20-25 µm at its maximal size. (H) The nucleus is 48 µm in the persistent larval salivary gland dissected from an adult similar to that in (I). *Sgs3*: *glueGRN* or *glueRED*. nucleus: nuclear-localized GFP driven by the *glueGal4* driver. Scale bars are 500 µm except in (G) and (H) where they are 25 µm. White arrowheads point to glue that is outside of the cuticle. Red arrows point to internal, larval salivary glands.
3.3.a.ii: Fat Body

The larval fat body undergoes a sequential dissociation beginning soon after puparium formation (Nelliot et al., 2006). At the prepupal to pupal transition (12 hrs APF), the fat body dissociates, forming clumps of individualized fat body cells (Figure 3.3C). When $E23^+$ or $EcR-DN$ is ectopically overexpressed, the fat body fails to dissociate by 12 hrs APF (Figure 3.3D). In addition, the percent of pupae that eclose (emerge from the pupal case) does not significantly differ between $EcR-DN$ and $E23^+$ when crossed to either fat body driver (Figure 3.3E and 3.4C). Furthermore, many pharate adults die as they are trying to emerge from their pupal cases, resulting in the phenotype depicted in Figure 3.4.

3.3.a.iii: Mushroom Body

In the developing brain, 20E triggers the degeneration of axons in the mushroom body as a pre-metamorphic response to the steroid (Lee et al., 2000b). Using a mushroom-body specific driver $OK107-Gal4$, we overexpressed $E23^+$ or $EcR-DN$ in the mushroom body during the third instar. What resulted was 20% viability with the majority of animals arresting during the pupal stage for both $E23^+$ and $EcR-DN$ (Figure 3.5D). However, no abnormalities in the mushroom body structure were detected during onset of pupariation using a low-resolution microscope (Figure 3.5A-C).
Figure 3.3: Ectopic overexpression of $E23^+$ or $EcR-DN$ blocks fat body dissociation. Confocal images of fat body labeled with a GFP marker were taken at 5x magnification. (A, C) The driver Cg-Gal4 was crossed to $w^{1118}$ parental or (B, D) $E23^+$. (A, B) The fat body forms flat, hexagonal sheets during WPP in both Cg-Gal4/$w^{1118}$ and Cg-Gal4>$E23^+$. (C) Normally, the flat cells become round and dissociate from each other by 12 hrs APF as in the control. (D) The cells fail to dissociate at 12 hrs APF in Cg-Gal4>$E23^+$. The scale bar in (C) is 100 µm. (E) Results from ANOVA comparing pupa-to-adult viability of control Cg-Gal4/$w^{1118}$, Cg-Gal4>$EcR-DN$, and Cg-Gal4>$E23^+$. Means +/- SEM are provided for three trials. Overall the difference in means was significant, and significant pairwise differences (Bonferroni; $\alpha=0.05$) are denoted by different letters. Cg-Gal4>$EcR-DN$ and Cg-Gal4>$E23^+$ are not significantly different. GAP: myristoylated UAS-GFP (plasma membrane marker).
Figure 3.4: The phenotypes of E23+ and EcR-DN are identical in the fat body. (A) Lsp2-Gal4>EcR-DN and (B) Lsp2-Gal4>E23+ arrest prior to and during eclosion as they struggle to emerge from their pupal cases. The adults in (A) and (B) are developmentally arrested. (C) Results from ANOVA comparing pupa-to-adult viability of control Lsp2-Gal[w118], Lsp2-Gal4>EcR-DN, and Lsp2-Gal4>E23+. Means +/- SEM are provided for three trials. Overall the difference in means was significant, and significant pairwise differences (Bonferroni; α=0.05) are denoted by different letters. Lsp2-Gal4>EcR-DN and Lsp2-Gal4>E23+ are not significantly different.
Figure 3.5: Overexpression of *E23*+ and *EcR-DN* in the mushroom body results in pupal lethality. Stereomicroscope images of mushroom body of (A) control *OK107/w^1118*, (B) *OK107>EcR-DN*, and (C) *OK107>E23*+ display no obvious defects at WPP. (D) Results from ANOVA comparing pupa-to-adult viability of control *w^1118*, *OK107>EcR-DN*, and *OK107>E23*+. Means +/- SEM are provided for three trials. Overall the difference in means was significant, and significant pairwise differences (Bonferroni; α=0.05) are denoted by different letters. *OK107>EcR-DN* and *OK107>E23*+ are not significantly different. The majority of *OK107>EcR-DN* and *OK107>E23*+ are lethal during the pupal stage. The scale bar in (A) is 100 µm.
3.3.a.iv: Eye

The *GMR* driver, which is expressed during the wandering phase of the third larval instar, causes abundant lethality after pupariation with no surviving adults for both *E23*+ and *EcR-DN* (Figure 3.6D). However, those pupae that develop into pharate adults can be dissected out of their pupal cases to reveal a striking, highly melanized-eye phenotype, similar to that seen in *EcR-DN* (Figure 3.6B, C), and as previously reported (Cherbas et al., 2003).

3.3.a.v: Ring Gland

The ring gland is a tissue that includes the prothoracic gland, the site of the synthesis of the 20E precursor, α-ecdysone. In addition, the ring gland is a polytenized tissue like many other larval tissues used in these experiments. The results for *EcR-DN* and *E23*+ do not differ significantly when driven in the ring gland with less than 30% of the animals from the cross surviving in adulthood (Figure 3.7A).

3.3.a.vi: Imaginal Discs

Four imaginal disc drivers crossed to *E23*+ mirrored *EcR-DN: Ser-Gal4, dpp-Gal4, Lz-Gal4, and GMR-Gal4* (as described above). All of these drivers are expressed in imaginal discs during the third instar. However, as shown in Table 3.1, expression of these drivers is not limited to the discs. The driver *dpp* is also expressed in the gut and salivary gland, and *Ser* drives *Gal4* expression in the proventriculus (Cherbas et al., 2003). *Lz-Gal4* has been reported to drive expression in the embryonic hemocytes and crystal cells in addition to the eye discs (Fossett et al., 2001). Survival to adulthood was
calculated at 50% and 60% using the Lz driver (Figure 3.7B). While there were no adult survivors using the Ser driver, 8% of the animals did survive when the dpp driver was used (Figure 3.7C-D). Both drivers crossed to E23+ and EcR-DN result in larval lethality that occurs prior to the third instar in many cases, raising the possibility that these drivers are expressed earlier than the third instar as reported (see Table 3.1 for references).

In summary, overexpression of E23+ is sufficient to inhibit pre-metamorphic 20E signaling in every larval tissue tested and in imaginal tissues using drivers that have promiscuous expression. Furthermore, the results closely phenocopy those of EcR-DN crossed to the respective driver.
Figure 3.6

Figure 3.6: Eye defects and lethality result when E23+ is overexpressed with the GMR driver. Stereomicroscope images of control (A) GMR/w^{1118} adult eyes or pharate eyes of (B) GMR>EcR-DN or (C) GMR>E23+. (D) Results from ANOVA comparing pupa-to-adult viability of control GMR/w^{1118}, GMR>EcR-DN, and GMR>E23+. Means +/- SEM are provided over three trials. Overall the difference in means was significant, and significant pairwise differences (Bonferroni; α=0.05) are denoted by different letters. GMR>EcR-DN and GMR>E23+ are not significantly different. The scale bar in (C) is 350 µm.
Figure 3.7: EcR-DN and E23+ arrest before adulthood when overexpressed by a variety of drivers. Results from ANOVAs comparing pupa-to-adult viability of controls to EcR-DN and E23+ crossed to drivers. Means +/- SEM are provided over three trials. Overall the difference in means was significant, and significant pairwise differences (Bonferroni; α=0.05) are denoted by different letters. The percent of pupa-to-adult survival does not significantly differ between EcR-DN and E23+ when crossed to (A) a ring gland driver Aug21, (B) an eye disc driver Lz, (C) a wing disc driver Ser, or (D) an imaginal disc driver dpp. See Table 3.1 for more specific descriptions of the domains of expression of these drivers.
3.3.b: Overexpression of E23+ Blocks 20E Signaling in the Mid-third Instar Salivary Gland

Next, we were interested in what would result if we overexpressed E23+ during periods with only small increases in the systemic 20E titer. A rise in 20E has been observed during the mid-third instar time period (Warren et al., 2006), which (in the salivary gland) triggers the expression of a glycoprotein glue mixture consisting of the Sgs gene products (reviewed in Lehmann, 1996). To test the hypothesis that E23 can negatively regulate 20E in the mid-third instar, we overexpressed E23+ before the pulse of 20E that initiates the synthesis of glue in the salivary gland. We predicted that this overexpression of the ABC transporter E23 would result in blocked responses to 20E signaling in the mid-third instar salivary gland.

Larval progeny of the cross between flies bearing UAS-E23+ and the salivary gland driver sgGal4 did not produce glue in the salivary gland during the mid-third instar when it is normally expressed (Figure 3.8). Instead, the cells were refractory to the 20E signal, and this result continued until pupariation. Furthermore, E23+ overexpression throughout larval salivary gland development produced a similar phenotype as that observed when EcR-DN was ectopically overexpressed with the same driver. Thus, the phenotypes of E23+ and EcR-DN are identical when expressed in the salivary glands with either sgGal4 or glueGal4, and the overexpression of E23+ appears to block 20E responses at times other than those occurring during metamorphosis.
**Figure 3.8: Overexpression of E23+ or EcR-DN in the early salivary gland blocks glue synthesis.** Presented are confocal images of cells of the salivary gland of different genotypes taken at the same stages. Control larvae from the w^{1118} parental cross (sgGal4/w^{1118}) are represented in the left column. These animals synthesize glue during the mid-third instar (D). The glue is packaged into vesicles (0.5-2 µm) that mature into granules (2-6 µm) as glue vesicles fuse. The later stages in the control w^{1118} are also shown for comparison including (G) late third instar secretion, (J) WPP, and (M) prepupal stages. The middle column represents images from the negative control for glue synthesis in which EcR-DN is ectopically produced (sgGal4>EcR-DN). The column on the right represents the experimental samples in which E23+ is ectopically overexpressed (sgGal4>E23+). Note that in the middle and right columns, animals do not synthesize glue at any of the developmental times shown. Sgs3: glueRED; nucleus: UAS-GFP.nls; L: lumen. The scale bar in (A) is 25 µm. All images were taken at the same magnification (630x with 1.5x optical zoom).
3.3.c: Overexpression of E23+ Results in Molting and Ecdysis Defects

Many experiments indicate that 20E is the molting hormone, but molting is a process that involves not only the building of the new cuticle and tracheae (e.g., molting) but also the programmed behavioral shedding of the old cuticle (e.g., ecdysis) (reviewed in Truman, 2005). Inka cells of the epitracheal glands respond to and secrete small peptide hormones to initiate ecdysis, and the larval epidermis must be replaced during each molting phase. We wanted to test whether E23+ could block molting and ecdysis by using drivers specific to the Inka cells and the larval epidermis.

Ecdysis was defective when E23+ was overexpressed using the ETH-Gal4 driver (King, 2012), which is expressed as early as embryonic development in the Inka cells. In the E23+ experimental group, fused dorsal bridges were apparent in surviving larvae (King, 2012), an indication of an ecdysis defect (Hall and Thummel, 1998). Therefore, E23+ is sufficient to negatively regulate the steroid hormone in the process of ecdysis. Also demonstrated were molting defects (such as first instar lethality and improperly formed cuticles) when E23+ was ectopically overproduced with the epidermis specific A58-Gal4 driver (King, 2012), which is expressed during the larval stages.

Molting also occurs during pupariation as a larva builds the cuticular structures required for the transition into the pupal state. The hormone 20E has a key role in this process, as a very large pulse is released during the larval to prepupal molt (Warren et al., 2006). In order to test whether overexpression of E23+ was sufficient to block molting at this stage, we used a cuticle driver, Eip-Gal4. The Eip driver causes arrest during pupariation with no animals surviving when E23+ or EcR-DN is overexpressed (Figure 3.9F). The majority of the larval cuticle fails to shorten and tan, and remains in a larval-
like state. At the extremities, the cuticle tans and shortly thereafter blackens (Figure 3.9). Thus, \( E23^+ \) overexpression is sufficient to block the formation of the new cuticle during the larval to prepupal molt, and there is a requirement for 20E signaling in both ecdysis and molting.
Figure 3.9

Figure 3.9: Overexpression of E23+ or EcR-DN in the third instar cuticle blocks pupal molting. Stereomicroscope images of (A-E) WPP and (A’-E’) age-matched prepupae (6 hrs later) of the listed genotypes crossed to the Eip driver. (A) Control Eip-Gal4/w1118; (B) Eip-Gal4>E23+; (C) Eip-Gal4>EcR-DN; (D) Eip-Gal4>EcRi; (E) Eip-Gal4>USPi. Arrows and arrowheads point to areas where the cuticle blackens, but the cuticle never hardens or tans and remains in a larval-like state (B’ and C’). (F) Results from ANOVA comparing pupa-to-adult viability of control Eip-Gal4/w1118, Eip-Gal4>EcR-DN, and Eip-Gal4>E23+. Means +/- SEM are provided over three trials. Overall the difference in means was significant, and significant pairwise differences (Bonferroni; α=0.05) are denoted by different letters. Eip-Gal4>EcR-DN and Eip-Gal4>E23+ are not significantly different. The scale bars represent 500 µm.
3.3.d: *E23* mRNA and Protein are Dynamically Regulated Throughout Development

While conducting our overexpression studies, we noticed an interesting trend. Some of the imaginal disc drivers crossed to *E23*+ did not result in identical phenotypes to that observed when *EcR-DN* is expressed with the same driver (Figure 3.10). In order to understand how this might occur, we conducted a literature search on all the drivers to find the more specific nature of their domains of expression (see Table 3.1 for references). We found that many of the lines drive expression of *Gal4* in other tissues not initially described. The lines that resulted in divergent outcomes between *E23*+ and *EcR-DN* have restricted expression in the imaginal discs; whereas, the imaginal disc drivers that resulted in the same phenotypes had additional expression in larval tissues. The one exception to this trend is that any of the “imaginal-restricted” drivers created from the *GawB* construct have spurious salivary-gland expression (Gerlitz et al., 2002); nevertheless, expression in the salivary gland does not affect the viability of the animal, so it was not factored into the resulting lethality of some of the imaginal drivers crossed to *E23*+ or *EcR-DN*.

Because the overexpression of *E23*+ did not have as severe an effect as *EcR-DN* in the imaginal discs but it did in larval tissues, we expected that *E23* might have a role in regulating the tissue- and temporally-specific responses to the 20E. One indication of this role would be differing levels of *E23* expression in larval and imaginal tissues. Thus, we wanted to see whether wild-type *E23* is differentially expressed in imaginal discs versus larval tissues and at different time periods throughout development, so we compared the mRNA and protein levels of individual tissues in similarly staged animals.
In order to test our hypothesis, we compared the transcriptional profiles for \textit{E23} from nine RNA-seq experiments of staged tissues from the modENCODE project (Graveley et al., 2011; www.modencode.org) (Figure 3.11A). We found a general trend in larval tissues is lower \textit{E23} mRNA levels during the late third-instar wandering stage than at WPP. However, only the late third instar \textit{E23} expression values for imaginal discs were available, so we were unable to compare these levels to WPP. \textit{E23} mRNA levels were lower in the imaginal discs during the late third instar in comparison to the larval tissues including the fat body, digestive tissues, and salivary gland. The highest \textit{E23} mRNA expression was in the third instar digestive system. Whereas, the levels of \textit{E23} mRNA in the salivary gland and fat body increased substantially at WPP. Thus, \textit{E23} levels differ over a wide range among tissues, and the imaginal discs have lower levels of \textit{E23} transcripts during the late third instar than the larval tissues.

