The *In Vivo* Function of Nuclear Receptors during *Drosophila* Development

by

Aleksandar Necakov

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Molecular Genetics

University of Toronto

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Abstract

Nuclear receptors (NR’s) comprise a large, ancient, superfamily of eukaryotic transcription factors that govern a wide range of metabolic, homeostatic, and developmental pathways, and which have been implicated in disease states including cancer, inflammation, and diabetes. The ability of NRs to activate or repress gene transcription is modulated through direct binding of small lipophilic ligands which induce conformational changes in their cognate receptor. These changes are structural in nature and lead to the recruitment of coactivator or corepressor complexes, ultimately regulating the expression of target genes to whose response elements NRs are bound. In Drosophila 18 NRs have been identified which have representative members belonging to each of the six major NR subfamilies, and which show a high degree of homology to their vertebrate counterparts. This fact, in addition to the power and ease of genetic manipulation, make Drosophila an excellent model system in which to study NR function. When I began my project, 17 of the 18 NRs in Drosophila were ‘orphan’ receptors for which no cognate ligand had been identified. As a first step in an effort to identify potential ligands for these 17 receptors I first set out to determine how, where and when nuclear receptors are
regulated by small chemical ligands and/or their protein partners. In order to do so I contributed to developing a ‘ligand sensor’ system to visualize spatial activity patterns for each of the 18 *Drosophila* nuclear receptors in live, developing animals. This system is based upon transgenic lines that express the ligand binding domain of each *Drosophila* NR fused to the DNA-binding domain of yeast GAL4. When combined with a GAL4-responsive reporter gene, these fusion proteins show tissue- and stage-specific patterns of activation. Analysis using this system has revealed the stage and tissue specificity of NR activation for each of the fly NRs. The amnioserosa, yolk, midgut and fat body, which play major roles in lipid storage, metabolism and developmental timing, were identified as frequent sites of nuclear receptor activity. Dynamic changes in activation that are indicative of sweeping changes in ligand and/or co-factor production are also a prominent feature that has been revealed using this approach.

In addition, I went on to characterize the ligand regulated function of a single *Drosophila* nuclear receptor, Ecdysone inducible protein 75 (E75). Previous work from our lab has demonstrated that E75 binds to heme, and that its function as a transcriptional repressor is regulated *in vitro* by binding of the small diatomic gases nitric oxide (NO) and carbon monoxide (CO) to its heme moiety. In an effort to validate and to further understand the *in vivo* relevance of E75 regulation by NO I used gain and loss of function transgenes, as well as tissues manipulated in culture to show that NO acts directly on the *Drosophila* nuclear receptor E75, reversing its ability to block the activity of its heterodimer partner *Drosophila* Hormone Receptor 3 (DHR3). By specifically focusing on the *Drosophila* larval ring gland, the principal endocrine organ responsible for the production of the metamorphosis-inducing hormone, ecdysone, I have shown that failure to produce NO and to inactivate E75 results in failure to recognize the signals that normally trigger metamorphosis.
This thesis is dedicated to Alison and our two sons Kieran and Stevan
Science is an endless search for truth. Any representation of reality we develop can be only partial. There is no finality, sometimes no single best representation. There is only deeper understanding, more revealing and enveloping representations.

Scientific advance, then, is a succession of newer representations superseding older ones, either because an older one has run its course and is no longer a reliable guide for a field or because the newer one is more powerful, encompassing, and productive than its predecessor(s).

- Carl Woese
Acknowledgments

I would like to begin by thanking my supervisor, Henry Krause, for all of the guidance, support, and patience that he has shown throughout the course of my degree. Henry has afforded me the freedom to make my own mistakes, and has continually provided me with insightful suggestions in helping me navigate my way through them. I am deeply grateful to him as this thesis would not have been possible without the freedom and opportunities that he has afforded me with.

I would also like to thank the members of my committee, Howard Lipshitz and Ulrich Tepass, who have been a great source of positive criticism and lively discussion, and who have helped to keep me on track through the years. Both have proven to be caring and knowledgeable mentors, and I have benefited tremendously from my contact with them.

This work would not have been possible without the contributions of Heidi Sampson, Ruoyu Ni, Chun Hu, Carol Schwartz, and Lucia Caceres. I am grateful to them for their help with experimental work, writing, and for critical discussions regarding the interpretation of data. Heidi, Ruoyu, and Chun were central contributors to the nuclear receptor ligand sensor paper, whereas the E75/NO story could not have been completed without the help of Carol and Lucia. I am thankful to them all for their contributions.

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I would like to thank my friends and family for their moral support and guidance through all of the tough times, and for being there to help whenever I needed them most. The nature of my work has in some ways resulted in the development of a conceptual distance between us, but you have all done such a good job of keeping me grounded and down to earth whenever my ideas got too big or too complicated, or when they so often failed. You may not have directly understood the hardship and heartache that I felt, but were always there to cheer me up when I needed you most.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Act5C</td>
<td>Actin 5C</td>
</tr>
<tr>
<td>AF1</td>
<td>Activation function 1</td>
</tr>
<tr>
<td>AF2</td>
<td>Activation function 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AKH</td>
<td>Adipo-kinetic hormone</td>
</tr>
<tr>
<td>AKHR</td>
<td>Adipo-kinetic hormone receptor</td>
</tr>
<tr>
<td>BPF</td>
<td>before puparium formation</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dare</td>
<td>Adrenodoxin reductase</td>
</tr>
<tr>
<td>alas</td>
<td>δ-aminolevulinate synthase</td>
</tr>
<tr>
<td>BH</td>
<td>Brain hemisphere</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CA</td>
<td>Corpora allata</td>
</tr>
<tr>
<td>CC</td>
<td>Corpora cardiaca</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin releasing hormone</td>
</tr>
<tr>
<td>CPR</td>
<td>Cytochrome P450 reductase</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>dib</td>
<td>disembodied</td>
</tr>
<tr>
<td>dsf</td>
<td>dissatisfaction</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DHR3</td>
<td><em>Drosophila</em> Hormone Receptor 3</td>
</tr>
<tr>
<td>dsRNAi</td>
<td>Double stranded interfering RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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<tr>
<td>E</td>
<td>Ecdysone</td>
</tr>
<tr>
<td>E75</td>
<td>ecdysone induced gene in 75</td>
</tr>
<tr>
<td>EcR</td>
<td>ecdysone receptor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERR</td>
<td>Estrogen related receptor</td>
</tr>
<tr>
<td>EID</td>
<td>Eye imaginal disc</td>
</tr>
<tr>
<td>FTZ-F1</td>
<td>Fushi Tarazu-Factor 1</td>
</tr>
<tr>
<td>GHRH</td>
<td>Growth hormone releasing hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>dHNF4</td>
<td>Hepatocyte nuclear factor-4</td>
</tr>
<tr>
<td>20-E</td>
<td>20-hydroxyecdysone</td>
</tr>
<tr>
<td>4HT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>IPC</td>
<td>insulin producing cell</td>
</tr>
<tr>
<td>JH</td>
<td>Juvenile hormone</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LBP</td>
<td>Ligand-binding pocket</td>
</tr>
<tr>
<td>LH</td>
<td>Leutinizing hormone</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver x receptor</td>
</tr>
<tr>
<td>MBIM</td>
<td>Modified basic incubation media</td>
</tr>
<tr>
<td>MODY1</td>
<td>Maturity-Onset Diabetes of the Young</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
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</tbody>
</table>
NO - Nitric Oxide
NOS - Nitric oxide synthase
NLS - Nuclear localization signal
NR - Nuclear Receptor
O₂ - Oxygen
phm - phantom
PG - Prothoracic gland
PTTH - Prothoracicotropic hormone
RE - Response element
RAR - Retinoic acid receptor
RXR - Retinoid X receptor
RNA - Ribonucleic acid
RNAi - RNA interference
sad - shadow
SCN - Suprachiasmatic nucleus
SF-1 - Steroidogenic factor 1
sGC - soluble Guanylate Cyclase
shd - shade
spk - spook
SVP - Seven-up
Tll - Tailless
TRH - Thyrotropin releasing hormone
3-D - Three dimensional
USP - Ultraspireacle
Chapter 1: An introduction to nuclear receptors and their role in

Drosophila melanogaster development

“If I have seen further it is by standing on the shoulders of giants.”

- Sir Isaac Newton

from a letter to Robert Hooke,

February 15th, 1676
Development in multicellular organisms is regulated by complex signaling systems capable of integrating information from a diverse range of nutritional and environmental inputs. In response, these systems direct coordinated tissue specific gene expression patterns throughout the organism. Knowledge gained from investigations aimed at identifying the underlying molecular determinants which regulate these complex gene expression hierarchies has demonstrated that nuclear receptors (NR’s) play a central role. NR’s belong to a large family of eukaryotic transcription factors whose structure is conserved from worms to humans (Maglich et al., 2001). NR’s have been shown to act as key regulators in a wide range of physiological and developmental processes including lipid metabolism, circadian rhythm, immune function, and puberty. In addition, it has been demonstrated that mutations in NRs result in a number of disease states such as cancer, inflammation, and obesity. The primary feature that distinguishes NR’s from other classes of transcription factors is that their activity is modulated by the binding of small lipophilic molecules such as steroids, thyroid hormone, retinoic acid and vitamin D (Mangelsdorf et al., 1995). Ligand binding induces conformational rearrangement in the 3-dimensional (3D) structure of NR’s, directly affecting their ability to recruit co-regulatory proteins, and ultimately resulting in changes in their ability to regulate the expression of downstream target genes.

In this thesis I have attempted to provide novel insights into the role of nuclear receptors in development by studying their function in Drosophila melanogaster through the development of an in vivo activity assay, and through a variety of molecular and genetic approaches aimed at demonstrating the regulation of the NR ecdysone inducible protein 75 (E75) by the small diatomic gas nitric oxide (NO) in vivo.
In this introductory chapter I will first provide an overview of NRs and their function, along with an outline of their involvement in *Drosophila* development. I will then provide background on several individual *Drosophila* NRs including ecdysone induced protein 75 (E75), *Drosophila* hormone receptor 3 (DHR3), and *Fushi Tarazu-Factor 1* (FTZ-F1), with a specific emphasis on the interactions between them, and regulation of these interactions by the small diatomic gas nitric oxide (NO).

1.1. **Nuclear Receptors**

1.1.1. **Nuclear Receptor Domain Architecture**

Members of the NR superfamily have a highly conserved structure that contains multiple functional domains (Figure 1.1) which are thought to have been incorporated into a single molecule as a result of a fusion event linking discrete, pre-existing, protein modules during the course of evolution (Barnett et al., 2000). NRs are characterized specifically by the presence of a highly conserved N-terminal DNA binding domain (DBD), and a less conserved C-terminal ligand-binding and dimerization domain (LBD) which contains a ligand-dependent activation domain known as the activation function 2 (AF2). The DBD and LBD are linked through a flexible hinge region which varies in length among members of the NR superfamily. In addition, NRs contain a diverse set of N-terminal domains (NTDs) which vary in length from a relatively short stretch preceding the DBD to hundreds of amino acids (Warnmark et al., 2003). In some NRs, such as the steroid hormone receptors, the NTD contains a ligand-independent activation domain termed activation function 1 (AF1), which is responsible for mediating transcriptional activation through ligand independent cofactor recruitment (Warnmark et al., 2003). In addition, some receptors contain a carboxyl-terminal domain, referred to as the F-domain, whose function
is not clear but which has been shown to mediate NR based repression (Olefsky, 2001). The presence of these multiple functions within the same molecule allows for a one-step response to a signal that results in direct effects on target gene expression.

1.1.2. The nuclear receptor DNA binding domain (DBD)

The DBD is composed of two highly conserved zinc fingers that set the nuclear receptors apart from other DNA-binding proteins (Berg, 1989; Klug and Schwabe, 1995). The DBD is responsible for targeting the receptor to highly specific DNA sequences comprising a hormone response element (RE) (Bourguet et al., 2000) by binding to small, 6 base pair, half sites through a region in the DBD called the P-box (Zilliacus et al., 1995a; Zilliacus et al., 1995b). The binding of NRs to these REs is central to their ability to regulate target gene expression.

The nuclear receptor superfamily has been organized into four categories based on both their DNA binding and dimerization properties (Mangelsdorf et al., 1995) (Figure 1.2). Class 1 receptors include the steroid hormone receptors, which function as homodimers and which bind to half-site RE inverted repeats. Class 2 receptors exist as heterodimers with retinoid X receptor (RXR) partners and function in a ligand dependent manner. Some of these receptors can also
bind DNA as homodimers and occasionally as monomers, but they preferentially heterodimerize in response to ligand and subsequently bind DNA elements consisting of half sites in direct repeats. Class 3 receptors include orphan receptors which function as homodimers binding to direct RE repeats. And Class 4 receptors bind DNA as monomers to single site REs.

Although the classical view of NR DNA binding holds that the DBD is responsible only for site-specific recognition and binding to DNA, it should be noted that recent evidence from structural studies has indicated that binding of the DBD to specific RE sequences may be more than just a mechanism for localizing the receptor to the correct DNA sequence (Thompson and Kumar, 2003). In fact, it has been hypothesized that the exact nucleotide sequence of the RE affects not only the overall affinity of the receptor for its RE site, but also influences the three dimensional (3-D) configuration of the receptor, thereby regulating NR activity through the binding of certain ancillary factors (Azoitei and Spindler-Barth, 2009; Thompson and Kumar, 2003).
Nuclear receptors can be grouped into four classes according to their ligand binding, DNA binding, and dimerization properties: steroid receptors, RXR heterodimers, homodimeric orphan receptors, and monomeric orphan receptors. Steroid receptors bind to DNA at inverted response element (RE) repeats as homodimers. RXR heterodimers bind to DNA at direct RE repeats. Homodimeric orphan receptors bind to DNA at direct RE repeats. Monomeric orphan receptors bind to single REs as individual monomers. Shown are representative receptors for each group with known ligands. Question marks refer to orphan receptors. Adapted from (Mangelsdorf et al., 1995)

1.1.3. The NR ligand binding domain (LBD)

The carboxyl-terminal half of the receptor encompasses the LBD (Figure 1.1). The LBD sets the nuclear receptor family apart from other classes of transcription factors in that it is capable of binding to small lipophilic molecules such as steroids, retinoids, and vitamins which regulate the activity of these receptors (Moras and Gronemeyer, 1998). In fact, it has been
suggested that the NRs evolved from a single ancestral orphan receptor that acquired the ability to bind ligands during the course of its evolution (Escriva et al., 1997). Unlike water-soluble peptide hormones and growth factors, which bind to their receptors sitting at the cell surface, the majority of nuclear receptor ligands are fat soluble and can therefore pass unhindered through the lipid bilayer of the cell membrane and interact with their cognate receptors in the nucleus.

In its simplest terms, the LBD can be thought of as a molecular switch that places the receptor in a transcriptionally active state in response to ligand binding. In fact, the availability of high resolution 3-D structures for several NR LBDs has provided insights into the structural basis for this switch (reviewed in (Bourguet et al., 2000; Egea et al., 2000; Kumar and Thompson, 1999; Li et al., 2003; McInerney et al., 1998). It has been well established that NR LBDs are globular in nature and that they typically consist of 11 or 12 alpha helices and 2 to 4 beta strands (Warnmark et al., 2003). In addition, the LBD is known to harbour a ligand-dependent activation function 2 (AF2) domain responsible for mediating both NR homo- and heterodimerization, and binding between NRs and a diverse set of coregulatory proteins in response to ligand binding (Bourguet et al., 2000). Both NR dimerization and binding of NRs by coregulators has been shown to rely, in large part, upon the physical interaction between NRs and short LXXLL motifs (where L corresponds to Leucine, and X corresponds to any amino acid) on the surface of coregulatory interacting proteins (Heery et al., 1997; Loinder and Soderstrom, 2004; Savkur and Burris, 2004).

Certain receptors such as thyroid and retinoic acid receptors (RARs) act as transcriptional silencers in their unliganded or antagonist bound state (Bourguet et al., 2000; Perissi and Rosenfeld, 2005) (Figure 1.3). Exceptions, however, have been reported (Perissi and Rosenfeld, 2005) and it is believed that some receptors may be capable of binding to a number of different
ligands with a range of individual receptor binding affinities, each capable of modulating the activity of the NR in subtle ways (Delage-Mourroux et al., 2000; White et al., 2004).

![Figure 1.3](image)

**Figure 1.3 - Schematic representation of three distinct conformational states of a nuclear receptor ligand-binding domain (LBD)**

(a) The unliganded [apo] retinoic acid receptor (RAR) LBD. (b) The agonist-bound [holo] RAR LBD. (c) The antagonist-bound RAR LBD. The a-helices (H1–H12) are depicted as rods whereas broad arrows represent the β-turn. The various regions of the LBD are coloured depending on their function: the dimerization surface is shown in green, the co-activator and co-repressor binding sites are shown in orange and the activation helix H12 that harbours the residues of the core activation function 2 (AF-2) activation domain (AD) is shown in red; other structural elements are shown in mauve. Abbreviation: LBP, ligand-binding pocket. Adapted from (Bourguet et al., 2000).

