THE TRANSCRIPTIONAL AND DEVELOPMENTAL REQUIREMENT FOR SOX14 AT THE ONSET OF *DROSOPHILA* DEVELOPMENT

by

AMANDA ROSE RITTER

(Under the Direction of Robert B. Beckstead)

ABSTRACT

The steroid hormone 20-hydroxyecdysone (20E), working through the nuclear receptor heterodimer complex, Ecdysone receptor (EcR) and Ultraspiracle (Usp), triggers key developmental transitions throughout the *Drosophila* life cycle through genetic regulation. The Sox14 transcription factor has been previously shown to be a primary response gene to the 20E/EcR/Usp complex. We show that animals mutant for *sox14* show prepupal and pupal lethality with defects in various 20E developmentally regulated pathways. Northern blot and genome-wide microarray analysis demonstrate that Sox14 is required for the proper expression of both 20E and non 20E-regulated genes at the onset of metamorphosis, expressed in a variety of larval and adult tissues corresponding with the Sox14 expression pattern. Thus, Sox14 is a critical transcription factor required for 20E signaling at the onset of metamorphosis.

INDEX WORDS: *Drosophila*, Sox14, EcR, ecdysone, 20E, metamorphosis

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by

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DEDICATION

I would like to dedicate this thesis to my mom and dad; two outstanding people whose unfailing belief in me and contagious optimism has propelled me forward in the pursuit of my goals. Thank you both for putting your faith in me, so much that it carried me when I lacked faith in myself. I love you both.

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going out, running our dogs to the dog park, taking gym breaks, etc. helped me to re-focus and not let work consume me. A little break can do wonders for your motivation once you return to the task.

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CHAPTER 1

INTRODUCTION

Steroid hormones control a wide range of biological pathways that occur in higher organisms, such as cellular differentiation and development. Members of the nuclear receptor superfamily act as hormone-responsive transcription factors that transduce hormone signals into tissue specific changes in gene expression. Extensive studies have elucidated many of the molecular mechanisms underlying nuclear receptor regulated transcription but how these changes in gene activity lead to the appropriate biological response during development is still not fully understood.

Drosophila melanogaster provide an ideal system to link steroid hormone signaling to specific developmental processes. Pulses of the steroid hormone, 20-hydroxyecdysone (20E), the biologically active form of ecdysone, function as critical temporal signals that direct each of the major developmental transitions in the *Drosophila* life cycle (Clever, 1964, Riddiford, 1993). 20E drives the morphogenetic movements that form the larvae during embryogenesis as well as triggering key developmental transitions as the animal pupariates and undergoes metamorphosis (Kozlova and Thummel, 2003; Thummel, 1996; Thummel, 1990). This single steroid hormone acts in a stage-specific manner to elicit different biological responses at each developmental stage. Emphasis has been focused on the early stages of metamorphosis, where 20E drives the total reorganization of the body plan resulting in the majority of the larval tissues being destroyed by programmed cell death and the formation of adult structures from clusters

of adult progenitor cells called imaginal discs (Robertson, 1936; von Kalm et al., 1995; Yin and Thummel, 2005).

Towards identifying and linking the genomic 20E-transcriptional cascade to biological responses regulated by this hormone, several microarray experiments were performed to identify genes regulated by 20E and its receptor complex which consist of the Ecdysone receptor (EcR) and ultraspiricle (Usp) (Beckstead et al., 2005; Li and White, 2003; White et al., 1999; Koelle et al., 1991). Through these studies, the transcription factor *sox14* was identified as a 20E-regulated, EcR-dependent gene that was upregulated in synchrony with both the late 3rd instar larval and prepupal pulses of 20E, defining *sox14* as a primary-response gene. The identification of Sox14 was of great interest due to the recognized roles of other Sox gene family members in many aspects of *Drosophila* and vertebrate development. In *Drosophila* there are eight Sox genes that exhibit diverse expression patterns in tissues like the brain and central nervous system, salivary gland, hindgut, testes, and ovaries (Cremazy et al., 2001; McKimmie et al., 2005). These gene family members have well defined roles in developmental events such as embryonic segmentation, nervous system specification, hindgut development, and oogenesis (Nambu and Nambu, 1996; Sanchez-Soriano and Russell, 2000; Buescher et al., 2002; Mukherjee et al., 2006). Sox14 is the first member of the family to be implicated in 20E signaling.

Recent work has supported the hypothesis that Sox14 is a key component of the 20E signaling pathway that regulates metamorphosis. Using an RNAi knockdown approach, Sox14 was shown to be required for 20E-mediated programmed cell death in the larval salivary gland and midgut (Chittaranjan et al., 2009). Phenotypic analysis of *sox14* mutant animals revealed a

role for Sox14 in the regulation of pruning of the class IV dendritic arborization neurons in response to 20E at the onset of metamorphosis (Kirilly et al., 2009). These studies support the hypothesis that Sox14 is a critical transcription factor functioning in the 20E genetic hierarchy but do not address the global role of Sox14 in this pathway.

To understand the global role of Sox14 in 20E signaling at the onset of metamorphosis, we generated a loss of function mutation in *sox14*. Phenotypic analysis of homozygous mutant *sox14* animals demonstrated that Sox14 is required for metamorphosis with mutant animals displaying defects in imaginal disc morphogenesis, programmed cell death, and differentiation of adult structures. Similar phenotypes have also been observed in animals that have mutations in known key components of the 20E signaling pathway, supporting the hypothesis that Sox14 is required for the proper timing and expression of key 20E-regulated genes. To identify other signaling pathways that Sox14 regulates, microarray analysis of control and *sox14* mutant animals was performed. This data suggests that Sox14 is required for the proper expression of genes that regulate hormone signaling, metabolism, immunity, and muscle development. Thus, our data supports a broader role for Sox14 in 20E signaling and suggests that understanding its role will provide key insights into how hormones regulate developmental processes.

CHAPTER 2

LITERATURE REVIEW

Drosophila as a Model Organism to Study Steroid Hormone Signaling

Drosophila melanogaster, more commonly known as the fruit fly, has been and continues to be one of the most widely used model organisms of scientists spanning fields varying from genetics to development. This popularity can be attributed to advantages like ease of animal husbandry, excellent visual traits, strong gene conservation, and numerous genetic tools and techniques available to analyze gene function. Pioneering work by Michael Ashburner in *Drosophila* demonstrated the role of steroid hormones in the regulation of transcription and established the fruit fly as a model in which to study classic hormone signaling pathways (Ashburner et al., 1972; Ashburner, 1974; Ashburner and Richards, 1976).

Recent advances in *Drosophila* molecular biology have established the fruit fly as a valuable tool to understand steroid hormone signaling and its impact on important signaling pathways also conserved in vertebrates. Sequencing of the *Drosophila* genome has shown that there are about 15,000 genes in the genome (Friedman and Hughes, 2001). Many of these genes have clear homologues in humans, 1,042 out of 2,753 known human disease genes identified as having a counterpart in *Drosophila* (Fortini et al., 2000; Feany and Bender, 2000). Several good examples of conserved developmental processes between fruit flies and vertebrates include both the dorsoventral (D/V) axes patterning and limb patterning (Holley et al., 1995). Molecular conservation exists as well, an example being the Hedeghog (Hh) signaling pathway in both *Drosophila* and vertebrates (Huangfu and Anderson, 2006). Thus, information gained from studies in the fruit flies can be used to model human disease states as well as to

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extensively study developmental processes common to both of these organisms, making *Drosophila* an ideal organism to use in the investigation of the many parallels between flies and higher organisms.

Drosophila has remained a powerful model system used to study gene function and to link signaling pathways to biological responses because of the many powerful genetic tools that are available and the high number of offspring that can be easily produced. In the fly, males completely lack meiotic recombination, enabling crosses to be performed without meiotic recombination occurring, while recombination in females can be inhibited through the use of balancer chromosomes (Rubin and Lewis, 2000). Balancer chromosomes are chromosomes whose normal sequence is scrambled through multiple breaks and repairs such that they are no longer capable of recombining with their normal homolog. Additionally, balancer chromosomes can be distinguished through dominant marker mutations and recessive mutations, allowing for their transmission to be tracked through generations of progeny. Balancer chromosomes are an extremely reliable tool in segregation analysis and prediction of genotype (Greenspan, 2004). These genetic characteristics allow for efficient and predictable mating schemes as well as for large-scale genetic screens to be performed in *Drosophila.* The mutants generated in these screens allow for scientists to identify genes involved in biological processes and to evaluate gene function. This approach has been referred to as 'forward functional genomics'. It is also possible to carry out reverse genetics in the fruit fly through homologous recombination, during which genes can be deleted and replaced with a known targeting construct (Gong and Golic, 2003).

In addition to loss of function mutations, overexpression of both *Drosophila* and human genes in fruit flies has provided new insight into gene function. This is primarily accomplished using the GAL4-UAS system. The GAL4-UAS system employs the yeast transcriptional activator Gal4 to regulate gene expression by inserting the upstream activating sequence (UAS) that it binds to next to any gene of interest. It is now possible to use one of the many 'enhancer-trap' lines that express GAL4 under the control of various enhancers in numerous cell types and tissues. This system also allows for the tissue specific expression of RNAi molecules that can reduce gene function in both a temporal and spatial manner. By combining loss of function and gain of function genetics, gene function can be more clearly defined and genetic pathways dissected.

Ecdysone Action

Ecdysone

Ecdysone (E), the immediate precursor of 20E, is secreted from the ring gland, the neuroendocrine organ in the fly (Gilbert et al., 2002). E is believed to be inactive, having a minimal to non-existent effect on the classic hormone induced transcriptional response in *Drosophila*. However, preliminary research suggests a role for E in other insects such as *Galleria mellonella, Chironomus tentans,* and *Manduca sexta* (Oberlander, 1972; Clever et al., 1973; Champlin and Truman, 1998). It is possible that E might play a role in the development of various organisms. However, this hypothesis has yet to be fully substantiated in *Drosophila* through molecular evidence (Beckstead et al., 2007). Unlike vertebrates who can synthesize cholesterol *de novo*, *Drosophila* must synthesize ecdysone from a dietary cholesterol or phytosterol (Clark and Block, 1959). Cholesterol is absorbed by the midgut and transferred to

surrounding tissues by a lipoprotein called lipophorin by way of the hemolymph (Soulages and Wells, 1994).

Members of the Cytochrome P450 (CYP) family convert cholesterol to 20E. P450 enzymes are monooxygenases, catalyzing the transfer of one atom of oxygen to a substrate and are found in most living organisms (Werck-Reichhart and Feyereisen, 2000). Five P450 enzymes called the Halloween genes are required for the synthesis of 20E. Mutations in any of these five enzymes, Spook (Spo), Phantom (Phm), Disembodied (Dib), Shadow (Sad), and Shade (Shd), disrupt 20E synthesis and these mutant animals show similar morphogenetic abnormalities in the embryo such as failure of head involution and cuticle formation, ultimately leading to death (Gilbert, 2004). *phm, dib,* and *sad* are all selectively expressed in the prothoracic gland cells of the ring gland, the source of ecdysone. The first step in the conversion process of ecdysone to 20-hydroxyecdysone is converting 7-dehydrocholesterol into ketadiol. This initial step has been dubbed the 'Black Box' due to the mystery surrounding the actual mechanism and how the products of *spo* (Spook) and *spok* (Spookier) function biochemically (Namiki et al., 2005; Ono et al., 2006). Ketadiol (2,22,25-trideoxyecdysone) is then converted to 22,2-dideoxyecdysone (ketotriol), 2-deoxyecdysone, and ecdysone through the products of *phm, dib,* and *sad* respectively. *shd* is expressed in the tissue peripheral to the prothoracic glands where ecdysone is converted to 20E (Rewitz et al., 2006). In *Drosophila* third instar larvae, *shd* is primarily expressed in the fatbody and gut but can also be detected in the Malpighian tubules and the epidermis (Petryk et al., 2003). The Halloween genes are found in most insect families, this conservation emphasizing the role that these genes play in the regulation and synthesis of 20E (Iga and Smagghe, 2010). Further research into how these genes are regulated will provide

insight into the modulation of ecdysteroidogenesis and how this impacts the process of metamorphosis in *Drosophila* (Rewitz et al., 2006). The release of E from the ring gland occurs under the control of the neuropeptide prothoracicotropic hormone (PTTH). PTTH stimulates secretion of E from the ring gland in response to cues like photoperiod, circadian factors, and the nutritional state of the animal (Iga and Smagghe, 2010; Gilbert, 2004). Ablation studies on PTTH indicate that PTTH exerts temporal control throughout an insect's larval stages, determining an animal's final body size by regulating the growth process through its control of ecdysteroid production (McBrayer et al., 2007).

Ashburner Puffing Model

Studies using *Drosophila* larval salivary gland polytene chromosome were the first to demonstrate that steroid hormones function through the regulation of gene expression. Through the use of cultured salivary glands from late third instar larvae, Ashburner and his colleagues were able to accurately reproduce the puffing response of the chromosomes seen in development by adding exogenous 20E (Ashburner, 1972). Based on these observations, they proposed a simple model for the genetic control of these puffing patterns by 20E. According to this model, ecdysone, upon binding to its specified receptor, has three main regulatory effects: 1) it induced the early puffs, 2) repressed some of the later puffs and, 3) repressed the puffs between larval molts. With the addition of cyclohexamide, a protein synthesis inhibitor, the intermolt and early puffs were still induced, classifying them as having a primary response to ecdysone. From this, Ashburner extrapolated that the protein products encoded for by the early puffs likely acted in a regulatory fashion, transducing and amplifying the ecdysone signal by inducing later puffs, called secondary response puffs. These early puff protein products were

also proposed to repress their own expression in a self-regulatory manner (Andres and Thummel, 1992), a discovery made through removing the hormone and observing the early puff genes immediate regression. These pioneer studies described a simple model of classic hormone signaling in the fly that could be studied further to gain a better understanding of genetic regulatory control. Over time, this model has become more complex as transcriptional targets in the hormonal cascade have been identified within the categories of primary or secondary response genes.