Next, we looked at E23 protein levels in salivary gland tissues that were precisely staged according the status of glue in the gland and the animal’s morphology. Because the salivary gland undergoes two temporally distinct responses to the same hormone (albeit at different titers of 20E), we were curious if E23 expression may also be variable at these times. We compared E23 protein levels at five stages of dissected salivary glands: the mid-third instar (the onset of glue expression), the late third instar wandering stage (prior to glue secretion), the late third instar (during secretion), the WPP stage, and 6 hrs APF. We found a robust and reproducible result in which E23 protein is highest during the late third instar and is virtually undetectable by WPP and 6 hrs APF in the salivary gland (Figure 3.11B).
Figure 3.10: *EcR-DN* has more severe phenotypes than *E23+* when overexpressed with imaginal-disc restricted drivers. Results from ANOVAs comparing pupa-to-adult viability of controls to *EcR-DN* and *E23+* crossed to drivers. Means +/- SEM are provided over three trials. Overall the difference in means was significant, and significant pairwise differences (Bonferroni; α=0.05) are denoted by different letters. (A-F) Drivers that are restricted to the imaginal discs result in pre-metamorphic lethality when crossed to *EcR-DN*, but there are lower percentages of pupa-to-adult lethality when the imaginal-restricted drivers are crossed to *E23+. Drivers: (A) 459.2: wing discs; (B) 30A: third instar imaginal discs; (C) 69B: ectoderm, imaginal discs; (D) 71B: imaginal discs; (E) c701b: eye discs, haltere discs, leg discs, wing discs, medial brain lobe; (F) T80: imaginal discs excluding the eye disc.
Figure 3.11: E23 mRNA and protein are dynamically expressed in larval and imaginal tissues and at different stages of development. In (A), the results of nine RNA-seq experiments for staged tissues from the modENCODE project are shown. Each stage is color-coded with black indicating larval, white indicating WPP, and gray indicating the pupal stage. The amount of E23 mRNA is represented as Reads Per Kilobase of transcript per Million mapped reads (RPKM) (Mortazavi et al., 2008). In (B), three separate western blots of E23 protein expression in the salivary gland are shown, demonstrating that the E23 protein is consistently undetectable at WPP in the salivary gland. W: Wandering; Sec: Secretion; L3: third instar larval; 6h: 6 hrs APF.
3.3.e: High Titers of 20E can Overcome the Block Induced by E23+ Overexpression

Because we were able to vigorously block any 20E response in larval tissues, we wondered whether the block could be overcome by supplying high levels exogenous 20E. While we expected that the block induced by EcR-DN would not be relieved by high titers of 20E, the E23-induced block should be overcome if the hormone flooded the cells and if the mechanism of E23 is to limit cellular exposure to the hormone. At a high-enough titer of 20E, the E23 transporters would be overwhelmed allowing 20E to leak into the cell, and the 20E response would be restored. One unexpected result hinted that this might be possible. Whenever we overexpressed E23+ using the sgGal4 driver, glue production, which was blocked during the last half of the third instar, was sometimes initiated (about 10% of the time) at the end of the instar during the time when the tissue was exposed to the large pre-metamorphic pulse of 20E (Figure 3.12). This new synthesis was cell autonomous because only a few cells within a gland would produce glue at this time.

Because this result could have been the consequence of the driver having diminished expression at that time, we analyzed the protein levels of E23 in the salivary glands of sgGal4>E23+ (Figure 3.12). We found that the protein levels were not greatly decreased by WPP, but they had declined by 6 hrs APF. Thus, it is plausible that the high titer of 20E was able to overwhelm the overexpressed E23 transporters.

In order to further test the idea that high titers of 20E can overcome the negative effect of highly expressed E23 transporters, we created a line of flies that produces mosaic tissue in vivo under the control of a heat-shock induced Flippase Recombinase (Flp-out method). We analyzed the salivary glands of larvae during the third instar and
prepupal stages to see whether varying the levels of $E23^+$ expression in adjacent cells would affect 20E-induced glue expression or secretion. In our experiments, the levels of $E23^+$ expression are correlated with the intensity of the GFP signal because both are expressed as $UAS$-transgenes regulated by the GAL4 transcription factor. Our results suggest that during the mid-third instar, high levels of $E23^+$ expression in individual cells result in no glue production, as shown previously (Figure 3.13G). Whereas, high levels of $E23^+$ expression 6 hrs prior to puparium formation result in glue synthesis in individual cells by the WPP stage (Figure 3.13I). The WPP stage is the improper timing of glue synthesis, indicating that the cell had blocked glue expression during the mid-third instar, but it was activated at the later, improper stage. The large pre-metamorphic pulse of 20E resulted in the activation of glue genes despite the high levels of E23. In summary, the negative regulation of 20E by the ABC transporter E23 can be overcome $in vivo$ when the endogenous levels of 20E are high.
Figure 3.12: Salivary gland glue is synthesized at the improper time when E23+ is ectopically overexpressed. Confocal images of (A, B) control and (C, D) sgGal4>E23+ show that glue is aberrantly synthesized in late third instar larvae and WPP of sgGal4>E23+ in about 10% of dissected salivary glands (C, D) when the 20E titer is high, but glue expression was never observed prior to the late larval stage of development (see Figure 3.8). (E) Western blot to show the levels of E23 protein in sgGal4>E23+. Scale bar in (B) is 25 µm. All confocal images were taken at 630x magnification with 1.5x zoom.
**Figure 3.13**: High endogenous titers of 20E are correlated with the reinitiation of glue expression in cells expressing high levels of E23+. Confocal images of salivary gland cells have glue labeled via glueRED and the nucleus with UAS-GFP.nls. (A-C) Controls of the same genotype as (G-I) were not subjected to heat shock and were imaged at the stages indicated on the left. (D-F) A second set of controls of the same genotype as (G-I) were heat shocked using the same conditions as in (G-I). Images were taken of cells in which mosaicism was not induced as indicated by the lack of GFP. (G-I) The experimental group hsflp/Act<cd2<Gal4; E23+, glueRED; UAS-GFP.nls was heat shocked to induce mosaic tissue. Increased GFP intensity correlates with increased E23+ expression. Yellow dotted lines (G, H) indicate the approximate cell boundary. L: lumen. The scale bar in (A) is 25 µm. All images were taken at the same magnification (630x magnification with 1.5x optical zoom).
3.4: Discussion

The data presented indicate the UAS-E23 reagent (E23+) is an effective and robust tool for negatively regulating 20E signaling during the larval stages. The most compelling evidence supporting this assertion is that the phenotypes of E23+ are virtually identical to UAS-EcR-DN when assayed with any larval tissue-specific driver. Ectopic E23+ overexpression not only results in the same stage of lethality as EcR-DN, but also in strikingly similar phenotypes. Furthermore, we have demonstrated that E23+ can inhibit 20E responses occurring at earlier developmental times and not just at the well-characterized metamorphic period occurring at the end of larval life.

Although EcR-DN already exists as a potent tool for studying 20E physiology, there are key differences between the use of E23+ and EcR-DN for blocking 20E responses. Recall that in order for target genes to be activated, EcR/USP must be bound to 20E. In some cases, this leads to the direct activation of the downstream gene. However, it has been demonstrated that in the wing disc, gene activation occurs through de-repression of EcR/USP when hormone titers are high (Schubiger et al., 2005). In the context of wing discs, once the hormone binds to the receptor, it has been postulated that the receptor is no longer bound and other activators are recruited to the regulatory region to initiate gene expression. Thus, EcR-DN is effective for preventing both responses that require the inhibitory function of the ecdysone receptor and those that require the activational function of the receptor because it cannot be activated or de-repressed. During the large pre-metamorphic pulse that both de-represses and activates genes, EcR-DN blocks all downstream responses. Whereas, E23+ can block the activational function of the ecdysone receptor in larval tissues, but it is not effective at blocking the inhibitory
function of the receptor, as shown by the near wild-type responses when \( E23 \) overexpression is limited to imaginal discs. In future experiments, expressing even higher levels of \( E23^+ \) during times lasting through the prepupal period can be used to block the de-repression of the ecdysone receptor in imaginal discs.

However, \( E23^+ \) has an advantage over \( EcR-DN \) concerning \textit{in vitro} experiments. Whereas, \( EcR-DN \) cannot be de-repressed or activated with high levels of exogenous 20E; the block induced by \( E23^+ \) can be overcome by titrating high concentrations of exogenous 20E to restore the 20E response. Using \( E23^+ \) and \( EcR-DN \) in this experimental context will help to tease apart the function of the ecdysone receptor as a repressor versus an activator in tissues that can be cultured.

More importantly, the data reveal that \( E23^+ \) participates in a powerful negative feedback loop that acts directly on the 20E signal. The inverse relationship between the mRNA and protein levels of E23 in the salivary gland at WPP suggests that E23 is regulated by another factor (such as a protease) that actively removes the protein during this stage. In addition, because the \textit{in vivo} block in 20E signaling can be overcome in the salivary gland by high levels of 20E, this suggests the ABC transporter directly acts on the hormone to negatively regulate downstream responses.

Mechanistically, the ABC transporter E23 could negatively regulate 20E in a variety of ways, with the simplest being extruding the steroid hormone from cells. Furthermore, several lines of evidence support this function of the transporter. Foremost, heat shock-driven expression of \( E23 \) inhibits 20E-mediated transcription of \textit{early} genes \( E74 \) and \( E75 \) and a lacZ reporter controlled by \( EcRE \) regulatory response elements (Hock et al., 2000). In addition, ABC transporters homologous to E23 form a group that pump primarily
lipophilic substances like steroid hormones, such as cholesterol and bile (Dean and Allikmets, 1995). Thus, we propose that E23 is a pump that selectively limits 20E by expelling it from cells in an ATP-driven active process. Thereby, the cellular exposure to 20E can rapidly be limited in periods when dramatic changes in gene expression are necessary, such as during metamorphosis, or when a tissue must limit its exposure to 20E. This would be an effective way to control unique genetic responses to the hormone during development and to rapidly diminish the whole-body titers of 20E. Thus, E23 could be central to controlling the timing of physiological events that require very precise levels of 20E. It will be informative to perform ex vivo cultures of salivary glands and titrate the levels of 20E to see whether the block by E23 can be lifted.

A question still arises as to what is the endogenous function of E23. One clue is that it is an early gene in the salivary gland puffing response induced by 20E (Ashburner, 1973), and as our data shows, it is effective at inhibiting tissue-specific physiology in the larval salivary gland and other larval tissues. Thus, it may have an important role in larval tissues that are polytenized or that have a major secretory or metabolic role. Furthermore, it may be instructive to look at how the sequential puffing of the polytene chromosomes is affected by E23+ overexpression, especially since the nuclear growth is robust in salivary gland cells even into adulthood as our data demonstrated.

A major advantage of contemporary science is that high-throughput mRNA sequencing is relatively inexpensive, and large transcriptome data sets are being made publicly available, such as through the modENCODE project (http://www.modencode.org; Graveley et al., 2011). Thus, we were able to observe that E23 mRNA and protein levels are inversely related in larval tissues. This points to a potent negative feedback
loop that limits E23 protein levels within only a few hours, suggesting a role for another factor that is specifically regulating E23 protein levels. It will be important to define the specific timing and tissues of E23 protein expression in future experiments to explore questions such as whether E23 is part of the imaginal disc developmental program.

Although we have focused on larval and pupal stages, the function of E23 in the adult is also important, as E23 consistently arises in genomic studies as one of the most highly expressed genes in the adult ovaries of Drosophila (Chintapalli et al., 2007; Cherbas et al., 2011). Because the steroid hormone 20E is required for the movement of the border cells within the egg chambers of the ovary at a defined time during the maturation of the egg, it is likely that E23 has a role in this event, likely through rapidly limiting 20E (Jang et al., 2009). A common theme seems to be that E23 is linked to the timing of 20E-regulated events.

Furthermore, recent work has pointed to E23 as being part of the circadian rhythm of Drosophila adults (Itoh et al., 2011). Because of the interplay between systemic-20E increases and growth and developmental timing, it follows that E23 has a role in controlling the circadian behavior through negative regulation of 20E. Perhaps E23 is necessary in the metabolic extrusion of 20E, so that it can be shuttled to a digestive organ and excreted, thereby allowing for periods of low systemic active hormone. Thus, the future is ripe with many possibilities for exploring the function of endogenous E23, perhaps with the most important being determining the specificity of the transporter for 20E as its substrate.
CHAPTER 4

A TRANSCRIPTOME ANALYSIS OF THE PREPUPAL SALIVARY GLAND:
THE ASHBURNER MODEL REVISTED

4.1: Introduction

The steroid hormone 20-hydroxyecdysone (20E) controls many global and tissue specific physiological and developmental processes in *Drosophila melanogaster*. For example, at metamorphosis, 20E initiates the timed destruction of larval tissues and generation of adult structures from precursor tissues (reviewed in Riddiford, 1993). We are interested in the genetic circuits that control these processes, and specifically, how a systemic steroid hormone signal can result in such divergent responses as development, reproduction, homeostasis, and even cell death.

One key to unraveling these processes is to identify all the genes that are regulated by 20E in a specific process or during a specific developmental stage. We used the larval salivary gland of *Drosophila* for this purpose because there are several temporally specific responses to the hormone that can be dissected genetically including the synthesis and secretion of glycoprotein glue (Zhimulev and Kolesnikov, 1975). In addition, the salivary gland is a powerful model system because the analysis of the 20E-regulated genetic cascade was first characterized in this tissue due to its ease of culturing and the presence of giant polytenized chromosomes that can be monitored for puffs as 20E-responsive genes are activated (reviewed in Zhimulev, 2004).

The Ashburner model was formulated from the 20E-induced puffing cascade of salivary gland polytene chromosomes (Ashburner et al., 1974). In this model, there are four sequential groups of puffs that are induced in response to the hormone along with
stage-specific puffs (see Table 4.1 and Thummel, 2002 for a review). The model was based on the timing of hundreds of puffs enlarging and regressing. It proposes that a small set of “early” puffs encode regulatory proteins that are capable of activating a larger set of “late” puff genes and repressing their own transcription (Ashburner, 1972). Thus, the early gene products were proposed to initiate a negative feedback loop causing their own regression. It was suggested that the functional ecdysone receptor consisting of a heterodimer EcR (Ecdysone Receptor) and USP (ultraspiracle) is necessary for the activation of the early genes and the early-late genes, but EcR/USP represses the late genes. Hence, the late genes require the protein products of the early genes for their activation (Ashburner, 1974). It was also noted that rising levels of 20E during the pre-metamorphic response in the salivary gland represses another set of puffs called the “intermolt” puffs encoding the salivary glue genes (Ashburner et al., 1974).

The Ashburner model has been tested extensively, but until the recent development of rapid next generation sequencing technology, scrutiny of the model has been labor-intensive (see Huet et al., 1993, 1995; Richards et al., 1999) or has relied on microarray analysis, a technique that is not as sensitive as RNA-seq (see Li and White, 2003 and Marioni et al., 2008). Thus, our methodology was simple. We sequenced the transcriptome from mid-third instar larval (mid-L3) salivary glands and white prepupal (WPP) salivary glands using the Illumina-based sequencing platform, and we analyzed and compared the levels of transcripts identified in the puffing cascade. Because there have been numerous studies that track the changes in mRNA levels of the 20E-induced puff genes, we compared our sequencing results to published studies (Huet et al., 1993, 1995; Richards et al., 1999; Li and White, 2003; Graveley et al., 2011).
Based on the Ashburner model, we made the following predictions: 1) If the *intermolt* genes are repressed by high levels of 20E, their expression should decrease from mid-L3 to WPP. 2) The *early* genes and *early-late* genes should have a moderate number of transcripts at WPP (due to the negative feedback), but overall their expression should increase from mid-L3 to WPP (because 20E levels increase at WPP, and because they are directly regulated by EcR/USP). 3) Lastly, the *late* genes should increase in expression from mid-L3 to WPP, and their transcript levels should be low during mid-L3 (because they require the early gene products for their expression).