The publication of several unique, liganded, structures of the estrogen receptor (ER) in complex with either the endogenous agonist, estrogen, or the xenobiotic antagonist, 4-hydroxytamoxifen (4HT), has provided one of the most elegant examples of the physical mechanism by which ligand binding induces a change in the 3-D structure of the NR LBD.
(Gangloff et al., 2001; Wang et al., 2006). Comparison between these structures has revealed that the estrogen receptor adopts unique conformations in response to the binding of structurally distinct ligands (Figure 1.4) in a manner consistent with the *induced fit* model of enzyme-substrate interaction first put forward by Daniel Koshlund (Mockrin et al., 1975). The most striking feature of this structural change is the dramatic repositioning in the LXXLL containing terminal helix (helix 12) of the estrogen receptor. The change in the position of helix 12 in response to has 4HT binding has been shown to be a central component in modulating the binding and recruitment of distinct coregulatory proteins by ER (Wang et al., 2006).
Figure 1.4 - Comparison of the structures of the human estrogen receptor in complex with estradiol and 4-hydroxytamoxifen

The heterodimeric ER LBD shown in complex with either the endogenous ER agonist estradiol (not visible, top), or the xenobiotic ER antagonist 4-hydroxytamoxifen (red, bottom). Atoms from each ER monomer are represented as either dark blue or light blue spheres, with residues from helix 12 shown in green. Adapted from RCSB PDB Molecule of the month, September 2003.
1.1.4. The mechanism of nuclear receptor action

The regulation of gene expression by NRs is a direct consequence of their ability to recruit a wide range of positive and negative coregulatory proteins referred to as coactivators and corepressors, respectively (Figure 1.5) (McKenna et al., 1999). Coactivator complexes contain factors that serve several important functions (Perissi and Rosenfeld, 2005). These functions include the ability to alter chromatin structure through ATP-dependent chromatin remodeling complexes, histone arginine methyltransferase activity, and histone acetyltransferase activity. In addition, it has been demonstrated that coactivator complexes contain components involved in RNA processing, and components of the Mediator complex responsible for mediating the interaction with the RNA polymerase II machinery. Corepressor complexes also include components with a range of important functionalities (Perissi and Rosenfeld, 2005). These include ligand-dependent corepressors such as LCoR and RIP140 which are capable of recruiting other members of the complex in a ligand-dependent manner. In addition, basal corepressors such as NCoR and SMRT serve as platforms for the assembly of a range of subcomplexes which are each able to remodel chromatin through histone deacetylase activity, and through ATP-dependent chromatin remodeling complexes. Taken together, it has become increasingly clear that there are a vast number of coregulatory proteins and complexes that function in a combinatorial manner, and which provide an additional level of regulation that functions downstream of NRs in the control of gene expression.
Figure 1.5 - Coactivator and corepressor complexes are required for nuclear receptor-mediated transcriptional regulation.

The regulation of a general transcription unit by nuclear receptors is dependent upon a large number of coregulatory complexes that have various functions and enzymatic activities. Coactivator complexes (shown in green) include factors that contain ATP-dependent chromatin remodelling activity, histone arginine methyltransferase activity, and histone acetyltransferase activity. Coactivator complexes also tend to contain factors involved in RNA processing as well as components of the Mediator complex. Conversely, corepressors (shown in red) include ATP-dependent chromatin remodelling complexes, basal corepressors (NCoR and SMRT), which function as platforms for the recruitment of various subcomplexes often containing histone deacetylase activity, and specific ligand-dependent corepressors, such as NCoR and RIP140. Adapted from (Perissi and Rosenfeld, 2005).
1.1.5. The Nuclear Receptor Superfamily

NRs comprise an ancient family of transcriptional regulators that have been found in clades as diverse as sponges, echinoderms, tunicates, arthropods and vertebrates, and are therefore believed to be present throughout the Metazoa (Escriva et al., 2004; Laudet, 1997; Thornton et al., 2003). In fact, the NR superfamily represents one of the largest families of transcriptional regulators in metazoans (Laudet, 1998).

Phylogenetic analysis of nuclear-receptor genes has revealed six distinct subfamilies, defined by conservation between both their DNA- and ligand-binding domains (Maglich et al., 2001). Although there are only 18 nuclear receptors in Drosophila, compared with 48 in humans and over 270 in Caenorhabditis elegans, these 18 receptors have representative members in each of the 6 human receptor subfamilies (Figure 1.6 and Table 1.1), making Drosophila a suitable model organism in which to conduct molecular and genetic studies into this diverse family of transcriptional regulators.
Figure 1.6 - Nuclear Receptor Phylogeny

The phylogenetic tree shown depicts the evolutionary relationship between human (shown in red) and *Drosophila* (shown in green) NRs. The six NR subfamilies are indicated on the right. Structures of several known ligands, with their corresponding receptor indicated in brackets, are shown. Adapted from (Maglich et al., 2001), (Robinson-Rechavi et al., 2003), and (King-Jones and Thummel, 2005)
Table 1.1 - *Drosophila* nuclear receptors and their human homologs

The 18 canonical *Drosophila melanogaster* nuclear receptors are listed and the ligands for the ecdysone receptor (EcR), and E75 are shown in parentheses. Subfamily designation and nuclear-receptor nomenclature is as described (Maglich et al., 2001; Ruau et al., 2004). The human nuclear receptors that display the highest percentage amino-acid identity with a fly NR DBD and/or LBD are listed along with a representative ligand, where known. The final column shows the percentage amino-acid identity between the homologous human and fly DBDs and LBDs. Adapted from King-Jones and Thummel (2005).

<table>
<thead>
<tr>
<th><em>D. melanogaster</em> receptor (ligand)</th>
<th>Subfamily</th>
<th>Nomenclature</th>
<th>Human orthologue (ligand)</th>
<th>DBD / LDB % identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E75 (heme)</td>
<td>1D/E</td>
<td>NR1D3</td>
<td>REV–ERB-A</td>
<td>80/25</td>
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<tr>
<td>E78</td>
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<td>NR1E1</td>
<td>REV–ERB-A</td>
<td>69/23</td>
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<td>1F</td>
<td>NR1F4</td>
<td>ROR-B (all trans retinoic acid)</td>
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</tr>
<tr>
<td>EcR (20-hydroxyecdysone)</td>
<td>1H</td>
<td>NR1H1</td>
<td>FXR (chenodeoxycholic acid)</td>
<td>72/28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LXR (22(R)-hydroxycholesterol)</td>
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</tr>
<tr>
<td>DHR96</td>
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<td>NR1J1</td>
<td>VDR (1α, 25-dihydroxyvitamin D3)</td>
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<td>HNF4A</td>
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<tr>
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<td>2B</td>
<td>NR2B4</td>
<td>RXR-A (9-cis-retinoic acid)</td>
<td>84/43</td>
</tr>
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<td>TR-2</td>
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<td>NR2E2</td>
<td>TLX</td>
<td>80/34</td>
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<td>88/28</td>
</tr>
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<td>62/25</td>
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<td>60/26</td>
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<td>6A</td>
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<td>GCNF</td>
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</table>
1.1.6. Nuclear receptors as targets for the treatment of human disease

As mentioned, NRs have been shown to act as key regulators in a diverse range of developmental and homeostatic pathways. These include embryogenesis, detoxification, circadian rhythm generation, glucose and lipid homeostasis, cell differentiation, and embryonic development (Chawla et al., 2001; Francis et al., 2003). Consistent with their playing a central role in a wide range of fundamental biological processes, mutations in NR genes have been implicated in a large number of human disease states including obesity, inflammation, cancer, and cardiovascular disease (Chawla et al., 2001; Francis et al., 2003). Only a relatively small number of all NRs, however, have a known natural ligand. As a consequence, significant attempts have been made towards the identification of both endogenous and synthetic ligands for individual members of the NR superfamily in an effort to provide novel therapeutics for a range of diseases in which NRs play a role.

Towards this goal, Drosophila melanogaster has served as an indispensable tool for the elucidation of the function and regulation of NRs, and has emerged as an ideal model system for characterizing their diverse biological roles during development (King-Jones and Thummel, 2005). Of the 18 identified nuclear receptors in Drosophila, a cognate ligand has been identified for only 2; EcR (Koelle et al., 1992) and E75 (Reinking et al., 2005). Identification of ligands for any of the remaining 16 orphan receptors would provide both key insights into the control of eukaryotic gene expression, and new insights into the complex regulatory pathways that these receptors and their human homologs participate in.
1.2. The hormonal control of Drosophila metamorphosis

“The butterfly's attractiveness derives not only from colors and symmetry: deeper motives contribute to it. We would not think them so beautiful if they did not fly, or if they flew straight and briskly like bees, or if they stung, or above all if they did not enact the perturbing mystery of metamorphosis: the latter assumes in our eyes the value of a badly decoded message, a symbol, a sign.

- Primo Levi

From the dawn of recorded history insect metamorphosis has been viewed by mankind with a sense of curiosity and wonder. Metamorphosis has been a central symbol of transformation and rebirth across numerous cultures and civilizations, and has served as a mirror into which human beings have gazed and come away from with a deeper understanding of both themselves and of the universe around them [reviewed in Berenbaum (1995)]. However, despite its prominent symbological role, and the considerable attention afforded by great thinkers such as Aristotle, Ovid, Charles Darwin, and August Weisman, the exact nature of the underlying determinants that govern insect metamorphosis remained elusive well into the Twentieth Century. In fact, the classical view of insect development, which saw the insect larva and pupa as active embryos, remained largely unchanged from Aristotle’s time through to the 19th Century. This view was challenged by Charles Darwin when he put forward his own interpretation of insect development. Darwin posited that the relationship of larva and pupa to the adult insect was that of a polymorphism with successive adaptive functions (Edwards, 1998). According to
Darwin, the larval stage involved sedentary feeding towards the acquisition and storage of nutrients, whereas the adult stage functioned primarily towards reproduction, with the pupal stage acting as a bridge between the two. It was not, however, until the early part of the twentieth century that this concept of metamorphosis as sequential polymorphism would take form.

1.2.1. The life cycle of Drosophila melanogaster

The fruit fly, *Drosophila melanogaster*, is a holometabolous insect which passes through four distinct life stages - as an embryo, a larva, a pupa and an imago (adult). *Drosophila* embryogenesis proceeds over the course of a day. Embryos hatch to produce a larva which progresses through three successive larval stages (instars) separated by intervals during which molting of the larval cuticle takes place. On the fifth day after egg laying (AEL), at the end of the third larval instar, metamorphosis begins. During this time, larvae cease feeding, wander away from the food, and attach themselves to a surface through a glycoprotein glue secreted by the salivary glands. These terminal larvae begin to form a hard, protective outer shell which will eventually form the pupal case inside which a dramatic reorganization of both cells and tissues takes place. During this time, larval tissues are broken down in a stereotypical fashion, and primitive adult structures, termed imaginal discs, begin to differentiate and evert, and will eventually contribute to the formation of an imago, or adult fly.

In humans, the passage from adolescence to adulthood is accompanied by rapid changes in growth and acquisition of sexual maturity (Navarro et al., 2007). Insect metamorphosis is a similar, albeit more dramatic, transformation which serves as a bridge between an adolescent stage, characterized by rapid growth and feeding, and the adult stage in which sexual maturity has been achieved. Although Swammerdam had provided detailed
descriptions of the life cycle of insects by 1669, and Darwin, amongst others, had provided
general theoretical interpretations of how genes exert their function in guiding metamorphosis,
the underlying regulatory mechanisms only began to be understood at the beginning of the
Twentieth Century. Since then it has become clear that hormonal signals are responsible for the
coordination and regulation of all aspects of *Drosophila* development. Through the seminal
transplantation and ligation experiments of early insect physiologists including Kopeč,
Wigglesworth, Bodenstein, Fraenkel, Hadorn, Vogt, Butenandt and Karlson (Kopec, 1922,
1996, Murray, 1994), it became clear by the middle of the twentieth century that hormones are
central in the control of insect development and metamorphosis, that the principal source of these
hormonal signals in *Drosophila* is a small endocrine organ called the ring gland, and that the
hormones signaled through NRs.

1.2.2. Identification of the molting hormone in insects

Around the same time that Wigglesworth and Bodenstein were conducting their
experiments on insect metamorphosis, the field of vertebrate endocrinology was beginning to
take form. In the late 1920’s Adolf Butenandt independently isolated the estrogenic compound
estrone through extraction from several thousand litres of urine (Karlson, 1995), and went on to
determine its structure in 1932 (Karlson, 1995). Although the molecular target of action was not
known at the time, Butenandt’s identification of estrone would come to be acknowledged as the
first identification of a nuclear receptor ligand. Knowledge gleaned from Butenandt’s early
experiments with estrone provided him with the tools that would later prove essential for the
identification of the molting hormone in insects. Despite the fact that the tissue of origin of the
insect molting hormone had been known by the late 1930’s, it was not until 1954 that the hormone itself was identified by Butenandt and Karlson (reviewed in Karlson (1996).

In 1954, in what has become a seminal work in insect endocrinology, α- and β-ecdysone were isolated from 500 kilograms of pupae of the silkworm Bombyx mori (reviewed in (Karlson, 1996). In a study that served as the first chemical identification of an insect hormone the isolated hormones turned out to be two slightly different steroid molecule variants with molt inducing activity, and whose structure Karlson later went on to determine (reviewed in (Karlson, 1996)). Subsequent analysis revealed that α-ecdysone was produced by the prothoracic glands and was not the agent directly responsible for molting but rather that it was a prohormone that was converted in peripheral tissues, such as the epidermis and fat body, to the highly active molting hormone β-ecdysone (20-hydroxyecdysone) (reviewed in (Karlson, 1996)).

The identification of ecdysone revolutionized insect endocrinology and insect molecular biology as it provided for the first time not only the identity of the molecule responsible for the onset of metamorphosis, but also a tool with which to probe the mechanism by which metamorphosis is regulated. Moreover, the realization that development in both insects and in humans was regulated by steroid hormones with astonishingly similar structures suggested that insects could serve as a model system in which to probe the hormonal regulation of gene expression.

1.2.3. *The Drosophila Ring Gland*

The *Drosophila* ring gland is a complex endocrine tissue which regulates postembryonic growth and development, and in particular the timing of both molting and metamorphosis, through the synthesis and secretion of steroid hormone ecdysteroids and sesquiterpenoid juvenile
hormone [reviewed in Berger and Dubrovsky (2005), Nijhout (2003), Riddiford (1993), and Riddiford (1994)].

The ring gland sits atop the brain and forms a ring that encircles the aorta, allowing for the secretion of hormones directly into the hemolymph for transport to distal sites. The ring gland is a large composite organ composed of three distinct cell types which include the the corpora allata (CA), the corpora cardiaca (CC), and prothoracic gland (PG) (Figure 1.7).

The CA lies at the centre of the ring gland and is responsible for the production of the sesquiterpenoid juvenile hormones (JHs), a group of acyclic sesquiterpenoids which regulate insect development, reproduction, and diapause, and which play a role in both promoting molting and in maintaining insects in their larval stages [reviewed in Nijhout (1994), Riddiford (1994)]. The role of JH in *Drosophila* development, however, has not yet been fully elucidated.

The CC is responsible for the production of the small peptide hormone adipo-kinetic hormone (AKH) which, through binding to the cell surface receptor AKHR, regulates the mobilization of lipid stores from the fat body for the production of circulating carbohydrates, and which is considered to be the *Drosophila* equivalent of vertebrate glucagon (Bharucha et al., 2008; Gronke et al., 2007; Kim and Rulifson, 2004; Lee and Park, 2004).

The PG is made up of approximately 50 large, polyploid cells whose principal function is to synthesize ecdysteroids, a class of polyhydroxylated steroidal hormones derived from either dietary cholesterol or phytosterols which are converted to the active, molt-inducing hormone 20-hydroxyecdysone (20-E) in peripheral tissues (Gilbert et al., 2002).
Figure 1.7 - The *Drosophila* 3rd instar larval ring gland

The brain-ring gland complex from a *Drosophila* 3rd instar larva is shown. Cells of the corpora allata (CA, yellow), prothoracic gland (PG, red), and corpora cardiaca (CC, green) are indicated with white arrows. The eye imaginal discs (EID), and brain hemispheres (BH) are also indicated. Nuclei (blue) are stained with DAPI.
1.2.4. *The ecdysone biosynthetic pathway in Drosophila*

“*Cholesterol is the most highly decorated molecule in biology*”

M.S. Brown and J.L. Goldstein, Nobel lecture 1985

The pioneering work of Hobson provided the first demonstration that a dietary supply of sterol was absolutely required for the growth of an insect [reviewed in Clayton (1964)] and led to the suggestion that a dietary requirement for sterol was likely a general one for all insects. Clayton also proposed that the ability of insects to synthesize sterols *de novo* was either entirely absent or insufficient in meeting the nutritional demands imposed during development. At almost the same time that Hobson was completing his experiments with insect sterols in the blowfly, Van’t Hoog reported similar findings for *Drosophila melanogaster* (Clayton, 1964). It has since been established that, despite the fact that many of the genes required for *de novo* cholesterol biosynthesis are both present and highly conserved, *Drosophila melanogaster* is a cholesterol auxotroph (Vinci et al., 2008).

Considering that the molting hormone identified by Butenandt and Karlson was a polyhydroxylated cholesterol derivative and that cholesterol was essential for insect development, the focus of insect endocrinologists began to shift towards identifying the components involved in the ecdysteroidogenic pathway. Since then it has become clear that all steroids are derived from cholesterol, which is an ancient and highly conserved compound fundamental in the structure and function of biological membranes [reviewed in Yamamoto (1985)]. Steroids are well suited as intercellular signaling molecules as they are small, stable, and lipophilic and can therefore cross cell membranes.
Steroid hormone biosynthesis starts with a hydroxylation and side-chain cleavage reaction that is dependent upon ATP, molecular oxygen, and cytochrome P450 (CYP450) enzymes (Gilbert et al., 2002). In fact, CYP450s play a central role in ecdysteroidogenesis. CYP450 monooxygenases comprise a large superfamily of enzymes that are found in all organisms and which catalyze a diverse range of biochemical reactions including, but not limited to, hydroxylation, epoxidation, aromatic and aliphatic oxidation, dehalogenation, dealkylation, desulphuration, and oxidative deamination (Agosin, 1985; Lewis, 1996). In accordance with the diverse range of chemistries in which they play a role, CYP450s have been recruited for the synthesis of compounds such as steroids, fatty acids, and prostaglandins and to metabolize xenobiotics such as drugs, carcinogens, and other environmental pollutants (Feyereisen, 1999; Gonzales, 1996; Maurel, 1996; Ronis, 1996; Schuler, 1996; Scott, 1999).

It is known that the conversion of dietary cholesterol into 20E is catalyzed by a minimum of seven discrete enzymatic steps, each of which involves hydroxylation by CYP450 enzymes (Gilbert et al., 2002). As a first step, dietary cholesterol (or plant sterol) precursors undergo dehydrogenation to 7-dehydrocholesterol in the endoplasmic reticulum and are then transported into mitochondria for subsequent oxidations (Lafont, 2004).