Nuclear Receptors – EcR/USP

Nuclear receptors (NR) are "ancient" ligand-regulated transcription factors that bind to and are regulated by small lipophilic compounds such as steroid hormones (i.e. ecdysone), fatty acids, and vitamins. Members of the nuclear receptor superfamily have a highly conserved structure consisting of a DNA-binding domain (DBD) as well as a less conserved C-terminal ligand-binding and dimerization domain (LBD). Nuclear receptors are key in many fundamental biological processes, including both signaling and metabolic pathways, influencing lipid metabolism and homeostasis, sex determination, and circadian rhythm and aging (Palanker et al., 2006; Francis et al., 2003). Additionally, nuclear receptors have been shown to have a strong regulatory role in human development. Through mutations of these receptors, common and lethal disorders such as cancer, diabetes, and heart disease can be linked to NR malfunction (Chawla et al., 2001; Francis et al., 2003). *Drosophila* only has 18 nuclear receptor genes. However, this number is deceptively small considering that these few genes represent all 6 subfamilies of vertebrate receptors (Laudet and Bonneton, 2005). Due to the nuclear receptor subfamily conservation as well as their more simplistic hormone signaling pathways, the fly is

an ideal organism in which to characterize nuclear receptor function and regulation in greater depth.

There have been numerous studies that provide molecular insight into the mechanisms by which nuclear receptors regulate transcription and a more detailed understanding has emerged regarding the way in which nuclear receptors convert a hormonal signal into genetic pathways (Chawla et al., 2001; Francis et al., 2003). Classical nuclear receptors are maintained in the cytoplasm by chaperone proteins. Upon binding to a hormone, a conformational change takes place releasing the chaperones and allowing the NR to translocate to the nucleus to regulate gene expression. Non-classical NRs function as heterodimers with the Retinoid X receptor (RXR) to regulate transcription of target genes. In the absence of the ligand, these receptors are bound to DNA act in a repressive fashion through recruitment of co-repressor complexes. Upon hormone binding, a conformational change takes place resulting in the release of co-repressors and the binding of co-activators to induce transcription (Mangelsdorf and Evans, 1995; Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). The ecdysone receptor (EcR) and Ultraspiracle (USP) are non-classical NRs with vertebrate orthologues consisting of the farnesoid X receptor (FXR) or liver X receptor (LXR) and RXR receptor, respectively. EcR and USP function in a similar way to their vertebrate counterparts, EcR binding 20-hydroxyecdysone (20E) upon heterodimerization with USP (Palanker et al., 2006; Koelle et al., 1991; Yao et al., 1993). Additionally, EcR has been shown to bind 20E alone but fails to optimally bind ecdysone response elements or activate transcriptional targets without the addition of USP (Grad et al., 2001; Grebe et al., 2004). The ecdysone transcriptional cascade is a classic example of nuclear receptor action, ecdysone binding to a nuclear receptor heterodimer

complex to activate the cascade of gene transcription (King-Jones and Thummel, 2005; Woodard et al., 1994; Horner et al., 1995).

Many nuclear receptors, including EcR, exist as functionally distinct isoforms that that are expressed in specific patterns and have different activation profiles (Hodin et al., 1989; Zelent et al., 1991; Giangrande and McDonnell, 1999; Flouriot et al., 2000; Mollard et al., 2000). In *Drosophila*, there are three EcR isoforms: EcR-B1, EcR-B2, and EcR-A, which can all actively bind USP (Koelle, 1992). These isoforms are derived from a single structural gene but differ in their N-terminal region due to alternative splicing and different promotor usage (Talbot et al., 1993). Genetic studies have indicated that isoforms have overlapping and/or distinct functions during fly development. Mutations that impair only one EcR isoform have distinct lethal phases in fly development as compared to loss of all isoforms (Bender et al., 1997; Schubiger et al., 1998). Studies aimed at understanding EcR isoform specific activity will provide new insight into 20E signaling pathways and how 20E specificity is established.

Unlike EcR, USP encodes for only one isoform with its primary role being the facilitation of EcR's DNA and ligand binding activities (Riddiford et al., 2000; Hu et al., 2003; Clayton et al., 2001; Buszczak and Segraves, 1998). Consistent with vertebrate RXR heterodimers that function as repressors in the unliganded state, genetic studies have revealed that USP can also function as a repressor (Zhang and Dufau, 2004). One such study involved the removal of USP from *Drosophila* wing imaginal discs, causing the premature activation of 20E-regulated target genes (Schubiger and Truman, 2000). This, as well as similar research, suggests that USP represses gene activity *in vivo* when ecdysteroid levels are low, preventing premature maturation as the animal goes through metamorphosis. Though USP is known to form a heterodimer with EcR, it

has also been suggested that *usp* does not appear to be required cell-autonomously for *Drosophila* metamorphosis and that EcR and USP may be able to interact with other nuclear receptors as well to control developmental processes. Studies have suggested that USP may also act as a juvenile hormone (JH) receptor capable of modulating EcR/USP complex activity (Oro et al., 1992; Buszczak and Segraves, 1998). Despite these recent postulations, the classic hormone transcriptional pathway that directs many known biological processes in a maturing organism works through the EcR/USP heterodimer.

Ecdysone Regulation of Drosophila Development

Steroid hormones are integral in the regulation of metabolism, reproduction, and development of higher eukaryotes, this regulatory function conserved in organisms as divergent as insects and humans (Gorbman et al., 1983). The primary steroid hormone 20-hydroxyecdysone (20E) has been shown to act as a temporal signal to coordinate cell and tissue-specific morphogenetic movements in *Drosophila*, including both molting and metamorphosis, triggering key developmental transitions throughout the embryonic, larval, prepupal, and pupal stages (Riddiford, 1993). The timing and titer of ecdysone pulses as the fly transitions between the different developmental stages to eventually eclose as a mature animal is instrumental to the proper body formation of a healthy and viable adult organism. Disruption of 20E pulses during the *Drosophila* life cycle can have drastic effects on the developing organism, affecting timing as well as adult structure morphagenesis.

A high titer pulse of 20E at the end of the third larval instar during the *Drosophila* life cycle drives the process of metamorphosis, through which larval tissues undergo programmed cell death and are replaced by adult structures that develop from discrete clusters of imaginal

progenitor cells, each imaginal disc destined ultimately to become a part of the adult body plan (Richards, 1981; Andres and Thummel, 1992). Examples of this can be seen in a variety of tissues occurring in synchrony with a rise in the ecdysone titer. Larval muscles undergo histolysis in the anterior portion of the prepupa, coinciding with the appearance of adult wing myocytes. During this time there are also changes in gut structure including the retraction of the gastric caecae and the proliferation of adult midgut cells. The larval salivary glands undergo histolysis while rudimentary adult structures initiate formation (Robertson, 1936; Bodenstein, 1965). Consistent with the flies' drastic body plan change during metamorphosis, programmed autophagy and apoptosis have been implicated as being under the regulation of ecdysone. Additionally, neuronal processes, metabolism functions, immunity, and muscle development have also been proposed to be influenced by 20E and the various target genes within the pathway (Changan et al., 1997; Yin and Thummel, 2005; Kirilly et al., 2009). Based on the broad spectrum of developmental processed already known to be 20E regulated, there is a high likelihood of discovering novel pathways in which 20E plays a role, directly or indirectly. A critical goal in research surrounding this hormonal cascade is to understand how this systemic 20E signal affects such a wide range of behavioral and physiological responses in the developing organism.

Ecdysone Signaling Cascade

The Ashburner puffing model provided novel insight into steroid hormone signaling and how 20E elicits a transcriptional response that dictates developmental transitions in *Drosophila* (Ashburner, 1972). This model has been expanded since its initial postulation through the discovery of the 20E receptor complex and cloning of early and late puff genes (Riddiford et al.,

2000; Andres et al., 1993). Key puff genes that have been identified include regulatory genes such as *EcR, usp, BR-C, E74A, E75A, DHR3,* and *FTZ-F1.* Molecular studies have demonstrated that 20E, acting through the EcR/USP receptor, directly induces *E74A* and *E75A* transcription. The protein products of these genes repress their own express and are required for the upregulation of early late genes such as *DHR3*. *DHR3* then induces *FTZ-F1* expression in the mid-prepupae stage (Baehrecke, 1996). Flies lacking function in any of these genes show misexpression of other 20E-regulated genes, highlighting their role in the Ashburner model of 20E signaling (Emery et al., 1994; Fletcher and Thummel, 1995). One observation regarding the 20E cascade and the primary response and secondary response genes involved is the similarity of the mutant phenotypes. This is clearly seen in *EcR, DHR3,* and *FTZ-F1* mutants, all of which show lethality at the prepupal to pupal transition and are defective in head eversion and gas bubble translocation (Bender et al., 1997; Lam et al., 1997; Yamada et al., 2000). All of these genes can be shown to faithfully reproduce the temporal expression patterns of 20E-regulated genes at the onset of metamorphosis through microarray screens and suggest that other genes that are regulated in the same manner may participate in 20E signaling (Beckstead et al., 2005; Arbeitman et al., 2002; Li and White, 2003; White et al., 1999). One gene identified in this way as a 20E-regulated gene within the hormonal transcriptional cascade is Sox14 (Beckstead et al., 2005). Identification and genetic characterization of these genes regulated by 20E in this pathway will serve to increase our understanding of 20E action in insect maturation.

Sox Gene Family

Sox box genes regulation of transcription

The cells within multicellular organisms are constantly undergoing lineage commitment, differentiation, proliferation, and death. Through this ongoing process, proper development of the organism is maintained. The decision made by cells to switch between states was once thought to be under the control of a master transcription factor. The current data suggests that several families of regulatory proteins function together to direct most developmental processes (Prior and Walter, 1996; Lefebvre et al., 2007). One such group is the Sox gene family, named for its shared HMG-like motif called the SRY box, a region showing homology to the DNA-binding domain of SRY, the mammalian sex determining gene (Gubbay et al., 1990; Sinclair et al., 1990). Sox proteins have 50% or higher similarity to the SOX domain of the Sry protein. Sox genes were originally thought to only exist in metazoans, but with the identification of Sox-like genes in the unicellular choanoflagellate, the data suggests that Sox protein origin predates the origin of multicellularity (King et al., 2008). The Sox gene family can be divided into eight groups designated SoxA through SoxH, with two B subgroups, B1 and B2. Proteins within the same group usually exhibit approximately 70% or greater amino acid similarity with one another. Sox proteins from different groups share only partial identity in the SOX domain and none outside of it.

Sox proteins contain properties of both classical transcription factors as well as architectural components of chromatin. The SOX domain is made up of about 80 residues twisted into an L-shape, containing three alpha helices and an N-terminal beta strand (Weiss, 2001). This unique shape allows for the SOX domain to bind DNA in the minor groove and

induces a bend in the DNA helix between 80° and 135° (Ferrari et al., 1992). The ability of SOX proteins to bend DNA gives this family a 'structural' mode of action, which is thought to promote various interactions amongst transcription factors, bound at different locations in promoter/enhancer regions (Grosschedl et al., 1994; Pevny and Lovell-Badge, 1997).

The SOX domain binds DNA at the common hexameric AACAAT sequence (Giese et al., 1992). Because this sequence is found randomly throughout the genome it is predicted that binding may be determined by the cooperation of various interacting proteins that bind DNA within proximity of the Sox binding sequence. Each Sox-partner pair governs a specific group of regulatory target genes responsible for directing different developmental processes (Kondoh and Kamachi, 2010). An example of this specificity is SOX2 interaction with PAX6, the pairing of which can specify the retina or lens in human eye development. The synergistic activity of this pairing activates *delta-crystallin* expression to elicit lens placodal development (Kamachi et al., 2001). The same Sox gene can regulate different sets of target genes in a cell type-specific manner dependent upon its partner(s) (Kamachi et al., 2000). We see this partner-specificity in the vertebrate Sox2 that is capable of binding with OCT 3/4, BRN2, and PAX6, each interaction resulting in different cell-type specification (Kondoh and Kamachi, 2010). It has also been suggested that while the SOX domain is required for protein-protein interactions, sequences outside of the SOX domain act to stabilize protein binding and promote binding specificity (Wilson and Koopman, 2002). Further identification of Sox protein binding partners will provide insight into how Sox family members can regulate various developmental pathways.

Sox box genes role in other organisms' development

Sox genes are best known for their roles in sex determination, gastrulation, nervous system development, chondrogenesis, lens development, and haemopoiesis. There have been 20 Sox proteins identified in both mouse and human. Besides the mouse model, analysis of many other model organisms including chicken, *Drosophila*, Xenopus, and Zebrafish exemplify the degree of conservation within the Sox gene family. The chicken in particular has become a useful model for studying various developmental processes. It is an ideal system to use to study both eye and cardiovascular development throughout the stages, especially later in development (Maschhoff et al., 2003). It is easy to visualize the various stages of chick development, allowing for parallels between chick maturation and that of other organisms to be investigated.

The chicken model shows great potential in the study of Sox genes due to its evolutionary conservation with humans. The chromosome location of the eight *SOX* family genes in chickens was determined by fluorescence in situ hybridization. This research demonstrated that these *SOX* genes form at least three clusters on chicken chromosomes. *SOX1, SOX2, SOX3, SOX5, SOX10, SOX14,* and *SOX21* are localized to chromosome segments with known homologies to human chromosomes. This is highly suggestive of the degree of conservation between chickens and humans (Kuroiwa et al., 2002). Studies done with the chicken δ1-*crystallin* illustrates the conserved functional regulatory elements present in both chicken and fruit fly genomes. δ1-*crystallin* is one of the best-characterized Crystallin genes, its lens-specific regulation controlled by the cooperative binding of the PAX6 and SOX2 transcription factors to the DC5 fragment present in the gene itself. Upon introduction of the DC5 fragment from the chicken into the fruit fly, there is cooperative binding of D-PAX2 and

SOXN (homologous to chicken PAX6 and SOX2 respectively) to regulate Crystallin secretion in the cone cells of the *Drosophila* eye (Bianco et al., 2005). This study, as well as others like it, demonstrates the advantages that studying biological and developmental processes in one organism can have on another. Using these different model organisms will help to elucidate the roles of transcription factors and Sox Box proteins in disease states and developmental processes (Chew and Gallo, 2009).