Although the Ashburner model helps to define the global response to 20E, the specific molecular mechanisms defining tissue-specific physiological responses are poorly understood. As a second goal of our analysis, we wanted to help define the steroid-triggered mechanism of glue secretion, a tissue-specific pre-metamorphic response in the salivary gland. Using our data set, we can look for genes that are not traditionally part of the puffing cascade, but their transcripts are either greatly enriched or depleted in the salivary gland during the time preceding glue secretion.

Previous studies have demonstrated that exogenous 20E is required to induce glue secretion in salivary glands *ex vivo*, and furthermore, the salivary gland must be exposed to the hormone for at least 2-3 hours for secretion to occur (Zhimulev and Kolesnikov, 1975; Boyd and Ashburner, 1977). In addition, if the protein synthesis inhibitor cyclohexamide is added to the *ex vivo* culture, secretion is completely blocked (Boyd and Ashburner, 1977). Thus, these results suggest that glue secretion is a secondary response to 20E and requires early gene products (presumably from the Ashburner cascade) to occur.
Interestingly, one of the early genes in the Ashburner cascade did not encode a transcriptional regulator but instead was a direct effector of salivary gland glue secretion. This key early gene is E63F-1 (E63-1), which is an EF-hand containing Ca$^{2+}$-binding protein that mediates glue secretion when Ca$^{2+}$ levels are elevated (Andres and Thummel, 1995; Biyasheva et al., 2001). Unlike the other early gene products, E63-1 provides a direct link between 20E signaling and the tissue-specific physiology of the salivary gland. However, null mutations in E63-1 do not have severely blocked glue secretion (Biyasheva et al., 2001), indicating that a redundant protein may be required for secretion to occur. Using our data set, we analyzed the mRNA levels of all of the Ca$^{2+}$-binding proteins in the salivary gland coincident with glue secretion, paying special attention to those with EF-hand domains. Previous work has demonstrated that E63-1 is likely functioning as a myosin light chain to secrete glue granules (Costantino, 2010). Thus, we predict that one or more of the EF-hand proteins from our analysis is also involved in glue secretion, and we predict that the most highly induced genes encoding EF-hand proteins at the time of granule delivery are the best candidates for this redundant role of E63-1. In future functional studies, we will test each of the candidates for glue secretion phenotypes, providing further evidence of a direct link between steroid hormone signaling and the distinct physiological response to the steroid in the salivary gland during the large pre-metamorphic pulse.
4.2: Materials and Methods

4.2.a: Drosophila Stocks and Culture

All stocks were maintained on standard cornmeal molasses media at room temperature (~22°C). The salivary glands used in the study were derived from the progeny of a cross between glueRED = w¹¹¹⁸, P[w+, Sgs3-DsRed] (described in Costantino et al., 2008) and w¹¹¹⁸ obtained from the Bloomington Drosophila Stock Center (BDSC, Bloomington, IN, USA).

4.2.b: Staging of Drosophila Larvae

Animals were precisely staged before isolating RNA from salivary glands. WPP were selected by morphological criteria as described in Bodenstein, 1994. To accurately stage mid-third instar larvae [10-18 hours (hrs) before puparium formation (BPF)], Drosophila cultures were raised on blue food (Andres and Thummel, 1994), and larvae with blue guts typically ranging from 10 to 18 hrs BPF were selected. We also used the glueRED marker to more precisely select mid-third instar larvae (mid-L3) based on their glue phenotype—animals in which only the posterior section of their salivary glands was filled with red glue. Such animals are more accurately staged as 20 hrs BPF.

4.2.c: RNA Isolation and Sequencing

Total mRNA was isolated via an RNeasy Micro Kit (Qiagen Sciences LLC, Germantown, MD, USA) from 25-50 pairs of salivary glands from either mid-L3 or WPP. Approximately 100 ng/μl total mRNA for each sample was sent to the Microarray Core Facility at the University of Utah for library preparation, bridge amplification, and
multiplex sequencing using an Illumina HiSeq 2000 analyzer (Illumina Inc., San Diego, CA, USA). The quality of the mRNA was verified prior to sequencing by a BioAnalyzer RNA 6000 (Agilent Technologies, Santa Clara, CA, USA).

4.2.d: Gene Analysis and Statistics

Single-end, 50 bp reads were aligned to the *Drosophila melanogaster* genome (Release 5.41, ftp://ftp.flybase.net/releases/current/dmel_r5.41/gff/). Reads that passed default parameters of the Illumina quality filter were mapped with Bowtie (Langmead et al., 2009); http://bowtie.cbcb.umd.edu). The reference sequence used to build an index for mapping was dm3 from the UCSC Genome Browser (Dreszer et al., 2011; http://genome.ucsc.edu), and the reference index was created using the Bowtie-build function with default parameters.

The read counts were normalized between samples using methodology described in Oshlack et al., 2010 and used in Marioni et al., 2008 based on library size. Briefly, the number of reads for each gene was normalized to the mRNA pool with the lowest percent of aligned reads (mid-L3). The aligned reads generated in the other sample (WPP) was multiplied by the product of the number of aligned reads from mid-L3 divided by the number of aligned reads in the WPP sample. Therefore, the raw reads for each gene in the WPP sample were multiplied by a factor of 1.35 to obtain the normalized read count. The fold change in reads from mid-L3 to WPP was log base 2 transformed before analysis of gene expression. To determine the genes with the highest expression changes in WPP versus mid-L3, transcript levels and log base 2 values were directly compared (see Supplemental Methods in Li and White, 2003). Transcripts were determined to be
enriched or depleted if the absolute value of the log base 2 transformation was greater than 1.5.

4.3: Results

4.3.a: RNA-Seq Results for Mid-L3 and WPP

Single-end, 50 bp-read sequencing resulted in 30,323,734 reads on average from each of our samples. The high quality, aligned reads for mid-L3 totaled 74.3%, and for WPP, 87.3%. After the reads were aligned, normalized, and log base 2 transformed, we analyzed our data sets for mRNA enrichment or depletion, specifically looking at the Ashburner puffing cascade genes. In Table 4.1, all of the puff genes and 20E-inducible genes that we used to test the Ashburner model are listed.

The strategy for the analysis is shown in the flow diagram (Figure 4.1). Briefly, there are two goals of this research. The first is to identify whether the predictions of the Ashburner model are upheld by our data set. The second is to identify candidates for the gene encoding the Ca\(^{2+}\)-binding protein that works in concert with the cascade gene \textit{E63-1} to carry out glue secretion (Andres and Thummel, 1995; Biyasheva et al., 2001), the primary physiological function of the salivary gland at WPP. These genes will be experimentally tested (via RNAi analysis, for example) to determine whether they are required for glue secretion.
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<td>rpr</td>
<td>Cell Death Induction</td>
<td>Jiang et al., 1997</td>
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<td>HR38</td>
<td>Nuclear Receptor</td>
<td>Baker et al., 2003</td>
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Figure 4.1: The strategy for RNA-seq analysis of the Ashburner model. (A) For the transcriptome analysis, we isolated RNA from glueRED salivary glands of animals at two stages: mid-L3 when glue is first produced but prior to the pre-metamorphic pulse of 20E, and WPP when the animal has been exposed to the pre-metamorphic pulse and glue has been secreted. We used the Illumina platform to sequence the salivary gland transcriptome. Between 12-25% of low quality reads were discarded, and the rest were aligned to the Drosophila melanogaster genome. (B) Bowtie was used to align the reads, and the reads were normalized across both groups as described in the methods. (C) The log 2 fold change in the reads at WPP for each of the genes in the Ashburner cascade was compared to published data both to validate our data set and to test the major tenets of the Ashburner model. (D) Finally, the entire data set was filtered to select all the genes encoding Ca$^{2+}$-binding proteins, and these candidates were sorted based on log base 2 fold changes in expression to find the most highly induced and repressed genes at WPP. In future experiments, individual gene candidates will be tested.
4.3.b: Gene Expression Analysis of the Ashburner Puff Genes

As a first step, we wanted to validate our results and analyze the Ashburner model with this very precise data set. Because there have been numerous studies of gene expression changes occurring in the salivary gland during metamorphosis and due to 20E signaling, we sought to compare our results to these published data. The key genes we explored were those found in the puffing cascade of the salivary glands. As previously mentioned, these genes fall into four groups: the intermolt puffs, the early puffs, the early-late puffs, and the late puffs (see Table 4.1; Ashburner et al., 1974). We compared the gene expression changes from mid-L3 to WPP in our data set for each of these groups of genes to an RNA-seq analysis of salivary glands that is publicly available through the modENCODE consortium (Graveley et al., 2011; www.modencode.org) and to a microarray analysis of salivary gland gene expression changes during metamorphosis (Li and White, 2003). We also used a very precise RT-PCR analysis of the available cascade genes to compare to our data set (Huet et al., 1993, 1995; Richards et al., 1999).

We chose the modENCODE data set because it was generated using the same Illumina sequencing methods that we used with one key difference; the third instar stage they chose is approximately 12 hours later than the mid-L3 stage that we used (Figure 4.2A). We selected these stages based on our research goal of finding genes that have the greatest differential expression at WPP as compared to a time when 20E levels are low. Thus, the Li and White microarray data was also used for comparison because they included salivary glands that were similarly staged to those in our study (Li and White, 2003). As reflected by the results, the microarray technique is not as sensitive as RNA-seq (see Figures 4.3-4.6 and Marioni et al., 2008). In the following sections, we
summarize the expression profiles for each of the four groups of genes and compare them to our results. The approximate timing and overview of the Ashburner model are shown in Figure 4.2B. In addition, the timing of the intermolt gene expression is depicted in Figure 4.2A.

4.3.b.i: Intermolt Puffs

The intermolt puffs are visible before the early puffs, but they regress quickly during the pre-metamorphic pulse when the 20E titer rises (Ashburner and Richards, 1976). As shown in Figure 4.3A and B, the trend among the intermolt genes (which are represented by the glue genes) is a large decrease in gene expression from the mid-L3 to WPP. All four studies had the same outcome with these genes showing a drastic reduction in expression by WPP. In the RT-PCR experiment, only the data for Sgs3 was available, but the results were consistent in that expression is greatly reduced by WPP (Figure 4.3B). We also included with this group EcR, which encodes one partner of the functional ecdysone receptor; it showed a decrease in all studies by WPP. Thus, these results are consistent with the idea that the glue genes are deactivated coincident with the large pre-metamorphic pulse of 20E, suggesting that high titers of 20E block further expression of the intermolt genes. These results also suggest that EcR may be downregulated in order to allow for late gene activation.

4.3.b.ii: Early Puffs and Early-Late Puffs

The early, early-late, and late puffs are induced in succession in the salivary glands in vivo in response to the pre-metamorphic pulse of 20E (Figure 4.2B). The early puffs are
induced very rapidly when polytene chromosomes are exposed to 20E in vitro
(Ashburner, 1972); whereas, the early-late puffs are induced when the early puffs have reached their maximal size (Ashburner, 1972; Ashburner et al., 1974). The changes in gene expression from mid-L3 to WPP corresponded very well for the early-late genes with the other studies (Figure 4.4B). In general, the early-late genes increased expression by WPP with only a moderate increase in reads, which supports the Ashburner model.

In addition according to the Ashburner model, early gene expression should only increase moderately because early genes are predicted to repress their own expression. For the most part, our data set is consistent with this prediction. E23, E63-1, and E75 increase in expression by WPP; whereas, BR-C and E74 appear to be static with slight decreases. It should be noted that BR-C and E74 have isoform-specific gene expression at various times between the mid-third instar and WPP in the salivary gland (Huet et al., 1993, 1995), and this is not discernable from our analysis.

Also, there were some differences between our results and modENCODE for the early genes (Figure 4.4A). The discrepancy lies in the changes in gene expression for E63-1, E74, and BR-C; whereas, our results for E23 and E75 were consistent with the other studies. However, our results for E63-1 are consistent with published molecular data in other studies; whereas, the modENCODE data are not (see Andres and Thummel, 1995). Nevertheless, the early gene expression in our study corresponded well with the Li and White study, which used identical staging to ours (Figure 4.4B). Both the early and early-late gene expression also corresponded well with the RT-PCR experiments of salivary glands (Figure 4.4C).
Figure 4.2: The Ashburner model tested by four different approaches. In order to validate our results, we compared gene expression changes in salivary glands at WPP among four salivary gland data sets: modENCODE (Graveley et al., 2011; downloaded from flybase.org), a microarray analysis (Li and White, 2003), an RT-PCR analysis (Huet et al., 1993, 1995; Richards et al., 1999), and our Illumina-based RNA-seq study. (A) The timing of selection for the isolation of salivary glands is depicted for each study in reference to the time from the third instar ecdysis. This image can be used as reference for the subsequent figures. In addition, the approximate timing of intermolt gene expression at 18-24 hrs BPF is shown. The 20E titers in the third instar are redrawn from Warren et al., 2006. (B) A simplified flow diagram of the Ashburner model is depicted for reference, and the timing of the puff cascade is shown. The puff cascade occurs in the late salivary gland during the large pre-metamorphic pulse of 20E.
Figure 4.3: RNA-seq results for the *intermolt* genes. (A) The *intermolt* genes show an identical trend of mRNA depletion at WPP among the studies. The reads (reads at mid-L3, reads at WPP) for our study and modENCODE are given next to each bar on the histogram. The log base 2 fold change in gene expression is plotted. (B) The results of an RT-PCR experiment for *Sgs3* are shown (Huet et al., 1993). The times correspond to the time after third instar ecdysis as in Figure 4.2A. The loading control *rp49* is also shown. L3: third instar.
Figure 4.4: RNA-seq results for the *early* and *early-late* genes. We compared gene expression changes at WPP among the four data sets for the *early* genes (A, C) and *early-late* genes (B, C) in the 20E puffing hierarchy. Our study corresponds to the microarray study, but there are some differences between our study and modENCODE (A) due to the timing of selection of salivary glands (see Figure 4.2A). Data points for E63-1 and E75 were not available from the published Li and White microarray data set in (A). (B) The *early-late* genes are all induced by WPP. In (C), the RT-PCR results for the *early* genes E74B and the Z3 isoform of BR-C (Huet et al., 1993) are shown along with the *early-late* genes E78B and DHR3 (Richards et al., 1999). The *early-late* genes show an overall increase in gene expression by WPP. Whereas, the *early* genes tend to diminish in expression by WPP. In (A) and (B) the reads (reads mid-L3, reads WPP) are listed next to the bars on the histogram. The log base 2 fold change in gene expression is plotted.
4.3.b.iii: Late Puffs

The late puffs are induced several hours after the early puffs and require protein expression and the withdrawal of the ecdysone receptor to reach their maximal size (Ashburner, 1974). The gene expression changes corresponded well among the three studies with large increases in the mRNA levels of the late genes at WPP (Figure 4.5A). The one exception is $L71-7$ ($Ee$) because this gene, although located in the 71E puff, encodes a glue gene with an expression profile similar to the intermolt genes (Restifo and Guild, 1986). These results also correspond with the Ashburner model when taken together with the early gene expression presented in Figure 4.4A. By WPP, the early genes would have already begun repressing their own expression and activating the late genes. The adjusted reads in mid-L3 and WPP are shown for the two RNA-seq experiments, and one difference between the studies is the larger number of adjusted reads in the modENCODE study than in our study for all the late genes. Again, at the later timing of the modENCODE study for the third instar, the late gene transcripts will have already begun to accumulate, so this could be reflected by this large number. In addition, the timing of the WPP stage in each study might be slightly different, indicating how rapidly mRNA transcripts accumulate or turnover during the pre-metamorphic response to 20E.