Recently, five genes that encode CYP450s that are essential for 20E biosynthesis have been identified in *Drosophila* (Chavez et al., 2000; Niwa et al., 2004; Ono et al., 2006; Petryk et al., 2003; Warren et al., 2002; Warren et al., 2004; Yoshiyama et al., 2006). These are: *phantom* (phm), *disembodied* (Petit-Bertron et al.), *shadow* (sad), *shade* (shd) and *spook* (spk), all of which encode cytochrome P450 mono-oxygenases, and which belong to the so-called Halloween gene family. Mutations in all five of these genes in *Drosophila* result in a failure to produce 20E and to induce ecdysone-responsive genes, and display both embryonic lethality and a failure to
secrete a first instar cuticle (Chavez et al., 2000; Ono et al., 2006; Petryk et al., 2003; Warren et al., 2002; Warren et al., 2004). In accordance with their role in ecdysone biosynthesis, the expression of these CYP450s has been shown to be temporally correlated with the ecdysteroid peaks during larval development and is restricted to the PG (Namiki et al., 2009; Niwa et al., 2004; Ono et al., 2006; Parvy et al., 2005; Rewitz et al., 2006; Warren et al., 2004).

In addition, recent work has resulted in the identification of the shade (shd) gene, which is responsible for catalyzing the final step in ecdysone biosynthesis, the conversion of ecdysone to 20E in peripheral tissues, and that it too is a CYP450 (Petryk et al., 2003; Rewitz et al., 2006).

Two common features shared by all of these ecdysteroidogenic CYP450s is that they localize either to the mitochondria or the endoplasmic reticulum (Gilbert, 2004), and that they require the transfer of an electron pair from NADPH and molecular oxygen in order to modify their substrates. In mitochondria transfer of these electrons is mediated by adrenodoxin reductase (DARE) which has been shown to play an essential role in ecdysteroid biosynthesis (Freeman et al., 1999), whereas in the ER this reaction is mediated by NADPH cytochrome P450 reductase (CPR) (Tijet et al., 2001).
1.2.5. The regulation of Drosophila development by 20E, JH, and PTTH

20E and JH are the two major hormones responsible for insect growth and development (Henrich et al., 1999; Riddiford, 1993). In Drosophila peaks of 20E determine the timing of all developmental transitions, from embryo to larva, from one larval instar to the next, from larva to pupa, and pupa to adult (Riddiford, 1993) (Figure 1.8). The synthesis and release of JH overlaps with 20E release in a dynamic manner during development (Figure 1.8). It has been shown that the balance of these two hormones defines the outcome of each developmental transition in insects (Henrich, 1995; Riddiford, 1993). Molting, whether it results in cuticle shedding or restructuring of the body plan, is initiated by 20E (Henrich et al., 1999; Riddiford, 1993), while the nature of the molt is determined by the level of JH (Riddiford et al., 2003). For molts between larval instars, a high JH titer is needed, whereas the metamorphic molt occurs only once the JH titer drops during the final instar (Riddiford, 1993).

Much of what is known about the regulation of metamorphosis and ecdysone biosynthesis in the ring gland comes from work done in the endocrine model insects Manduca sexta and Bombyx mori, and although the mechanistic details of metamorphosis differ between species, it has become clear that the overall pattern of hormone action is remarkably similar to that in Drosophila melanogaster.
Figure 1.8 - Ecdysone and Juvenile hormone titers in *Drosophila* development

Ecdysone titers (shown as a solid line) and juvenile hormone titers (shown as a broken line) determine the normal course of *Drosophila* development, shown in days at bottom, from an embryo to an adult fly. Adapted from (Riddiford, 1994).

The production and release of ecdysone is initiated by the small, secreted neuropeptide known as prothoracicotropic hormone (PTTH) (Kim et al., 1997; McBrayer et al., 2007; Nagasawa et al., 1984). PTTH is synthesized and secreted by a specialized class of bilateral neurosecretory cells which innervate the ring gland and whose axon terminals make contact with PG cells (Siegmund and Korge, 2001) which respond to PTTH through its receptor the receptor tyrosine kinase Torso (Rewitz, 2009). In addition, PTTH-secreting cells are subject to circadian regulation (McBrayer et al., 2007) and have been shown to participate in a direct, physical interaction with circadian pacemaker neurons through an overlapping dendritic field (Siegmund and Korge, 2001).
Studies in Lepidoptera and in several other insect groups have demonstrated that PTTH release is regulated by a number of environmental factors including animal crowding, nutritional status, temperature, and circadian rhythm [reviewed in Gilbert et al. (2002), Mirth et al.(2005)]. PTTH is thus thought to serve as an integrative output for a complex set of developmental cues, one of the most important of which is nutritional in nature. It is known that in larvae, a complex nutritional assessment is made which is responsible for ensuring that an appropriate size has been reached and that sufficient nutritional stores have been acquired in order for the animal to survive the ensuing period of starvation and to meet the energetic demands of metamorphosis [reviewed in Edgar (2006), Nijhout (2003)]. Two such distinct size thresholds exist during the terminal instar of larval development. The first threshold is termed ‘minimal viable weight’ which is the first checkpoint that must be attained for survival through metamorphosis. Starvation before this time results in a prolonged larval stage and eventual death without pupariation [reviewed in Nijhout (2003)]. The second threshold is termed ‘critical weight’ and represents a time at which starvation no longer affects the time to pupariation [reviewed in Nijhout (2003)]. It is important to note that the establishment of critical weight is not a result of the accumulation of a sufficient amount of stored nutrients for survival through metamorphosis, but rather it has been proposed to represent a developmental switch in the response of larvae to starvation (Mirth and Riddiford, 2007). Once critical weight has been achieved, there exists a photoperiod gating mechanism which restricts the release of PTTH to a specific eight-hour window each day (Truman, 1972; Truman and Riddiford, 1974). If critical weight is achieved outside of this time-frame, PTTH is not secreted, ecdysone production remains low, and the larvae continue to feed until the next photoperiod gate is reached the following day. This PTTH photoperiod gating mechanism exists in *Drosophila* as well (McBrayer et al., 2007; Mirth et al., 2005). Specifically, the circadian pacemaker neurons with which the PTTH secreting neurons
make contact have been shown to negatively regulate PTTH signaling at night, either at the level of transcript initiation or message stability (McBrayer et al., 2007).

It has recently been shown that the *Drosophila* PG plays a critical role in the assessment of growth status in determining when critical weight has been attained and that insulin-dependent growth of the PG is a critical factor in determining the timing of the onset of metamorphosis (Mirth et al., 2005). It was specifically demonstrated that the duration of the larval stage can be shortened or lengthened by either precocious or delayed ecdysone release through the perturbation of insulin signaling in the PG (Mirth et al., 2005). As a consequence, premature ecdysone release results in the early development of small adults, whereas delayed ecdysone release results in the delayed emergence of large adults (Caldwell et al., 2005; Mirth et al., 2005).

Interestingly, it has been demonstrated that the nuclear receptor DHR4, a repressor of early ecdysone-inducible genes, is both expressed exclusively in the PG of feeding 3rd instar larvae, and plays an important role in determining critical weight (King-Jones et al., 2005). DHR4 mutant larvae behave similarly to larvae in which insulin signaling has been perturbed in that they reach apparent critical weight more rapidly in comparison with their wild-type counterparts, and initiate metamorphosis precociously, resulting in small adults (King-Jones et al., 2005). This is an interesting finding in that it provides the first demonstration that nuclear receptor function in the ring gland is directly involved in the determination of critical weight.

The ring gland, however, does not elicit its effects on growth on its own. It is well known that cell-non-autonomous growth regulation occurs in response to the release of insulin-related growth factors. For example, in *Drosophila* it has been demonstrated that ablation of
neurosecretory cells responsible for expressing insulin-like peptides (ILPs) results in growth defects (Rulifson et al., 2002).

It is now known that 20E signaling negatively controls growth rates by interfering with insulin signaling and that the larval fat body, which functions similarly to the vertebrate liver, is a critical element in relaying ecdysone-dependent growth inhibition (Colombani et al., 2005). This has led to the suggestion that ecdysone counteracts the growth-promoting action of insulins, forming a humoral regulatory loop responsible for the determination of organismal size (Colombani et al., 2005). The following model has therefore been put forward (Mirth et al., 2005): accelerated growth of PG cells in response to increased insulin signaling results in a precocious increase in their basal level of ecdysteroid biosynthesis. Once systemic ecdysteroid concentrations surpass a certain threshold, critical weight is reached following which the endocrine events responsible for the termination of the growth phase of larval development and for allowing the larva to begin metamorphosis are set into motion. Ultimately, these signals coordinately determine developmental timing and final body size.

This has led to the suggestion that insect metamorphosis can in some ways be considered to be similar to mammalian reproductive development (McBrayer et al., 2007). In particular, it was suggested that the regulation of developmental timing through ecdysone synthesis and release resembles the way in which the synthesis and secretion of kisspeptin peptide, a G-protein receptor ligand that triggers gonadotropin releasing hormone (GnRH) secretion in humans at puberty, in conjunction with input from nutritional and metabolic sensors, regulates the gating of the timing of puberty through its action on cells in the hypothalamus (Fernandez-Fernandez et al., 2006; Navarro et al., 2007; Smith and Clarke, 2007). Taken together, the regulation of
*Drosophila* metamorphosis is an emerging model system with which it may be possible to glean insights into the regulation of vertebrate development.
1.2.6. Genetic responses to ecdysone – the Ashburner model

Building upon the observation, originally made by Becker, that the salivary gland polytene chromosomes of *Drosophila* larvae exhibit highly reproducible and temporally stereotypical puffing patterns (Becker, 1959), it was demonstrated that salivary gland puffing could be induced through the application of ecdysone (Clever, 1960). It was also demonstrated that ecdysone induced puffing results in the expression of genes whose products would subsequently activate the expression of another set of genes (Clever, 1964). The assay used would come to serve as a powerful model in probing the molecular nature of the transcriptional response to ecdysone. In fact, using this assay Ashburner was able to elaborate upon the concept that steroids elicit their physiological effects by regulating gene expression. Through careful examination of the morphological changes in the structure of the salivary gland polytene chromosomes in response to the application of ecdysone, Ashburner was able to demonstrate that ecdysteroids trigger chromosomal puffing at specific sites, with a distinct temporal order, in a highly reproducible manner (Ashburner et al., 1974). Through these experiments, what has come to be known as the Ashburner Model for the genetic control of polytene chromosome puffing by ecdysone was developed (Figure 1.9) (Ashburner et al., 1974, Riddiford, 1993). This model put forward the idea that ecdysone functions through its receptor protein to activate the expression of a set of ‘early’ genes and to suppress the premature activation of another set of ‘late-late’ genes. It also suggested that the protein products encoded by the early genes repress their own expression - a function critical to the timing of their regulatory function – and that they go on to both transduce and to amplify the ecdysone signal by inducing the expression of a set of ‘late’ genes in a precisely timed manner. It was also proposed that the ‘late’ gene products act as critical regulators of morphogenesis in distinct target tissues during the early stages of metamorphosis. Further refinement of this model has resulted in the identification of numerous
genes belonging to each class of the ecdysone-induced salivary gland puffing hierarchies. It should come as no surprise that a number of genes involved in regulating the salivary gland chromosome puffing hierarchies are nuclear receptors.

Figure 1.9 - The Ashburner model for the genetic control of polytene chromosome puffing in response to ecdysone.

The ecdysone-receptor protein complex is hypothesized to both directly activate the early genes and the early late genes, and to repress the late-late puff genes. A small set of early genes encode regulatory proteins that repress their own expression and induce a large set of both early-late and late-late puff genes which are thought to play a key role in the initiation of metamorphosis. Adapted from (Thummel, 1990)
1.2.7. NRs are induced in response to ecdysone

Ashburner’s work had shown that six puffs in the salivary gland polytene chromosomes of Drosophila form within minutes after the addition of ecdysone, and that these so-called ‘early’ puffs peak in size after approximately four hours and then they regress (Ashburner et al., 1974).

The first indications that nuclear receptors may be involved in ecdysone signaling in Drosophila were provided by a study that identified a 50-kb ecdysone-inducible gene, E75, located within the early puff locus at 75B (Segraves and Hogness, 1990). Two products of the E75 gene, E75A and E75B, were initially identified, and were shown to encode members of the steroid receptor superfamily (Segraves and Hogness, 1990). Functional studies on the E75A protein indicated that the E75 proteins are not ecdysone receptors, but cloning of the E75 gene proved essential in the identification of two other Drosophila nuclear receptor genes; the bona fide ecdysone receptor itself (Koelle et al., 1991) and DHR3 (Koelle et al., 1992).

1.2.8. Identification of the target of ecdysone action – the ecdysone receptor

The identification of the gene coding for EcR in Drosophila came in 1991 through work done in the Hogness lab (Koelle et al., 1991). EcR was identified by screening cDNA libraries under low stringency conditions with a probe specific to the previously identified ecdysone inducible Drosophila steroid hormone receptor E75 (Segraves and Hogness, 1990). It was demonstrated that EcR protein binds both active ecdysteroids and to DNA at ecdysone response elements with high specificity, that EcR is nuclear and found in all ecdysone target tissues they examined, and that EcR is expressed at each developmental stage marked by a pulse of ecdysone. In addition, they went on to show that ecdysone-responsive cultured cells express EcR, whereas their ecdysone-resistant counterparts are deficient in EcR, and that expression of
EcR in these ecdysone-resistant cells by transfection restores their responsiveness to the hormone (Koelle et al., 1991).

Several studies in Drosophila have demonstrated that EcR does not function on its own, but rather that the ecdysone receptor is a heterodimeric complex of the nuclear receptors EcR and ultraspiracle (USP), the Drosophila homolog of the vertebrate RXRs (Hall, 1998; Thomas et al., 1993; Yao et al., 1992). Heterodimerization between USP and EcR has been shown to be essential for proper transduction of the ecdysone signal at the onset of metamorphosis (Hall, 1998; Schubiger and Truman, 2000), and is regulated in large part through the binding of ecdysteroids (Bergman, 2004). In addition, recent reports describing the three-dimensional structure of the heterodimeric EcR/USP ecdysone receptor in complex with the ecdysteroid ponasterone-A (Billas et al., 2003) and the molting hormone 20-hydroxyecdysone (Browning et al., 2007) (Figure 1.10) have provided insights into the mechanistic basis by which ligand binding regulates both heterodimerization and receptor activity. These structural studies have shed light on the function and physical interaction of the vertebrate counterparts of USP and EcR - RXR and LXR.

The importance of the identification of the ecdysone receptor cannot be understated as it provided for the first time the identity of the molecular target of ecdysone action, thereby revolutionizing the field of insect endocrinology. More importantly, the identification of the ecdysone receptor as a member of the nuclear receptor superfamily suggested a universal role of these receptors in animals and provided clear evidence that the receptors evolved prior to the divergence of vertebrates and invertebrates, reinforcing the importance of Drosophila as a model system with which to better understand the molecular determinants involved in vertebrate endocrinology.
A heterodimer of the ligand binding domains of EcR (black) and USP (light blue) from *heliothis virescens* in complex with 20-hydroxyecdysone (blue) and phosphatidyl ethanolamine (red), respectively. The receptor is shown in two orientations, differing by a 90 degree rotation around the x-axis. Images of the EcR crystal structure PDB ID 2R40 (Browning et al., 2007) were produced using PyMol (Delano Scientific).

1.2.9. *Nuclear receptors play a central role in the ecdysone signaling hierarchy*

Since the discovery of the ecdysone receptor, it has become clear that the expression of at least half of the members of the *Drosophila* NR superfamily are induced directly or indirectly by ecdysone. These are EcR, βFTZ-F1, E75, E78, DHR3, DHR4, DHR39, DHR78, and DHR96 (King-Jones et al., 2005; King-Jones and Thummel, 2005; Thummel, 1995). In fact, with the exception of DHR4 which was first identified in *Manduca sexta* in 2001 (Weller et al., 2001), most of these genes were discovered in a relatively short period of time (1990 to 1995) through efforts aimed at understanding how ecdysone directs the early stages of metamorphosis (Ayer et al., 1993; Fisk and Thummel, 1995; Horner et al., 1995; King-Jones et al., 2005; Koelle et al.,
1992; Lavorgna et al., 1991; Ohno and Petkovich, 1993; Palli et al., 1992; Segraves and Hogness, 1990; Stone and Thummel, 1993). Of the remaining 9 NRs, USP and DHR38 are thought to function in transducing the ecdysone signal (Baker et al., 2003; Yao et al., 1992). It has yet, however, to be determined whether the remaining NRs including Dissatisfaction (DSF), Tailless (TLL), Estrogen related receptor (ERR), Hepatocyte nuclear factor-4 (dHNF4), DHR83, DHR51, or Seven-up (SVP) are also either regulated by or responsible for transducing the ecdysone signal.

A recent study describing the developmental expression profiles for all of the *Drosophila* NRs over the course of embryonic and larval development (Sullivan and Thummel, 2003) has confirmed these results and has served to provide insights into the dynamic temporal fluctuations of these genes during development (Figure 1.11). The discovery that NRs are critical determinants in the ecdysone induced gene expression hierarchies that govern *Drosophila* development has provided support for the Ashburner model and has served to further elucidate the molecular basis for its regulation.
Figure 1.11 - The temporal patterns of nuclear receptor expression during Drosophila larval development.

Gene-expression patterns, depicted in schematic form, are shown for the individual mRNA isoforms of seven Drosophila nuclear receptors, including EcR, E75, E78, DHR3, DHR4, ftz-f1, and DHR39, from the second larval instar to the pupal stage. Ecdysone pulses are shown at top in red, in reference to the main developmental transitions — molts, puparium formation and head eversion, along with developmental stage and time in hours after egg laying for larvae, or hours after puparium formation for prepupae and pupae.

Adapted from (King-Jones and Thummel, 2005; Sullivan and Thummel, 2003)
1.2.10. The Drosophila NR E75

E75 is a complex gene with four transcripts that encode distinct protein isoforms (E75A, E75B, E75C and E75D) (Segraves and Hogness, 1990). These three isoforms share a common C-terminal domain which encompasses the LBD, and differ in that each contains a unique N-terminal exon. The first exon of both E75A and E75C contains an intact DBD, whereas E75B lacks one of the two zinc fingers in the DBD, and is therefore unable to bind to DNA on its own (Segraves and Hogness, 1990).