Sry, the decisive factor for male sex determination is the prototype of the entire Sox protein family (Wegner, 1999). It has been proposed that Sry evolved from its putative ancestor, Sox3. Additionally, Sox9 shows expression in the genital ridge and is proposed to function as a critical Sertoli cell differentiation factor (Kent et al., 1996; daSilva et al., 1996). Quail and duck *Sox9* expression patterns also serve to highlight gene conservation amongst Sox proteins within and between species. *Sox9* acts in much the same way as *Sry* does in mammals, associated with testis differentiation and sex determination in avian species (Takada et al., 2006). This defines a role for Sox genes in vertebrate testis formation and sex determination.

Sox proteins also play many roles in early embyrogenesis. The group B protein Sox2 is transiently expressed in the inner cell mass and epiblast of mouse blastocysts as well as later in the formation of the neuroepithelium (Collignon et al., 1996; Yuan et al., 1995). Mouse Sox2 is similar to the *Drosophila* Dichaete, showing a 42% sequence identity on the amino acid level. Additionally, biochemical properties indicate conservation, both proteins showing similar expression patterns in their respective organisms (Nambu and Nambu, 1996; Russell et al., 1996). Another example is *Xenopus* proteins, Xsox17α and Xsox17β, both proteins showing sequence similarity to the group F mouse protein Sox17. The expression of both proteins is

endoderm specific and remains as such throughout early development up until the tailbud stages (Hudson et al., 1997).

Sox proteins have been strongly implicated in neural development as well as neural crest development. In *Xenopus* embryos, SoxD expression is found in the prospective ectoderm and has been shown through research to be an essential mediator of neuralization pathways (Mizuseki et al., 1998). Sox2 has also been found to play a role in neural development not only in *Xenopus* but also in the chicken and the mouse. It is believed to be one of the earliest pan-neuronal markers, strengthening neural cell fate and assisting cells in their specificity (Uwanogho et al., 1995; Collignon et al., 1996; Mizuseki et al., 1998; Rex et al., 1997). Similar to the mouse, chicken *Sox4* is highly expressed in the developing nervous system and is implicated in craniofacial development (Maschhoff et al., 2003). Group B and Group C Sox proteins also show overlapping expression patterns and may function redundantly in the developing central and peripheral nervous system (Kuhlbrodt et al., 1998). A Group E protein, Sox10, shows strong expression in the neural crest, contributing to the formation of the both the peripheral and central nervous system (Kuhlbrodt et al., 1998). Mutations in one SOX10 allele in humans confer a congenital aganglionic megacolon (Hirschsprung disease) associated with a combination of pigmentation defects and deafness (Waardenburg syndrome) (Kuhlbrodt et al., 1998; Pingault et al., 1998).

Sox9, a Group E protein previously linked to sex determination, impacts chondrogenesis. The mouse Sox9 expression pattern implicates Sox9 in the developing brain, optic vesicle, urogenital system, lung, and heart (Ng et al., 1997; Wright et al., 1995). Sox9 is pivotal to chondrocyte formation, showing strong expression in the mesenchymal condensations from

which the skeleton develops. In humans, a mutation in one SOX9 allele causes autosomal sex reversal in male individuals. All carriers suffer from severe skeletal malformations as well as possible heart and renal malformations, the absence of olfactory bulbs, defects of the tracheo-pulmonary system, deafness, and mental retardation. This autosomal dominant condition is known as campemelic dysplasia and it affects about 1 in 20,000 births (Foster et al., 1994; Wagner et al., 1994).

The processes of lens development and haemopoiesis are also under the influence of Sox proteins. In chickens, Sox1, Sox2, and Sox3 are co-expressed during chick lens development. In the mouse model, Sox1 is the predominant protein in the developing lens, with Sox2 having little involvement and Sox3 having none (Kamachi et al., 1998). Sox4, a mouse Sox protein, shows widespread expression in the brain, gonads, lung, heart, and thymus. It has also been shown to be an active transcription factor in lymohocytes of B- and T-cell lineages (van de Wetering et al., 1993). Through targeted deletion studies in the mouse, Sox4 has been shown to be essential to the developing circulatory system and cardiac structures and necessary for B-cell development (Schilham et al., 1996).

Sox box genes role in Drosophila development

The *Drosophila* genome encodes eight Sox genes, four genes showing homology to Group B and four genes showing homology to Groups C-F (Clark et al., 2007). As in vertebrate development, Sox genes in the fly are thought to act as key regulators of early developmental events and in the differentiation and proliferation of numerous cell types (Cremazy et al., 2001). Genes representative of all of the groups have also been shown to be involved in

processes such as neurogenesis, dorsal-ventral patterning, and segmentation, processes conserved between vertebrates and invertebrates (Wilson and Dearden, 2008).

In *Drosophila* there are four Group B genes: *SoxNeuro* (*SoxN*), *Dichaete* (*D*), *Sox21a*, and *Sox21b*. Similar to vertebrates, these Group B genes have established roles in the specification and development of the central nervous system. In 1915, the discovery of the wing and bristle mutation, *Dichaete*, was later identified as one of the first known Sox genes (Bridges and Morgan, 1923). *Dichaete Sox* shows an expression pattern in the female germline (Mukherjee et al., 2006), is required for hindgut morphogenesis (Sanchez-Soriano and Russell, 2000), and is widely expressed in the developing CNS. *SoxN* is also dynamically expressed during embryogenesis, first expressed in all cells of the neurogenic region and then in just the delaminated neuroblasts (Cremazy et al., 2000). Additional roles for both *Dichaete* and *SoxN* have been identified in larval cuticle patterning (Chao et al., 2007; Overton et al., 2007) and can sometimes play partially redundant roles in development, compensating for one another (Overton et al., 2007). *Sox21a* shows expression in the foregut and hindgut and *Sox21b* shows expression in the hindgut as well in a segmented pattern running along the ventral epidermis (Cremazy et al., 2001; McKimmie et al., 2005).

The other Sox family members include Groups C-F, Sox14 identified as being the only member of the Group C subfamily. It shows ubiquitous expression throughout embryogenesis and gut/digestive system expression during the larval/pupal stages (Cremazy et al., 2001; Chintapelli et al., 2007). Group D includes *Sox102F,* which shows expression late in embryogenesis in the brain as well as cells in the ventral CNS (Cremazy et al., 2001). Inducible RNAi studies resulted in *Sox102F* phenotypes showing disruptions in CNS morphology, implying

a role in glial lineages (Phochanukul and Russell, 2009). Additionally, *Sox102F* is also found in the posterior mesodermal cells that contribute to the gonad. Consistent with this, null *Sox102F* mutants show an almost complete failure in testis morphogenesis during the pupal stages. These mutants show no embryonic or larval phenotype abnormalities but die as pharate adults, attributed to defects in fatbody morphogenesis (Nanda et al., 2009). Group E includes *Drosophila Sox100B* and is dynamically expressed during embryogenesis in the gut, Malpighian tubules (analogous to the vertebrate kidney), anal pads, and gonads (Loh and Russell, 2000). *Sox15* is the sole *Drosophila* Group F gene. It shows expression in a subset of the embryonic PNS as well as the proximal portion of the third larval instar leg and wing imaginal discs (Cremazy et al., 2001). Functional studies have shown that mutants null for this gene die as pupae, this gene required in adult mechanoreceptor socket cells and hair/bristle formation (Miller et al., 2009). Based on expression and mutational analysis *Drosophila* Sox genes participate in the regulation of most developmental processes making Drosophila a good model organism to understand their molecular regulation.

In order to identify genes regulated by Sox family members several genome-wide techniques like ChIP, ChIP-seq, DamID, and microarrays have been employed. These techniques have proven themselves useful in the analysis of *in vivo* binding locations of Sox proteins and provide key information into the pathways they regulate (Girard et al., 2006). As research using invertebrate models continues, the role of the Sox family will be defined more clearly and expanded upon and the impact that Sox genes have on insect metamorphosis as well as vertebrate development will be better understood.

Sox Group C, Sox14, and its homologues in other organisms

Drosophila Sox14 is a member of the SOX Group C genes and appears to have similar developmental roles as its vertebrate homologues. In the mouse, both Sox4 and Sox11 show expression in the oligodendrocyte precursors in the developing central nervous system. A gain of function study done in the mouse suggests that these genes function in maintaining cells of the oligodendrocyte lineage in an immature state, playing a role in when the onset of oligodendrocyte differentiation begins (Potzner et al., 2007). *sox14* also shows homology to two human Sox genes, Sox11 and Sox22. However, much less is known about the how these transcription factors function in humans and what role they may play in developmental processes. This lack of current research highlights the importance of Sox14 studies in other organisms that show homology. Research studying Sox Group C genes in organisms such as the fruit fly will be able to shed light on the potential roles for homologous Sox Group C genes, Sox11 and 22, in human development and disease states. The *Drosophila Sox14* gene and its homologues in other known sequenced insects representative of Sox Group C show ubiquitous embryonic expression and during gut morphogenesis in larval, pupal, and adult animals (Cremazy et al., 2001; Chintapalli et al., 2007). The honeybee orthologue of *Drosophila sox14, amSoxC,* shows ubiquitous expression in late embryonic stages as well as in the adult brain (Wilson and Dearden, 2008). This is interesting given the role that *Drosophila sox14* plays in dendrite pruning in the larval brain and its role in the vertebrate nervous system (Kirilly et al., 2009; Cremazy et al., 2000). A single gene occurring in the *C. elegans* genome has also been identified as being part of Sox Group C. Research has associated this gene with the *sem-2* locus, a mutation that affects different aspects of embryonic muscle development (Bieri et al., 2007).

Sox Group C genes have also been identified in more obscure organism such as the coral, *A. millepora,* and the sea anemone. Coral's *AmSoxC* is zygotically expressed in the ectodermal region, later contributing to the sensory neurons. This expression pattern is similar for the sea anemone's *NvSoxC* gene, providing another example of Sox Box gene conservation among lower metazoans (Shinzato et al., 2008). Research in organisms showing homology to Sox14 allows for comparative studies between previously observed Sox Group C genes expression profiles and the pathways in which the proteins function to that of the *Drosophila* Sox Group C.

Sox14 was identified through microarray screens and organ culture experiments as being EcR-dependent and 20E-regulated, altering its expression pattern in synchrony with the pulses of 20E occurring throughout development. Sox14 acts as a primary-response/target gene within this signaling pathway (Beckstead et al., 2005). These experiments definitively classified Sox14 as being a primary target gene within the 20E EcR-mediated hormonal cascade and are the first experiments to link a Sox Group C family member to steroid hormones signaling.

Within the past year, two studies have been conducted that have shed light on the role Sox14 is playing in fruit fly development. Through cell viability, cell proliferation, and apoptosis assays, Sox14 was identified within a set of pro-death related genes. These studies demonstrated that overexpression of Sox14 reduced cell viability and induced apoptosis in cell culture. Sox14 expression was also analyzed *in vivo* using a GAL4 driver to ubiquitously express Sox14 dsRNA, mutant animals displaying three distinct lethal phenotypes during pupariation with defects in tracheal formation, distorted folding, collapsed and blackening of the cuticle, and persistent larval midguts and salivary glands. Thus, a reduction in Sox14 levels result in inhibition or a severe delay of midgut and salivary gland cell death, functioning as a positive

regulator of this process during the larval to pupal transition (Chittaranjan et al., 2009). This study establishes a role for Sox14 in *Drosophila* programmed cell death and implicates its function as a regulator of already known 20E-mediated pathways.

During *Drosophila* metamorphosis, the class IV dendritic arborization neurons (ddaC) undergo pruning to remove larval dendrites in preparation for adult structure formation. These larval neurons go through extensive remodeling by apoptosis but survive to form the adult nervous system before eclosion at the end of the fruit fly life cycle. 20E regulates this pruning event in the larval dendrites as well as the apoptosis of select larval neurons, acting via the 20E transcriptional hierarchy. A recent study investigated the role Sox14 plays within this cascade and has linked this gene to the neuronal remodeling event. Sox14 was found to be both necessary and sufficient to induce dendrite severing in the pruning process, mediating the event by promoting the expression of its target gene, *Mical* (Kirilly et al., 2009). This research not only redefines Sox14 as a primary target gene in the 20E cascade but also defines its regulatory role in developmental events needed for proper metamorphosis.

Both of the studies performed within the last year have begun to characterize a role for Sox14 in several *Drosophila* developmental processes already known to be 20E-mediated. These studies serve to reinforce our own data as well as demonstrate additional roles for Sox14 outside the scope of our study. Sox14 functioning as a pro-death gene is consistent with a known role for 20E in the induction of programmed cell death, autophagy, and apoptosis (Chittaranjan et al., 2009). The association identified between Sox14 and Mical is not surprising given Sox14's known action as a transcription factors and its likely interaction with other proteins in addition to those currently identified. Additionally, Sox14's position within the

proposed two-step hierarchy as a primary target gene suggests that Mical might be only one of many potential target genes that Sox14 acts on to regulate many different developmental events.