4.3.b.iv: Stage Specific Puffs and 20E Inducible Genes

Finally, we validated our data by looking at two stage-specific puffs ($E93$ and $\beta Ftz-F1$) and a set of known 20E-inducible genes (see Table 4.1 for References). $\beta Ftz-F1$ is highly expressed when the hormone titer is low (Woodard et al., 1994;
Broadus et al., 1999), so in the three studies the expression of this gene should be low or absent, which is manifested by the low number of total reads (Figure 4.6A). $E93$ is part of the second wave of early gene expression that occurs in response to the large pulse of 20E approximately 12 hours after puparium formation, and $E93$ is required for salivary gland histolysis (Lee et al., 2000a). The expression of $E93$ increases during the WPP period in our study even though the transcript levels are relatively low compared to the late genes (Figure 4.6A). Overall, the trend was an increase in gene expression at WPP for the 20E-inducible genes.

Thus, for the early-late, intermolt, and late genes, there was a clear pattern of gene expression from the third instar to WPP that correlated well among all studies. The early genes showed a slightly inconsistent pattern when comparing our study to modENCODE, but in general the reads were consistently lower for the early genes than the early-late and late genes at WPP, suggesting that we isolated WPP salivary glands at a time when the early-late and late genes are undergoing heightened expression; whereas, the early gene expression is diminished.
Figure 4.5: RNA-seq results for the late puff genes. (A) We compared gene expression changes at WPP among three data sets for the late genes in the 20E hierarchy. The general trend is an increase in gene expression for the late genes at WPP. *E71-7 (Ee)* is an exception because it was found to encode a glue gene with more similarities to the intermolt puff genes (Restifo and Guild, 1986). The adjusted reads (reads mid-L3, reads WPP) for our study and modENCODE are given next to each bar on the histogram. The log base 2 fold change in gene expression is plotted. Below, an outline of the puff cascade is provided for reference.
Figure 4.6: RNA-seq results for 20E primary response genes, *E93*, and *ftz-F1*. The log base 2 fold change in gene expression at WPP is plotted for genes directly induced by 20E, i.e., primary response genes and for the stage specific puffs containing *E93* and *ftz-F1*. Data points for some genes were not available from the published Li and White microarray data set. Overall, these genes increased in expression at WPP. However, as indicated by the adjusted reads (reads mid-L3, reads WPP), the reads for some of the genes were minimal at this time, indicating that these genes may not be expressed at high levels in the salivary gland, e.g., *ImpL1*. 
4.3.c: Transcriptome Analysis of Genes Encoding Ca\(^{2+}\)-binding Proteins in the Salivary Gland

In addition to analyzing the known genes in the Ashburner model, our transcriptome data set is informative for discovering salivary gland specific mediators of secretion and other physiological events specific to the salivary gland. The product of \(E63-1\) (the *early* gene encoding a Ca\(^{2+}\)-binding protein) is predicted to have a redundant role with another EF-hand, Ca\(^{2+}\)-binding protein in the process of salivary gland glue secretion. Using the RNA-seq data, we analyzed the expression changes of all genes encoding Ca\(^{2+}\)-binding proteins from mid-L3 to WPP (Figure 4.7). In order to find all of the genes with predicted Ca\(^{2+}\)-binding domains, we conducted a systematic search using GO-terms (Ashburner et al., 2000) and InterPro domains (Hunter et al., 2011). We predict that the expression of the Ca\(^{2+}\)-binding protein(s) involved in secretion will be considerably elevated because of the immense amount of glue granules that will be exocytosed by WPP.

Therefore, we separated the genes into groups based upon their total number of reads (Reads mid-L3 + Reads WPP). A total of 20 genes had greater than 10,000 reads in the salivary gland (Figure 4.7A), and 12 of these have EF-hand domains. There are 72 genes having between 1,000 and 10,000 reads, one of which is \(E63-1\), and 38 of these have EF-hand domains (Figure 4.7B). There are 75 genes having between 100 and 1000 reads (Figure 4.7C), and 40 genes having less than 100 reads in the salivary gland (Figure 4.7D). Finally, a number of genes encoding Ca\(^{2+}\)-binding proteins were not detected in our experiment (Figure 4.7E).
Figure 4.7: Changes in the expression of genes encoding Ca\(^{2+}\)-binding proteins at WPP. Genes with the molecular function: [Ca\(^{2+}\)-ion binding (GO:0005509)] and/ the InterPro domains [IQ motif, EF-hand binding site (IPR000048)] and/ [EF-hand like (IPR011992)] are represented by their log base 2 fold change in gene expression at WPP. (A) Twenty genes have over 10,000 reads in the salivary gland at WPP. (B) Genes having between 1,000 and 10,000 reads. (C) Genes having between 100 and 1,000 reads. (D) Genes having less than 100 reads in the salivary gland. (E) Genes with no detectable reads at mid-L3, WPP, or both. Genes encoding Ca\(^{2+}\)-binding proteins with an EF-hand like domain or binding site are indicated by an asterisk.
4.3.d: Transcriptome Analysis of Genes Encoding Immunity-Related Proteins in the Salivary Gland

The salivary gland secretes glycoprotein glue that, once expectorated, affixes a prepupa to a surface during metamorphosis. Even though this is the major known function of the salivary gland, it has also been postulated that antimicrobial peptides and other immune related proteins are loaded into the prepupal salivary gland in order to prevent bacterial and fungal infections during metamorphosis (Wright et al., 1996). Thus, we used flybase.org (Crosby et al., 2007) to search for immunity related genes and defense genes using the GO term: Defense response (Ashburner et al., 2000) and InterPro domain: Immunoglobulin-like fold (Hunter et al., 2011). We recovered 67 genes encoding proteins with an immunoglobulin-like fold, and 79 genes involved in defense responses that are expressed in the salivary gland. Then, we plotted their log base 2 fold change from our transcriptome analysis. We excluded genes that were not detected in the salivary gland at mid-L3 and WPP. While only 38 of these genes decrease in expression by WPP, 105 of them amplify their expression, indicating that one or more of these genes may be important for immune function during metamorphosis.
Figure 4.8: RNA-seq results for genes encoding immunity-related proteins. Genes with the molecular function: [GO:0006952: Defense response] or the InterPro domain: [IPR007110: Immunoglobulin-like fold] are represented by their log base 2 fold change in gene expression at WPP. (A) Genes with immunity-related functions that have a log base 2 fold change of 1.5 or higher in the salivary gland are represented. (B) Genes involved in defense response that have a log base 2 fold change of 1.5 or higher are shown. (C, D) Genes involved immunity and defense response that have a log base 2 fold change of -1.5 or lower are shown. (E, F) Genes with an absolute value of a log base 2 fold change of less than 1.5 are shown. These are genes that did not experience a large change in gene expression between L3 and WPP. The genes with no detectable reads in the salivary gland are not shown.
4.4: Discussion

Our next-generation transcriptome analysis comparing mid-L3 to WPP salivary glands allowed us to apply a very precise measure of mRNA transcript abundance to a widely tested model of steroid hormone signaling called the Ashburner model. Several predictions were made concerning the levels and changes in mRNA expression for the various groups of genes in the puffing hierarchy of the polytene chromosomes in the salivary glands at the two times we isolated. These predictions, if confirmed, would support the Ashburner model. In sum, we found that the expression profiles of every gene in the Ashburner puffing cascade corresponded to previously published data. This is important for several reasons. The first is that it strengthens and validates our results even in the absence of biological replicates. The second is that it provides additional support for the Ashburner model.

Nonetheless, several inconsistencies must be addressed. First, neither the mid-L3 nor WPP data set produced any reads that aligned to one of the *intermolt* puff genes *Sgs4*. One explanation could be that the reads for this gene were of low quality and discarded prior to the Bowtie alignment algorithm. For this reason, a second analysis of the data using less restrictive settings and a biological replicate will be useful. Second, the reads for many of the puff genes at WPP did not correspond well with the modENCODE RNA-seq data. Because we are comparing a snapshot of mRNA abundance at a time period that is 12 hours earlier than the time period in the modENCODE study, rapid changes in gene expression could account for the discrepancies in reads at mid-L3. The WPP stage lasts about 2 hours, so it is striking that the reads could be as much as an order of magnitude different in such a short time. Another possibility is that many of the reads
were not aligned and were discarded. Though, for the WPP sample, only 12.8% of the reads were of low quality, making this a remote possibility. We would also like to expand our differential expression analysis by looking at splice variants. This will help to clarify the reads and the changes in gene expression since many of the genes have isoform-specific expression at differing times in the salivary gland.

The correspondence between our results, previously published data, and the Ashburner model surpasses these minor inconsistencies. Foremost, our data support the idea that while one battery of genes (e.g., the intermolt puff genes) are downregulated during the high pre-metamorphic titer of 20E, other groups of genes (e.g., the late genes) are upregulated. These results support the Ashburner model, asserting that the intermolt genes are repressed by rising levels of 20E; whereas, there is a lag before the late genes are able to be expressed.

As a second goal of our analysis, we looked for enriched and depleted genes in order to compile a list of candidates potentially involved in salivary gland glue secretion or immunity and defense. We found several hundred interesting candidates that may encode the protein that is redundant with E63-1 or may be enriched in the salivary gland to provide immune defense during metamorphosis. Ultimately, each gene will have to be experimentally confirmed as being required for secretion or immunity via functional experiments. Currently, a systematic study using RNA interference is underway to determine if any of the genes encoding the Ca$^{2+}$-binding proteins are required for glue secretion.
5.1: Introduction

Cellular machines operate on two levels. On one level, constitutively active gene expression invokes the minimal activity required to keep the cell alive. On another level, specialized gene activity determines the unique physiology of a group of cells. Interestingly, endocrine signals can initiate specialized and often contradictory responses in different groups of cells, or tissues. For example, in humans parathyroid hormone (PTH) causes the kidney to reabsorb $\text{Ca}^{2+}$, but it triggers bone to release $\text{Ca}^{2+}$ into the bloodstream. As such, we are interested in how systemic steroid hormone signals direct one group of cells to behave in a particular way and another group of cells to behave differently. Specifically, we want to understand the mechanism of steroid hormone signaling that allows distinct responses to occur.

The larval salivary gland of *Drosophila melanogaster* is uniquely fitted for studying this question because it responds in three distinct and easily observable ways to three separate systemic pulses of the steroid hormone signal, 20-hydroxyecdysone (20E), namely: glue production, glue secretion, and gland histolysis (see Chapter 1; Zhimulev and Kolesnikov, 1975; Yin and Thummel, 2005). To ascertain how this occurs at the molecular level, one approach is to block 20E signaling in the salivary gland, and to find which genes are not induced or repressed. These genes can then be tested individually to see if they are required for the specific salivary gland physiology at the time being tested. Fortunately, decades of research have gone into identifying the battery of genes regulated
by 20E in the salivary gland (see Chapter 4), but large genomic studies predict 20E regulates many more genes than what are currently known (for example, see Beckstead et al., 2005).

In addition, in the salivary gland, the known 20E-regulated genes have been studied mostly in the context of their roles in salivary gland histolysis and the control of the sequential puffing activity of polytene chromosomes (reviewed in Thummel, 2002 and Zhimulev et al., 2004). However, 20E acts at an earlier time in the salivary gland to initiate a totally distinct physiological response from the cell death of the gland. In effect, a small increased titer of 20E during the mid-third instar results in the production of a glycoprotein glue mixture in the salivary gland that is later secreted in response to the larger pre-metamorphic 20E pulse. However, the mid-third instar pulse of 20E has been problematic to measure due to the difficulty of precisely synchronizing larvae at a specific developmental stage during the third instar. Yet the existence of the mid-third instar 20E pulse has been supported by developmental studies of gene expression because there is a “transition” that occurs in the mid-third instar that is marked by rapid, coordinate changes in transcription (see Andres et al., 1993).

Thus, in order to look at global transcriptional changes during the mid-third instar, we took a next generation sequencing approach of the transcriptome of the larval salivary gland at two distinct times, the mid-third instar (mid-L3) and the white prepupal (WPP) developmental stage. The crucial component of our analysis was that we were able to very precisely stage mid-L3 larvae because we used an endogenously-tagged glue protein under its own regulatory control (glueRED) to synchronize animals based on the status of the glue in their salivary glands. In order to block 20E signaling during mid-L3, we
overexpressed a gene encoding the ABC transporter, *E23* (Hock et al., 2000), using a salivary gland specific driver that is expressed during all larval stages of *Drosophila* development (Costantino et al., 2008). Ectopic overexpression of *E23* negatively regulates all three 20E responses in the larval salivary gland (see Chapter 3). We sequenced the transcriptome from mid-third instar larval salivary glands with blocked 20E signaling and from mid-L3 and WPP controls. Therefore, we were able to compare the differential gene expression among the three groups in an effort to uncover novel 20E-regulated gene candidates in the salivary gland. We discovered over 100 genes that are strong candidates for 20E-regulated expression during the mid-third instar. Among these candidates are genes that logically would be necessary for the physiological responses induced by 20E, including secretory products and the secretory apparatus. In addition, genes that have already been characterized as 20E-regulated were identified by our approach, further validating our methodology.

With this set of genes on hand, we hope to experimentally determine their involvement in distinct salivary gland physiological processes and to confirm their regulation by 20E. Using this data and previously published results, we will find the key differences initiated by 20E during the mid-third instar, which will help to account for the divergent temporal responses to the hormone in the salivary gland and will help us to understand how a systemic signal can be so versatile.
5.2: Materials and Methods

5.2.a: Drosophila Stocks and Culture

All stocks were maintained on standard cornmeal molasses media at room temperature. The control genotype (sgGal4, glueRED/+ ) were the progeny of the cross between w^{118}; P[w^{+}, sgGal4], P[ w^{+}, glueRED] (described in Costantino et al., 2008) and w^{118} (Bloomington Drosophila Stock Center). The experimental genotype (sgGal4, glueRED/UAS-E23) was produced from the cross between sgGal4, glueRED and P[ w^{+}, UAS-E23]2 (a generous gift from Dan Garza, and hereafter referred to as E23+). In summary, three groups will be compared in this analysis: mid-L3/control, WPP/control, and mid-L3/E23+.

5.2.b: Staging of Drosophila Larvae

Animals were precisely staged before isolating RNA from salivary glands. White prepupae were selected by morphological criteria as described in Bodenstein (1994). To accurately stage mid-third instar larvae (18-24 h before puparium formation), animals were raised on blue food (Andres and Thummel, 1994), and larvae with blue guts ranging from 10-18 hrs before puparium formation (BPF) were selected. We also used the glueRED marker to select mid-third instar larvae (mid-L3) in the control genotype (progeny of sgGal4, glueRED/+ ). For mid-L3, the salivary gland cells are partially filled with red glue indicating a more precise developmental stage of 20 hours prior to puparium formation.
5.2.c: RNA Isolation and Sequencing

Total mRNA was isolated via the RNeasy Micro Kit (Qiagen) from 25-50 pairs of salivary glands from mid-L3/control, WPP/control, and mid-L3/E23+. Approximately 100 ng/µl total mRNA for each sample was sent to the Microarray Core Facility at the University of Utah for cDNA conversion, library preparation, bridge amplification, and multiplex sequencing using a HiSeq 2000 analyzer (Illumina Inc.). The quality of the mRNA was verified beforehand by a BioAnalyzer RNA 6000 (Agilent Technologies).

5.2.d: Gene Analysis and Statistics

Single-end, 50 bp reads were aligned to the *Drosophila melanogaster* genome (Release 5.41, ftp://ftp.flybase.net/releases/current/dmel_r5.41/gff/). Reads that passed default parameters of the Illumina quality filter were mapped with Bowtie (Langmead et al., 2009; http://bowtie.cbcb.umd.edu). The reference sequence used to build an index for mapping was dm3 from the UCSC Genome Browser (Dreszer et al., 2011; http://genome.ucsc.edu), and the reference index was created using the Bowtie-build function with default parameters.