Both an E75 deficiency and isoform-specific null mutations have been generated and characterized (Bialecki et al., 2002). Investigation into these mutations has demonstrated that E75 is required for normal development as a loss of all three E75 isoforms results in early larval lethality. Out of the three isoform specific mutants, E75A shows the most dramatic phenotype with defects in molting, and both delays and arrests in development. Approximately 20% of E75A mutant second instar larvae survive for several days and then undergo pupariation without molting to the third instar (Bialecki et al., 2002; Sliter, 1992). The molting defects and developmental delays are suggestive of an underlying defect in ecdysone signaling. In accordance with this suggestion, ecdysone feeding is capable of rescuing E75A mutant second instar larvae such that they are able to molt to the third instar indicating that E75A is likely involved in regulating a feedforward pathway responsible for increasing the levels of ecdysone (Bialecki et al., 2002). It has also been suggested that E75A may regulate ecdysone biosynthesis by regulating the expression of specific enzymes in the ecdysteroidogenic pathway exclusive of the genes *dare* or *disembodied*, as the expression levels of these two CYP450 genes is unaffected in E75A mutants (Bialecki et al., 2002).
In contrast, it has been demonstrated that E75B is dispensable for normal development. This finding is somewhat surprising given that E75B has been shown to play a critical role in the regulation of the expression of FTZ-F1 (White et al., 1997), which plays an essential role in regulating ecdysone biosynthesis (Parvy et al., 2005). This inconsistency has been attributed to functional redundancy between E75 isoforms. However, this hypothesis has not been adequately tested as yet.

E75C has been shown to be required to regulate the expression of a subset of genes during the prepupal to pupal transition but is not essential to larval or pupal development as a large proportion of E75C mutants eclose but display severe defects in coordination and fertility. It has been proposed that the survival of E75C mutant animals can be accounted for by redundancy with E75A during metamorphosis.

The temporal expression profiles of E75A, E75B, and E75C mRNA are distinct (Sullivan and Thummel, 2003). E75B mRNA is first expressed in early embryos and thought to be maternally deposited (Dubrovskaya et al., 2004). This expression disappears until a brief pulse of E75B mRNA expression appears during mid-embryogenesis (Sullivan and Thummel, 2003). In contrast, E75A mRNA expression correlates inversely to that of E75B mRNA at mid-embryogenesis (Sullivan and Thummel, 2003). No expression of E75C mRNA is detected during embryogenesis and is detectable only during later stages. The complementary expression of E75A and E75B mRNA is seen again during the second larval instar (Sullivan and Thummel, 2003). Over the course of the third larval instar, E75C mRNA expression gradually increases whereas, in contrast, E75A and E75B mRNA expression is restricted to the end of the instar. When 3rd instar larvae begin to pupariate, E75B mRNA levels increase for 4 hours then drop. E75A mRNA, however, is not seen until 10 hrs after puparium formation (APF), while E75C is
expressed throughout the prepupal period. Consistent with the ecdysone-inducibility of E75, these expression patterns correlate with the well documented changes in the 20-E titer over the course of development (Sullivan and Thummel, 2003). However, it should be noted that the levels of each transcript reflect whole-larval titres and, therefore, individual tissues could vary considerably in terms of transcript expression.

The role of E75 in the regulation of developmental timing has been well characterized, and it has been shown that E75 acts in conjunction with its heterodimeric interaction partner DHR3 to control ecdysone-induced molting, pupariation, and eclosion at least in part through its ability to regulate the expression of the NR βFTZ-F1 (White et al., 1997). During the early prepupal period expression of DHR3 is induced, however ability to activate βFTZ-F1 expression begins only subsequent to the disappearance of E75B (White et al., 2004). In addition, based on their findings that E75B and DHR3 interact directly, and that E75B interferes with the ability of ectopic DHR3 to induce precocious βFTZ-F1 expression, a model was put forward in which E75B regulates the timing of βFTZ-F1 expression by binding to DHR3 (White et al., 2004). Consistent with a role for E75 in regulating ecdysone titers, recent work in Drosophila has shown that βFTZF1 expression in the PG is critical for ecdysone biosynthesis, through its ability to directly regulate the expression of the ecdysteroidogenic enzymes phm and dib (Parvy et al., 2005)

Although E75 was originally defined as an orphan nuclear receptor, E75 function appears to require heme as a requisite ligand (Reinking et al., 2005). In addition, E75 stability is dependent upon heme-binding, and that the E75-heme complex is stable and does not easily dissociate, leading to the suggestion that heme is an obligate component of E75 (Reinking et al., 2005).
Heme-containing proteins are involved in numerous fundamental biological processes such as the transport and storage of oxygen, redox reactions, electron transfer, and NO transport. It has been shown that heme can also act as a biosensor for gaseous ligands such as NO, CO, and O₂, in enzymes, and in transcription factors containing PAS domains (Ponting and Aravind, 1997).

Consistent with these abilities of other heme-proteins, in vitro assays have demonstrated that E75-heme is also capable of binding to the small diatomic gases NO and CO (Reinking et al., 2005). A functional consequence of both NO and CO binding is that they prevent both the physical interaction between E75 and DHR3, and the E75-mediated transcriptional repression of βFTZ-F1 (Reinking et al., 2005). In addition, in vitro data suggest that the mechanism which underlies the gas regulated control of E75 function is based upon the ability of NO and CO to disrupt the interaction of E75 with helix 12 of DHR3 (Reinking et al., 2005).

More recent work has confirmed that E75 is a thiolate hemoprotein and that it undergoes redox-dependent ligand switching, CO- and NO-induced ligand displacement, and exhibits NO and CO binding characteristics that are similar to those of other known redox and gas sensors (de Rosny et al., 2006; Marvin et al., 2009; Pardee et al., 2009). These results strengthen the argument that E75 may function in heme-, redox-, or gas-regulated control of cellular function.

The mammalian homologs of E75, the NR Rev-erb proteins, are known to function as transcriptional repressors (Forman et al., 1994; Giguere, 1999; Guillaumond et al., 2005; Harding and Lazar, 1995). It has also been shown that they are capable of binding heme, NO and CO in vitro, that the presence of NO interferes with their ability to recruit co-repressors, and that their ability to mediate gene repression is responsive to altered levels of heme (Marvin et al.,
2009; Pardee et al., 2009; Rogers et al., 2008; Yin et al., 2007) and NO (Pardee et al., 2009) in cultured cells.

Considering that the responses of both E75 and Rev-erb to NO and CO exhibit striking similarities, the study of E75 in *Drosophila* promises to provide valuable insights into the function of its vertebrate counterparts. However, given that all of these effects have been identified using *in vitro*, bacterial and cell culture models, their relevance to E75/Rev-erb functions *in vivo* remains poorly understood and thus requires further investigation.

1.2.11. *Drosophila* Nitric Oxide Synthase (*dNOS*)

Nitric Oxide (NO) is a highly diffusible intercellular signaling molecule. NO is produced by the enzyme Nitric oxide synthase (NOS) by the conversion of L-arginine to L-citrulline through the utilization of NADPH. Vertebrates have 3 distinct NOS proteins that vary in their tissue specificity and localization, these being neuronal, endothelial, and macrophage NOS.

In *Drosophila*, there exists only one NOS gene, *dNOS*, which shows a high degree of homology to vertebrate neuronal NOS and which, like its vertebrate counterpart, has been shown to be dependent upon both Ca\(^{2+}\) and calmodulin for the production of NO (Regulski and Tully, 1995). Recently, dNOS has been shown to play an essential role in development as *dNOS* null mutants are embryonic lethal (Regulski et al., 2004). In addition, dNOS appears to be subject to complex regulation as truncated dNOS isoforms act as dominant-negative regulators of dNOS activity (Stasiv et al., 2004).

It has previously been shown that NO serves as a switch to arrest cell growth during differentiation of neuronal cells (Peunova and Enikolopov, 1995) and that *in vitro* inhibition of
NOS activity results in the overgrowth of retinal axons (Gibbs et al., 2001). Consistent with these findings and with a role in the regulation of cell growth and proliferation, inhibition of dNOS activity in larvae through injection of NOS-specific inhibitors results in hypertrophy of larval organs and resulting segments in adult flies, whereas ectopic overexpression of NOS has the opposite effect (Kuzin et al., 1996).

dNOS is expressed in a dynamic pattern throughout embryonic, larval, pupal, and adult development (Wildemann and Bicker, 1999). Interestingly, it has been shown that dNOS is expressed in the 3rd instar larval ring gland (Wildemann and Bicker, 1999), suggesting that NOS may participate in the regulation of ecdysteroidogenesis through a direct role in E75 signaling in the ring gland.
Overview of Thesis

Traditional forward endocrinological approaches to NR biology have relied on prior knowledge of a ligand directly capable of regulating gene expression. For instance, radiolabelling of ligands has been successfully used to purify binding proteins which were identified as NRs (Jensen and DeSombre, 1973). In recent years, however, this approach has changed significantly. Bioinformatic analyses of entire genomes has identified a large number of previously unknown genes belonging to the NR superfamily (Maglich et al., 2001), very few of which have known endogenous ligands. Knowledge of the gene sequences encoding these ‘orphan’ receptors has ushered a new era of reverse endocrinology (Kliewer et al., 1999).

Reverse endocrinology revolves around the identification of novel biologically relevant NR ligands by taking advantage of NR sequence data in order to identify novel ligands through both purification based techniques and the generation of NR activity based bioassays (Solomin et al., 1998).

In this thesis I describe a novel tool for the visualization of endogenous patterns of nuclear receptor activity throughout development, and discuss its application in the identification of cognate ligands for orphan nuclear receptors in Drosophila. I then provide evidence that the Drosophila nuclear receptor E75 is regulated by NO in vivo, and describe the biological role of this interaction in Drosophila development.
Chapter 2: Dynamic regulation of *Drosophila* nuclear receptor activity

*in vivo*

A similar report was published in Laura Palanker*, Aleksandar S. Necakov * (* these authors contributed equally to this work), Heidi M. Sampson, Ruoyu Ni, Chun Hu, Carl S. Thummel and Henry M. Krause.


Laura Palanker contributed the larval patterns of activity and larval organ culture experiments. Heidi Sampson contributed the ecdysone biosynthesis mutant analysis in *Drosophia* embryos and helped in the characterization of embryonic ligand sensor patterns. Ruoyu Ni and Chun Hu generated all of the HA-tagged chimeric ligand sensor lines.
2.1. Abstract

Nuclear receptors are a large family of transcription factors that play major roles in development, metamorphosis, metabolism and disease. To determine how, where and when nuclear receptors are regulated by small chemical ligands and/or protein partners, we have used a ‘ligand sensor’ system to visualize spatial activity patterns for each of the 18 Drosophila nuclear receptors in live developing animals. Transgenic lines were established that express the ligand binding domain of each nuclear receptor fused to the DNA-binding domain of yeast GAL4. When combined with a GAL4-responsive reporter gene, the fusion proteins show tissue- and stage-specific patterns of activation. We show that these responses accurately reflect the presence of endogenous and exogenously added hormone, and that they can be modulated by nuclear receptor partner proteins. The amnioserosa, yolk, midgut and fat body, which play major roles in lipid storage, metabolism and developmental timing, were identified as frequent sites of nuclear receptor activity. We also see dynamic changes in activation that are indicative of sweeping changes in ligand and/or co-factor production. The screening of a small compound library using this system identified the angular psoralen angelicin and the insect growth regulator fenoxycarb as activators of the Ultraspiracle (USP) ligand-binding domain. These results demonstrate the utility of this system for the functional dissection of nuclear receptor pathways and for the development of new receptor agonists and antagonists that can be used to modulate metabolism and disease and to develop more effective means of insect control.

2.2. Introduction

Nuclear receptors (NRs) are ligand-regulated transcription factors that share a common domain architecture. DNA binding is achieved via a highly conserved zinc-finger motif. C-terminal to the DNA binding domain (DBD) is a flexible hinge region of variable length followed by a structurally conserved ligand binding domain (LBD) composed of 10-12 alpha
helices (reviewed in (Robinson-Rechavi et al., 2003)). Ligand binding alters the LBD structure, leading to changes in subcellular localization, DNA binding, dimerization, cofactor binding and/or transcriptional activity (reviewed by (Nagy and Schwabe, 2004)). Nuclear receptor ligands tend to be small lipophilic compounds such as steroids, fatty acids and vitamins. Despite extensive studies of NR structure, function and regulation, approximately half of the 48 human NRs remain orphan receptors – receptors for which no ligand has been identified.

NRs feature in most fundamental biological processes, functioning as key control points in diverse signaling and metabolic pathways, including electrolyte homeostasis (reviewed by (DeLuca, 2004; Pearce, 2001)), lipid metabolism and homeostasis (reviewed by (Chawla et al., 2001)), sex determination (reviewed by (Iyer and McCabe, 2004)), circadian rhythm and aging (reviewed in (Pardee et al., 2004)). NRs also play a central role in sensing xenobiotic compounds and coordinating an appropriate detoxification response (Willson and Kliewer, 2002). Accordingly, NR mutations are associated with many common and lethal human disorders, including cancer, diabetes and heart disease (Agoulnik et al., 2004; Alcalay et al., 1991; Barroso et al., 1999; Culig et al., 2000; Gurnell et al., 2003; Sarraf et al., 1999). Thus, understanding NR function, and the ligands that regulate their activity, provides an important opportunity to understand central aspects of growth, metabolism, development and disease.

The fruit fly, *Drosophila melanogaster*, has 18 genes that encode NRs. In spite of this relatively small number, the fly NRs span all major subclasses of vertebrate receptors (King-Jones and Thummel, 2005). Close fly orthologs of key vertebrate NRs include DHR3 (ROR family members in vertebrates), DHR38 (NGFIB/NURR1), DHR78 (TR2/TR4), Dissatisfaction (Dsfl) and Tailless (Tll) (both orthologous to vertebrate Tlx), E75 (Rev-Erb family members), ERR, DHR51 (PNR), FTZ-F1 (SF-1, LRH-1), HNF4, Seven-up (SVP) (COUP-TF in
vertebrates) and Ultraspiracle (USP) (RXR in vertebrates). These features establish *Drosophila* as an ideal model system for defining NR regulation and function. Although developmental and genetic studies have been conducted on the majority of these NRs, ligands have only been identified for two: E75, which binds heme and can use this prosthetic group to exchange small diatomic gases (Reinking et al., 2005); and the ecdysteroid receptor EcR, which binds 20-hydroxyecdysone (20E) as a heterodimer with USP (Riddiford et al., 2000). Although not capable of direct hormone binding, DHR38 can also be activated by ecdysteroids in combination with an activated form of USP (Baker et al., 2003). 20E directs the major developmental transitions in *Drosophila*, including molting and metamorphosis (reviewed by (Riddiford, 1993; Thummel, 2001). Many NRs are transcriptionally induced by the 20E/EcR/USP complex and play crucial roles during the larval-to-adult transition (King-Jones and Thummel, 2005). Most *Drosophila* NRs, however, are also expressed in embryos, larvae and adults – stages at which their functions are relatively poorly understood (Sullivan and Thummel, 2003).

As part of an effort to gain comprehensive insights into NR regulation and function, we have used an in vivo ligand detection system to follow NR LBD activation patterns in intact developing animals. This bipartite detection system consists of the LBD of each *Drosophila* NR fused to the DNA-binding domain of yeast GAL4, along with a GAL4 UAS-controlled reporter gene. As originally reported in cultured cells, in mouse tissues (Mata De Urquiza et al., 1999; Solomin et al., 1998) and later in *Drosophila* (Han et al., 2000; Kozlova and Thummel, 2002; Osterwalder et al., 2001; Roman et al., 2001), this system can respond properly to activating hormones. Here, a heat-inducible promoter is used to drive ubiquitous expression of the transgenic fusion proteins at different stages in an effort to document the normal patterns of LBD activation during development, with the goal of using these patterns to guide future studies of
NR regulation and function. In addition, a number of hypotheses were tested, leading to both suspected and unexpected findings.

Among the results obtained, we find that half of the 18 GAL4-LBD fusion proteins show no detectable activity patterns, suggesting that these function only as repressors. The other half reveal a variety of developmentally regulated patterns of activity, with dynamic changes in activation in specific cell types. In several cases, fusion proteins are active in the same tissues, revealing common or related functions. As expected, we show that the activation pattern of GAL4-EcR in early *Drosophila* embryos is dependent on the ecdysteroid biosynthetic pathway and that it responds to exogenously added ecdysone. By contrast, GAL4-DHR38 activity, which also responds to exogenous ecdysone, continues to function in the absence of ecdysone, suggesting that EcR and DHR38 respond to distinct hormonal signals at this stage in development. We test the hypothesis that xenobiotic agonists will activate DHR96, which was recently shown to contribute to insect xenobiotic responses (King-Jones et al., 2006). In addition, we test the hypothesis that the ligand sensor system can be used to reveal regulatory interactions between NR partner proteins. We further demonstrate that this system can be used to screen for new NR agonists and antagonists in live embryos and cultured larval tissues, identifying two new agonists for USP.

2.3. *Materials and Methods*

2.3.1. *Embryo collection, permeabilization, fixation and staining*

For visualization of ligand sensor activation patterns, embryos were collected and aged to 2-7 hours AEL, 6-11 hours AEL, 10-15 hours AEL or 14-17 hours AEL and heat treated for 35 minutes, recovered at room temperature for 4 hours, dechorionated and then mounted on slides in halocarbon oil. For all other experiments, overnight embryo collections were heat shocked for 60
minutes, recovered at room temperature for 4 hours, dechorionated and then mounted on slides in halocarbon oil. It should be noted that the final staining pattern reflects a cumulative pattern of ligand sensor activation that occurs from the time of heat treatment until the animals are fixed and stained. See Kozlova and Thummel (Kozlova, 2003) for a detailed description of the ligand sensor system in *Drosophila*, including a discussion of interpreting activation patterns and the spatial and temporal resolution of this system. For dib mutant analyses, embryos were fixed in 4% paraformaldehyde prior to staining. Reporter gene expression was detected using rabbit anti-GFP (Abcam, 1:500) or mouse anti-β-galactosidase antibodies (Promega, 1:750). Secondary antibodies used were Cy5-conjugated goat anti-rat IgG (Abcam, 1:1000), Cy5-conjugated goat anti-mouse IgG (Jackson Immunolabs, 1:1000) or Alexa 488-conjugated donkey anti-rabbit IgG (Molecular Probes, 1:1000). DAPI (4,6-diamidino-2-phenylindole, Sigma, 0.1 µg/ml) or propidium iodide (Sigma, 0.5 µg/ml) was used as a nuclear counterstain.