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CHAPTER 3

SOX14 IS REQUIRED FOR TRANSCRIPTIONAL AND DEVELOPMENTAL RESPONSES TO 20-HYDROXYECDYSONE AT THE ONSET OF *DROSOPHILA* METAMORPHOSIS[1]

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Abstract

The steroid hormone 20-hydroxyecdysone (20E), by means of a heterodimer consisting of two nuclear receptors, the Ecdysone receptor (EcR) and Ultraspiracle (Usp), triggers the major developmental transitions in the Drosophila life cycle through the regulation of genetic hierarchies. We have previously demonstrated that the Sox14 transcription factor is a primary response gene to 20E/EcR/Usp complex. In this study, we show that mutations in sox14 result in prepupal and pupal lethality with animals displaying a multitude of defects in 20E developmentally regulated pathways. In addition, through Northern blot and microarray analyses of sox14 mutant animals, we demonstrate that Sox14 is required for the proper expression of 20E- and non–20E-regulated genes at the onset of metamorphosis. We also show that the Sox14-regulated gene set correlates well with Sox14 expression in a variety of larval and adult tissues. Thus, Sox14 is a critical transcription factor required for 20E signaling at the onset of metamorphosis.

Key words: Drosophila; Sox14; EcR; ecdysone; metamorphosis

Introduction

Steroid hormones control a wide range of biological pathways that occur in higher organisms, such as cellular differentiation and development. Members of the nuclear receptor superfamily act as hormone-responsive transcription factors that transduce hormone signals into tissue-specific changes in gene expression. Extensive studies have elucidated many of the molecular mechanisms underlying nuclear recep- tor-regulated transcription but how these changes in gene activity lead to the appropriate biological response during development is still not fully understood.

Drosophila melanogaster provide an ideal system to link steroid hormone signaling to specific developmental processes. Pulses of the steroid hormone, 20-hydroxy-ecdysone (20E), the biologically active form of ecdysone, function as critical temporal signals that direct each of the major develop- mental transitions in the Drosophila life cycle (Clever, 1964). The 20E drives the morphogenetic movements that form the larvae during embryo- genesis (Kozlova and Thummel, 2003). A pulse of 20E during the first and sec- ond larval instars initiate molting and allow the larvae to grow, while a high titer pulse of 20E at the end of the third-instar triggers puparium forma- tion, signaling the onset of metamorphosis and the beginning of prepupal development. At 10 hr post pupariation, the prepupal pulse of 20E ini- tiates head eversion, marking the prepupal-to-pupal transition and the beginning of the differentiation of adult tissues (Thummel, 2001). This single steroid hormone acts in a stage- specific manner to elicit different biological responses at each developmental stage. Emphasis has been focused on the early stages of metamorphosis where 20E drives the total reorganization of the body plan resulting in the majority of the larval tissues being destroyed by programmed cell death and the

formation of adult structures from clusters of adult pro- genitor cells called imaginal discs (von Kalm et al., 1995; Yin and Thummel, 2005).

An early understanding of the molecular mechanisms of 20E action in insects was derived from the characterization of puffing patterns of the giant larval salivary gland polytene chromosomes (Becker, 1959; Clever, 1964; Ashburner, 1972, 1974; Ash- burner et al., 1974). These studies used an organ culture system that allows ectopic treatment with hormone and cycloheximide, a protein synthesis inhibitor, to distinguish between primary and secondary responses to 20E. Results from these studies suggested that in the salivary gland, 20E induces approximately six early puff genes (primary-response). The protein products encoded by these genes were proposed to repress their own expression and induce numerous late puff genes (secondary-response). It was assumed that the protein products encoded by the late puff genes would carry out the biological responses to the hormone. This proposed 20E two-step genetic regulatory cascade has been supported and extended by identification and characterization of a 20E receptor, along with several early and late puff genes. Like vertebrate steroid hormones, 20E directly mediates its effects on gene expression by binding to a nuclear receptor heterodimer, which consist of the Ecdysone receptor (EcR) and Ultraspiracle (Usp)- orthologs of the vertebrate LXR and RXR receptors respectively (Koelle et al., 1991; DiBello et al., 1991; Thomas et al., 1993; Yao et al., 1993).

Toward identifying and linking the genomic EcR/Usp/20E-transcriptional cascade to biological responses regulated by the hormone and its receptor, several microarray experiments were performed to identify direct targets of the 20E/EcR/Usp nuclear receptor complex (Beckstead et al., 2005). Through these studies, the transcription factor sox14 was

identified as 20E-regulated, EcR-dependent gene that was up-regulated in synchrony with both the late third-instar larval and prepupal pulses of 20E. Thus, sox14 behaves as other known 20E primary-response genes. The identification of Sox14 was of great interest due to the recognized roles of other Sox gene family members in many aspects of Drosophila and vertebrate development. In Drosophila there are eight Sox genes that exhibit diverse expression patterns in tissues like the brain and central nervous system, salivary gland, hindgut, testes, and ovaries (Cremazy et al., 2001; McKimmie et al., 2005). These gene family members have well defined roles in developmental events such as embryonic segmentation, nervous system specification, hindgut development, and oogenesis (Nambu and Nambu, 1996; Sanchez-Soriano and Russell, 2000; Buescher et al., 2002; Mukherjee et al., 2006).

Recent work has supported the hypothesis that Sox14 is a key component of the 20E signaling pathway that regulates metamorphosis. Using an RNAi knockdown approach, Sox14 was shown to be required for 20E- mediated programmed cell death in both the larval midgut and salivary gland thus defining it as a pro-apoptotic gene (Chittaranjan et al., 2009). Phenotypic analysis of sox14 mutant animals has also revealed a role for Sox14 in the regulation of pruning of the class IV dendritic arborization neurons in response to 20E at the onset of metamorphosis (Kirilly et al., 2009). These studies support the hypothesis that Sox14 is a critical transcription factor in the 20E genetic hierarchy.

Here, we analyze the global role of Sox14 at the onset of metamorphosis. We observe that mutations in sox14 result in defects in metamorphosis with all animals failing to eclose. These phenotypes are consistent with the widespread expression pattern of Sox14 and its requirement for the proper timing and expression of key 20E-regulated genes. Microarray

analysis of sox14 mutant animals suggests that Sox14 participates in both EcR- dependent and -independent genetic pathways and regulates key genes in hormone signaling, metabolism, immunity, and muscle development.

Results

Loss-of-Function Mutations in sox14 Are Lethal

To test the effects of loss of sox14 expression throughout development, we generated a deletion in the sox14 gene using ends-out homologous recombination (Gong and Golic, 2003). As shown in Figure 1A, upon recombination between the *sox14* locus and the targeting construct, exon 1 and 400 bp of the upstream promoter sequence was deleted and replaced by the *mini-white* gene. Four targeting events were identified and subject to further genetic and molecular characterization. Because each targeted insertion results in the same genomic deletion/insertion, we chose to focus our analysis on one insertion event that we named $sox14^{L1}$.

To characterize the nature of the $sox14^{L1}$ insertion/deletion, long-range polymerase chain reaction (PCR) using a primer set that hybridizes to sequences outside of the region of homology used in the targeting construct was performed to amplify the *sox14* genomic region in both the w^{1118}; +/+ control and w^{1118}; $sox14^{L1}/sox14^{L1}$ background. This PCR reaction amplifies a 9,732 base pair fragment from the control genomic DNA and a 12,928 base pair fragment in the $sox14^{L1}$ mutant genomic DNA (Fig. 1B). Insertion of the *mini-white* gene in the *sox14* locus results in four *SacI* sites in the amplified PCR fragment as compared to two found in the w^{1118} control PCR fragment. As seen in Figure 1A,B, PCR products of the predicted size and expected restriction patterns were observed for both the w^{1118} and $sox14^{L1}$ DNA, confirming the

targeting of the *sox14* locus. Sequence analysis of the $sox14^{L1}$ PCR fragment confirmed the PCR and restriction analysis results (data not shown).

To test the effect of the $sox14^{L1}$ mutation on *sox14* mRNA expression, Northern blot analysis was performed using a full-length sox14 cDNA probe on RNA isolated from w^{1118}; +/+ control and w^{1118}; $sox14^{L1}/sox14^{L1}$ animals that were staged at pupariation. As seen in Figure 1C, no full-length *sox14* transcript was detected in the mutant animals, thus suggesting that $sox14^{L1}$ will behave as a strong loss- of-function mutation. Of interest, two low molecular weight transcripts were observed in the w^{1118}; $sox14^{L1}/sox14^{L1}$ background when the Northern blot was overexposed (Fig. 1C). Using a DNA probe consisting of the first exon and a DNA probe to the second exon of *sox14*, we determined that this transcript corresponds to expression of exon 2 (data not shown). It is possible that these transcripts could encode for a truncated Sox14 protein consisting of the HMG domain and thereby change the nature of the $sox14^{L1}$ mutant animals, see Figure 4A.

To determine the effect of a loss of *sox14* on Drosophila development, lethal phase analysis was performed on w^{1118}; sox^{L1}*/CyO, kr-GFP*, w^{1118}; $sox14^{L1}/sox14^{L1}$, and w^{1118}; $sox14^{L1}$/ *Df(2R)BSC136* (*Df*) animals. Mutant animals were detected by the absence of GFP. As compared to the $sox14^{L1}$*/ CyO, kr-GFP* animals, we observed that the $sox14^{L1}/sox14^{L1}$ mutant animals pupariated 97% of the time and $sox14^{L1}$*/Df* mutant animals pupariated 94% (n = 319 and 217, respectively). All $sox14^{L1}/sox14^{L1}$ and $sox14^{L1}$*/Df* mutant animals died before eclosion. To further analyze the role of sox14 during metamorphosis, we determined the stage of lethality during metamorphosis for both $sox14^{L1}/sox14^{L1}$ and $sox14^{L1}$*/Df* mutant animals. As shown in Figure 2, $sox14^{L1}/sox14^{L1}$ and $sox14^{L1}$*/Df* animals died either as early prepupae (37% and 41%),

with clear morphological defects and displayed defects in anterior spiracle eversion, as pupae (26% and 26%) that were often cryptocephalic with a failure of the legs and wings to elongate properly and in many cases a failure to tan, or pharate adults (37% and 33%) which display similar phenotypes as the earlier stages in addition to a pinhead phenotype. In the majority of the cases, terminal differentiation of the abdomen was not observed. Internally, we also observed a similar delay in the destruction of the larval midgut and salivary glands as reported by the sox14 RNAi experiments (Chittaranjan et al., 2009). Thus, loss of *sox14* results in defects in several 20E-mediated events. To confirm that the phenotype that we observed is due to loss of *sox14*, we tested the ability of an 8,080 base pair construct that contained the *sox14* gene and 2kb of upstream sequence, presumably containing key enhancers, to rescue the mutant animals. This construct was targeted using PhiC31-mediated trangenesis to insert at cytological location 22A3 containing the *PBac{yellow[þ]- attP-3B}VK00037*. Insertion of the *sox14* genomic rescue construct to the left arm of the second chromosome ensured that the rescue construct was a similar distance from the telomere, thus placing the genomic rescue construct in a similar genomic context as the endogenous *sox14* gene. The *sox14* genomic rescue construct was recombined onto the second chromosome containing the $sox14^{L1}$ allele to generate the $sox14^{L1}$ rescue line. Inclusion of the *sox14* genomic construct completely rescued the $sox14^{L1}/sox14^{L1}$ and $sox14^{L1}/Df$ lethal phenotype. Thus, it appears that the allele that we engineered behaves as a strong loss-of-function mutation and suggests that *sox14* is playing a critical role at the onset of metamorphosis, but that zygotic expression of *sox14* is not required for developmental stages before metamorphosis.

Sox14 Is Required for Proper Expression of 20E-Regulated Genes

Loss-of-function analysis of *sox14* mutants along with previously published microarray analysis suggests that Sox14 may play a key role in the regulation of the 20E-genetic hierarchy at the onset of metamorphosis (Beckstead et al., 2005; Chittaranjan et al., 2009; Kirilly et al., 2009). To determine if this is the case, Northern blot analysis was performed on RNA isolated from whole animals staged at - 18, - 4, 0, 2, 4, 6, 8, 10, 12, and 14 hr relative to pupariation in both w^{1118} control and w^{1118}; $sox14^{L1}/sox14^{L1}$ animals. This time period allows the assessment of genes regulated by the late larval and prepupal pulses of 20E as well as the expression of *EcR* and *usp*. As shown in Figure 3, the *EcR* transcripts are expressed at wild-type levels in the sox14 mutant animals, while the *usp* transcript is significantly reduced. Although reduced expression of *usp* would suggest a disruption of the 20E-genetic hierarchy regulated by the EcR/Usp/20E nuclear receptor complex, we observed that the 20E-primary response genes *E74* and *E75* show proper induction in response to the late-larval pulse of 20E. However, down-regulation of both *E74* and *E75* fails to occur until the 6-hr times point in the $sox14^{L1}$ mutant animals. This 2- hr delay in expression is also observed in *ImpL1*, a gene that is expressed exclusively in imaginal discs. In agreement with previously published real-time PCR data (Chittaranjan et al., 2009), all *BR-C* transcripts showed a slight up-regulation at 0 and 2 hr after pupariation when Sox14 function is lost. Additionally we observed premature down-regulation of the Z2 and Z3 isoforms at 4 hr and a severe reduction of the Z1 isoform at 14 hr after pupariation in the $sox14^{L1}/sox14^{L1}$ animals. Thus, loss of *sox14* expression results in numerous defects in both the timing and level of expression of key 20E- regulated genes.