The read counts were normalized using methodology described Oshlack et al., 2010 as normalization between samples based on library size. Briefly, the number of reads for each gene was normalized to the mRNA pool with the lowest percent of aligned reads (in this case, mid-L3/control). The aligned reads generated in the other samples (mid-L3/E23+ and WPP/control) were multiplied by the product of the number of aligned reads from mid-L3/control divided by the number of aligned reads in the second sample (mid-L3/E23+ or WPP/control). Therefore, the raw reads for each gene in the
WPP/control sample were multiplied by a factor of 1.35, and the raw reads for each gene in the mid-L3/E23+ sample were multiplied by a factor of 1.27 to obtain the normalized read count. The fold change from mid-L3/control to mid-L3/E23+ and WPP/control was log base 2 transformed before analysis of gene expression. The reads presented in the tables are the normalized read values.

To determine the genes with the highest expression changes in WPP/control or mid-L3/E23+ versus mid-L3/control, transcript levels and log base 2 values were directly filtered and compared (for example, see the Supplemental Methods in Li and White, 2003). Genes were determined to be enriched or depleted if the absolute value of the log base 2 transformation was greater than 1.5. These criteria were chosen because they successfully identified all 20E-regulated intermolt and late genes in the mid-L3/control versus WPP/control group (see Chapter 4 for intermolt and late gene descriptions). The DAVID Clustering algorithm was used to find groups of significantly enriched genes based on shared gene ontology (Huang et al., 2009a, 2009b). Venn diagrams comparing gene sets were generated with the open access software GeneVenn (Pirooznia et al., 2007).
Figure 5.1: The strategy used for RNA-seq-based discovery of 20E-gene candidates. The high-quality reads were aligned via Bowtie (A) resulting in a read count per gene (B). The reads per isoform were not calculated for this study (C), but this approach will be taken in future experiments using Tophat and DeSeq software. The reads per gene were normalized and compared to published accounts of gene expression for validation of our results (see Chapter 4). Normalized reads were log base 2 transformed when comparing the fold change of gene expression among groups. A list of 20E-regulated gene candidates was generated by comparing the most highly enriched and depleted transcripts of genes from the mid-L3/E23+ group and the mid-L3/control group (D). Finally, we will use the power of *Drosophila* genetics to verify the function of the individual candidates experimentally (E).
5.3: Results

5.3.a: An Overview of the RNA-Seq Results and Strategy for Discovering Novel 20E-Regulated Genes

The high quality, aligned reads for mid-L3/control totaled 74.3%; the white prepupae (WPP/control) sample totaled 87.3%; and for mid-L3/E23+, they were 84.2%. Our strategy for finding 20E-gene candidates is shown in Figure 5.1. The overall idea is that we first validate our results by confirming that mid-third instar genes with known expression profiles are behaving in their predicted way. Specifically, we examined a majority of the genes that have been characterized in the puffing cascade of the polytene chromosomes in the salivary gland because their changes in expression have been well characterized (see Chapter 4).

Next, we filtered our results to find the most highly induced or repressed genes whenever \( E23 \) is ectopically overexpressed. Because ectopic \( E23 \) can negatively regulate 20E signaling in the salivary gland, the genes that are controlled by 20E are predicted to have significant changes in gene expression when \( E23 \) is ectopically expressed at a time when it can block a specific 20E peak. In particular, we predicted that 20E-regulated genes of the mid-third instar would not be induced or repressed when their mid-L3/E23+ expression profiles are compared to their mid-L3/control expression profiles.

As another way of refining the list of gene candidates, we compared the log base 2 values for the mid-L3/control versus the WPP/control to the mid-L3/control versus mid-L3/E23+. Note that mid-L3/E23+ salivary glands were isolated at the identical stage to the mid-L3/control salivary glands. By making this comparison for known 20E-regulated genes in the mid-third instar, we were able to use the pattern of gene expression to
identify genes from our candidate list that had the most similar expression profiles to
genes that are known to be regulated by 20E in the mid-third instar salivary gland. Thus,
we would be able to focus on a subset of the larger list of genes for the initial functional
tests of each gene candidate.

5.3.b: 20E-Regulated Gene Candidates

In order to find novel 20E targets, we filtered the log base 2 fold change in mRNA
levels for mid-L3/control versus mid-L3/E23+. We identified the genes that had the
greatest enrichment or reduction in transcript levels when 20E was overexpressed, as
defined by the absolute value of a log base 2 fold change of 1.5 or greater. We identified
357 transcripts that are repressed and 1280 transcripts that are induced in the
mid-L3/E23+ group versus the mid-L3/control group. Because E23 negatively regulates
20E signaling, the genes with transcripts that are depleted in mid-L3/E23+ are predicted
to be activated normally by 20E; whereas, those that are induced in mid-L3/E23+ are
predicted to be repressed normally by 20E. Furthermore, many known 20E-regulated
genomes were identified including *mmp2* (Llano et al., 2002; Bond et al., 2011), the nuclear
hormone receptors *Hr4* (Lam et al., 1997) and *DHR3 (Hr46)* (Fisk and Thummel, 1995),
and the heat shock genes *Hsp23* (Mestril et al., 1986) and *Hsp27* (Antoniewski et al.,
1996), suggesting that our candidate list contains actual targets of 20E signaling in the
salivary gland.

Using a clustering algorithm, we next looked for significantly enriched groups of
genomes based on gene ontology in order to identify related groups of genes that may be
upregulated or downregulated in the mid-third instar salivary gland (Figure 5.2). The
most highly enriched groups of genes that are repressed by $E23$ overexpression are those that encode proteins that bind sugars, are involved in vesicle transport, and those that function as different types of transporters (Figure 5.2A). In addition, the \textit{glue} genes were found to be repressed when $E23$ is overexpressed. Also, among the repressed are genes encoding proteins involved in salivary gland histolysis. Some examples of the cell death regulators found include \textit{sav}, which is part of the hippo-warts signaling pathway, promoting apoptosis and preventing cell growth (Tapon et al., 2002; Wu et al., 2003), and \textit{santa-maria}, which was found in a genome-wide study of regulators of autophagic cell death (Gorski et al., 2003).

Of the genes that are induced in mid-L3/$E23^+$, the most enriched functions include RNA processing, oxidoreductases, cytochrome P450s, a variety of metabolic activities, immunity and defense related, and salivary gland histolysis (Figure 5.2B). Notably, genes encoding transcriptional activators and repressors are absent in both groups (Figure 5.2A, B).

\textbf{5.3.c: Comparison of 20E Gene Candidates with Other Studies}

Other researchers have used similar methodologies to uncover novel 20E targets including the use of RNAi directed against \textit{EcR} (Beckstead et al., 2005) and culture conditions of Kc167 cells or salivary glands where high levels of exogenous hormone are applied (Gauhar et al., 2009; Gonsalves et al., 2011). Another study looked at the transcriptional response of the mid-third instar salivary gland compared to the whole animal at the same stage (Li and White, 2003). These studies were useful for comparison
with our list of gene candidates (Figure 5.3) because they may direct us to the most viable 20E-regulated candidates from a list of over 1600.

We implemented a Venn analysis of each of the gene lists generated from the four studies and compared them to the gene candidates from our study either having extremely depleted amounts of transcripts in the mid-L3/E23+ experimental group or greatly induced amounts of transcripts in the mid-L3/E23+ group. Transcripts that are depleted in mid-L3/E23+ are predicted to be normally activated by 20E; whereas, those that are enriched in mid-L3/E23+ are predicted to be normally repressed by 20E. The Li and White microarray identified 394 genes with transcripts that showed significant changes in the salivary gland 10-18 hrs BPF. There was an overlap of 58 genes that are predicted to be activated by 20E (Figure 5.3A) and 35 genes that are predicted to be repressed by 20E (Figure 5.3A’) with the Li and White study. Of the 743 genes found to change in expression in the Beckstead et al. study, 39 overlapped with those that are predicted to be activated by 20E (Figure 5.3B), and 89 overlapped with those that are predicted to be repressed by 20E (Figure 5.3B’). From the Gauhar et al. study which used Kc167 cells, of 897 genes, there was a greater overlap (152 genes) with the 20E-candidates that are predicted to be repressed by the hormone (Figure 5.3C’) than those (21 genes) that are activated by 20E (Figure 5.3C). Finally, in Gonsalves et al. that used both Kc167 cells and salivary glands in culture, of 516 genes identified, there was an overlap of 17 genes that are predicted to be activated by 20E (Figure 5.3D) and 47 genes that are predicted to be repressed by 20E (Figure 5.3D’).

Because these overlapping gene sets may be useful in identifying bona fide targets of 20E, we identified the genes that were found in at least two of the studies along with our
study. The genes with transcripts that are extremely reduced in mid-L3/E23+ and are found in more than two studies are listed in Table 5.1 along with their biological functions, and those that are induced in mid-L3/E23+ and found in more than two studies are listed in Table 5.2. Many genes that are known 20E-targets from other studies were found in both groups (genes marked with an asterisk in Tables 5.1 and 5.2). Furthermore, it is interesting to note that many more genes are activated by the withdrawal of 20E (indicated by induced mRNAs in mid-L3/E23+) than by the addition of 20E (indicated by depleted mRNAs in mid-L3/E23+).
Figure 5.2

Depleted in mid-L3/E23+: (genes normally induced by the mid-L3 20E pulse)

357 genes

(A) Genes with depleted mRNA transcripts (log base 2 of -1.5 and below) in mid-L3 when E23 was ectopically overexpressed as compared to mid-L3/control are clustered based on gene ontologies, pathways, and domains. Of 357 genes, 70 clusters were recovered. The 17 most enriched clusters are shown in the chart. The enrichment score is presented for each group.

Figure 5.2: Groups of genes with depleted or enriched transcripts in mid-L3/E23+.
Figure 5.2 (continued): Groups of genes with depleted or enriched transcripts in mid-L3/E23+. (B) Genes with enriched mRNA transcripts (log base 2 of 1.5 and above) in mid-L3/E23+ versus mid-L3/control are clustered. Of 1280 genes, 181 clusters were recovered, and the top 31 enriched clusters are shown. The enrichment score is presented next to each group on the chart.
**Figure 5.3**

A comparison of the 20E-regulated gene candidates versus four similar studies. The Venn diagrams were generated using the 20E candidate gene lists from each of the four studies. (A-D) A comparison of the gene candidates from the studies (both enriched and depleted) that overlapped with the genes candidates from our study with the most depleted transcripts in mid-L3/E23+ is shown. (A’-D’) A comparison is presented for the gene candidates from the studies (both enriched and depleted) that overlapped with genes with the most enriched transcripts in mid-L3/E23+ from our study. The methodology among the studies differed. Our study used mRNA extracted directly from the salivary glands of precisely staged living animals at 20 hrs BPF, WPP, and 20 hrs BPF with E23 overexpressed. (A) Li and White used precisely staged salivary glands at 10-18 hrs BPF and identified enriched or depleted transcripts relative to whole animals (2003). (B) Beckstead et al. used *ex vivo* salivary glands exposed to 5 µM 20E as compared to treatments without 20E or with 20E and cyclohexamide (a protein synthesis inhibitor). They identified 743 genes with significant changes in expression (2005). (C) Gauhar et al. used Kc167 cells treated with 0.5 µM 20E for up to 24 hrs as compared to controls at 0 hrs after 20E treatment (2009). (D) Gonsalves et al. used Kc167 cells and *ex vivo* salivary glands from late third-instar larvae exposed to 0.5 µM 20E up to 4 hrs as compared to untreated controls (2011). The number below the Venn diagrams represents the percentage of genes in our sample that overlapped with the indicated study.
**TABLE 5.1**

Transcripts that are depleted in mid-L3/E23+ and found in more than one study

<table>
<thead>
<tr>
<th>Gene</th>
<th>GO Term: Biological Process</th>
<th>Log₂(Fold Change) mid-L3/control vs. mid-L3/E23+</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Eig71Ee&quot;</td>
<td>puparial adhesion ; GO:0007594</td>
<td>-10.4</td>
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<tr>
<td>&quot;Eip74EF&quot;</td>
<td>salivary gland cell autophagic cell death ; GO:0035071</td>
<td>-1.5</td>
</tr>
<tr>
<td>&quot;Hr46 (DHR3)&quot;</td>
<td>steroid hormone mediated signaling pathway ; GO:0043401</td>
<td>-3.2</td>
</tr>
<tr>
<td>&quot;Hr78&quot;</td>
<td>salivary gland cell autophagic cell death ; GO:0035071</td>
<td>-2.0</td>
</tr>
<tr>
<td>&quot;Hsp23&quot;</td>
<td>response to heat ; GO:0009408</td>
<td>-1.8</td>
</tr>
<tr>
<td>&quot;Hsp27&quot;</td>
<td>response to heat ; GO:0009408</td>
<td>-2.2</td>
</tr>
<tr>
<td>&quot;ImpL3&quot;</td>
<td>glycolysis ; GO:0006096</td>
<td>-2.2</td>
</tr>
<tr>
<td>&quot;Sgs1&quot;</td>
<td>puparial adhesion ; GO:0007594</td>
<td>-10.7</td>
</tr>
<tr>
<td>&quot;vri&quot;</td>
<td>imaginal disc-derived wing hair organization ; GO:0035317</td>
<td>-2.3</td>
</tr>
<tr>
<td></td>
<td>circadian rhythm ; GO:0007623</td>
<td></td>
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<tr>
<td>*biot&quot;</td>
<td>morphogenesis of an epithelium ; GO:0002009</td>
<td>-2.6</td>
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<td>CG10157</td>
<td>oxidation-reduction process ; GO:0055114</td>
<td>-5.2</td>
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<td>CG10688</td>
<td>cellular response to hypoxia ; GO:0071456</td>
<td>-1.5</td>
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<tr>
<td>CG13784</td>
<td>-</td>
<td>-3.5</td>
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<tr>
<td>CG13950</td>
<td>-</td>
<td>-2.3</td>
</tr>
<tr>
<td>CG7656</td>
<td>neurogenesis ; GO:0022008</td>
<td>-1.6</td>
</tr>
<tr>
<td>*cindr&quot;</td>
<td>compound eye morphogenesis ; GO:0001745</td>
<td>-1.7</td>
</tr>
<tr>
<td></td>
<td>actin filament organization ; GO:0007015</td>
<td></td>
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<tr>
<td>*GstD3&quot;</td>
<td>response to heat ; GO:0009408</td>
<td>-2.4</td>
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<td>Hsc70-3</td>
<td>response to heat ; GO:0009408</td>
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<td>response to heat ; GO:0009408</td>
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<tr>
<td>Nopp140</td>
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<td>*pnut&quot;</td>
<td>apoptotic process ; GO:0006915</td>
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<td></td>
<td>cytokinesis ; GO:0000910</td>
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<td>Prx2540-2</td>
<td>hydrogen peroxide catabolic process ; GO:0042744</td>
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<td>Sep2</td>
<td>GTPase activity ; GO:0003924</td>
<td>-2.5</td>
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<tr>
<td>Tg</td>
<td>hemolymph coagulation ; GO:0042381</td>
<td>-2.4</td>
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</tbody>
</table>

The genes listed had depleted transcripts in mid-L3/E23+ in our study, and they were identified in at least two other studies as candidates for being 20E-regulated.