Embryo permeabilization, using heptane, was performed as described previously (Schreuders and and Mazur, 1996; Strecker, 1994) with the following modifications. Ligand sensor embryos were heat shocked to induce transgene expression, dechorionated rinsed with water and then transferred to scintillation vials containing 2 ml of modified basic incubation media (MBIM) (Strecker et al., 1994) and 6 ml of heptane. Embryos were then swirled gently for 2 minutes and then transferred in ~100 µl heptane to deep well slides. The excess heptane was removed and the embryos allowed to air dry just long enough to allow the remaining heptane to evaporate. Embryos were then immediately covered with MBIM containing 5.0 x 10^{-6} M 20-hydroxyecdysone, CITCO, PCN or TCPOBOP (all compounds from Sigma; 100x stocks were dissolved in ethanol). Embryos were incubated for 15 minutes at 25°C, the MBIM subsequently removed and the embryos covered with Halocarbon oil and allowed to develop for a minimum of 2 hours prior to observation.
2.3.2. Larval and prepupal staging, fixing and staining

Animals carrying both the GAL4-LBD and UAS-nlacZ transgenes were maintained on food containing 0.5% bromophenol blue (Andres and Thummel, 1994). Vials were heat treated in a water bath at 37°C for 30 minutes and allowed to recover for 6-7 hours at 25°C. Partial blue gut larvae were selected from this population of heat-treated animals to assess activation during the late third instar, prior to the high titer ecdysone pulse (Andres and Thummel, 1994). White prepupae were identified after 3-4 hours of recovery time and aged an additional 2-3 hours to assay GAL4-LBD activation in early prepupae, for a total of 6-7 hours after heat treatment. For earlier timepoints, animals were staged at the L2-L3 molt (−48 hours) or as fully grown, blue gut animals upon harvest (−24 hours). Animals were fixed in 1% glutaraldehyde (Sigma) in PBS for 20 minutes and stained in 0.2% X-gal (Roche) for 15 minutes to overnight at 37°C, depending on the strength of activation. Negative lines were stained overnight in an attempt to reveal low levels of activation, and very strongly activating lines were limited to short staining times to see cell-autonomous stains and overall tissue structure. Mid-third instar larvae carrying the hs-Gal4-DHR3, UAS-nlacZ, and hs-E75B transgenes were heat treated, staged and assayed as described above. hs-Gal4-DHR3, UAS-nlacZ animals lacking the hs-E75B construct were tested in parallel as a control.

2.3.3. Organ culture

Mid-third instar (blue gut stage) (Andres and Thummel, 1994) hs-GAL4-USP; UAS-nlacZ larvae were heat treated in a water bath at 37°C and allowed to recover for 3-6 hours at 25°C before dissection. They were bisected and the anterior half was rinsed in PBS + 0.1% Triton-X, everted, and placed in a glass nine-well glass dish in oxygenated Grace’s Insect Medium (Invitrogen). Compounds were administered at 1-100 µM in freshly oxygenated Grace’s medium with appropriate solvent controls. For juvenile hormone treatment, glass dishes were treated with 20%
PEG 20,000 (Fluka) and rinsed before treatment, to prevent the hormone from sticking to the dish. Animals were cultured at room temperature overnight in an oxygenated chamber, and fixed and stained in the morning as described above. Selected data are depicted in Figure 2.7 for each tissue because, as found in our earlier studies, not all tissues of a particular animal show a response (Baker et al., 2003; Kozlova and Thummel, 2002).

2.4. Results

2.4.1. Ligand sensor constructs and lines

The GAL4-LBD ‘ligand sensor’ system involves the use of transgenic Drosophila that have two P element insertions, as shown schematically in Figure 2.1. The first P element carries a heat-inducible hsp70 promoter upstream from a gene encoding the yeast GAL4 DNA binding domain (residues 1-147) fused to the C-terminal coding region of each fly NR. The NR sequences start just downstream from the DBD and include the hinge region and full length LBD. An HA-tag was added to the N terminus of each construct to facilitate fusion protein detection (with the exception of GAL4-FTZ-F1 and the previously established GAL4-EcR and GAL4-USP constructs). The hsp70 promoter was selected in order to provide precise temporal control, reducing potential lethality that might be caused by constitutive expression of the GAL4-LBD fusion proteins. In addition, the hsp70 promoter directs widespread GAL4-LBD expression upon heat induction, allowing an assessment of LBD activation throughout the organism.

Transcriptional activation by these fusion proteins will only occur at times and in places where the appropriate hormonal ligand and/or protein partners are present (Figure 2.1). Multiple transgenic lines were isolated for each construct. These lines were then crossed to one of two reporter lines that carry a GAL4-responsive UAS promoter driving the expression of either a lacZ or GFP reporter gene (UAS-nlacZ or UAS-nGFP). Both reporter proteins carry a nuclear localization signal to facilitate their detection in transgenic animals. Western blot analysis of
protein extracts using an antibody directed against the HA epitope revealed that all transgenic lines express heat-inducible full-length GAL4-LBD protein, as expected (data not shown). Previous studies have shown that GAL4-EcR and GAL4-USP are activated in an overlapping pattern at the onset of metamorphosis, in tissues that are known to respond to 20E, representing the expected response for a 20E receptor (Kozlova and Thummel, 2002).

Figure 2.1 - The ligand sensor system

A schematic representation of the two transgenes that comprise the ligand sensor system is depicted. Upon heat treatment, the hsp70 promoter directs widespread expression of the GAL4 DBD fused to a nuclear receptor LBD. This fusion protein is able to bind to a GAL4 UAS response element on a second transgene, activating reporter gene expression in cells that contain the necessary ligands and/or co-factors. Reporter genes that encode nuclear GFP or β-galactosidase are used to monitor GAL4-LBD ligand sensor activity in a cell-autonomous manner.
2.4.2. **Ligand sensor activity patterns**

The temporal and spatial patterns of ligand sensor activation were determined at two stages in the life cycle when the animal undergoes major developmental changes – embryogenesis and the onset of metamorphosis. Embryos collected over a 4-hour interval were aged appropriately, heat-treated to induce ligand sensor expression, and the patterns of GFP reporter expression were documented. Studies at the onset of metamorphosis were conducted at three developmental stages: (1) in feeding, metabolically active mid-third instar larvae; (2) in late third instar larvae (at ~8-14 hours before pupariation, just prior to the high titer 20E pulse that triggers pupariation); or (Phillips et al.) as 2 hour prepupae. The tissue- and stage specificity of the activation patterns are summarized in Table 1 and examples are shown in Figures S1 (embryos) and S2 (larvae) in the supplementary material.

Nine out of the 18 ligand sensors displayed temporally and/or spatially restricted activation patterns: EcR, USP, E78, ERR, HNF4, FTZ-F1, DHR3, DHR38 and DHR96. Each of these patterns was consistent in multiple transgenic lines and when tested with different reporters. The remaining ligand sensors did not display detectable activation at the times or stages tested: DHR4, DHR39, DHR51, DHR78, DHR83, DSF, E75, SVP and TLL. This lack of activation cannot be attributed to an absence of expression because widespread GAL4-LBD fusion protein can be detected in all of these ligand sensor lines following heat induction (data not shown). Rather, their lack of activity is probably due to these NRs functioning as repressors. Earlier studies have demonstrated repressive functions for E75 (White et al., 1997), SVP (Zelhof et al., 1995a), TLL (Yu et al., 1994), DSF (Pitman et al., 2002), DHR4 (King-Jones et al., 2005) and DHR78 (Zelhof et al., 1995b). DHR51 and DHR83 are also likely to act as repressors based on studies of their closest vertebrate
homologues (Chen et al., 2005). A different method will be required to examine the regulatory activities of these NRs. GAL4-EcR activity is ligand-dependent and responsive to hormone during embryogenesis. The GAL4-EcR fusion protein exhibits transcriptional activity during mid-embryogenesis in the amnioserosa (Figure 2.2A) (Kozlova, 2003). To determine whether this activity pattern reflects the presence of ligand, we tested the dependence of this localized GAL4-EcR transcriptional activity on the biosynthesis of \( \alpha \)-ecdysone (E), which is the immediate precursor of 20-hydroxyecdysone (20E), the active ecdysteroid in insects (Gilbert et al., 2002). This was achieved by crossing EcR ligand sensor flies with flies carrying a mutation in the disembodied (Petit-Bertron et al.) gene, which encodes a cytochrome P450 enzyme required in the penultimate step of E biosynthesis (Chavez et al., 2000; Warren et al., 2002). In a \( \textit{dib} \) mutant background, GAL4-EcR activity in the amnioserosa is no longer detectable, confirming that this response is 20E dependent (Figure 2.2B). By contrast, no effects were observed on any of the other positively acting ligand sensor lines when tested in the \( \textit{dib} \) mutant background (Figure 2.2E,F,J; data not shown). Together, these results show that EcR LBD activation in the embryonic amnioserosa is dependent on zygotic ecdysteroid biosynthesis and that ligand sensor fusion proteins function in a ligand-dependent and ligand-specific fashion. GAL4-EcR can be activated by exogenously added hormone during embryogenesis. To test whether ligand sensor proteins can be used to detect exogenously added ligands, GAL4-EcR embryos were permeabilized and allowed to develop in media supplemented with 20E. Fig. 2D shows a typical 20E-treated embryo, displaying widespread GFP expression that extends significantly beyond the response to endogenous 20E in the amnioserosa (Figure 2.2A). By contrast, GAL4-FTZ-F1 shows no changes from the untreated control (Figure 2.2G,H).
### Table 1. Patterns of GAL4-LBD activation during *Drosophila* development

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Tissues exhibiting ligand sensor activity are listed at the top, and the NR-LBD ligand sensors tested are listed on the left. Ligand sensors were monitored throughout embryogenesis (indicated by an E preceding the age of the collection in hours AAE), within several hours of hatching (L1), in late third instar larvae (L3) or in newly formed prepupae (PP). Tissues include the amnioserosa, CNS, Malpighian tubules and peripheral nervous system (PNS). A plus sign (+) indicates significant detectable activity; +/- denotes activation that is slight or partial but greater than background control. NA, not applicable; ND, not determined.
Figure 2.2 - Ligand regulation of GAL4-LBD fusion protein activity

GAL4-LBD activation patterns are shown for three receptors in a wild-type background (wt; A,E,I) or disembodied mutant background (dib; B,F,J), in culture in either the absence (C,G,K) or presence of 5 µM 20-hydroxyecdysone (20E; D,H,L). GAL4-EcR is active in the amnioserosa of stage 14 wild-type embryos (A), but not in dib mutant embryos (B). Culturing in the presence of 20E induces ectopic activation in the epidermis (C,D). GAL4-FTZ-F1 is active in the yolk nuclei of embryos at stage 13 (E,F) and stage 16 (G,H) and is unaffected in a dib mutant background (F) or by the presence of exogenous 20E (H). GAL4-DHR38 is active in the epidermis and amnioserosa of stage 13 embryos (I,J) and is not affected in a dib mutant background (J, compare with K). The activity of GAL4-DHR38 in stage 17 cultured embryos is upregulated by exogenous 20E (L). The activity of GAL4-DHR96 in stage 13 embryos (M) is significantly increased by the addition of 5x10^{-6} M CITCO (N).
These results, which are similar to the previously published effects of 20E on GAL4-EcR and GAL4-USP activity in cultured larval organs (Baker et al., 2003; Kozlova and Thummel, 2002) show that the co-factors required for EcR ligand sensor activity are not temporally or spatially limiting, and that the presence of ligand is sufficient for ectopic activation. By exploiting high-throughput screening strategies, it should therefore be possible to expand this effort by testing large compound libraries for their effects on ligand sensor activities. GAL4-DHR38 can be activated by 20E but is not dependent on dib. DHR38 has previously been shown to be activated by a set of ecdysteroids that are distinct from those that significantly activate EcR, although this effect appears to be achieved through a novel mechanism that does not involve direct ligand binding (Baker et al., 2003). Like the EcR ligand sensor, GAL4-DHR38 is also active in the amnioserosa (Figure 2.2I). Interestingly though, this activity begins at an earlier stage than that of the EcR ligand sensor (see Figure 2.4, Table 2.1). In addition, no effects were observed on DHR38 ligand sensor activity in dib mutant embryos (Fig. 2.2J), possibly owing to its activation by maternally provided ecdysteroids other than 20E. The DHR38 ligand sensor embryos treated with 20E do, however, exhibit a modest but reproducible increase in their activation pattern. The embryo in Figure 2.2L shows typical patches of responding cells, which, unlike the epidermal cells that respond to endogenous ligand (Figure 2.2K), tend to be contiguous and display weaker GFP fluorescence. Thus, although GAL4-DHR38 is not dependent on E biosynthesis, it can respond to the addition of exogenous 20E, although not as robustly as EcR, consistent with the weak 20E activation of DHR38 previously seen in transient transfection assays (Baker et al., 2003).
2.4.3. **GAL4-DHR96 is activated by the selective CAR agonist CITCO**

Like its vertebrate orthologs SXR/PXR and CAR, DHR96 has been recently shown to act in insect xenobiotic responses, providing resistance to the sedative effect of phenobarbital and lethality caused by chronic exposure to DDT (King-Jones et al., 2006). DHR96 is also required for the proper transcriptional response of a subset of phenobarbital-regulated genes. Accordingly, we used the ligand sensor system to determine if known mammalian xenobiotic agonists could activate the DHR96 LBD. Embryos expressing GAL4-DHR96 were treated with the PXR-selective agonist PCN and the CAR selective agonists TCPOBOP and CITCO (Blumberg et al., 1998; Maglich et al., 2003; Tzameli et al., 2000). Of these, only CITCO gave reproducible, strong activation of the DHR96 ligand sensor, indicating that the activation status of the DHR96 LBD can be regulated by xenobiotic compounds in a manner similar to that of its vertebrate orthologs (Figure 2.2M,N). Interestingly, CITCO had no effect on GAL4-DHR96 subcellular localization, as it does with CAR (Maglich et al., 2003). Thus, the CITCO effect on the DHR96 LBD most probably occurs at the level of co-activator recruitment.

2.4.4. **ERR displays widespread and dynamic switches in ligand sensor activity.**

Although several ligand sensor lines showed shifts in their spatial and temporal patterns of activation, the most dramatic changes were observed with the ERR ligand sensor. GAL4-ERR activity is initially detected during mid-embryogenesis in a subset of myoblasts (Figure 2.3A, 6-11 hours). Its activity then shifts to a different cell type at 14-17 hours after egg laying (AEL) – the central nervous system (CNS) and a few cells in the peripheral nervous system (Fig. 3A). Interestingly, the timing of this shift in ERR tissue activity coincides with a switch in ERR transcript sizes that occurs at 14-18 hours AEL (Sullivan and Thummel, 2003).
Figure 2.3 - Dynamic changes in the spatial and temporal patterns of ERR LBD activation.

GAL4-ERR activation patterns are shown during embryogenesis (A) and third instar larval and prepupal stages (B). (A) ERR activation switches from myoblasts (6-11 hours AEL) and muscle (10-15 hours AEL) to predominantly CNS cells (14-17 hours AEL) in the late embryo. (B) In larvae, transient and widespread activation of GAL4-ERR occurs in the mid-third instar (mid-L3) in the muscle, CNS, midgut and fat body. Background bacterial β-galactosidase expression is seen in the larval midgut lumen of early third instar larvae (early L3). Background β-galactosidase expression is also present in the optic lobes of the CNS from larvae and early prepupae.
Remarkably, the muscles and CNS also display GAL4- ERR activity in third instar larvae, along with restricted activation in the midgut (Figure 2.3B). Moreover, the ERR ligand sensor shows a dramatic switch in its activation pattern at this later stage in development. GAL4-ERR activity is undetectable in early third instar larvae, peaks at ~24 hours after the L2-to-L3 molt, and then rapidly drops to background levels again by late third instar (Figure 2.3B). This type of widespread transient LBD activation has only been seen for the EcR and USP ligand sensors at puparium formation, in response to the high titer late larval pulse of 20E. Thus, ERR appears to be responding to a widespread, temporally restricted activating signal that occurs in the mid-third instar.

2.4.5. Temporally distinct patterns of ligand sensor activation in the amnioserosa

One of the advantages of studying all of the Drosophila nuclear receptors in parallel is that common as well as unique features become apparent. For example, although most tissues appear to support ligand sensor activity at some stage, several tissues are particularly prevalent sites of activity. These include the amnioserosa, yolk, regions of the midgut and fat body. In some cases, the dynamics of these activity patterns suggest that different ligand sensors may be responding to related sets of ligands or act in functional hierarchies. The patterns of E78, DHR38, DHR3, HNF4 and EcR ligand sensor activation in the amnioserosa provide one such example (Figure 2.4). The amnioserosa is a dorsally located sheet of extra-embryonic polyploid cells that controls essential morphogenetic movements such as retraction of the germ band and dorsal closure (Kozlova and Thummel, 2003a; Narasimha and Brown, 2004; Reed et al., 2004; Scuderi and Letsou, 2005). Interestingly, the E78 ligand sensor, which is active in most embryonic and larval tissues, displays its first high level of activation at about stage 9 in the amnioserosa (Figure 2.4A, arrowheads). The DHR38 and DHR3 ligand sensors respond at about the same time or soon after (Figure 2.4D,G, arrowheads), with downregulation of E78, DHR38, and DHR3 ligand sensor activity at this stage.
activity in the amnioserosa at stages 13-14 (Figure 2.4C,F,I). By contrast, GAL4-HNF4 is active in yolk nuclei at early times (Fig. 4J, arrows), only switching to the amnioserosa at stage 12 (Figure 2.4K,L, arrowheads). The EcR and USP ligand sensors are the last to display activity in the amnioserosa, beginning at about stage 13 (arrowheads in Figure 2.4O for EcR; Table 2.1 and data not shown). Thus, not only is the amnioserosa a hotspot for ligand sensor activation, but the different timing of these responses may be due to distinct threshold responses to the same or related set of ligands or to hierarchical interactions between NRs and/or co-factors.
Figure 2.4 - Distinct temporal patterns of GAL4-LBD activation in the amnioserosa.