Sox14 Protein Is Expressed in a Dynamic Pattern

Previous published in situ hybridization and immunohistochemical experiments demonstrate that Sox14 is expressed in the embryo, salivary gland, midgut, and class IV dendritic arborization neurons (Sparkes et al., 2001; Kirilly et al., 2009). To determine the Sox14 protein expression pattern at the onset of metamorphosis, we generated a polyclonal antibody to the last 491 amino acids of Sox14 in guinea pig. This part of the protein corresponds to exon 2 of the *sox14* gene and includes the HMG DNA binding domain. Therefore, this antibody will detect if exon 2 is expressed in the $sox14^{L1}$ mutant background that lacks exon 1, but contains exon 2. To test this, immunohistochemistry was performed on - 18 hr and - 4 hr w^{1118} larvae and - 4 hr $sox14^{L1}$ mutant animals. Consistent with the low level of *sox14* mRNA expression in the - 18 hr larvae only a few cells expressed Sox14 in the fat body at this time. As predicted by the *sox14* mRNA expression pattern, Sox14 protein expression was seen in all cells of the fat body in the - 4 hr larvae (Fig. 4). Sox14 levels were severely reduced in the $sox14^{L1}$ mutant larvae, but expression was still observed. We believe that the low level of staining may be due to the expression of a transcript from exon 2 (Fig. 1C). Immunocytochemical staining in the 0 hr pupae reveals a dynamic expression pattern. We observed Sox14 expression in the nucleus of the cells in the fat body, salivary gland, hindgut, a subset of cells in the midgut, a subset of cells in the epidermis, and the peripodial membrane surrounding the imaginal disc, as shown for the leg disc (Fig. 5).

Loss-of-Function Mutations in sox14 Results in Disruption of Several Key Regulatory Pathways at the Onset of Metamorphosis

Sox14 has been shown to be required for proper expression of 20E-regulated genes,

genes required for programmed cell death, and *Mical*, a gene required for neuronal remodeling (Chittaranjan et al., 2009; Kirilly et al., 2009). Phenotypic and immunohistochemical analysis suggests that Sox14 may play a critical role in other 20E-regulated events. To identify signaling pathways in which Sox14 is functioning, microarray analysis was performed using RNA isolated from both w^{1118}; $sox14^{L1}/sox14^{L1}$ mutant and w^{1118} control animals that were staged at pupariation. RNA was labeled and hybridized to Affymetrix GeneChip Drosophila Genome Arrays 2. Comparison of the data revealed 827 genes that changed their expression two-fold or greater, with 487 genes being up- regulated and 340 genes being down- regulated in the *sox14* mutant animals (Fig. 6A).

Published data suggests that sox box family members acquire transcriptional specificity through interaction with protein binding partners and post- translational modifications (Ambrosetti et al., 1997; Kamachi et al., 2000; Bernard and Harley, 2010). To determine if Sox14 displays tissue-specific patterns of gene regulation indicative of this model, FlyAtlas was used to identify genes found in the Sox14-regulated data set that had tissue-specific enrichment values greater than or equal to four-fold in larval tissues that express Sox14 (Chintapalli et al., 2007). We identified 45 Sox14-regulated genes expressed in the larval salivary gland, 204 in the larval midgut, 109 in the larval hindgut, and 116 in the larval fat body (Fig. 6B). In agreement with the models of cell-specific regulation of gene expression by sox box transcription factors, we observed 18 genes unique to the larval salivary gland, 144 to the larval midgut, 45 to the larval hindgut, and 73 to the larval fat body in the Sox14 data set (Supp. Tables S1– S4, which are available online). Thus, it appears that Sox14 is required for the regulation of distinct sets of genes in each tissue.

To understand the role of Sox14 in the 20E-signaling pathway, we compared the Sox14-regulated genes with those genes that had previously been shown to be misregulated in *EcRi* animals (Beckstead et al., 2005). The EcR- regulated gene sets represent three col- lection times covering the late third- instar pulse of 20E; those 4 hr before pupariation, those at pupariation (0 hr), and those 4 hr after pupariation. We compared Sox14-regulated genes with the entire set of EcR-regulated genes and a subset of those genes specifically regulated at the 0 hr time point, the same stage when the $sox14^{L1}$ microarray was performed. We observed an overlap of 202 genes between the Sox14-regulated and total EcR-regulated genes, with the majority of the genes showing a similar up- or down- regulation profile (Fig. 6C). In a similar manner, statistically significant overlaps were only observed between the data sets from *EcRi* 0 hr and $sox14^{L1}$ where the gene expression profiles were similar. Visual inspection of the list revealed well-studied 20E-regulated genes such as *usp, EIP78C, EIP63E, EIG71-K, EIG71-H, EIG71-EF, EIG71-EI, IMPL3, SGS4,* and *reaper*. These results support the hypothesis that Sox14 participates in the regulation of a subset of EcR-regulated genes. It also suggests *sox14* regulates a set of genes independent of 20E signaling.

To identify biological pathways that Sox14 regulates at the onset of metamorphosis and to gain insight into the *sox14* mutant phenotype, we analyzed Sox14-regulated genes based on gene ontology, mutant phenotype, and expression analysis. We observed that 31% of the up-regulated genes were either involved in metabolism or digestion/proteolysis. In a similar manner, 24% of the down-regulated Sox14-regulated genes were involved in digestion and DNA and hormone metabolic processes. These data suggest that defects in metabolism may contribute to the sox14 mutant phenotype. In agreement with the suggestive role of Sox14 as a

pro-apoptotic gene, we observed the down-regulation of 13 genes involved in cell death or autophagy. Of interest, we also observed misregulation of 91 genes involved in immunity and 22 genes involved in muscle development, suggesting that multiple signaling pathways are affected in the *sox14* mutant animals. In addition to these biological pathways, 22 transcription factors were misregulated, suggesting that other transcriptional cascades downstream of Sox14 are affected in the mutant background.

DISCUSSION

The original hierarchical model of ecdysone action proposed by Ashburner and colleagues postulated that a limited number of primary response genes would be induced and the protein products of these genes would repress expression of early regulatory genes and up-regulate the expression of secondary response genes (Becker, 1959; Clever, 1964; Ashburner, 1972, 1974; Ashburner et al., 1974). We propose that Sox14 is one of the ecdysone primary response transcription factors that function to regulate a subset of the ecdysone transcriptional response at the onset of metamorphosis. Not only have we previously shown that *sox14* expression is induced by 20E in the presence of cycloheximide and that *sox14* requires EcR for its proper expression at the onset of metamorphosis, but we demonstrate in this study by Northern blot and microarray analysis that known 20E-regulated genes require Sox14 for their proper expression at this stage of development. We observed a statistically significant overlap with Sox14-regulated genes and genes that required EcR for their proper expression at the onset of metamorphosis. Importantly, the majority of these overlapping gene sets demonstrate similar patterns of expression in both the *EcRi* and *sox14* mutant animals, further supporting the position of Sox14 in this genetic hierarchy.

Microarray expression data also suggested that Sox14 activity is not confined to the 20E-signaling hierarchy. In agreement with these data, Northern blot analysis and immunohistochemistry show that *sox14* is expressed at a low level both before and after the late-larval pulse of 20E. From the immunohistochemistry, we observe that up-regulation of Sox14 in response to 20E results in increased expression in cells already expressing Sox14 as well as new expression patterns. Previous research has demonstrated in vitro that increasing the concentration of Sox14 protein results in multiple Sox14 proteins binding to a single target sequence. As a consequence, the Sox14 induced DNA bending increases from a 48.6° to 54° (Sparkes et al., 2001). Thus, the increased levels of Sox14 protein that are observed may have functional consequences in the regulation of gene expression in those cells.

Using the ends-out homologous recombination method, we removed exon 1 and 400 bp of upstream sequence (Gong and Golic, 2003). Based on lethal phase analysis, $sox14^{L1}$ acts as a strong loss-of-function mutation. Of interest, the $sox14^{L1}$ mutation that we generated is similar to the $sox14^{\&Dgr;13}$ and $sox14<^{15}$ P-element excision mutants in that all three alleles remove the first exon as well and part of the first intron (Kirilly et al., 2009). Thus, one would predict that they would have similar lethal phases. We observed that in addition to the late pupal lethality that was observed by Kirelly et al., 37% of the $sox14^{L1}$ homozygous and 42% of the $sox14^{L1}/Df$ animals died as prepupa. As with the late pupal lethality, this prepupal lethality was rescued by a genomic construct containing the *sox14* gene and approximately 2,000 base pairs of upstream sequence, confirming that the earlier phenotype we observed is due to loss of *sox14*. The ability of the *sox14* genomic construct to completely rescue the *sox14* mutant phenotype suggests that we have identified the *sox14* regulatory elements. Future characterization of regulatory

elements could provide insight into how 20E, a systemic hormone, induces the expression of Sox14 in a tissue-specific manner.

Earlier phenotypic analysis of loss of *sox14* has suggested a role in 20E directed larval midgut and salivary gland cell death and the regulation of pruning of the class IV dendritic arborization neurons (Chittaranjan et al., 2009; Kirilly et al., 2009). Of interest, analysis of our microarray data suggests several genes whose misregulation in the *sox14* mutant background may lead to these phenotypes. We observe a down-regulation of *reaper*, a gene involved in 20E-regulated programmed cell death, and *REP4*, a DFF/CIDE homolog, and *mats*, a tumor suppressor that is defective in apoptosis (Inohara et al., 1998; Lai et al., 2005). Of interest, autophagy genes *apg7, apg9*, and *apg18* were also down-regulated as well, suggesting defects in both autophagy and programmed cell death. In terms of the neuronal pruning phenotype, we also observed a down-regulation of two other genes known to be involved in dendrite morphogenesis, *tricornered* and *nanos* (Emoto et al., 2004; Ye et al., 2004). These results suggest that Sox14 may have a larger role in dendrite morphogenesis. Based on FlyAtlas analysis, *Mical* is expressed at high levels in the larval fat body. These data, along with our microarray analysis, suggest that Sox14 may be regulating *Mical* expression in several different target tissues (Kirilly et al., 2009).

Our mutational analysis of Sox14 gene function suggests that like other 20E-regulated transcription factors, Sox14 plays a critical role in regulating several developmental and physiological pathways at the onset of metamorphosis. In addition, analysis of the microarray data suggests that Sox14 regulates gene expression in a tissue- specific manner. This is consistent with the proposed requirement of cooperative binding partners to determine DNA

target specificity. Thus, future studies of Sox14 should yield insight into the specificity of steroid hormone signaling, Sox protein target specificity, and the role of Sox14 in 20E regulation of metamorphosis.

Experimental Procedures

Generating sox14 Knockout and Genomic Rescue Lines

To generate the *sox14* targeting construct PCR was performed using primer5'-ctcgagaagttacttcaggtatgcggacca-3'and 5'-ctcgagcacagctgttcaagtgaactgactg-3' to amplify 2,968 base pairs of sequence upstream of the *sox14* gene with attached *XhoI* restriction sites and primers 5'-ggatccgtgagtaacactgtctacactgag-3' and 5'-ggatccgcgaagccagaaatgacacgacca-3' to amplify 3,120 base pairs of internal sequence with attached *BamHI* restriction sites. These sequences were cloned into TV3- laxP-whs construct using *XhoI* and *BamHI* respectively (gift from Kent Golic). BestGene, Inc. was used to generate transgenic flies containing the targeting vector. Crosses for targeting were performed as previously published (Gong and Golic, 2003). To confirm *sox14* targeting event, PCR analysis was carried out using primers 5'-cccacgtacacacaaagtctttaccc-3' and 5'-atc agcgatcattccactacgcag-3' and TaKaRa La Taq (Takara Bio, Inc.). In a similar manner, primers 5'-ggcgcgcctagcgagatatagagtccaggcac-3' and 5'-ttaattaactgc gtagtggaatgatcgctgat-3' were used to generate an 8,080 base pair DNA fragment that contained the *sox14* gene and 2,000 base pairs of upstream sequence with attached *PacI* and *AscI* restriction sites. The rescue construct was cloned into the P[acman]-attb construct using *PacI* and *AscI* restriction sites (Venken et al., 2006). BestGene, Inc was used to generate transgenic flies containing the *sox14* genomic rescue vector using PhiC31-mediated trangenesis and targeting the *PBac {yellow[þ]-attP-3B}VK00037* stock at cytological location 22A3.

Animals, Staging, and Phenotypic Analysis

w^{1118} animals were used for phenotypic, array, and Northern blot studies. w^{1118}; *sox14*^{L1}*/CyO, kr-GFP* and w^{1118}; *Df(2R)BSC136/CyO kr-GFP* animals were used to analyze *sox14* function. Third-instar larvae were staged as previously described by adding 0.05% bromophenol blue to the food (Andres, 1994). Prepupae and pupae were staged by synchronizing animals at pupariation. *sox14*^{L1} mutant animals were identified by the loss of the *kr- GFP* marker that is associated with the *CyO* balancer chromosome.

Northern Blot Hybridizations

Total RNA from staged animals or organ cultures was isolated using Trizol (Gibco), fractionated by formaldehyde gel electrophoresis, transferred to nylon membranes, and probed with radioactively labeled DNA probes. To facilitate comparisons, w^{1118} blots and *sox14*^{L1}, and *hs-EcRi-11* blots were probed, washed, and exposed together. *rp49* was used as a loading control. Probes for *sox14, E74, E75, DHR3, FTZ-F1, EcR, usp, BR-C, IMP-L1*, and *rp49* were used as previously described (Andres et al., 1993; Beck- stead et al., 2005).

Microarray and Cluster Analysis

All microarray analysis experiments were performed independently and in triplicate to facilitate statistical analysis. Total RNA was isolated using Tri-Pure (Roche, Indianapolis, IN) followed purification with RNAeasy columns (Qiagen, Valencia, CA). Probe labeling, hybridization to Affymetrix GeneChip Drosophila Genome Arrays 2 (Affymetrix, Santa Clara, CA) and scanning, were performed by Emory University Biomarker Core facility. RMA was used to normalize the raw data and determine gene expression values (Bolstad et al., 2003; Irizarry et al., 2003). Statistically significant changes between sample sets were identified using

significance analysis of microarray (SAM; Tusher et al., 2001). Further analysis and comparisons between datasets were performed using Access (Microsoft Corporation, Redmond, WA). Microarray data from this study can be accessed at the National Center for Biotechnology In- formation Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.- gov/geo/) with accession number GSE23355. A cutoff of two-fold change in expression for genes both up and down-regulated in the absence of *sox14* was chosen to reflect those genes most significantly affected by loss of Sox14, resulting in 487 genes showing up-regulation and 340 genes showing down-regulation. Of those genes showing at least a two-fold change in expression, further analysis was performed using the GOstat program (Beissbarth and Speed, 2004). Sox14-regulated genes were classified according to known expression in particular larval tissues using FlyAtlas (Chinta- palli et al., 2007). Genes showing an enrichment value greater than or equal to four in a specific larval tissue were further analyzed.