*Genes already established experimentally as being regulated by 20E.
### TABLE 5.2

Transcripts that are enriched in mid-L3/E23+ and found in more than one study

<table>
<thead>
<tr>
<th>Gene</th>
<th>GO Term: Biological Process</th>
<th>Log$_2$(Fold Change) mid-L3/control vs. mid-L3/E23+</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eip71CD</em></td>
<td>sulfur amino acid metabolic process ; GO:0000096</td>
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<td><em>Ama</em></td>
<td>cell adhesion ; GO:0007155</td>
<td>1.8</td>
</tr>
<tr>
<td><em>bmm</em></td>
<td>positive regulation of lipid storage ; GO:0010884</td>
<td>1.8</td>
</tr>
<tr>
<td><em>brat</em></td>
<td>negative regulation of cell proliferation ; GO:0008285</td>
<td>1.9</td>
</tr>
<tr>
<td><em>cact</em></td>
<td>innate immune response ; GO:0045087</td>
<td>2.1</td>
</tr>
<tr>
<td><em>Eip71CD</em></td>
<td>Toll signaling pathway ; GO:0008063</td>
<td></td>
</tr>
<tr>
<td><em>CG10444</em></td>
<td>transmembrane transport ; GO:0055085</td>
<td>2.3</td>
</tr>
<tr>
<td><em>CG10824</em></td>
<td>gluconeogenesis ; GO:0006094</td>
<td>1.8</td>
</tr>
<tr>
<td><em>CG11089</em></td>
<td>purine nucleotide biosynthetic process ; GO:0006164</td>
<td>1.8</td>
</tr>
<tr>
<td><em>CG15739</em></td>
<td>metabolic process ; GO:0008152</td>
<td>2.8</td>
</tr>
<tr>
<td><em>CG15820</em></td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td><em>CG31919</em></td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td><em>CG5059</em></td>
<td>positive regulation of apoptotic process ; GO:0043065</td>
<td>1.8</td>
</tr>
<tr>
<td><em>CG5522</em></td>
<td>small GTPase mediated signal transduction ; GO:0007264</td>
<td>1.8</td>
</tr>
<tr>
<td><em>CG5840</em></td>
<td>oxidation-reduction process ; GO:0055114</td>
<td>1.6</td>
</tr>
<tr>
<td><em>CG7224</em></td>
<td>-</td>
<td>2.7</td>
</tr>
<tr>
<td><em>CG7324</em></td>
<td>regulation of Rab GTPase activity ; GO:0032313</td>
<td>1.5</td>
</tr>
<tr>
<td><em>CG9416</em></td>
<td>proteolysis ; GO:0006508</td>
<td>3.6</td>
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<tr>
<td><em>clu</em></td>
<td>translational initiation ; GO:0006413</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Cpr49Ac</em></td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td><em>Cpr67B</em></td>
<td>-</td>
<td>2.2</td>
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<td><em>CREG</em></td>
<td>negative regulation of transcription from RNA polymerase II promoter ; GO:000122</td>
<td>2.5</td>
</tr>
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<td><em>Gapdh2</em></td>
<td>glycolysis ; GO:0006096</td>
<td>1.5</td>
</tr>
<tr>
<td><em>Gel</em></td>
<td>phagocytosis, engulfment ; GO:0006911</td>
<td>1.6</td>
</tr>
<tr>
<td><em>Ho</em></td>
<td>home oxidation ; GO:0006788</td>
<td>1.5</td>
</tr>
<tr>
<td><em>Hsp60</em></td>
<td>response to heat ; GO:0009408</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Idgf2</em></td>
<td>imaginal disc development ; GO:0007444</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Idgf3</em></td>
<td>imaginal disc development ; GO:0007444</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Keap1</em></td>
<td>response to oxidative stress ; GO:0006979</td>
<td>1.6</td>
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<tr>
<td><em>LanA</em></td>
<td>imaginal disc-derived wing morphogenesis ; GO:0007476</td>
<td>1.6</td>
</tr>
<tr>
<td><em>Mec2</em></td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td><em>Men</em></td>
<td>regulation of cell death ; GO:0010941</td>
<td>1.7</td>
</tr>
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<td><em>MFS10</em></td>
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<tr>
<td><em>Nop56</em></td>
<td>neurogenesis ; GO:0022008</td>
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<td><em>Papss</em></td>
<td>imaginal disc-derived wing morphogenesis</td>
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</tr>
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<td><em>pdgy</em></td>
<td>negative regulation of insulin receptor signaling pathway ; GO:0046627</td>
<td>2.1</td>
</tr>
<tr>
<td><em>Pdp1</em></td>
<td>circadian rhythm ; GO:0007623</td>
<td>3.4</td>
</tr>
<tr>
<td><em>Pect</em></td>
<td>ethanolamine-containing compound metabolic process ; GO:0042439</td>
<td>1.9</td>
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<td><em>Reg-2</em></td>
<td>metabolic process ; GO:0008152</td>
<td>2.1</td>
</tr>
<tr>
<td><em>Reg-5</em></td>
<td>circadian rhythm ; GO:0007623</td>
<td>1.8</td>
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<tr>
<td><em>Scp</em></td>
<td>muscle system process ; GO:0003012</td>
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<td><em>spi</em></td>
<td>oenocyte development ; GO:0007438</td>
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<tr>
<td><em>Vago</em></td>
<td>defense response to virus ; GO:0051607</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The genes listed had enriched transcripts in mid-L3/E23+ in our study, and they were identified in at least two other studies as candidates for being 20E-regulated.

*Genes demonstrated experimentally to be regulated by 20E.
5.3.d: Gene Candidates with Similar Patterns of Expression to the Glue Genes

Our next step was to filter our results further by identifying the specific pattern of gene expression changes that occurred in the known 20E-induced puffing cascade genes in the WPP/control versus the mid-L3/E23+ group (see Chapter 4 for the groups of 20E-induced cascade genes). The intermolt glue genes had a clearly defined pattern, which we used as baseline for filtering the RNA-seq results (Figure 5.4). The intermolt genes encode glue proteins that are downregulated by the pre-metamorphic pulse of 20E. Therefore, their normal expression levels should be higher in the mid-L3/control samples compared to the WPP/control (Velissariou and Ashburner, 1980; Garfinkel et al., 1983; Guild and Shore, 1984; Hansson and Lambertsson, 1989; Hofmann et al., 1991; Roth et al., 1999). When mid-L3/controls are compared to WPP/controls, that downregulation is apparent (Figure 5.4 and see Chapter 4). However, when mid-L3/controls are compared to mid-L3/E23+, the transcripts of the intermolt genes are severely depleted in the mid-L3/E23+ group (Figure 5.4), an observation that is consistent with the evidence that a small pulse of 20E induces the expression of these glue genes, and that E23+ blocks that 20E pulse and the subsequent gene induction.

After having established this pattern, we filtered our results for transcripts that were depleted in the WPP/control and mid-L3/E23+ groups as compared to the mid-L3/control group to screen for genes that would normally be induced by the mid-larval 20E pulse. Our cutoff was a log base 2 of -1.5 fold or lower as an indication of a significant reduction in transcript levels. In establishing these criteria, we did not include Sgs4 because none of the reads were aligned to Sgs4 in any of the samples even though the Sgs4 is downregulated similarly to the other glue genes at WPP (see discussion). Thus,
in total we extracted 100 candidates that had similar profiles to the intermolt genes (Figure 5.5). Because these criteria were very specific, we reduced the number of gene candidates to characterize in initial follow-up studies from over 1600 to 100, which represents less than 1% of the genes in the *Drosophila* genome.

As a validation of our methodology, the genes vrille (*vri*) and Mef2 were identified as having a pattern similar to the glue genes (Figure 5.5), and these genes have been characterized as 20E-regulated in studies of circadian rhythm (Itoh et al., 2011) and muscle development (Lovato et al., 2005), respectively. Overall, among the genes with a pattern similar to intermolt genes, the most significantly enriched group was that of carbohydrate metabolism and sugar binding (p<0.001, Table 5.3). This is important in that the known intermolt genes encode glue glycoproteins, and perhaps the enriched aforementioned group of proteins are necessary for transporting and modifying these highly glycosylated glue proteins. Other significantly enriched groups of genes in the mid-third instar include those encoding proteins required from defense and immunity and vesicle-mediated transport (p<0.05, Table 5.3).
Figure 5.4

A

Figure 5.4: The transcriptional response of the glue genes in mid-L3/E23+. (A) The intermolt genes are normally downregulated when mid-L3/control (more reads per gene) is compared to WPP/control (fewer reads per gene). When mid-L3/E23+ is compared to mid-L3/control, the transcripts of the intermolt genes have greatly reduced abundance. (B) The normalized transcript reads in mid-L3/control, WPP/control, and mid-L3/E23+ salivary glands are given for comparison and as an illustration of the extremely low read counts for the glue genes when E23 is overexpressed in the salivary gland.

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sgs1</td>
<td>$1.3 \times 10^6$</td>
<td>1747</td>
<td>771</td>
</tr>
<tr>
<td>Sgs3</td>
<td>$1.3 \times 10^7$</td>
<td>$2.2 \times 10^4$</td>
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<td>Sgs5</td>
<td>$3.8 \times 10^5$</td>
<td>9904</td>
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<td>Sgs7</td>
<td>$6.6 \times 10^5$</td>
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<td>555</td>
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<td>Sgs8</td>
<td>$1.4 \times 10^5$</td>
<td>5360</td>
<td>135</td>
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</tbody>
</table>
Figure 5.5: Potential 20E-target genes with an *intermolt* gene pattern of expression.

The genes in lists (A) and (B) show a pattern similar to the *intermolt glue* genes (see Figure 5.4). Transcripts from the genes are highly depleted with a log base 2 of -1.5 or lower in both WPP/control and mid-L3/E23+ groups compared to mid-L3/controls. The genes in (A) have a log base 2 fold change of -2.5 and below in the mid-L3/E23+ group. Those in (B) have a log base 2 fold change of -1.5 to -2.5 in the mid-L3/E23+ group as compared to mid-L3/control. Recall that in the mid-L3/E23+ group, the *intermolt* gene transcripts were depleted as much as a log base 2 fold change of -11. The genes that are marked with an asterisk have been previously implicated as 20E targets, but they are not *intermolt puff* genes.
## Table 5.3

Enriched groups of 20E-inducible-gene candidates that exhibit a pattern similar to the glue genes

<table>
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<tr>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate metabolism and sugar binding (p&lt;0.001)</td>
<td>GalNAc-T1</td>
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<td>1020</td>
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<td>CG13950</td>
<td>791</td>
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<td>161</td>
<td>FBgn0031289</td>
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<tr>
<td>Defense Response and Immunity (p&lt;0.05)</td>
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<td>Vesicle-mediated transport, ER, Golgi (p&lt;0.05)</td>
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*Genes demonstrated to be regulated by 20E. See the text for references. The p-values were generated by the DAVID clustering analysis algorithm by comparing the gene-list input to the entire *Drosophila melanogaster* genome as background (Huang 2009a, 2009b).
5.3.e: *In silico* Evidence of 20E-Regulation of the Gene Candidates

Finally, we wanted to test whether any of the gene candidates could be linked to ecdysone receptor regulation *in silico* as a first step in experimentally confirming these genes as actual targets of 20E signaling. Thus, we used a data set from modENCODE that mapped EcR (part of the ecdysone receptor heterodimer) bound to the genome during the mid-third instar or WPP in a ChIP-Seq experiment (www.modencode.org). If EcR were present in the vicinity of the gene, it was a preliminary indication that the gene may be regulated by the nuclear hormone receptor, and thus, the hormone 20E as well.

For the 100 gene candidates with similar expression profiles to the *glue* genes, we looked for evidence of EcR binding from 10 kb upstream of the 5’ UTR to 10 kb downstream of the 3’ UTR. Though a 10 kb buffer is a large span, many of the known 20E targets showed evidence of EcR binding greater than 10 kb away with no other known 20E targets in that region, and most enhancers will fall within 10 kb when examining larger patterns of genome architecture (Levine and Tijan, 2003; Gauhar et al., 2009). Of the 100 genes that had a similar pattern to the *intermolt* genes, 33 (33%) of them showed evidence of EcR binding. In the genes where EcR was found, a pattern emerged where the binding occurred either at WPP or L3 but not both times (Figure 5.6). Because the ecdysone receptor can repress gene expression (Schubiger et al., 2005), the observed binding might not be an indication of the activation of the gene. This could explain why the receptor is bound to the regulatory region of some of the gene candidates when they are expected to only have a low level of activation, but it is not found there when they are highly activated.
**Figure 5.6:** EcR is bound near the gene candidates. The predicted locations of EcR via a Chip-seq experiment (www.modencode.org) are indicated by the highlighting (green) underneath each transcription unit. Blue bars represent enriched genomic DNA fragments recovered after immunoprecipitation with an antibody specific to EcR. In (A), several examples of gene candidates are shown that have evidence of EcR bound during L3. In (B), examples of gene candidates are shown with EcR bound during WPP. The images were redrawn from data publicly available at www.modencode.org.
5.4: Discussion

Our attempt at identifying novel 20E targets during the mid-third instar 20E response in the salivary gland has opened many doors for experimental confirmation of over 1600 gene candidates, and our strategy to use the ectopic expression of the ABC transporter *E23* to abolish 20E-induced gene expression was very useful in facilitating the discovery of these genes. We were also able to find genes that were identified as being regulated by 20E in several studies and those that had similar transcript profiles to known 20E-regulated *glue* genes in the mid-third instar. Therefore, we can initially test this subset of about 200 genes for their involvement in mid-third instar salivary gland physiology since they are strong candidates.

In addition, some interesting candidates were uncovered that also had evidence of EcR binding within the vicinity of their transcription unit. One of these genes is *Past1*. The *Past1* protein contains a Ca\(^{2+}\)-binding domain and is important for intracellular membrane trafficking and interacts with Rab GTPase effectors (Olswang-Kutz et al., 2009; Mottola et al., 2010). Because the function of the salivary gland is a secretory tissue that is involved in the production and secretion of glycoprotein glue as well as antimicrobial peptides (Zhimulev and Kolesnikov, 1975; Korge, 1977; Wright et al., 1996), there is a necessity for a high level of expression of endosomal machinery such as *Past1*. In fact, another known 20E target, *E63-1*, encodes a Ca\(^{2+}\)-binding protein that is directly involved in secretion (Andres and Thummel, 1995). However, *E63-1* null mutants still secrete a small amount of glue, and it is likely that another protein plays a redundant role with E63-1. It will be interesting to test the double mutant of *E63-1* and
*Past1* (or drive *RNAi* constructs against both in the tissue) to see if glue secretion is impacted.

In addition, many of the gene candidates uncovered fell into predictable gene ontologies based on the secretory function of the salivary gland, including vesicle delivery and immunity defense. Whereas, gene ontologies for transcriptional regulatory proteins were not highly enriched. Thus, it is likely that many of the gene candidates are direct effectors of the physiological responses of interest as opposed to regulators of a subsequent set of genes that cause the physiological response. This is surprising given that the Ashburner model predicts that the genes that are directly activated by 20E are transcription factors and other regulatory proteins that initiate their own repression and also activate a larger battery of genes that would act as the direct effectors.

It is the combinatorial control of gene expression which makes teasing apart the regulation of our gene candidates a challenge. For example, is it the EcR component of the ecdysone receptor bound by 20E that results in ramping up gene expression, or is it the de-repression of the ecdysone receptor and recruitment of other activators that results in the increase in gene expression? Many of the gene candidates were also affected by ectopic expression or loss of *forkhead* (*fkh*) in the salivary gland in another transcriptome study (Liu and Lehmann, 2008). The gene *fkh* encodes a transcriptional activator that is required for the stage-specific expression of some 20E-regulated transcripts such as the intermolt gene *Sgs4* (Lehmann and Korge, 1995). Thus, it is likely that many of our gene candidates are not primary targets of 20E, but instead are secondary response genes. This would explain why many of the gene candidates are not transcription regulators.
One weakness of our methodology was that there might have been a large number of false negatives, especially since less than half of the candidates overlapped with studies using similar approaches (Li and White, 2003; Beckstead et al., 2005; Gauhar et al., 2009; Gonsalves et al., 2011). The genes in common among the studies are very likely strong candidates for 20E regulation, and the general lack of overlap could mean that there are flaws in the criteria that were used to select the 20E gene candidates or that we isolated a very specific set of genes involved in salivary gland physiology that went undetected in the other studies. Interestingly, two genes \textit{hsp27} and \textit{brat} overlapped among all five studies including ours. The translational repressor \textit{brat} has been implicated in wing development (Sonoda and Wharton, 2001; Edwards et al., 2003; Ren et al., 2005). It is notable that \textit{brat} is superinduced by \textit{E23} overexpression; whereas, \textit{hsp27} encodes a stress response protein that is considerably reduced when \textit{E23} is overexpressed, and it is a known target of 20E (Huet et al., 1996).