GAL4-LBD activation patterns are shown for five receptors: E78 (A-C), DHR38 (D-F), DHR3 (G-I), HNF4 (J-L) and EcR (M-O). The earliest activation in the amnioserosa is detected in stage 9-10 GAL4-E78 (A), GAL4-DHR38 (D), and GAL4-DHR3 (G) embryos. HNF4 embryos (J) show activation in the yolk nuclei at this stage (arrows). At stage 12, activation is detected in the amnioserosa of GAL4-E78 (B), GAL4-DHR38 (E), GAL4-DHR3 (H) and GAL4-HNF4 (K) embryos. At stage 13-14, activation in the amnioserosa is detected in all lines and becomes visible in GAL4-EcR embryos (O). The amnioserosa is indicated with arrowheads.
2.4.6. Restricted patterns of ligand sensor activation in the yolk and midgut

Another major site of ligand sensor activity is the yolk, consistent with its role in providing nutrition for the developing embryo and its abundance of lipophilic compounds. The E78, DHR38, HNF4 and FTZ-F1 ligand sensors are all active in the yolk at early stages, with initial activation of GAL4-DHR3 in the yolk at mid-embryogenesis (arrowheads in Figure 2.5C,E,G,I,J; Table 1). This continues through stage 14 when the polyploid yolk nuclei become engulfed within the developing midgut (Figure 2.5D,F,H; see also Fig. S1 in the supplementary material). Interestingly, the E78, DHR3, DHR38 and HNF4 ligand sensors display a transition during stages 15-17 from activity within the yolk to the gut epithelia that surround the yolk, suggesting that this response could be due to one or more yolk-derived ligand(s) (Figure 2.5D,F,H).

Following hatching and the onset of feeding, DHR3, DHR38, HNF4 and FTZ-F1 ligand sensor activities continue within regions of the midgut (Figure 2.5K-T). Of these, GAL4-DHR3 has the highest and most uniform pattern of activity, spanning most of the midgut and gastric caeca (Figure 2.5M-N). Although DHR3 ligand sensor activity is evident in the proventriculus of the midgut of third instar larvae, it is downregulated at puparium formation (Figure 2.5M,N arrow). By contrast, DHR38 and HNF4 ligand sensor activities are restricted to the cells that span the junction of the midgut, proventriculus and gastric caeca (Figure 2.5O-R), suggesting that these receptors may be responding to similar signal(s). Interestingly, GAL4-FTZ-F1 is activated in the midgut only after feeding has ceased, at puparium formation (Figure 2.5S-T).
Figure 2.5 - The yolk and midgut are hotspots for ligand sensor activation.

GAL4-LBD activation patterns are depicted for the yolk and midgut during embryogenesis (A-J) and in the midgut at the onset of metamorphosis (K-T). Representative embryos are shown for control (A,B), DHR3 (C,D), DHR38 (E,F), HNF4 (G,H), and FTZ-F1 (I,J) ligand sensors. The yolk is a major site of activation for GAL4-DHR3 (C), GAL4-DHR38 (E), GAL4-HNF4 (G) and GAL4-FTZ-F1 (I) embryos at stages 14-15 (arrowheads). Yolk activation remains prominent for GAL4-FTZ-F1 during stages 16-17 (J), but switches to the gut epithelium (arrows) for GAL4-DHR3 (D), GAL4-DHR38 (F) and GAL4-HNF4 (H). At later stages, GAL4-DHR3 displays strong and widespread activation in the proventriculus and midgut of late third instar larvae (M), and selectively reduced activation in the proventriculus after pupariation (arrow in N), while activation in the rest of the midgut is maintained. GAL4-DHR38 and GAL4-HNF4 display spatially restricted activation at the junction of the midgut, proventriculus (small arrow in Q) and
gastric caeca (arrowheads in Q) (O-R). The FTZ-F1 ligand sensor is activated in the anterior midgut in a spatially and temporally specific fashion at puparium formation (S,T).

2.4.7. Dynamic changes in ligand sensor activity in the larval fat body

As expected, the EcR and USP ligand sensors both display widespread transient activation at puparium formation, following the high titer pulse of 20E (Table 2.1; see also Fig. S2 in the supplementary material). This response includes activation in the larval fat body, as shown for the USP ligand sensor in Fig. 6. Curiously, however, the DHR3, DHR38 and HNF4 ligand sensors show an opposite pattern in the fat body, with dramatic downregulation of activity at puparium formation (Figure 2.6). This switch correlates with the cessation of feeding that occurs at the end of larval development, a time when the animal stops using food as a nutrient source and begins to use stored carbohydrates and fatty acids. Thus, the activities of GAL4-DHR3, GAL4-DHR38 and GAL4-HNF4 in the fat body reflect the metabolic status of the animal, suggesting that the corresponding receptors may respond to nutrients or metabolites.
Figure 2.6 - Dynamic changes in GAL4-LBD activation patterns in larval fat bodies at the onset of metamorphosis.

As expected, GAL4-USP is activated in larval fat bodies by the 20E pulse at puparium formation. By contrast, the DHR3, DHR38 and HNF4 ligand sensors are active in the larval fat bodies of feeding third instar larvae and show reduced activation after pupariation.
2.4.8. **Identification of new receptor agonists by compound screening**

Larval organ culture provides an accurate and simple means of testing compounds for hormonal activity within a normal physiological context (Ashburner, 1972). We thus asked whether larval organ culture could be combined with our ligand sensors to identify novel receptor agonists, screening for activation of GAL4-USP. Two properties of USP make it a good prototype for this study. First, like its vertebrate ortholog RXR, USP can dimerize with multiple *Drosophila* nuclear receptors (Sutherland et al., 1995), increasing the likelihood of obtaining a positive response to a new compound. Second, the ability of GAL4-USP to interact with EcR, and to activate reporter gene expression in the presence of the EcR ligand 20E, permits the use of 20E as a positive control (Kozlova and Thummel, 2002).

USP ligand sensor third instar larvae were heat-treated to induce GAL4-USP expression, allowed to recover for 3-6 hours, and then dissected for organ culture in the presence of different compounds. Consistent with our earlier studies, the addition of 20E led to efficient activation of GAL4-USP, showing robust expression of either β-galactosidase or GFP (Figure 2.7Q-T; data not shown) (Kozlova and Thummel, 2002). Over 40 other compounds were also tested for their ability to activate GAL4-USP in this assay (Table 2.2). We focused on a range of known and potential insect hormones and plant-derived compounds, including ecdysteroids, juvenoids, plant hormones and psoralen-derived xenochemicals. We also tested two forms of vitamin D as well as fatty acids and xenobiotics, which can regulate vertebrate nuclear receptors (Chawla et al., 2001). Each assay was repeated at least twice, and 20E was included in each experiment as a positive control.

As expected, GAL4-USP is activated by ecdysteroids from several insect and plant species, including α-ecdysone, 2-deoxy-20- hydroxyecdysone, 20,26-dihydroxyecdysone, 20-
hydroxyecdysone 22-acetate and makisterone A (Table 2.2), consistent with an earlier study of compounds that activate the EcR/USP heterodimer in cultured cells (Baker et al., 2000). The ability of α-ecdysone to activate GAL4-USP is most probably due to its conversion to 20E (Petryk et al., 2003). The plant ecdysteroids ajugasterone C, azadirachtin, cyasterone, muristerone A, polypodine B and ponasterone A are also able to activate GAL4-USP in this system, consistent with their ecdysteroid properties and earlier cell culture studies (Baker et al., 2000). Although linear psoralens and brassinosteroids did not activate GAL4-USP in this assay, an angular psoralen, the furanocoumarin angelicin, is a relatively strong activator of GAL4-USP (Figure 2.7E-H). Interestingly, angelicin has no effect on GAL4-EcR (data not shown).

Juvenile hormone (Nijhout) and several JH analogs were also tested for their ability to activate GAL4-USP, following up on studies suggesting that USP is a receptor for this insect hormone (Petryk et al., 2003). However, JHI, JHII, JHIII and two well-studied JH analogs, pyriproxifen and methoprene, were unable to activate GAL4-USP (Table 2.2). Curiously, however, we observed weak activation by the insecticide fenoxycarb in tissues from some animals but not others, suggesting that activation may be influenced by developmental stage or the physiological state of the animal (Figure 2.7I-L). Fenoxycarb is a carbamate insecticide that mimics the action of JH on several physiological pathways, including molting and reproduction. Given the absence of an effect with natural JH, pyriproxifen or methoprene, however, the observed activation by fenoxycarb may represent a xenobiotic response, rather than an effect caused by its JH-like activity. Neither angelicin nor fenoxycarb had a significant effect on the DHR96 ligand sensor in larval organ culture, suggesting that USP either responds directly to these compounds, or acts as a heterodimer with another NR (L.P. and M. Horner, unpublished).
Figure 2.7 - GAL4-USP is activated by 20-hydroxyecdysone, angelicin and fenoxycarb in cultured larval organs.

Organs dissected from hs-GAL4-USP; UAS-nlacZ (A-L) or hs-GAL4-USP; UAS-nGFP (M-T) mid-third instar larvae were cultured with either no hormone (control; A-D,M-P), 10 µM angelicin (Kakimura et al.), 100 µM fenoxycarb (I-L) or 5 µM 20E (Q-T). Activation is seen in the oenocytes (E,I), the fat body (F,J,R), the epidermis (Q), the proventriculus of the midgut (G,K,S), the larval salivary glands (T) and the CNS (H,L).
2.4.9. A repressive heterodimer partner can regulate ligand sensor activity

Earlier studies have shown that DHR3 induces βFTZ-F1 at the onset of metamorphosis, and that the activation function of DHR3 in cultured cells can be blocked by heterodimerization with E75B, an isoform of E75 that is missing its DNA binding domain but contains an intact LBD (Segraves and Hogness, 1990; White et al., 1997). We used the ligand sensor system to test if this functional interaction also occurs in vivo and to determine if the ligand sensor system can be used to monitor repressive protein-protein interactions. As described above, GAL4-DHR3 is widely active in late third instar larval tissues (Figure 2.5M,N, Fig. 6, Fig. 8A; see also Figure S2 in the supplementary material). This pattern changes dramatically, however, following ectopic co-expression of full-length E75B protein, with a significant reduction in DHR3 ligand sensor activity in the epidermis, proventriculus of the midgut, CNS and fat body (Fig. 8B). Importantly, this pattern reflects that normally seen in early prepupae with GAL4-DHR3 (Fig. 8C), suggesting that the change in DHR3 ligand sensor activation during the onset of metamorphosis can be accounted for by ecdysone-induced expression of endogenous E75B at puparium formation. This observation supports previous evidence that E75B is sufficient to block the activation function of DHR3 at the onset of metamorphosis (White et al., 1997). In addition, the specific inability of E75B to block GAL4-DHR3 activity in the larval midgut suggests that either the DHR3/E75B heterodimer cannot form in this tissue or, more likely, that modifying ligand(s) or co-factors may block the repressive function of E75B in this tissue. Given the recent discovery that E75 binds heme and responds to diatomic messenger gases, it is possible that E75B activity may be differentially regulated in a tissue-specific manner (Reinking et al., 2005). Moreover, this experiment demonstrates that ligand sensor fusion proteins can be used to assess regulatory responses due to protein partners and cofactors, as well as to detect ligand-regulated responses.
Figure 2.8 - A repressive heterodimer partner can down-regulate GAL4-LBD activation.

(A) GAL4-DHR3 is active in many tissues of a late third instar larva, including the epidermis, midgut, central nervous system (CNS) and fat body. (B) Ectopic expression of E75B results in a significantly reduced level of activation at this stage in development, recapitulating the pattern normally seen in early prepupae (C), in the presence of endogenous E75B expression. E75B-mediated downregulation of GAL4-DHR3 activity in the midgut is restricted to the proventriculus (compare arrows).
Table 2.2 - Compounds tested for their ability to activate GAL4-USP in organ culture

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<td></td>
</tr>
<tr>
<td>Juvenile hormone I (racemic, -78%)</td>
<td>–</td>
<td>2, 20</td>
<td>Angelicin</td>
<td>++</td>
<td>10, 100</td>
</tr>
<tr>
<td>Juvenile hormone II (racemic, -78%)</td>
<td>–</td>
<td>2, 20</td>
<td>5-methoxypsoralen</td>
<td>–</td>
<td>10, 100</td>
</tr>
<tr>
<td>Juvenile hormone III (R, -98%)</td>
<td>–</td>
<td>5</td>
<td>8-methoxypsoralen</td>
<td>–</td>
<td>10, 100</td>
</tr>
<tr>
<td>Insect hormone analogs</td>
<td></td>
<td></td>
<td>Vitamin D complex</td>
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<tr>
<td>Fenoxycarb</td>
<td>++</td>
<td>10, 100</td>
<td>Cholecalciferol</td>
<td>–</td>
<td>1, 10</td>
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<tr>
<td>Halofenazide (RH-0345)</td>
<td>++</td>
<td>10, 100</td>
<td>Ergocalciferol</td>
<td>–</td>
<td>1, 10</td>
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<tr>
<td>Tebufenozide (RH-5992)</td>
<td>++</td>
<td>10, 100</td>
<td>Insecticides</td>
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<tr>
<td>S-hydropropene</td>
<td>–</td>
<td>1, 10</td>
<td>DDT</td>
<td>–</td>
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<tr>
<td>Methoprene acid</td>
<td>–</td>
<td>10, 100</td>
<td>Malathion</td>
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<td>Pyriproxyfen</td>
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<td>1</td>
<td>Phenobarbital</td>
<td>–</td>
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<td>Plant hormones</td>
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<td>10, 100</td>
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<td>Ajuasterone C</td>
<td>+++</td>
<td>1, 10</td>
<td>Chenodeoxycholic acid</td>
<td>–</td>
<td>10, 100</td>
</tr>
<tr>
<td>Azadirachtin</td>
<td>++</td>
<td>1, 10</td>
<td>Linoleic acid</td>
<td>–</td>
<td>10, 100</td>
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<tr>
<td>Cystasterone</td>
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<td>1, 10</td>
<td>γ-linolenic acid</td>
<td>–</td>
<td>10, 100</td>
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<tr>
<td>Muristerone A</td>
<td>+++</td>
<td>1, 10</td>
<td>Oleic acid</td>
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<td>10, 100</td>
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<tr>
<td>Polypropene B</td>
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<td>Palmitic acid</td>
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<td>10, 100</td>
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<tr>
<td>Ponerasterone A</td>
<td>+++</td>
<td>1, 10</td>
<td>Postesterone</td>
<td>–</td>
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Organs were dissected from mid-third instar GAL4-USP, UAS-nlacZ larvae and cultured with the indicated compounds for 12-18 hours, using appropriate solvent controls. Activation is reported for the highest concentration tested, as relative β-galactosidase expression.
Table 3. List of primers used to construct the ligand sensor GAL4 fusion genes

<table>
<thead>
<tr>
<th>Nuclear receptor</th>
<th>Primers used</th>
<th>Restriction sites</th>
<th>Size (bp)</th>
<th>Source</th>
<th>Number of transgenic lines obtained and tested</th>
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<tr>
<td>ERR Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>NotI/NotI</td>
<td>972</td>
<td>EST clone</td>
<td>8</td>
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<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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<td>DHR51 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>NotI/SpeI</td>
<td>662</td>
<td>RT-PCR</td>
<td>4</td>
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<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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<td></td>
<td></td>
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<tr>
<td>HNF4 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>NotI/XbaI</td>
<td>1524</td>
<td>W. Zhong</td>
<td>9</td>
</tr>
<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
<td></td>
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<tr>
<td>DHR3 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>NotI/XbaI</td>
<td>1143</td>
<td>C. Thummel</td>
<td>7</td>
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<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DHR39 Fwd</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
<td>XbaI/StuI</td>
<td>1126</td>
<td>M. Petkovich</td>
<td>9</td>
</tr>
<tr>
<td>Rev</td>
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<td></td>
<td></td>
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<tr>
<td>DHR4 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>XbaI/BamHI</td>
<td>3881</td>
<td>RT-PCR</td>
<td>10</td>
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<tr>
<td>Rev</td>
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<tr>
<td>DHR78 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>NotI/XbaI</td>
<td>1486</td>
<td>C. Thummel</td>
<td>11</td>
</tr>
<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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<td></td>
<td></td>
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<tr>
<td>DHR96 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>NotI/XbaI</td>
<td>2000</td>
<td>C. Thummel</td>
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<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>DHR83 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>NotI/SpeI</td>
<td>744</td>
<td>RT-PCR</td>
<td>4</td>
</tr>
<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DSR Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>XbaI/StuI</td>
<td>1885</td>
<td>M. McKeown</td>
<td>12</td>
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<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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</tr>
<tr>
<td>E75 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>NotI/XbaI</td>
<td>2811</td>
<td>C. Thummel</td>
<td>5</td>
</tr>
<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E78 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>XbaI/StuI</td>
<td>1348</td>
<td>EST clone</td>
<td>5</td>
</tr>
<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
<td></td>
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<tr>
<td>FTZ-F1 Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>XbaI/StuI</td>
<td>1387</td>
<td>C. Schwartz</td>
<td>2</td>
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<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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<td>SVP Fwd</td>
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<td>NotI/XbaI</td>
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<td>Y. Hiromi</td>
<td>18</td>
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<tr>
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</tr>
<tr>
<td>TLL Fwd</td>
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<td>NotI/XbaI</td>
<td>1089</td>
<td>J. Lengyel</td>
<td>7</td>
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<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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<td>EcR Fwd</td>
<td>5'-ATAAGAATGCGGCCGCGCGATGTCAGAAGGAGGGTGT</td>
<td>XbaI/StuI</td>
<td>1683</td>
<td>C. Thummel</td>
<td>13</td>
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<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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</tr>
<tr>
<td>USP Fwd</td>
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<td>NotI/XbaI</td>
<td>1054</td>
<td>EST clone</td>
<td>6</td>
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<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
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<td></td>
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</tr>
<tr>
<td>DHR38 Fwd</td>
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<td>NotI/XbaI</td>
<td>834</td>
<td>T. Kozlova</td>
<td>7</td>
</tr>
<tr>
<td>Rev</td>
<td>5'-GCTCTAGACAGATAATTGCGGCGCGATA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nuclear receptors are listed on the left. Primer sequences, restriction sites, fragment sizes, sources of cDNA clones and the number of transgenic lines obtained and tested are indicated across the top.