Immunohistochemistry

To generate the Sox14 antibody, a DNA fragment corresponding to amino acids 179–669 was cloned into the pMAL-c2X expression vector (New England BioLabs). This region encompasses the HMG domain and C-terminal portion of the protein. The purified fusion protein was injected into guinea pigs to produce antiserum (Rockland Immunochemicals). Antiserum was affinity-purified as described (Carroll, 1987), using a Sox14 protein containing amino acids 179-669. Affinity-purified anti-Sox14 antibodies were used at a 1:50 dilution for immunofluorescence and Cy3-conjugated donkey anti- guinea pig IgG (Jackson ImmunoRe- search) was used at 1:1000. Nuclei were counterstained with DAPI (40,6- diamidine-2- phenylidole-dihydrochlor- ide; Invitrogen). Larval tissues were isolated by dissection and fixed

in 4% formaldehyde/1X phosphate buffered saline on ice for 20 min. Animals were imaged on a Leica MZ12.5 dissecting microscope, equipped with a Cool- SNAP-Pro cf COLOR camera (Media Cybernetic). Tissues were imaged on a Zeiss Axioskop 2 Plus microscope using the same camera.

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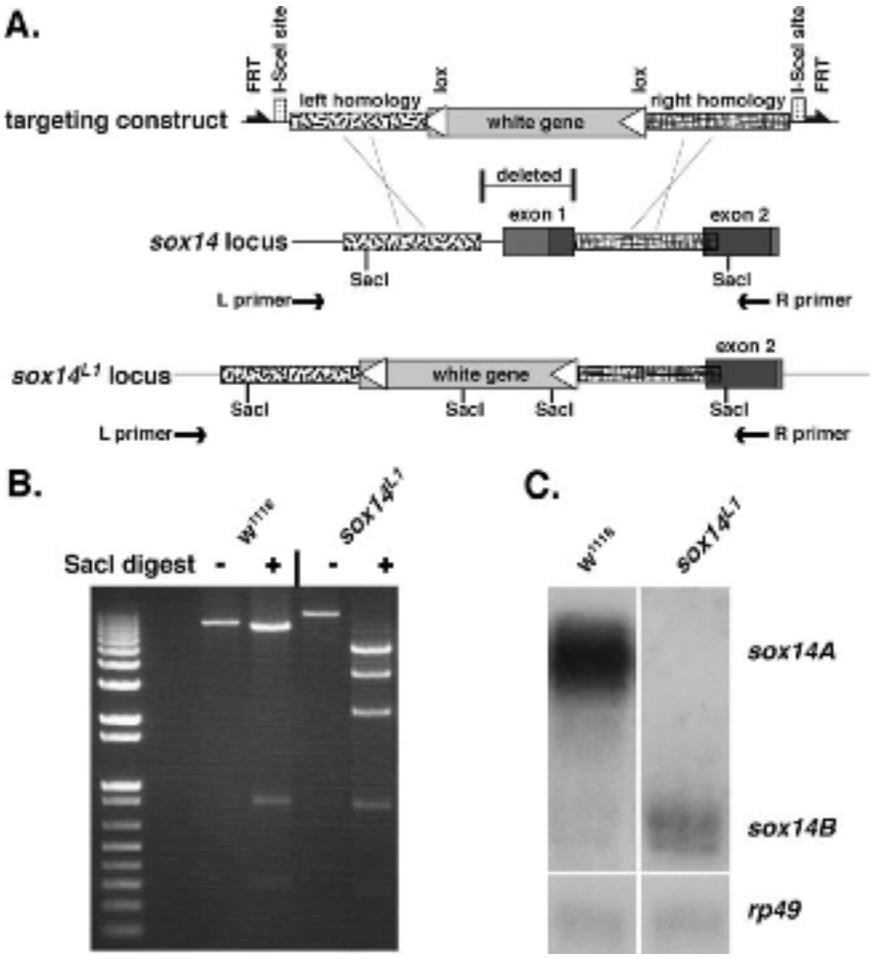


Fig. 1. A: Schematic diagrams representing the *sox14* targeting construct, the *sox14* genomic locus, and the resulting sox14L1 mutation locus. SceI and SacI are restriction endonuclease sites and FRT represents flip-recombinase targets. L and R arrows represent location of primers used to amplify regions of the genomic DNA. **B:** Image of gel electrophoresis of PCR fragments amplified from w^{1118} control and $sox14^{L1}$ genomic DNA and cut with the SacI restriction endonuclease. **C:** Northern blot analysis using a full-length sox14 cDNA or *rp49* probe and mRNA isolated from w^{1118} control and sox14L1 animals staged at pupariation. *rp49* acts as a loading control. Engineering of a *sox14* mutation through use of ends-out homologous recombination.

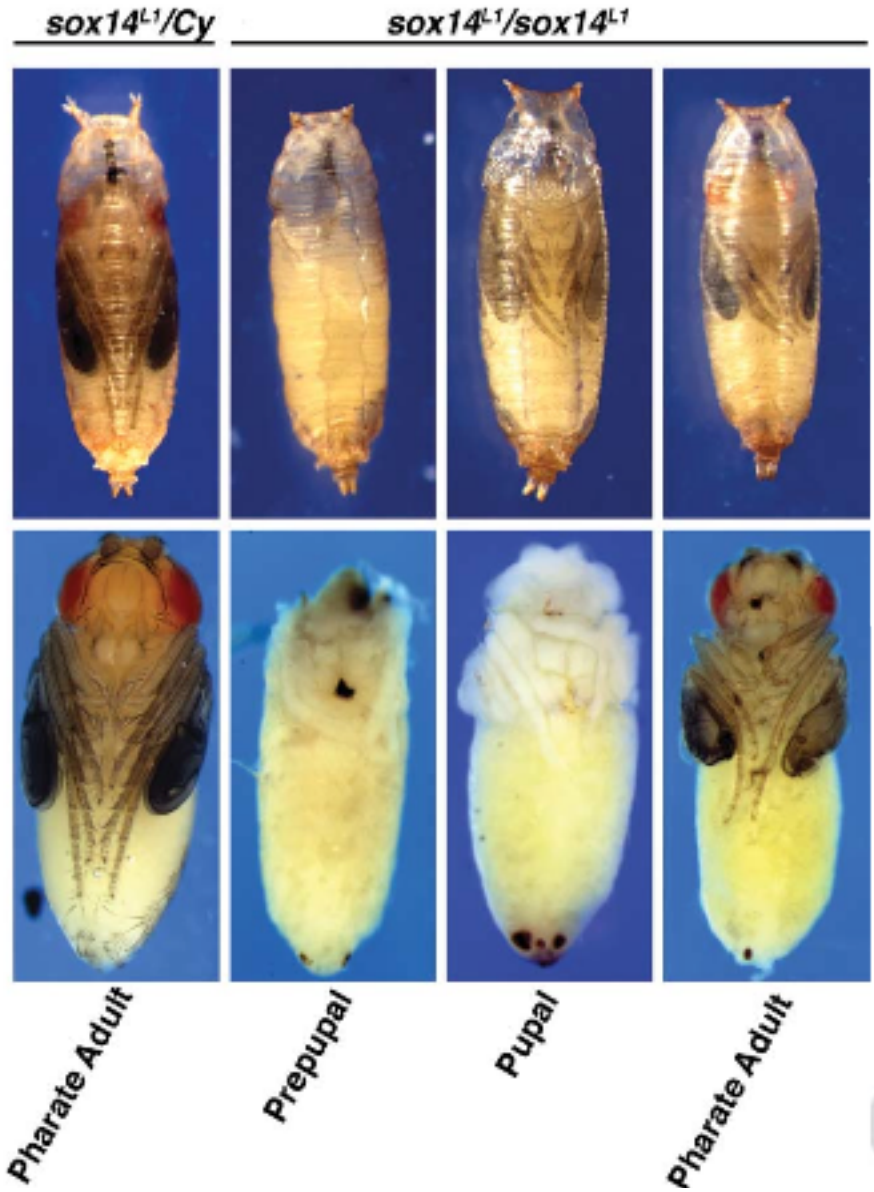


Fig. 2. Loss-of-function *sox14*^{L1} mutations are lethal. Terminal phenotypes observed in control *sox14*^{L1}*/CyO* and *sox14*^{L1}*/sox14*^{L1} animals. Images are representative of three phenotypic classes that were observed in the mutant animals.

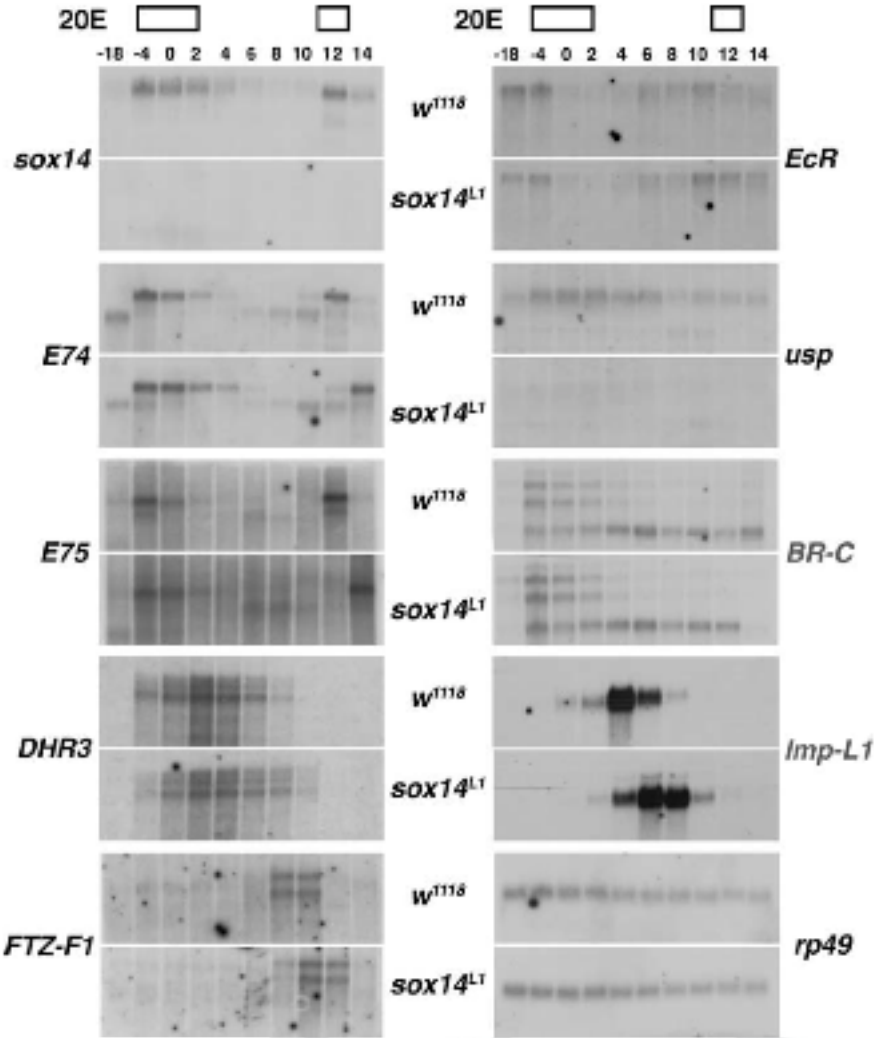


Fig. 3. Mutations in *sox14* result in disruption of several key regulatory genes in the 20E transcriptional cascade. Northern blot analysis on mRNA obtained from whole w^{1118} control and $sox14^{L1}/sox14^{L1}$ animals staged -18, -4, 0, 2, 4, 6, 8, 10, 12, and 14 hr relative to pupariation. Expression patterns for key transcription factors (*sox14, E74, E75, DHR3, FTZ-F1, EcR, usp, BR-C*) and *Imp-L1* and *rp49* are shown. *rp49* acts as a loading control. Boxes signify presence of 20E.

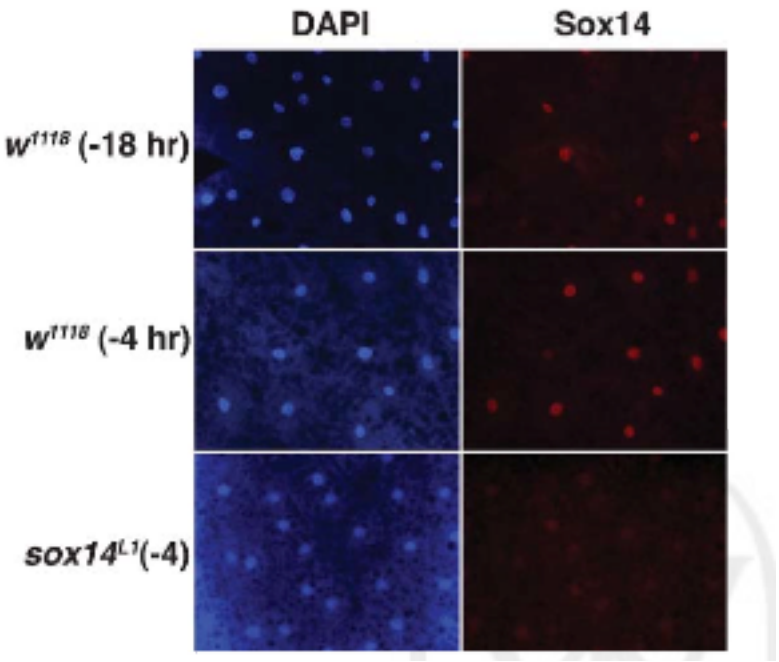


Fig. 4. Sox14 protein is induced in concert with pulse of 20E. Immunolocalization experiments in fat body from w^{1118} control and $sox14^{L1}/sox14^{L1}$ animals using an antibody against Sox14 (red) and nuclear stain DAPI (blue; 40,6-diamidine-2- phenylidole-dihydrochloride).