On the other hand, many false positives are often uncovered in these large genomic studies (Oshlack et al., 2010). For example, the Beckstead et al. study found over 700 genes that changed expression levels in response to 20E (2005), and we found over 1600 that affected by the loss of 20E. It is likely that many of the genes in theses studies are not primary targets of the hormone. Nevertheless, our filtering criteria for the genes with expression profiles similar to the \textit{glue} genes limited the list to 100 candidates, potentially reducing the number of false positives. In order to determine whether these are actual 20E targets, more experimentation will be necessary including \textit{individual} manipulation and detection of the gene of interest in conditions where 20E signaling is altered. In future studies, we will expand our criteria by using the gene expression patterns of more
known 20E-regulated genes in order to eliminate the false negatives problem; however, loosening the criteria will also come at the cost of finding more false positives.

Finally, there were weaknesses in the strategy and outcome of our experiment that must be reconciled. First, one of the known 20E gene targets, Sgs4, that is an intermolt gene in the puffing hierarchy was inexplicably absent from the aligned reads. We think that this could be attributed to problems with the alignment algorithm (Bowtie), and we will correct this once we prepare a biological replicate of our experiment.

Another major challenge is that the 20E-induced genetic cascade contains many negative feedback loops, which make it difficult to decipher to what degree E23 overexpression is negatively affecting 20E gene candidates. In some cases, ectopic E23 might actually result in a slight increase in the expression of genes that are normally repressed by EcR/USP because of the negative feedback. For example, even though E23 is an early gene in the ecdysone cascade, its overexpression had little effect on some of the other early genes, especially BR-C and E74. However, these genes have several isoforms that have temporal and tissue specific expression, so it will be necessary to look at isoform-specific expression of these genes in future studies to determine the specific effects of ectopic E23.

Despite a few shortcomings, our RNA-seq methodology using E23+ was a good strategy for finding genes in the 20E cascade given that E23 is most likely negatively regulating the hormone itself and not any of the downstream effectors of hormone signaling. Thus, ectopic E23 should be blocking 20E-regulated gene expression from the very top of the cascade. By combining the results of our analysis with the results of other gene expression studies that look at the targets of early, primary response genes encoding
transcription regulators (such as BR-C, E74, and E75), we can more easily place each of our candidates into the ecdysone signaling hierarchy as primary or secondary response genes. What has emerged from this analysis and others is that it may be the lack of hormone that allows for the full activation of a subset of 20E-regulated genes. We speculate that these genes may also require the absence of EcR/USP binding for full activation based on the \textit{in silico} analysis of receptor binding. It will be important to clarify the mechanism of de-repression in future analyses since it appears to be more prominent than direct activation by 20E mediated through EcR/USP (see Johnston et al., 2011).
6.1: The Regulation of Glue Production and Glue Secretion

At the most fundamental level, molecular geneticists are interested in the complex interactions that dictate when and where a gene will be turned on and ultimately what the product of the gene does. I have presented the results of three projects that on the surface seem relatively distinct, but they converge around a fundamental question of developmental biology: How can a single signaling molecule cause such diverse outcomes in different cells and at different times?

To approach this question, we built a solid model system using the salivary gland of *Drosophila melanogaster*. Our signaling molecule is the steroid hormone 20-hydroxyecdysone (20E). Signaling by this single molecule results in two physiological responses separated in time: salivary gland glue synthesis and salivary gland glue secretion. What is happening molecularly at each time period to result in such different responses to the same signal? In Chapter 1, I discussed several levels of control that may be functioning to regulate the spatial and temporal responses to the hormone including the structure of the EcREs, the cellular exposure to 20E, and the constellation of coactivators, corepressors, and other regulatory proteins present in a cell at a given time. I also noted that we were interested in the intersection between the Ashburner model and tissue-specific regulation of intracellular 20E concentrations. Below I summarize what we have found regarding what determines the distinct regulation of glue production and glue secretion in the salivary gland, and I propose key experiments that
we will need to accomplish in order to further our understanding of these 20E-regulated processes.

6.1.a: The Role of ChA and Lgr3 in Glue Secretion

In Chapter 2 we described the results of an EMS-induced loss-of-function screen for glue expulsion defects on the third chromosome, and we genetically mapped two mutations to the genes Choline Acetyltransferase (ChA) and Lgr3 via complementation testing. ChA and Lgr3 were previously not known to be involved in the response triggering glue secretion and expulsion. The genetic evidence of one of the mutant alleles (DmB^{k67}) mapping to ChA is compelling because the offspring from a cross between an EMS-induced lesion in ChA and DmB^{k67} are completely lethal (which is identical in phenotype to the homozygous DmB^{k67}). Whereas, the genetic evidence identifying Lgr3 as the location of the other mutation (DmA^{k46}) is not as robust because the offspring of a cross between DmA^{k46} and a P element insertion that disrupts Lgr3 does not result in a complete block in expulsion, and some offspring appear wildtype. Thus, the next important step will be to sequence the mutants compared to wildtype in order to locate the molecular lesions of the mutations and in order to infer the resultant amino acid changes in the polypeptides encoded by the mutant alleles of ChA and Lgr3. If the mutations are located in a controlling region of the genes, qRT-PCR can be used to see whether mRNA expression of ChA or Lgr3 is affected. Detecting changes in mRNA expression will be critical in the event that the molecular lesions are not in coding regions of the genes because mutations in introns and non-coding sequences will be difficult to locate by sequence alignment alone.
Once the nature of the mutant alleles have been established, it will be important to test whether the genes that encode the enzyme Choline Acetyltransferase (ChA) and the G Protein-Coupled Receptor Lgr3 are directly controlled by 20E. Using qRT-PCR to quantify the mRNA expression of these genes in an EcR-DN (20E-signaling deficient) background in the salivary gland will help to define whether the genes are activated by 20E via the nuclear hormone receptor EcR. Testing whether Lgr3 and ChA are directly regulated by 20E is important because our hypothesis is that secretion can occur at the WPP stage (and not earlier) in response to 20E due to the coordination of multiple signaling pathways that are temporally specific to the late third instar salivary gland (Figure 6.1A, B).

Presently, our model is that neurotransmitters acting non-neuronally such as acetylcholine (ACh) induce a nongenomic pathway in the salivary gland, which may be mediated through a GPCR, in order to elevate Ca\(^{2+}\) levels (reviewed in Wessler et al., 1998). Lgr3 may not be the GPCR through which Ach signals, so a genetic experiment in which we overexpress the wild-type allele of one gene in the mutant background of the other gene will help to define their interaction. We can also compare intracellular Ca\(^{2+}\) levels in wild-type salivary glands versus our secretion mutants to test whether Ca\(^{2+}\) elevation is absent in the mutants, and we can look at transcriptome profiles of salivary glands in controlled culture conditions when ACh is added in order to rule out a genomic response to ACh triggering secretion. We propose that in a synergistic pathway to ACh signaling, 20E bound to EcR/USP activates genes that are effectors of secretion, such as E63-1. We can perform a much larger loss-of-function screen saturating all four chromosomes to find other 20E-regulated genes encoding effector proteins necessary for
secretion. Finally, components of the secretion apparatus such as myosin motor proteins and E63-1 respond to the elevated cytoplasmic Ca\(^{2+}\) levels resulting in the delivery and exocytosis of glue granules to the lumen. We can find other genes encoding proteins required for glue secretion by using RNA interference against the EF-hand domain proteins from our transcriptome data in Chapter 4. Our transcriptome data set can be further mined for upregulated or downregulated salivary gland genes at WPP encoding any of the groups of known secretion effectors including, for example, SNARES, SNARE regulators, Rabs, and Rab effectors (reviewed in Burgoyne and Morgan, 2003).

6.1.b: The Role of E23 in 20E-Regulated Physiology

In Chapter 3 we provided thorough evidence consistent with the prediction that the ABC transporter E23 negatively regulates 20E signaling. In effect, overexpression of EcR-DN and E23 resulted in identical phenotypes when expressed in the larval fat body, eye disc, larval salivary gland, larval mushroom body, larval cuticle, and larval ring gland.

However, there were differences in the mean percent of pupal-to-adult viability when E23 or EcR-DN was overexpressed in a subset of imaginal discs. Recall that in order for target genes to be activated, EcR/USP must be bound to 20E. In some cases, this leads to the direct activation of the downstream gene. However, it has been demonstrated that in the wing disc, gene activation occurs through de-repression of EcR/USP when hormone titers are high (Schubiger et al., 2005). In the context of wing discs, once the hormone binds to the receptor, it has been postulated that the receptor is no longer bound and other activators are recruited to the regulatory region to initiate gene expression. Thus,
EcR-DN is effective for preventing both responses that require the inhibitory function of the ecdysone receptor and those that require the activational function of the receptor because it cannot be activated or de-repressed. During the large pre-metamorphic pulse that both de-represses and activates genes, EcR-DN blocks all downstream responses. Whereas, E23+ can block the activational function of the ecdysone receptor in larval tissues, but it is not effective at blocking the inhibitory function of the receptor, as shown by the near wild-type responses when E23 overexpression is limited to imaginal discs. In future experiments, expressing even higher levels of E23+ during times lasting through the prepupal period can be used to block the de-repression of the ecdysone receptor in imaginal discs. However, E23+ has an advantage over EcR-DN concerning in vitro experiments. Whereas, EcR-DN cannot be de-repressed or activated with high levels of exogenous 20E; the block induced by E23+ can be overcome by titrating high concentrations of exogenous 20E to restore the 20E response. Using E23+ and EcR-DN in this experimental context will help to tease apart the function of the ecdysone receptor as a repressor versus an activator in tissues that can be cultured.

An important first step in this analysis will be to characterize the endogenous expression patterns of E23. Thus, we would like to know whether endogenous E23 protein is found at lower or higher levels in imaginal discs versus larval tissues. With the E23 antibody, we can use tissue staining or western blots of individual staged tissues to characterize the protein localization. In addition, culture experiments comparing the response of imaginal discs to larval tissues when E23 or EcR-DN is overexpressed and high levels of exogenous 20E are applied will help to define whether 20E-regulated physiological responses can be restored even when E23 is found at high levels. We
predict that EcR-DN should completely block 20E-regulated physiology and no amount of exogenous 20E should restore blocked responses. However, kinetically high enough levels of 20E can potentially outcompete the negative regulatory role of E23, given our model that E23 actively pumps 20E extracellularly. This experiment is key because it is a direct test of our model (Figure 6.2).

Via western blots and RNA-seq, in Chapter 3, we demonstrated the dynamic expression of E23 mRNA in larval tissues and imaginal discs and of E23 protein during each of the different salivary gland responses to 20E. Intriguingly, in the salivary gland, E23 mRNA levels did not predict protein levels (i.e., when E23 mRNA was higher, E23 protein was absent). It will be interesting to study how rapid E23 protein turnover is in many different tissues because E23 was reproducibly absent in WPP salivary glands when it had been present in larval salivary glands only four hours prior. We hypothesize that a protease is rapidly turning over the E23 protein. Because there are so many genomic tools available, we can generate a list of all the known proteases in Drosophila that are expressed in the larval salivary gland (derived from our transcriptome data of WPP), and we can detect E23 protein expression when each of these proteases is compromised by way of loss-of-function analysis. Again, the RNAi toolkit will be the most rapid way of screening each of these proteases. Yeast-two-hybrid experiments can also be performed to look for specific interactions between E23 and the protease of interest. Furthermore, bioinformatic analysis will be important to identify conserved sequences in the E23 amino acid sequence that predict proteasome-mediated degradation.
6.1.c: The Role of \textit{E23} in Glue Synthesis, Glue Secretion, and Salivary Gland Histolysis

In terms of glue production and secretion, if we overexpress \textit{E23} to eliminate the 20E signal, we block both glue production and secretion. Nevertheless, glue synthesis is reinitiated at a later improper time when the endogenous, high-titer 20E signal returns at that later time. The reinitiation of glue synthesis may be dependent on a low intracellular concentration of 20E, setting in motion gene expression that typically occurs during the mid-third instar. An important follow-up experiment will be an \textit{ex vivo} culture of the salivary gland with high levels of E23. The hormone 20E can then be titrated until the exact concentration of 20E necessary to reinitiate glue expression is determined. This experiment will accomplish two aims. First, it will indicate whether the reinitiation of glue actually depends on lower concentrations of 20E, and second, it will support the role of \textit{E23} as a pump.

Another interesting result of \textit{E23} overexpression was that adults emerge from their pupal cases with glue-filled larval salivary glands stuffed into their heads, thoraxes, or abdomens. We need to do further characterization of these adults to determine whether the larval salivary gland histolyzes as it would in prepupae. Furthermore, it would be interesting to compare the transcriptome profile of a larval salivary gland that persisted to one of a prepupa in which salivary gland histolysis is imminent. The results may provide a larger picture of what genes were never activated or repressed, which resulted in the persistent adult salivary gland, and we may find new genes involved in the genetic pathway required for salivary gland histolysis.

Although ectopic \textit{E23} expression has a potent negative regulatory effect on 20E-initiated physiology in the salivary gland, a null allele of \textit{E23} both produced and secreted
glue (Biyasheva et al., 2001). This result indicates that E23 is dispensable for both glue production and secretion and raises the possibility that E23 is not required in the larval salivary gland. On the other hand, targeted expression of an RNAi against E23 in the larval salivary gland revealed that E23 was required for glue production, but glue secretion was nearly wildtype (E.P., unpublished observation). These contradictory results will need to be reconciled in future analyses by confirming that there are no off-target effects of the RNAi directed against E23. We can see whether the block in glue production is “rescued” when we overexpress E23+ and E23-RNAi in the same animal to check for off-targets because glue should be produced if the RNAi reagent is specifically knocking-down E23. In addition, a complete analysis of the null to determine whether it is a molecular null will be important.

In addition, determining whether 20E is the specific substrate for E23 will help to clarify the role of E23 in the salivary gland. Biochemical experiments involving inside-out membrane preparations with varying levels of E23 and exogenous radiolabeled hormone will be the most direct method of testing whether E23 is specific for 20E. However, competition experiments in which we add several different steroid hormones to our membrane preparations will also elucidate the specificity of the transporter for 20E. Mutational analysis of the specific ATP-binding domains within the E23 amino acid sequence could be combined with the inside-out membrane experiments because currently the presence of these domains and homology with other ABC pumps are the only evidence that E23 is actually a transporter capable of exporting a substrate.
6.1.d: Genes Implicated in the Mid-Third Instar Salivary Gland Response and Salivary Gland Glue Secretion

In Chapter 5 we used a transcriptome analysis to identify genes that form a primary or secondary response to the hormone during the mid-third instar during the time of glue production. Many of these are interesting candidates for triggering temporally-specific responses to the hormone. As expected, we identified the glue genes that were upregulated during the mid-third instar. However, we also demonstrated that genes that are involved in immunity are upregulated at this time. The salivary gland may be producing highly glycosylated mucoproteins for functions other than adhering a pupa to a solid surface. Perhaps antimicrobial peptides and other immunity defense proteins are loaded with the glue in the salivary gland and have a protective function against microbial infection during metamorphosis. Our transcriptome data set is laden with many gene candidates that we can test for involvement in the mid-third instar glue response via loss-of-function analysis.

Thus, one of the major findings that emerged from this work is that the complex regulatory circuit in the salivary gland is not as neat and simple as the Ashburner model predicted. We discovered that many genes that are primary targets of the hormone are likely to be direct effectors of downstream physiological responses because they do not encode transcription factors that could potentially regulate other genes as the Ashburner model would have predicted. Further characterization of these 20E-regulated genes will help to define what precise molecular machinery is required for the delivery and exocytosis of the glue cargo in the pre-metamorphic salivary gland. Furthermore, because RNA interference reagents are available for nearly every gene in the Drosophila
genome (Dietzl et al., 2007), the assessment of the gene candidates for glue secretion phenotypes and the mid-third instar response promises to be fruitful.