2.5. Discussion

Nine GAL4-LBD ligand sensor lines described here show tissue-specific patterns of activity during development: EcR, USP, ERR, FTZ-F1, HNF4, E78, DHR3, DHR38 and DHR96. These transgenic lines will serve as valuable tools for the genetic and molecular dissection of the receptors they represent, the pathways they regulate and the upstream factors and co-factors that modulate their activity. Specifically, the data reported here show that these
lines can be used to: (1) indicate tissues and stages in which the corresponding NRs are likely to function; (2) indicate where endogenous ligands and cofactors are likely to be found; (Phillips et al.) suggest NR biological functions; (4) suggest possible NR-NR interactions, cascades and target genes; (5) evaluate putative co-factors and ligands; (6) screen chemical compound libraries for new agonists and antagonists; and (7) screen genetically for new pathway components. The results of these studies will also provide important insights into the ligands, cofactors and functions of their vertebrate NR homologues.

2.5.1. Hormonal regulation of GAL4-LBD activation in the amnioserosa and yolk

Examination of the nine active ligand sensor lines provided a number of insights into possible relationships between their corresponding NRs. For example, although each of these ligand sensors displays unique temporal and spatial patterns of activity, activation in specific tissues and stages is common to many. These common sites of LBD activity may indicate shared functions, hierarchical or physical interactions, or related ligands. Examples of tissues that represent hotspots for GAL4-LBD activation include the amnioserosa, yolk, midgut and fat body. Each of these tissues, and the stages at which they score positively, correlates well with the presence of putative ligands. The yolk, for example, is believed to act as a storage site for maternally provided ecdysteroids during embryogenesis. Work with other insects has shown that these ecdysteroids are conjugated in an inactive form to vitellin proteins via phosphate bridges (Hoffmann, 1985). Around mid-embryogenesis, these yolk proteins and phosphate bonds are cleaved, thereby releasing what are presumed to be the earliest biologically active ecdysteroids in the embryo (Bownes et al., 1988). Interestingly though, GAL4-EcR activation in the amnioserosa depends on dib function (Fig. 2B), suggesting that the final steps in the linear E biosynthetic pathway are required for EcR function in this tissue (Chavez et al., 2000; Warren et al., 2002) and contradicting the prediction that this activity would be dependent on maternal ecdysteroids.
and independent of the zygotic biosynthetic machinery (Kozlova and Thummel, 2003). The mechanisms by which dib exerts this essential role in providing an EcR ligand, however, remain to be determined. The response of the EcR and USP ligand sensors in the adjacent amnioserosa tissue shows that active ecdysteroids are not present until the hormone reaches the amnioserosa. A recent study of yolk-amnioserosa interactions has revealed dynamic transient projections that emanate from one tissue and contact the other, suggesting that there may be functional interactions between these two cell types (Reed et al., 2004). It is possible that these projections mediate the transfer of lipophilic ligand precursors from the yolk to the amnioserosa. This transfer, in turn, could determine the proper timing of EcR activation in the amnioserosa, thus triggering the major morphogenetic movements that establish the body plan of the first instar larva (Kozlova and Thummel, 2003). Studies of the DHR38 receptor have demonstrated that it can be activated by a distinct set of ecdysteroids from those that activate EcR, through a novel mechanism that does not involve direct ligand binding (Baker et al., 2003). The activation of GAL4-DHR38 that we observe in the embryonic amnioserosa is consistent with this model of DHR38 regulation. First, exogenous 20E can only weakly activate GAL4-DHR38, relative to the strong ectopic activation seen with 20E on the EcR ligand sensor (Figure 2.2L,D). This correlates with the weak ability of 20E to activate DHR38 in cell culture transfection assays relative to the strong 20E activation of EcR (Baker et al., 2003). Second, the DHR38 ligand sensor is activated in the amnioserosa earlier than the EcR construct, suggesting that it is responding to a different signal (Figure 2.4). It is possible that this signal is an ecdysteroid precursor that can act on DHR38 but not EcR – paralleling the ability of DHR38 to be activated by E, the precursor to 20E, which activates EcR. This putative ecdysteroid must be produced in a manner independent of the conventional ecdysteroid biosynthetic pathway, however, as a zygotic dib mutation has no effect on GAL4-DHR38 activation in the amnioserosa. Rather, this early
activation may be due to maternal ecdysteroids that are conjugated and inactive in the yolk and transferred to the amnioserosa. These studies highlight the value of combining mutations in hormone biosynthesis with ligand sensor activation as a powerful means of dissecting hormone signaling pathways. Further studies of DHR38 function and regulation in embryos could help clarify the potential significance of this distinct activation response.

2.5.2. DHR3, DHR38 and HNF4 ligand sensors appear to respond to metabolic signals

Interestingly, the midgut continues to be a hotspot for ligand sensor activity long after it has engulfed the yolk during embryogenesis. This seems logical, as the midgut is responsible for most lipid absorption and release, and many vertebrate NRs are involved in fatty acid, cholesterol and sterol metabolism and homeostasis (Chawla et al., 2001). The observed restriction of ligand sensor activity to a narrow group of cells located at the base of the gastric caeca is of particular interest (Figure 2.5M-R). This is the site where nutrients in a feeding larva are absorbed into the circulatory system (Chapman, 1998). The activation of DHR3, DHR38 and HNF4 ligand sensors in this region of the gastric caeca suggests that these receptors are activated by one or more small nutrient ligands (Figure 2.5M-R). Moreover, this suggests that the corresponding receptors may exert crucial metabolic functions by acting as nutrient sensors.

Further evidence of metabolic functions for DHR3, DHR38 and HNF4 arises from their ligand sensor activation patterns in the embryonic yolk and larval fat body (Figure 2.4, 2.6). The yolk is the main nutrient source for the developing embryo and represents an abundant source of lipids, correlating with specific activation of DHR3, DHR38 and HNF4 ligand sensors in this cell type during embryogenesis (Figure 2.4C,E,G). Upon hatching into a larva, the fat body acts as the main metabolic organ of the animal, functionally equivalent to the mammalian liver. Upon absorption by the gastric caeca, nutrients travel through the circulatory system and are absorbed by the fat body, where they are broken down and stored as triglycerides, glycogen and trehalose.
Once again, the efficient activation of the DHR3, DHR38 and HNF4 ligand sensors in the fat body of metabolically active third instar larvae, and lack of sensor activity in non-feeding prepupae, supports the model that the corresponding NRs operate as metabolic sensors (Figure 2.6). This proposed function is consistent with the roles of their vertebrate orthologs. Mammalian ROR, the ortholog of DHR3, binds cholesterol and plays a crucial role in lipid homeostasis (Kallen et al., 2004; Lau et al., 2004). Similarly, mammalian HNF4 can bind C14-18 fatty acids, is required for proper hepatic lipid metabolic gene regulation and lipid homeostasis, and is associated with human Maturity-Onset Diabetes of the Young (MODY1) (Dhe-Paganon et al., 2002; Shih et al., 2000; Stoffel and Duncan, 1997; Wisely et al., 2002). The studies described here suggest that DHR3 and HNF4 may perform similar metabolic functions in flies, defining a new genetic model system for characterizing these key NRs.

2.5.3. New insights into the regulation of Drosophila xenobiotic responses

Several vertebrate NRs play a central role in xenobiotic responses by directly binding toxic compounds and inducing the expression of key detoxification enzymes such as cytochrome P450s and glutathione transferases (Willson and Kliewer, 2002). Ligand sensor activation observed in the gut, epidermis, tracheae or fat body could represent xenobiotic responses insofar as toxic compounds could enter the organism through any of these tissues. Directed screens that test xenobiotic compounds for their ability to activate Drosophila NR ligand sensors will provide a means of identifying potential xenobiotic receptors. Understanding these response systems, in turn, could facilitate the production of insect resistant crops and the development of more effective pesticides. In this regard, we have shown that DHR96, which is required for proper xenobiotic responses in Drosophila, can be activated by the CAR-selective agonist CITCO, suggesting that it may be regulated in a manner similar to that of the vertebrate xenobiotic receptors (Figure 2.2M,N). It is also interesting to note that angelicin was found to activate the
USP ligand sensor fusion (Figure 2.7E-H). Angelicin is an angular furanocoumarin that has the furan ring attached at the 7,8 position of the benz-2-pyrone nucleus. Detailed studies have shown that insects have adapted to the presence of furanocoumarins in their host plants by expressing specific cytochrome P450 enzymes that detoxify these compounds (Hung et al., 1996). In the black swallowtail butterfly (*Papilio polyxenes*), furanocoumarins induce the transcription of P450 genes through an unknown regulatory pathway, thereby aiding in xenobiotic detoxification (Berenbaum, 2002). Our observation that angelicin, and not the linear furanocoumarins 8-methoxypsoralen (xanthotoxin) or 5-methoxypsoralen (bergapten), can activate GAL4-USP suggests that NRs may mediate this detoxification response and may be capable of distinguishing between the linear and angular chemical forms. It is possible that USP may mediate this effect on its own or, more likely, as a heterodimer partner with another NR. Similarly, the activation of GAL4-USP by fenoxycarb may represent a xenobiotic response (Figure 2.7I-L). This activation, however, is weaker and more variable than the activation we observed with angelicin.

Identifying other factors that mediate xenobiotic responses in *Drosophila* would provide a new basis for dissecting the control of detoxification pathways in higher organisms.

2.5.4. **ERR activity appears to be regulated by a temporally restricted and widespread signal**

GAL4-ERR displays a remarkable switch in activity during mid-embryogenesis, from strong activation in the myoblasts to specific and strong activation in the CNS (Figure 2.3A). The ERR ligand sensor also shows widespread transient activation in the mid-third instar (Figure 2.3B), a time when larval ERR gene expression begins (Sullivan and Thummel, 2003), together with a global switch in gene expression that prepares the animal for entry into metamorphosis 1 day later (Andres et al., 1993; Cherbas et al., 2003). This so-called mid-third instar transition includes upregulation of EcR, providing sufficient receptor to transduce the high titer late larval 20E hormone pulse (Talbot et al., 1993), upregulation of the Broad-Complex, which is required
for entry into metamorphosis (Kiss et al., 1988), and induction of the genes that encode a polypeptide glue used to immobilize the puparium for metamorphosis (Lehmann, 1996). The signal and receptor that mediate this global reprogramming of gene expression remain undefined. The widespread activation of GAL4-ERR at this stage raises the interesting possibility that it may play a role in this transition. Moreover, given that the only ligand sensors to display widespread transient activation are EcR and USP, in response to 20E, it is possible that this response reflects a systemic mid-third instar pulse of a ERR hormone. Vertebrate members of the ERR family can bind the synthetic estrogen diethylstilbestrol and the selective ER modulator tamoxifen, as well as its metabolite, 4-hydroxytamoxifen, suppressing their otherwise constitutive activity in cell culture (Coward et al., 2001). This is notably different from the highly restricted patterns of ERR ligand sensor activity that we detect in Drosophila, which suggests that it does not function as a constitutive activator in vivo. Rather, we envision that the patterns of ERR activation are precisely modulated by protein co-factors and/or one or more ligands to direct the dynamic shifts in activation that we detect during embryogenesis and third instar larval development. Functional studies of the Drosophila homolog of the ERR receptor family may provide a basis for understanding these dynamic shifts in LBD activation, as well as revealing a natural ligand for this NR.

2.5.5. Future directions

This study provides, for the first time, a comprehensive analysis of the activation patterns of NR LBDs in a developing organism, uncovering a wide range of dynamic and localized changes in activity that occur as the animal undergoes massive developmental and physiological changes during embryogenesis and early metamorphosis. Our data provide a foundation for biochemical and genetic studies aimed at defining the molecular and functional basis for these LBD activation responses. We anticipate that this work will lead to new insights into NR
regulation and function, including the discovery of new NR partner proteins and endogenous ligands. In addition, extensions of this work could have practical consequences by identifying novel agonists and antagonists that could be used for insect population control, potentially impacting deadly insect-borne human diseases such as malaria and providing more effective means of crop protection. Finally, characterization of the activity patterns described here should lead to novel insights into embryonic patterning, metabolic control, xenobiotic metabolism, immunity, circadian rhythms and aging, with direct implications for how these pathways might be controlled by orthologous NRs in humans.
2.6. **Supplemental Data**

## Embryonic Activity Patterns

### Control

![Stage 10](image)

![Stage 17](image)

![Stage 11](image)

![Stage 13](image)

![Stage 17](image)

### DHR3

![Stage 10](image)

![Stage 9](image)

![Stage 12](image)

![Stage 13/14](image)

![Stage 17](image)

![Stage 15](image)

![1st Instar](image)

![Stage 17](image)

### DHR38

![Stage 12](image)

![Stage 13](image)

![Stage 13](image)

![Stage 17](image)

![1st Instar](image)
Embryonic Activity Patterns

DHR96

Stage 16/17
Stage 17
1st Instar

E78

Stage 10
Stage 13/14
Stage 14
Stage 16
Stage 17
Stage 17
Stage 17
1st Instar

EcR

Stage 13/14
Stage 17

ERR

Stage 16
Stage 14
Stage 14
Stage 16
Stage 16
Stage 17
Stage 17
1st Instar
Embryonic Activity Patterns

FTZ-F1

Stage 14/15
Stage 15
Stage 16
Stage 17

Stage 14/15
Stage 15
Stage 16
1st Instar

HNF4

Stage 9
Stage 12
Stage 13/14
Stage 16
Stage 17
Stage 17
Stage 17

USP

Stage 13/14
Stage 17
Chapter 3 : Nitric oxide signaling controls the nuclear receptor-directed transition from growth to metamorphosis

A similar report was submitted to Cell in December, 2009 titled:

‘Nitric oxide signaling controls the nuclear receptor-directed transition from growth to metamorphosis

Aleksandar S. Necakov, Lucía Cáceres, Carol Schwartz, Sandra Kimber, Ian J. H. Roberts, and Henry M. Krause

Ian Roberts and Sandra Kimber produced the UAS-RNAi::NOS and hsNOS transgenic lines.

Lucia Caceres produced the ring gland western blot data, lipid droplet, protein and triacylglyceride analysis in larvae, and the developmental profile data.

Carol Schwartz contributed by establishing the protein and triacylglyceride analysis in larvae, by providing preliminary developmental profile data, and by providing useful suggestions to the completion of the manuscript.
3.1. Abstract

A burgeoning collection of work has shown that the diatomic gas Nitric Oxide (NO) acts as a short-range signaling molecule in a vast array of important physiological processes. While these responses include major changes in cellular gene expression, the mechanisms by which they are transmitted are poorly understood. Here, using gain and loss of function transgenes, as well as tissues manipulated in culture, we show that NO acts directly on the Drosophila nuclear receptor E75, reversing its ability to block the activity of its heterodimer partner DHR3. Focusing on the gland that produces the metamorphosis-inducing hormone ecdysone, we show that failure to produce NO and to inactivate E75 results in failure to recognize the signals that normally trigger metamorphosis. Instead, larvae continue to eat for up to several weeks resulting in a morbid increase in lipid content. Given the similarity between NOS and E75 phenotypes, and those of their human counterparts, this family of receptors may be the main mediators of NO function in the nucleus.

3.2. Introduction

In all metazoans, the nuclear receptor family of transcription factors play a major role in coordinating growth, metabolism, development and sexual maturation [reviewed in (Francis et al., 2003; He et al., 2009; McElreavey and Fellous, 1999)]. Incumbent on these roles is control over the physical and behavioral transformations that take place between the growth and reproductive phases of the life cycle. Although much is known about the endocrine processes that manage these transformations, relatively little is known about the actual cues that initiate them. In this study, we show that the Drosophila nuclear receptors Ecdysone Induced Protein 75 (E75) and Drosophila Hormone Receptor 3 (DHR3, also known as HR46) (Tweedie et al., 2009) play major roles in mediating the transition between the growth and reproductive phases of the
life cycle, as may their respective vertebrate counterparts, the Rev-erbs and Retinoid-related orphan receptors (RORα, RORβ, RORγ).

Nuclear receptors (NRs) are hormonally regulated by small lipophilic compounds. Upon binding, these small molecules trigger structural changes that alter the binding of necessary cofactors, chaperones and post-transcriptional modifiers, thereby changing the localization, function and stability of the bound receptors (Chawla et al., 2001; Owen and Zelent, 2000). In most organisms, however, the majority of NR ligands have yet to be identified. In *Drosophila*, ligands have been identified for only two of the 18 NRs encoded in the genome, the Ecdysone receptor (EcR) and E75 (Koelle et al., 1991; Reinking et al., 2005).

In previous work, we have shown that E75 expressed in bacteria, cultured insect cells or whole animals contains heme constitutively bound to the ligand binding domain (LBD) via coordinate bonds (Reinking et al., 2005). As heme is required for E75 stability, levels of E75 expression are proportional to levels of available heme (Reinking et al., 2005). *In vitro* analyses have shown that the amino acids coordinately bound to the heme iron can be exchanged or displaced by the presence of NO, carbon monoxide (CO) or redox state (Marvin et al., 2009; Pardee et al., 2009; Reinking et al., 2005). Thus, E75 LBD stability and structure can be modulated by heme, NO, CO and redox status, providing E75 with a number of potential cellular responses and functions. Like E75, the mammalian Rev-erb proteins are also capable of binding heme, NO and CO *in vitro*, and have been shown to respond functionally to altered levels of heme (Marvin et al., 2009; Pardee et al., 2009) and NO (Pardee et al., 2009) in cultured cells.

For E75, one effect of NO binding *in vitro* is disruption of the ability of E75 to interact with helix 12 of DHR3, thereby blocking DHR3-mediated target gene activation (Reinking et al., 2005). In the case of the Rev-erbs, the presence of NO interferes with the ability of the receptor
to recruit co-repressors (Pardee et al., 2009). Although it is unclear if these different insect and mammalian responses to NO are unique or shared, both result in derepression of E75/Rev-erb, DHR3/ROR, coregulated target genes in cultured cells. Given that all of these effects have been delineated using \textit{in vitro}, bacterial and cell culture models, their relevance to E75/Rev-erb functions \textit{in vivo} are unknown.