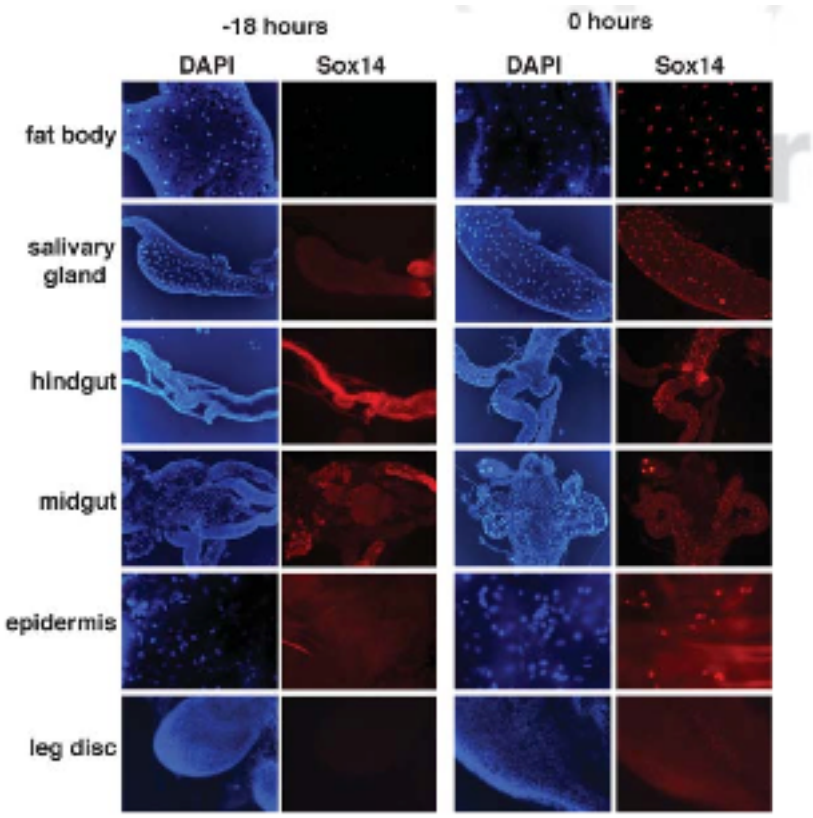
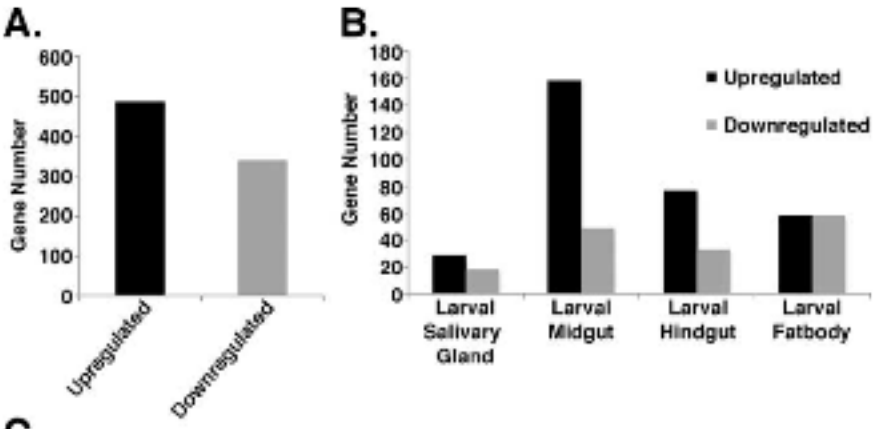


Fig. 5. Sox14 exhibits a dynamic expression pattern. Immunolocalization experiments against animals staged at -18 and 0 hr relative to pupariation using an antibody against Sox14 (red) and nuclear stain DAPI (blue; 40,6-diamidine-2-phe- nylidole-dihydrochloride). Note Sox14 expression in the fat body, salivary gland, hindgut, midgut, epidermis, and leg imaginal disc.



C.

Microarray	*sox14*^{L1} (n=827)	*sox14*^{L1}↑(n=487)	*sox14*^{L1}↓(n=340)
EcRi (n=1546)	202 (2.1E-36)	121 (7.1E-23)	61 (2.0E-07)
EcRi ↑ (n=852)	123 (1.2E-46)	105 (2.5E-72)	18 (5.9E-01)
EcRi ↓ (n=928)	84 (2.9E-05)	20 (2.2E-02)	64 (7.0E-20)
EcRi (0 hr)↑ (n=276)	58 (2.1E-40)	57 (1.5E-55)	11 (9.1E-02)
EcRi (0 hr)↓ (n=389)	48 (5.4E-08)	6 (3.4E-02)	42 (1.9E-27)

D.

GO Term Categories (Upregulated)	Gene Count (487)
Metabolism	95
Immunity	68
Digestion	59
Muscle Development	22
Chromosome Puffing	5

GO Term Categories (Downregulated)	Gene Count (340)
DNA Metabolic Process	49
Digestion	29
Immunity	23
Cell Death/Autophagy	13
Hormone Metabolism	5

Fig. 6. Analysis of genes that require Sox14 for proper expression at the onset of metamorphosis. **A:** Graphical representation showing the number of genes both up (487) and down-regulated (340) by two-fold or greater in the *sox14*^{L1}*/sox14*^{L1} mutant animal at pupariation. **B:** Graphical representation of a subset of *Sox14*-regulated genes with genes that have enrichment value of four-fold or greater in the larval salivary gland, larval midgut, larval hindgut, and larval fat body based on FlyAtlas. **C:** Table depicting the overlap between Sox14-regulated (microarray *sox14*^{L1}) and previously published EcR-regulated gene sets with microarray *EcRi* representing those genes misexpressed at -4, 0, or 4 hr relative to pupariation and microarray *EcRi* 0 hr representing only a subset of genes misregulated at pupariation. Each gene set is divided into up- regulated or down-regulated genes as represented by the arrows, with the number of genes in each dataset represented by ‘‘(n =)’’. The first number in each cell represents the number of overlapping genes between the two datasets being compared. The numbers within the parentheses in each cell represent a P value based on the x2 test that accounts for the differences between the observed and expected numbers. **D:** Table depicting gene ontology (GO) categories identified most often in the *Sox14*-regulated gene set.

Tble 1-4: Genes identified as misregulated in the *sox14*^{L1}/*sox14*^{L1} zero hour prepupae and showing an enrichment value greater than or equal to four in a the larval salivary gland, midgut, hindgut, and fatbody based on FlyAtlas. * Denotes genes that are enriched in only one of the specific larval tissue tested.

Table 1

Gene ID	**Fold Change *sox14*^{L1}**	**Larval Salivary Gland Expression Profile**
GstD4	16.16	4.7
Hsp70Ba	14.19	29.58
Hsp70Aa	13.27	15.99
CG13822	9.16	4.26
Hsp70Bc	7.91	33.66
CG31530	6.23	4.62
Cyp9c1	5.96	5.52
CG15404	5.01	974.64
Sgs4	4.80	4887.36
CG8160	4.23	7.59
CG31698	3.83	3286.31
Mthl4	3.45	15.21
CG9826	2.86	4.61
*CG30411**	2.74	11.29
*net**	2.60	28.17
*Pvf2**	2.56	9.41
CG31439	2.52	22.68
CG33469	2.33	10.19
*CG15822**	2.31	15.73
*CG12918**	2.21	6.28
*CG31496**	2.18	79.41
CG5002	2.16	8.26
*nes**	2.11	6.79
*CG30371**	2.10	20.88
*JIL-1**	2.09	6.52
CG13461	2.07	1269.41
*CG31809**	2.02	37
*CG18343**	-2.02	4.17
*CG5039**	-2.07	12.68
*CG9149**	-2.09	7.13
*CG12081**	-2.18	4.71
unc-115	-2.26	5.8
*CG10918**	-2.34	1077.79
*CG9186**	-2.45	6.4

*mura**	-2.47	4.81
CG18557	-2.51	4.04
Nplp4	-2.65	125.82
CG14258	-2.70	9.14
CG4839	-2.97	14.52
CG40294	-3.56	14.01
CG12643	-3.62	7.93
*p24-2**	-3.99	5.24
CG3117	-5.55	28.79
Cyp6d2	-23.79	10.34
CG13962	-87.63	7.62

Table 2

Gene ID	**Fold Change *sox14*^{L1}**	**Larval Midgut Expression Profile**
*CG5246**	52.13	11.49
*GstD5**	16.16	4.18
*CG10943**	27.46	9.5
*Jon44E**	27.00	17.35
*CG13324**	22.01	19.96
*CG8562**	21.94	9
*Amyrel**	21.03	15.26
*CG31343**	19.62	5.79
*CG5892**	17.80	33.67
GstD4	16.16	12.66
*LysS**	12.72	252.55
*CG9394**	11.71	5.86
*CG6403**	11.20	71.85
*CG6296**	10.29	65.97
CG10300	9.80	25.78
CG17570	9.53	7.32
*CG34005**	9.26	4.95
*CG17475**	8.39	6.06
*PH4alphaPV**	8.10	8.94
CG15829	7.89	44.19
*CG31446**	7.84	24.97
Cyp6a13	7.68	9.06
*CG9682**	7.54	24.42
vanin-like	6.82	15.72
*CG1941**	6.41	7.09
*CG30043**	6.09	23.31
*CG13492**	5.97	14.55

Cyp9c1	5.96	7.56
*CG8693**	5.54	4.61
*CG33127**	5.36	4.48
*CG33510**	5.22	7.16
*CG15784**	5.15	4.17
*CG9673**	5.13	5.86
*CG31267**	4.96	5.42
*Cht4**	4.93	10.94
Sgs4	4.80	13.59
*CG18745**	4.77	27.12
*CG11201**	4.76	14.52
*CG3987**	4.69	14.29
*MtnD**	4.64	9.48
*CG14500**	4.62	17.69
CG32284	4.52	45.48
*CG34301**	4.46	71.03
*Cyp4ad1**	4.44	14.74
Zip3	4.43	4.2
Cyp6a21	4.38	9.9
*Cht9**	4.37	31.66
Prx2540-2	4.35	11.19
*CG30340**	4.34	4.92
*MtnB**	4.26	8.7
CG15818	4.25	65.73
*CG5765**	4.21	768.56
*mex1**	4.00	13.53
*CG8661**	3.93	18.14
CG31789	3.89	2985.11
*CG8773**	3.87	14.28
*CG13323**	3.79	14.86
*CG7876**	3.76	1320.58
*CG1942**	3.71	13.5
CG6933	3.69	227.2
*CG15423**	3.66	27.69
*CG10827**	3.58	14.53
CG9555	3.55	9.37
CG6041	3.54	15.43
CG32633	3.53	35.46
*CG31758**	3.47	6.81
*CG3884**	3.44	6.03
*CG31148**	3.43	6.61
*CG6834**	3.37	8.31

*CG8560**	3.37	16.17
CG10910	3.27	19.58
*CG7252**	3.25	542.61
*CG5767**	3.25	22.21
*CG34176**	3.24	7.72
*CG42249**	3.21	5.2
*CG10062**	3.20	6.04
*CG10182**	3.19	72.46
*CG18748**	3.19	7.25
*CG18747**	3.10	10.59
*CG8664**	3.07	612.52
*CG15347**	2.98	6.33
*CG13704**	2.97	5.43
*CG5932**	2.91	22.42
*CG7916**	2.91	7.57
*CG3868**	2.89	6.9
*CG14457**	2.89	8.84
*CG11453**	2.88	21.18
*m1**	2.88	20.07
*CG30160**	2.87	7.04
*CG8852**	2.86	8.25
CG9826	2.86	36.43
*CG18585**	2.78	8.3
*obst-G**	2.77	655.46
*CG16732**	2.76	9.29
*CG3344**	2.75	23.72
*CG17147**	2.73	215.03
*Tsp2A**	2.71	6.89
CG14302	2.70	403.3
CG33013	2.70	13.14
*CG34330**	2.69	4.97
CG1246	2.68	15.06
*Jon66Cii**	2.67	153.75
CG5156	2.65	5.8
*CG8353**	2.64	6.53
CG32302	2.57	675.81
*CG16771**	2.55	9.18
CG31439	2.52	7962.97
*CG10116**	2.52	4.47
CG32107	2.51	5.56
Cyp9f3Psi	2.47	8.27
*betaInt-nu**	2.46	8.52

*CG13511**	2.44	13.87
*CG18746**	2.43	10.71
*CG13512**	2.42	13.07
*Tsp42Ec**	2.40	17.41
*pc1**	2.40	20.07
*decay**	2.39	12.03
*CG15043**	2.39	5.4
*CG7678**	2.38	35.74
*CG14131**	2.35	10
CG15678	2.34	9.8
*CG6996**	2.34	50.86
*CG17571**	2.34	6.66
*CG12813**	2.34	10.45
CG33469	2.33	5.21
CG31087	2.32	12.53
CG17119	2.31	7.98
GstD10	2.31	4.94
*CG11961**	2.25	18.16
*Ugt86Dj**	2.24	10.11
*Ptp52F**	2.24	10.19
*CG10086**	2.23	7.09
CG9486	2.22	10.16
*Dh31**	2.22	5.94
*Ag5r2**	2.21	11.73
*CG15422**	2.21	14.43
*CG12951**	2.21	22.3
*CG1809**	2.20	12.59
*CG34026**	2.20	8.74
*CG15044**	2.20	8.54
*CG7290**	2.17	789.45
*CG9380**	2.16	6.34
AR-2	2.16	7.74
*CG11470**	2.15	752.91
CG31439	2.14	2833.67
*CG13211**	2.11	4.5
CG12602	2.11	11.64
*kappaTry**	2.11	4.19
CG31683	2.09	5.31
*Ugt86Dh**	2.09	14.24
*tgy**	2.08	6.08
*CG6164**	2.05	16.95
*Ugt35a**	2.04	10.62