Yet, despite the complexities that complicate the Ashburner picture of steroid-regulated gene expression, in general the model holds up quite well across many studies as demonstrated in Chapter 4. One of the most incredible findings that developed from comparing our transcriptome data to the publicly available modENCODE data set is how rapidly gene expression is changing during the short period of time prior to pupariation. Even though in general RNA-seq experiments are very precise (Marioni et al., 2008), we found differences as great as 10 fold in the reads of late genes for WPP that could be separated developmentally by no more than 2 hours in our study versus modENCODE. Thus, gene expression may be dynamic and mRNA turnover may be very rapid at this time.

6.1.e: Does E23 Play a Role in Temporally and Spatially Specific Responses to 20E?

Finally, since the inception of the Ashburner model, many research groups have demonstrated that their gene of interest has a critical role in controlling temporally and spatially specific responses to the hormone. We do not claim that E23 is the ultimate gene solely responsible for conferring specificity, but it does have an important part especially if it is limiting the cellular exposure to 20E. We have seen how critical 20E thresholds are for proper gene expression. Thus, if E23 is regulating intracellular concentrations of 20E, it could allow for the rapid changes in gene expression we observed at WPP (Figure 6.2). Again, controlled culture conditions of salivary glands
and other tissues with E23 expressed at different levels and with exogenous hormone applied may provide the best means of testing E23 as a pump.

In terms of known critical regulators of temporal responses to the hormone, the early gene encoding the nuclear receptor E75A was recently shown to compete with EcR/USP for enhancer binding (Johnson et al., 2011), and E75A represses 20E target genes such as BR-C (Dubrovsky et al., 2004; Johnson et al., 2011). Thus, with the aid of E23 limiting cellular exposure to 20E, E75A competition for EcREs may provide the critical switch that turns the glue genes off (Figure 6.1C and Figure 6.2). In addition, research from our lab points to the requirement for EcR but not USP in glue gene expression, suggesting the existence of a novel partner for EcR (Costantino et al., 2008). Perhaps the EcR/NR-X partnership binds to specific EcREs and competes with E75A, and perhaps the kinetics of the hormone receptor binding depends on the amount of 20E that is available (Figure 6.1C and Figure 6.2). Nevertheless, even the glue genes are not regulated by identical factors (Lehmann, 1996), so it may be worthwhile to look at each glue gene individually to determine whether E75A in concert with E23 provide a critical switch.
Figure 6.1: Models of glue secretion and synthesis in the salivary gland.

(A) Secretion is induced during the pre-metamorphic pulse of 20E. The high titer of 20E activates EcR/USP and results in the expression of regulated secretory apparatus such as E63-1 (Andres and Thummel, 1995). Other secretory apparatus may be expressed via a separate signaling pathway. (B) Concomitant with the pre-metamorphic pulse, the enzyme Choline Acetyltransferase (ChA) produces acetylcholine (ACh) which signals through a GPCR to increase intracellular Ca^{2+} in the salivary gland. Lastly, Ca^{2+} interacts with secretory apparatus including E63-1 to secrete glue granules to the lumen of the tissue.
Figure 6.1 (continued): Models of glue secretion and synthesis in the salivary gland. (C) Glue production is induced by the pulse of 20E that occurs 24 hrs before puparium formation (Biyasheva et al., 2001; Warren et al., 2006). BR-C is required for glue gene expression, and BR-C is regulated by EcR/USP bound by 20E. 20E also activates E74B, which helps to activate the glue genes (Fletcher et al., 1997), and E75A, which likely has a role in shutting off the glue genes either directly or by negatively regulating BR-C (see Johnson et al., 2011). In addition, a different functional heterodimer hormone receptor consisting of EcR and an unknown partner (NR-X) may have a direct role in activating the glue genes (Costantino et al., 2008). Other transcription activators like fkh (Mach et al., 1996) and SEBP-3 (Lehmann and Korge, 1995) were shown to regulate the glue genes as well. Thus, the glue genes achieve full activation when all of the activating factors are present, but they are completely shut off if just EcR is mutated because that would result not only in the loss of EcR/NR-X but also BR-C. Loss of USP may not affect glue gene expression because the NR-X partner for EcR along with fkh and other factors may allow for full activation even in its absence.
Figure 6.2: A model for E23 action in the salivary gland during the mid-third instar. (A) During the mid-third instar, there are low levels of E23 protein, and so 20E is not rapidly expelled and is able to contact a nuclear hormone receptor consisting of EcR/NR-X in order to activate the glue genes. NR-X when bound to EcR and 20E would bind tightly to the glue gene EcREs. (B) The levels of E23 protein would increase rapidly during the late third instar as the pre-metamorphic titer of 20E is rising. Ultimately, the glue genes will be completely shut off at WPP due to the following: 1) E75A protein levels would increase and EcR would decrease; 2) The higher levels of E23 would result in rapid extrusion of 20E; 3) Once the amount of intracellular hormone was limited, E75A would out-compete EcR/NR-X for some EcREs and shut off the glue genes; 4) E23 protein would be rapidly degraded at WPP allowing for the hormone to activate another group of genes.
APPENDIX A

DROSOPHILA PHOSPHATE BUFFER SOLUTION RECIPE

2x Salts:
3.04g NaCl (104 mM)
2.98g KCl (80 mM)
2.38g HEPES (20 mM)
0.30g MgSO₄ x 7H₂O (2.4mM)
0.24g MgCl₂ x 6H₂O (2.4mM)
0.28g Na₂HPO₄ (4mM)
0.054g KH₂PO₄ (0.8 mM)
d-e-ionized water to 500ml

1. Add all ingredients to a 1L beaker on a stir plate.
2. Add de-ionized water up to 500 ml and stir until solids are dissolved.
3. Aliquot in 25-ml lots in 50ml Falcon tubes and freeze -20°C.

1x complete DPBS (50mls):
25mls 2x salts
1.54g sucrose (90 mM)
0.09 g glucose (10 mM)
CaCl₂ to 1mM
d-e-ionized water to 50ml

1. Thaw 2x salts at 4°C overnight.
2. The next day, in an autoclaved 100 ml beaker, add the 25ml 2x Salts
   on a stir plate with a stirring rod.
3. Add the sucrose, glucose, and CaCl₂ while stirring.
4. Add de-ionized water up to 50ml.
5. Adjust the pH to 7.2 with HCl and NaOH.
6. Filter sterilize the solution into a 50ml Falcon tube.
7. Store at 4°C.
APPENDIX B

E23 WESTERN BLOT PROTOCOL 7.5% GEL

1. Preparing the Gels

1. Clean the gel plates with EtOH and assemble apparatus for pouring the gel.

2. Obtain a 10-ml mini beaker and a 15-ml beaker. Add all the ingredients listed below to each of the beakers, stopping after adding the SDS. Cover the 10-ml beaker with parafilm (containing what will be the stacking gel). Add the remaining ingredients (APS and Temed) which will start the polymerizing reaction to the separating/resolving gel in the 15-ml beaker. Immediately use a plastic pipette to carefully pour the separating gel into the glass plates. Leave about 1 cm space below the comb for stacking gel. You’ll need about 4 ml of separating gel/ minigel. Overlay carefully with dH₂O using a disposable plastic pipette, and let polymerize for about 20-30 minutes.

3. Pour off the H₂O and dab with a kimwipe. Add the APS and Temed to the stacking gel beaker. Pipette the stacking gel, and insert the comb. Less then 1 ml stacking gel is necessary/minigel. Let polymerize for about 20-30 minutes before running the gel. Alternatively, wrap in wet paper towel and Saran Wrap and store at 4°C. I have stored gels for 1 week successfully.

Recipe for 10 ml of 7.5% resolving gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>3.3</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>4.0</td>
</tr>
<tr>
<td>1.5 M Tris, pH 8.8</td>
<td>2.5</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
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</tbody>
</table>

Recipe for 4 ml of stacking gel

<table>
<thead>
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<th>Component</th>
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</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>2.7</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>0.67</td>
</tr>
<tr>
<td>1 M Tris, pH 6.8</td>
<td>0.5</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.04</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.04</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Solutions:
- 30% acrylamide: purchased from Biorad, stored in 4°C fridge.
- 1.5M Tris, pH 8.8
- 1 M Tris, pH 6.8
- 10% SDS: dissolve 10 g of SDS in 90 ml of H₂O, bring the volume to 100 ml, store at room temperature.
- 10% APS: dissolve 10 g of APS in 100 ml of H₂O, bring the volume up to 100 ml, aliquot in 1 ml aliquots, and store in -20°C.
- TEMED: purchased from GIBCO, stored at 4°C.
2. Running the Samples in the Gels

1. Assemble apparatus for running samples. Remove samples from freezer and thaw on ice. Heat samples for 5 minutes at 100°C. Store on ice until ready to load gel. Prepare rainbow marker (sometimes it is necessary to add 2x Laemmli’s buffer and heat. Read manufacturer’s instructions.) Store Rainbow Marker on ice until ready to load gel.

2. Make 1L of Running Buffer by diluting 100 ml of 10x Running Buffer in up to 1000 ml of dH₂O. This can be prepared directly in a 1L beaker and homogenized by covering with parafilm and inverting several times.

   - 10x Running buffer:

<table>
<thead>
<tr>
<th></th>
<th>1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>800 ml</td>
</tr>
<tr>
<td>Tris-base</td>
<td>30.3 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>144.2 g</td>
</tr>
<tr>
<td>SDS</td>
<td>10 g</td>
</tr>
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</table>
   - Adjust pH to 8.3
   - Adjust volume to 1 L
   - Store at RT.

3. Cover central chamber containing the gels with 1x Running buffer. Fix leaks before pouring the remaining running buffer into the outer portion of the apparatus. Load samples. Place lid with power cord on samples.

4. If you are running two gels at the same time, start at 36 mA. After samples enter the separating gel, increase to 46 mA or more, depending on how fast you want to be done (with 36mA --> 46 mA current, you will be done in about 1 hour).

3. Blotting

1. Take down the gel, cut off the stacking part, and soak in blotting buffer for about 15-30 minutes. Too long might be bad, because proteins can start to leach out (at least that’s what I’ve heard).

2. While the gel is soaking, cut out a piece of membrane exactly the size of a gel (for our minigel, its about 5.2x8.1 cm, depending on the size of your stacking gel). If working with nitrocellulose, soak briefly in dH₂O, and then in blotting buffer till ready to assemble the sandwich. If using the PVDF membrane (Immobilon P), soak in methanol/5 seconds, then dH₂O/5 minutes, then blotting buffer/15 minutes or till ready to assemble sandwich.

3. Cut out two pieces of Whatman 3MM paper slightly larger then gel 6x9cm(it really doesn’t matter, as long as they fit into the blotting unit). Soak the sponges in blotting buffer.

4. Sandwich. Remember to keep track of the position of gel and membrane (gel must be closer to the - electrode, while membrane to the +.) Wet one piece of Whatman 3MM paper and slide the gel onto it. Place on the sponge. Place the membrane over the gel; roll over with a glass pipette to get rid of air bubbles. Wet the second piece of a Whatman 3MM paper and place on top. Roll over with a pipette. Top with second sponge and place the assembled sandwich into the blotting unit.
5. Blot at 50V/1.5 hr. E23 can be blotted for 2 hours. For E63-1, this time ensures complete transfer of the protein from the gel to the membrane. 30 minutes is sufficient to get enough E63-1 for immunodetection, but at least half of the protein is left behind in gel (you can check by staining the gel with Coomassie blue). For CaM, 30 minutes is sufficient for complete transfer. You don’t have to worry about using cold room under these conditions; the unit will not overheat if you start with cold buffer.

- Western blotting buffer (can be reused):

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<thead>
<tr>
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<th>3 1</th>
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<tbody>
<tr>
<td>Tris</td>
<td>9.09 g</td>
<td>3.03 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>43.23 g</td>
<td>14.41 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>600 ml</td>
<td>200 ml</td>
</tr>
<tr>
<td>H2O</td>
<td>up to 3l</td>
<td>up to 1l</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Store at 4°C.</td>
<td></td>
</tr>
</tbody>
</table>

3. Immunodetection

1. Take down the blot. If you are using pre-stained MW standards, you will be able to tell whether protein transfer to the membrane was complete. While the membrane is still laying on top of a gel, use a black “BICK” pen to mark positions of MW standards (the blue color tends to fade during following procedures. This can be used later to determine which is the “protein side” of the membrane.)

2. Block 1 hr/RT in 5% (w/v) dry milk in TPBS [PBS with 0.05% (v/v) Tween 20]. Alternately, block overnight at 4°C on a belly dancer.

Next day->

3. Wash 3x5 minutes in TPBS.

4. 1° Ab overnight 4C belly dancer. For E23 (3F5-4F10), use 1:50 dilution in 5% dry milk in TPBS. Try to limit to less than 20 mL total due to limited antibody supply (200 uL in 10 mL). For alpha tubulin (mouse), use 1:15,000 (1 uL in 15 mL).

5. Wash 3x10 minutes in TPBS.

6. 2° Ab 30 min/RT. For GAM-HRP (Jackson), use 1:25,000 dilution in TPBS.

7. Wash 3x15 minutes in TPBS, then 2x15 minutes in PBS.

- 10x PBS

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>80 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>14.4 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.4 g</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Dissolve in approximately 900 ml H₂O, adjust pH to 7.2. Adjust volume to 1 liter. Divide into 4x 250 ml aliquots (in 500 ml bottles), and autoclave. Store at RT.
- 10x TPBS: add 2.5 ml of Tween 20 to 500 ml of 10x PBS. Store at room temperature.

4. ECL+ detection (GE Healthcare)

1. In 15-ml falcon tube, pipette 4 ml ECL+ Solution A. Cover falcon tube with foil to block light.

2. When ready to image*, mix solutions A and B (1:40 dilution). For 4 ml, add 100 ul solution B.

3. Remove blot from PBS with tweezers and dry excess PBS on kimwipe. Place the blot into a small container (protein side up) and pour over it 2 ml of mixed solutions onto each blot. Gently swirl. Make sure the whole surface of the blot is covered.

4. Remove blot with tweezers, dry excess solution with kimwipe. Place blot, protein side down on the Typhoon 9410.

5. Click Scan. Save. Image can be adjusted using Image Quant.

6. Note: Save the blot wrapped at 4°C. As long as it doesn’t dry out, it can be stripped and re-probed with a different antibody (see Stripping protocol).

*Preparing the Typhoon 9410 for use:
1) Turn on. Click Scanner on desktop.
2) Acquisition: Choose Fluorescence.
   Setup-Emission Filter: 520 BP 40 Cy2 Blue “Ecl+”
   Laser Blue 1 [457]
   Even if it says “not optimal”, click ok.
3) Clean the glass on the Typhoon with dH2O and a kimwipe.

**Lysis Buffer**
1% NP-40
0.5% Deoxycholic acid
0.1% Triton-X-100
100 mM NaCl
0.1 mM CaCl2
2 mM MgCl2
water to preferred amount
pH 7.6
*can be stored up to 1 year*
LITERATURE CITED


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Honorable Mention, GPSA Research Forum, UNLV  Spring 2011
GPSA Fellowship, UNLV, $675  Fall 2010
Summer Session Scholarship, UNLV, $2000  Summer 2010
Golden Key Graduate Scholarship, UNLV, $250  Summer 2010
2nd Place, Best Platform Presentation at the GPSA Research Forum, $150  Spring 2010
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Golden Key International Honour Society, UNLV  2009-2012
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GPSA Fellowship, UNLV, $550  Spring 2007
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Graduate Faculty Representative, Ernesto Abel-Santos, Ph. D.