*CG10650**	2.04	10.52
*CG7248**	2.03	276.35
*CG17906**	2.03	6.34
CG5897	2.02	16.12
*CG5399**	2.00	4.08
*mth114**	-2.01	6.9
*PGRP-SC2**	-2.02	5.09
*Su(dx)**	-2.04	5.72
CG34112	-2.06	13.13
Cyp12d1-d	-2.09	4.18
*CG15170**	-2.10	36.41
*Adgf-A**	-2.11	6.56
CG4893	-2.17	5.22
*CG6283**	-2.19	10.38
CG13796	-2.25	9.31
unc-115	-2.26	4.31
*Sply**	-2.26	6.99
Rgk3	-2.26	5.9
*Jon25Biii**	-2.27	4.63
*CG5828**	-2.27	4.67
*Arc1**	-2.38	8.09
*CG12963**	-2.42	8.93
*Cyp6a14**	-2.48	10.59
CG12824	-2.50	26.3
*CG5770**	-2.51	137.18
Lerp	-2.56	16.8
*Cyp4d8**	-2.67	15.47
*CG1698**	-2.71	6.41
Rhp	-2.79	4.68
*CG1837**	-2.83	8.63
CG33514	-2.90	9.66
CG4839	-2.97	4.79
*Dip-C**	-3.04	7.05
Mdr50	-3.41	11.98
Hsp67Bc	-3.54	5.45
*CG1399**	-3.55	4.53
CG40294	-3.56	4.38
CG12643	-3.62	12.9
*Tsp42Er**	-3.64	10.11
*CG18622**	-3.75	6.5
CG3589	-3.80	4.21
CG4484	-3.88	8.29

shd	-4.10	6.63
*CG17754**	-5.12	7.05
*CG5853**	-5.13	7.1
CG7860	-5.25	7.2
CG11192	-5.55	7.49
*CG7953**	-5.89	9.16
Sk1	-6.04	7.51
*Ugt36Bc**	-7.19	4.56
*CG18179**	-7.81	5.67

Table 3

Gene ID	**Fold Change *sox14*^{L1}**	**Larval Hindgut Expression Profile**
GstD4	16.16	6.78
Hsp70Ba	14.19	11.94
Hsp70Aa	13.27	8.98
*CG34426**	12.60	5.26
CG10300	9.80	7.49
CG17570	9.53	7.2
Hsp70Bc	7.91	12.84
CG15829	7.89	7.22
Cyp6a13	7.68	5.88
vanin-like	6.82	10.17
*CG10806**	6.39	11.67
CG31530	6.23	31.59
Cyp9c1	5.96	11.08
*CG8066**	5.66	4.54
*nahoda**	5.59	4.7
*CG9877**	5.47	50.7
*CG16884**	5.01	12.89
*Mp20**	4.83	4.48
*CG6337**	4.71	22.64
CG32284	4.52	32.53
Zip3	4.43	8.71
Prx2540-2	4.35	4.02
CG15818	4.25	31.65
*CG14285**	4.17	6.31
*1(2)efl**	4.12	7.43
*CG16885**	4.02	12.81
Cyp9h1	3.99	13.79
CG31789	3.89	7.64
CG31698	3.83	4.53

CG6933	3.69	57.76
*Tm2**	3.68	4.37
CG9555	3.55	5.94
CG6041	3.54	18.25
CG32633	3.53	10.15
*CG5177**	3.49	5.77
mth14	3.45	4.93
CG10910	3.27	8.15
*CG33143**	2.93	5.44
*Tm1**	2.92	4.15
*CG16820**	2.72	5.96
*Prm**	2.70	5.09
CG33013	2.70	14.15
CG1246	2.68	4.21
*CG8888**	2.61	5.01
*CG5391**	2.53	31.68
*ImpL3**	2.53	7.76
CG31439	2.52	8.84
CG32107	2.51	5.66
*CG34165**	2.51	4.49
CG15282	2.49	18.97
*CG13403**	2.47	10.94
*Cyp12a5**	2.42	4.78
*CG1143**	2.40	45.33
*CG13616**	2.39	26.61
CG15678	2.34	5.78
*fau**	2.34	4.93
CG31087	2.32	11.93
CG17119	2.31	4.49
GstD10	2.31	10.61
*CG10211**	2.28	24.42
*up**	2.28	4.72
He	2.25	4.83
CG9486	2.22	9.59
*TpnC73F**	2.22	19.94
*Lep65Ac**	2.20	13.26
*CG13075**	2.16	133.25
CG5002	2.16	11.29
AR-2	2.16	14.7
*CG10184**	2.14	4.56
CG31439	2.14	6.3
*CG17107**	2.12	5.82

CG12602	2.11	7.28
*CG5023**	2.06	4.56
CG5897	2.02	11.33
Lep3	2.01	558.91
*CG11854**	2.00	64.41
CG34112	-2.06	4.58
grass	-2.08	6.31
*CG3264**	-2.11	4.01
*CG3599**	-2.11	8.09
*CG3588**	-2.13	27.12
CG4893	-2.17	8.46
CG7016	-2.17	4.47
unc-115	-2.26	4.37
*rpr**	-2.30	12.55
*CG17105**	-2.39	17.02
CG12824	-2.50	8.76
Lerp	-2.56	4.66
Np1p4	-2.65	18.58
CG14258	-2.70	82.13
Rhp	-2.79	5.95
*CG33144**	-2.88	4.36
CG33514	-2.90	4.29
PGRP-SA	-3.16	5.55
*CG8034**	-3.23	6.04
Tsf3	-3.26	7.24
Mdr50	-3.41	7.19
Hsp67Bc	-3.54	10.48
CG40294	-3.56	10.11
CG12643	-3.62	4.66
CG17754	-3.81	6.42
shd	-3.85	10.17
CG4484	-3.88	12.59
*CG32415**	-5.14	5.54
CG7860	-5.25	4.52
CG3117	-5.55	23.38
Sk1	-6.04	4.46
*CG6231**	-6.73	7.56
CG13962	-87.63	11.87

Table 4

Gene ID	**Fold Change *sox14*^{L1}**	**Larval Fatbody Expression Profile**

*w**	123.01	11.4
*Cypg1**	95.93	11.2
*CG14527**	40.88	7.6
GstD4	16.16	28.8
*CG2177**	13.66	5.4
Hsp70Aa	13.27	4.5
CG13822	9.16	9
*Tequila**	8.79	29.5
Hsp70Bc	7.91	4.9
*GstE3**	7.44	11.1
Cyp9c1	5.96	6.3
*Hsp68**	5.82	9.3
*Cyp28d1**	5.44	9.8
CG15404	5.01	14.9
Sgs4	4.80	514.6
*CG30083**	4.77	37.2
*CG33307**	4.49	11.4
*CG14332**	4.45	12.5
Cyp6a21	4.38	4.6
*CG14642**	4.28	5.2
CG8160	4.23	297.4
Cyp9h1	3.99	15
CG31789	3.89	48.6
CG31698	3.83	77.1
*Glut1**	3.62	4.4
*CG33333**	3.53	8
*CG5840**	3.50	5.8
mth14	3.45	4.6
*CG4500**	3.21	117.2
*Cyp6t1**	2.85	4.5
*CG5322**	2.77	16
*bw**	2.76	10.8
CG14302	2.70	20.8
*CG5321**	2.66	6.8
CG5156	2.65	4.3
CG32302	2.57	6.4
*ry**	2.55	4.1
CG31439	2.52	8.8
Cyp9f3Psi	2.47	5.1
*CG4398**	2.44	8.5
*CG5255**	2.44	4
*Mtk**	2.40	5.7

*Odc1**	2.37	9.6
*rgr**	2.35	11.6
*CG30098**	2.31	4
GstD10	2.31	97.3
*Glllspla2**	2.26	6.7
*CG34104**	2.26	7.3
*CG33458**	2.25	13.6
He	2.25	23.1
CG9486	2.22	77.4
*CG14528**	2.20	15
*CG9455**	2.14	11.1
CG31683	2.09	11.6
*CG9812**	2.07	8.5
CG13461	2.07	4.3
*Adgf-D**	2.04	11.9
Lep3	2.01	7.8
*Lsd-1**	-2.03	18.6
*Fmo-1**	-2.04	13.8
*Eip78C**	-2.08	21.2
grass	-2.08	17.8
Cyp12d1-d	-2.09	4.4
*Sucb**	-2.11	15.4
*CG6415**	-2.14	8.5
*CG34376**	-2.15	6.8
*CG5270**	-2.17	4.5
*Eig71Ef**	-2.17	19.2
CG7016	-2.17	8.4
*Tsp42Ej**	-2.18	5.1
*CG5493**	-2.22	32.7
CG13796	-2.25	66.1
Rgk3	-2.26	9.4
*CG32762**	-2.32	26.1
*CG30090**	-2.42	21.2
*CG10874**	-2.45	4.6
*Cyp4ac1**	-2.50	4.5
CG18557	-2.51	28.3
*CG12012**	-2.54	4.5
*CG6385**	-2.69	45.2
*Mical**	-2.78	5.3
*CG18249**	-2.81	19.5
*CG30431**	-2.86	4.3
*CG1827**	-3.04	4.6

*CG8112**	-3.10	8.7
*MESR6**	-3.10	5.5
PGRP-SA	-3.16	24.1
*CG13531**	-3.19	4.2
Tsf3	-3.26	5
*CG5302**	-3.30	7.2
CG40294	-3.56	7.9
*CG8157**	-3.59	88.3
CG12643	-3.62	21.6
CG3589	-3.80	5.5
CG4484	-3.88	18.4
shd	-4.10	16.7
*CG5262**	-4.18	5.8
*CG10621**	-4.40	22.7
*CG32185**	-4.45	16.5
*DptB**	-4.63	9.3
*CG8678**	-4.87	6.7
CG7860	-5.25	8.7
*CG8745**	-5.33	16.3
*CG4650**	-5.37	230.1
CG3117	-5.55	1345.6
CG11192	-5.55	4.3
*Cyp6d5**	-6.56	4
*Idgf5**	-8.28	14.2
*PGRP-SB1**	-9.45	10.3
*CG30148**	-10.84	58
*CG17110**	-11.04	4.6
*CG2277**	-12.43	7.5
*Cyp12d1-d**	-13.61	5.5
Cyp6d2	-23.79	48.2
CG13962	-87.63	648.6

CHAPTER 4

CONCLUSIONS

The major objective of this research was to define the role Sox14 plays in the 20E hormonal transcriptional cascade at the onset of *Drosophila* metamorphosis. Through analysis of Sox14, we ascertained how Sox14 acts in cell specific transcriptional responses to 20E, providing insight into how larval tissue transmits its response to 20E to direct adult tissue development in the fruit fly. Previous research has identified the Sox14 transcription factor as a primary target gene that is directly regulated in response to the 20E/EcR/Usp complex (Beckstead et al., 2005). Our research demonstrates that a mutation of *sox14* results in prepupal and pupal lethality in the developing fruit fly with animals displaying various defects in known 20E developmentally regulated pathways.

Our Northern blot and microarray analyses suggest that the Sox14 transcription factor functions in the 20E hormonal cascade by regulating a subset of the 20E transcriptional response during development. Our Northern blot analysis demonstrates the requirement of Sox14 for the proper expression and timing of known 20E-regulated genes. Sox14-regulated genes and EcR-regulated genes also show a significant overlap in our microarray analysis as well as mutant animals showing similar expression patterns. These studies in addition to immunohistochemistry performed suggest a role for Sox14 outside of the traditional 20E-signaling cascade, low levels of *sox14* being expressed both before and after as well as during the late-larval pulse of 20E.

Our mutational analysis of Sox14 gene function suggests that like other 20E-regulated transcription factors, Sox14 plays a critical role in regulating several developmental and

physiological pathways at the onset of metamorphosis. In addition, analysis of the microarray data suggests that Sox14 regulates gene expression in a tissue- specific manner. This is consistent with the proposed requirement of co- operative binding partners to deter- mine DNA target specificity. Thus, future studies of Sox14 should yield insight into the specificity of steroid hormone signaling, Sox protein target specificity, and the role of Sox14 in 20E regulation of metamorphosis.

Previous phenotypic analysis of *sox14* mutants suggested a role in 20E-mediated events such as larval midgut and salivary gland cell death as well as regulation of larval neuronal pruning (Chittaranjan et al., 2009; Kirilly et al., 2009). Our microarray analysis supports this supposition through our identification of the misregulation of various programmed cell death, apoptosis, and autophagy genes, including the downregulation of reaper, REP4, and mats, and ap7, 9, and 18. . Genes like tricornered and nanos, which are known to be involved in dendrite morphogenesis were also identified in our screen, suggesting that Sox14 may play a larger role in dendrite morphogenesis. These genes could be involved in the larval neuronal remodeling process, functioning much like the known Sox14 target gene, Mical.

Our lethal phase analysis suggests that Sox14 functions like other primary target genes regulated by 20E, playing a role in regulating several developmental pathways at the onset of *Drosophila* metamorphosis. Our microarray data suggests that this regulation may occur in a tissue-specific manner, affecting developmental processes such as muscle development, immunity, programmed cell death, neuronal remodeling, metabolism, and hormone signaling. Future studies would give greater insight into specific Sox14 function in these various tissues as well as steroid signaling in general.