One of the best characterized roles of E75 and DHR3 is within the NR cascade initiated by the production of ecdysone (Figure 3.1). Upon binding ecdysone, the Ecdysone Receptor (EcR) acts as a heterodimer with a second NR called Ultraspiracle (USP) to activate transcription of the DHR3 and E75 genes (Bialecki et al., 2002; Koelle et al., 1991; Lam et al., 1999; Lam et al., 1997; White et al., 1997). DHR3 autoregulates its own expression as well as that of the E75 splice variant E75B and the downstream nuclear receptor gene $\beta$Ftz-F1 (Lam et al., 1997; White et al., 1997). $\beta$Ftz-F1, in turn, activates the expression of ecdysone synthetic enzyme genes (Parvy et al., 2005), resulting in the next round of ecdysone production.

Four E75 gene splice variants have been identified [E75A-D (Tweedie et al., 2009)]. Except for the B isoform, each encodes a repressor with the same DNA and ligand binding domains but different N-terminal extensions. Although the E75B splice variant lacks a functional DNA binding domain, it is nevertheless still able to bind and repress DHR3 on target gene promoters (Reinking et al., 2005; Segraves and Hogness, 1990; White et al., 1997). As transcriptional repressors, E75 proteins play a major role in controlling the progression of this cascade. For the pathway to proceed, posttranscriptional regulatory mechanisms are required to inactivate E75 isoforms so that DHR3 can drive the pathway forward. A possibility suggested by our earlier \textit{in vitro} studies was that E75 inactivation \textit{in vivo} may be controlled by the production or delivery of NO gas. Consistent with a possible role for NO in ecdysone production, previous
work has shown that *Drosophila* Nitric Oxide Synthase (NOS), the enzyme that produces NO, is expressed in the ring gland (Wildemann and Bicker, 1999), which is where ecdysone is produced (Lam et al., 1997; Sullivan and Thummel, 2003). The role of NOS in the ring gland, however, has not been addressed, due in part to the embryonic lethality of available mutations (Regulski et al., 2004). Interestingly, microinjection and organ culture experiments with NOS inhibitors results in tissue overgrowth (Gibbs et al., 2001; Peunova and Enikolopov, 1995), whereas ectopic NOS expression has the opposite effect (Gibbs et al., 2001), consistent with numerous other studies showing that NOS and NO act as inhibitors of cell growth and division, with potential for use in cancer therapy (Contestabile, 2008; Villalobo, 2006).

We show here that DHR3 and E75 are also expressed together with NOS in the *Drosophila* ring gland, and that all three are required for *Ftz-F1* expression and ecdysone production. Furthermore, using gain- and loss-of-function analyses, along with small molecule manipulation, we show that NOS activation and E75 neutralization are indeed required for the proper timing of ecdysone production. Depending on the nature of these manipulations, they lead either to premature cessation of feeding and growth or, conversely, to a greatly extended feeding phase with massive lipid buildup and eventual lethality. Given that vertebrate NOS inactivation or mutation also leads to obesity and many other Rev-erb-related effects, we suggest that the E75/Rev-erb family of nuclear receptors are likely to be major mediators of NO action on gene expression. A review of the literature suggests that processes regulated by this pairing include feeding, metabolism, circadian rhythm, immunity, hypertension, sexual maturation, depression and aging.
3.3. Results

3.3.1. Expression of E75, NOS, DHR3, and FTZ-F1 in the ring gland

To see if E75, DHR3 and NO are all co-expressed in the prothoracic gland (PG) portion of the ring gland (Figure 3.1B), which is where ecdysone is produced, mRNA and protein expression patterns were analyzed. To simplify these analyses, two time points were selected. The first, ~18 hr before puparium formation (BPF), is well before the first major peak of ecdysone production. The second, ~8hr BPF, corresponds with the onset of expression of the E75/DHR3 target gene $\beta$Ftz-F1, which in turn activates the expression of genes that encode ecdysone synthesis enzymes. Hence, we expected that levels of DHR3, E75 and NOS would be low or undetectable during the earlier time point (18hr BPF) and relatively high during the latter time point (8hr BPF).

As stated earlier, the E75 gene produces two major mRNA and protein isoforms. The E75A isoform contains the conserved DNA binding domain, while the E75B isoform does not (Segraves and Hogness, 1990). Both, however, can interact with DHR3 and block its ability to activate $\beta$Ftz-F1 transcription (Figure 3.1A) (White et al., 1997). Immunohistochemistry reveals that both E75 isoforms appear to be present at very low levels in the PG at 18hr BPF, but increase dramatically by 8hr BPF (Figure 3.1C-F). Interestingly, E75B appears to be enriched perinuclearly in these cells. Like the E75 proteins, DHR3 also shows increased expression at the later time point (Figure 3.1G, H). These results are consistent with previous Northern blot analyses of E75A, E75B, and DHR3 transcript levels in whole larvae at these stages of development (Sullivan and Thummel, 2003).

Previous studies using NADPH Diaphorase, a histochemical dye that detects NOS reductase activity, showed staining in the ring gland of third instar larvae, suggesting a role in
ring gland function (Wildemann and Bicker, 1999). Consistent with this finding, we see that NOS protein is expressed in the ring gland, also increasing dramatically between the 18hr and 8hr BPF time points (Figure 3.1I, J).

As might be expected, expression of βFtz-F1 mRNA (and protein, data not shown) also ramps up in response to the increased expression of E75, DHR3 and NOS proteins observed in the 8hr BPF PG (Figure 3.1K, L). Thus, the spatial and temporal expression profiles of E75A, E75B, DHR3, NOS and their mutual target βFTZ-F1, are consistent with our hypothesized roles in ecdysone production.
Figure 3.1 - E75, NOS, DHR3, and FTZ-F1 are expressed in the larval ring gland

A) Diagramatic representation of the ecdysone-dependent nuclear receptor signaling pathway during larval development. B) Depiction of the larval *Drosophila* ring gland. CA, corpus allatum; PG, prothoracic gland; CC, corpus cardiaca. C-L) Ring glands stained for E75A protein (Green; C-D), E75B Protein (Green; E-F), DHR3 Protein (Green; G-H), NOS Protein (Green; I-J), or FTZ-F1 mRNA (Green; KL). Nuclei were stained with DAPI (blue). Dashed white lines outline wild-type larval ring glands from dark gut, ≥ 18 hrs BPF larvae (left column), or clear gut, 0-8 hours BPF larvae (right column). Bar = 100 µm.
3.3.2. *NOS expression in the ring gland can be depleted by RNAi*

The *Drosophila* genome contains a single NOS gene, which when mutated is embryonic lethal (Regulski et al., 2004). Hence, to test if the NOS expression seen in the larval ring gland is required for E75/DHR3-regulated ecdysone production, we attempted to knock down PG NOS expression using targeted RNAi-mediated repression. Four GAL4-UAS-regulated NOS-RNAi transgene lines were obtained. Two of the transgenes, UAS-NOSRNAi IR (2 lines; inserts on chromosomes II and X) and UAS-NOS-RNAi VDRC carry inverted repeats of exon 16 of the NOS gene, while the third transgene, UAS-NOS-RNAi NIG carries inverted repeats derived from exon 2 (Figure 3.2A). The 4 lines derived from these 3 transgenes will be referred to hereafter as NOS-RNAi IR-X, NOS-RNAi IR-II, NOS-RNAi VDRC and NOS-RNAi NIG. Three different GAL4 driver lines were used to induce tissue-specific expression of these RNAi transgenes. The Phantom-GAL4 (*phm*-GAL4) and Amnesiac-GAL4 (Amn-GAL4) drivers are expressed primarily in the PG of the ring gland (Mirth et al., 2005; Warren et al., 2004), whereas the third driver, Tubulin-GAL4 (Tub-GAL4) delivers fairly ubiquitous GAL4 expression.

As a first determinant of the effectiveness of the RNAi transgenes, we examined the levels of NOS protein in dissected ring glands using Western blot analyses (Figure 3.2B). The NOS antibody recognizes a ~100 kDa protein in wild-type ring glands. Expression levels of this protein were reduced in the ring glands of all GAL4-induced NOS-RNAi transgenic lines, although with significantly different efficiencies. The NOS-RNAi IR transgene, when located on the X chromosome (NOS-RNAi IR-X), consistently yielded the strongest reduction in protein expression (~90%), with the NOS-RNAi NIG and NOS-RNAi VDRC lines showing progressively weaker effects (45% and 15% respectively; Figure 2B). In terms of the GAL4 drivers, Tub-GAL4 was strongest, followed by *phm*-GAL4 and lastly Amn-GAL4.
Further validation of these RNAi effects was obtained via immuno-histochemical and biochemical analyses of larval ring glands (Figure 3.2B). As was seen in the Westerns, NOS antibodies revealed a strong decrease in ring gland antigen detection following induction of the RNAi transgene in the PGs of NOS-RNAiIR ring glands (Figure 3.2CI-II). Similarly, using the dye 4,5-diaminofluorescein diacetate (DAF-2 DA), a NOS-specific fluorescent indicator (Blute et al., 2000; Schuppe et al., 2002), the strong signal detected in wild-type ring glands was no longer detected (Figure 3.2CIII-IV). Taken together, these data show that NOS is expressed in the PGs of the ring gland, that the protein appears to be producing NO at the 8hr BFP time point, and that the RNAi lines used are capable of substantially reducing these levels of expression and activity.
Figure 3.2 - NOS is required in the Ring gland

A) Schematic diagram of NOS gene organization and regions targeted by RNAi knockdown constructs. Colored vertical lines indicate the locations of the sequences targeted by the NOS-RNAi\textsuperscript{NIG} (Green line), NOS-RNAi\textsuperscript{IR} (Blue line), and NOS-RNAi\textsuperscript{VDRC} (Red line) transgenes. B) Western blot of NOS protein from wild-type and Tub-GAL4>NOS-RNAi knock-down ring glands. The arrowhead highlights the 100 kDa NOS protein isoform. The bottom panel is the loading control for each genotype. C) NOS protein (panels I, II), DAF-2 DA (panels III, IV), and βFTZ-F1 protein (panels V, VI) in the ring glands of wild-type (left column) and NOS-RNAi\textsuperscript{IR-II} larvae (right column). The phm-GAL4 driver was used in panels II, VI and Tub-GAL4 in panel IV. Nuclei are stained with DAPI (blue). Ring glands are outlined by dashed white lines. Bar = 100 µm.
3.3.3. **NOS is required in the ring gland for βFTZ-F1 expression**

Having shown that the NOS RNAi lines obtained are active, our next objective was to see if their expression affected E75 activity. This was assayed first by examining their effects on expression of the E75/DHR3 target gene βFtz-F1. If the repressive function of E75 on DHR3 is incapacitated by NO, as previously seen *in vitro* and in cultured cells (Reinking et al., 2005), then reduction of the levels of NO via NOS RNAi should result in decreased expression of βFTZ-F1. Whole-mount immuno-stainings against βFTZ-F1 protein in 8hr BPF ring glands showed strong nuclear and cytoplasmic protein accumulation in the wild-type PGs (Figure 3.2C-V). As expected, βFTZ-F1 expression was lost in the PGs of *phm*-GAL4>NOS-RNAiIR-X ring glands (Figure 3.2C-VI).

3.3.4. **Ectopic NOS reverses E75-mediated βFTZ-F1 repression**

To test whether the effects of NOS reduction on βFtz-F1 expression are mediated directly via E75, ectopic expression assays were used to increase the levels of E75B, DHR3 and NOS within the ring gland at 18hr BPF when the expression of all four genes is normally very low. This was achieved using transgenic lines containing E75B, DHR3, and/or constitutively active NOS (mouse macrophage NOS) cDNAs under the control of a heat-inducible promoter. As predicted, boosting the levels of DHR3 expression at 18hr BPF resulted in greatly increased levels of βFtz-F1 transcripts (Figure 3.3F). Conversely, ectopic expression of E75B, alone or together with DHR3, resulted in levels of βFTZ-F1 expression below the background levels normally seen at this time (Figure 3.3L and data not shown). However, by co-expressing macrophage NOS, the complete repression mediated by ectopic E75B was largely reversed (Figure 3.3O). Thus, the overall levels of βFTZ-F1 expression in the ring gland appear to depend on the relative stoichiometries of DHR3, E75 and active NOS.
Figure 3.3 - E75B mediated repression is sensitive to NO

≥18 hour BPF larvae were collected from (A-C) wild-type, (D-F) hs-DHR3, (G-I) hs-DHR3; hs-NOS, (J-L) hs-DHR3; hs-E75B, and (M-O) hs-DHR3; hs-E75B; hs-NOS. Ring glands were subsequently stained for DHR3 protein (Blue; A, D, G, J, and M), E75B protein (Red; B, E, H, K, N), and FTZ-F1 mRNA (Green; C, F, I, L, O). Bar = 100 µm.
3.3.5.  *E75-mediated βFtz-F1 repression can be reversed by NO gas*

As an alternative means of providing NO gas, a variation of the previous experiment was conducted using ring glands in which DHR3 and E75B expression were ectopically induced and then dissected and treated in culture with NO donor compounds. As seen in the previous experiment, 18hr BPF ring glands overexpressing DHR3 produced increased levels of βFTZ-F1 transcripts (Figure 3.4F), and co-expressing DHR3 with E75B reversed this induction (Figure 3.4L). In the presence of the NO donors diethylenetriamine-NO (DETA-NO; Figure 3.4O), or SNAP (data not shown), the repression mediated by E75B was reversed. Taken together, our genetic and chemical manipulations are consistent with NOS acting through E75 via the production of NO gas.
Figure 3.4 - E75B mediated repression is sensitive to NO

≥ 18 hour BPF larvae were collected for (A-C) wild-type, (D-I) hs-DHR3, or (J-O) hs-DHR3; hs-E75B. After heat-treatment larvae were cultured in the absence (A-F, J-L) or presence (G-I, M-O) of 100 µM DETA-NO. Ring glands were subsequently stained for DHR3 protein (Blue; A, D, G, J, M), E75B protein (Red; B, E, H, K, N), and FTZ-F1 mRNA (Green; C, F, I, L, O). The strong nuclear foci detected in panels C-O are nascent FTZ-F1 mRNA detected using Tyramide signal amplification (see Materials and methods). Bar = 100 µm.
3.3.6. *E75B mediated repression of DHR3 activity is reversed by NO*

Another way to assess the effects of NO on E75 activity is to visualize DHR3 activity *in vivo*. In previous work, a fusion protein comprised of the ligand binding domain of DHR3 and the DNA binding domain of GAL4 was shown to be capable of activating the transcription of a UAS-GFP reporter transgene in larvae, and that this activity could be repressed by ectopically expressing E75B (*hs-E75B*) (Palanker et al., 2006). To see if this E75-repressible activity could be reinstated by NO, we used the constitutively active macrophage NOS (*hs-NOS*) to produce NO in 18hr BPF larvae. As shown in Figure 3.5.I, the DHR3 fusion protein was active when viewed in 8hr BPF ring glands (Figure 3.5.II), and this activity was repressed by overexpression of E75B protein (Figure 3.5.III). As expected, the E75-repressible activity of DHR3 could be restored by co-expressing active NOS (Figure 3.5.IV), consistent once again with NOS affecting the ability of E75 to interact with and block the transcriptional activity of DHR3 through the production of NO gas.
Figure 3.5 - E75B mediated repression of DHR3 activity is sensitive to NO

A) Diagramatic representation of the scheme used to monitor DHR3 LBD activity. Transgenic larvae containing fusion protein composed of the DHR3 LBD and the DNA binding domain of yeast GAL4 (hs-GAL4::DHR3) was co-expressed with a reporter construct containing a nuclear GFP encoding gene driven by a UAS_{GAL4}-dependent promoter (UAS-EGFP_{NLS}). This line produces GFP in the PG of clear gut larvae and prepupae.

B) Effects of E75 and NO on DHR3 activity. (I) hs-GAL4::DHR3>UAS-EGFP_{NLS}, (II) hs-GAL4::DHR3>UAS-EGFP_{NLS}; hs-NOS, (III) hs-GAL4::DHR3>UAS-EGFP_{NLS}; hs-E75B, (IV) hs-GAL4::DHR3>UAS-EGFP_{NLS}; hs-E75B; hs-NOS. Nuclei are stained with DAPI (blue).
3.3.7. Down-regulation of NOS in the ring gland prevents the onset of metamorphosis

The reversal of E75-mediated repression of DHR3 by NOS results in βFTZ-F1 expression, which, in turn, leads to the production of ecdysone and entry into metamorphosis. Conversely, knocking down NOS expression by RNAi should result in delayed metamorphosis and prolonged larval stages of development. Wild-type third instar larvae normally enter metamorphosis on the fifth day after egg laying (AEL), and begin to eclose as flies on the ninth day AEL (Figure 3.6A). In contrast, the majority of NOS-RNAi larvae failed to pupariate on time and continued to feed. In the case of phm-GAL4>NOSRNAiIR-X animals, 50% remained as larvae 12 days AEL (Figure 3.6B). Although these animals all eventually died as larvae, some managed to survive for as long as 30-40 days AEL. Those that did begin metamorphosis between days 6 and 12 exhibited defects and 10 failed to eclose as adults. A small percentage of phm-GAL4>NOS-RNAiIR-X (< 20%) escapers did manage to pupariate on time, and the vast majority of these produced normal adult flies.

Importantly, the penetrance and severity of these effects varied in proportion to the effectiveness of the RNAi transgenes and GAL4 drivers used. For example, virtually all phm-GAL4>NOS-RNAiVDRC animals developed normally and produced adults, whereas phm-GAL4>NOS-RNAiIR-II and phm-GAL4>NOS-RNAiNIG animals showed intermediate levels of success (Figure 3.6SA-C). Phenotypic severity could also be increased or decreased by rearing the animals at higher or lower temperatures, respectively (data not shown).

A second method of NOS knockdown was attempted using the NOS-specific chemical inhibitor NG-nitro-L-arginine methyl ester (L-NAME) (Rees et al., 1990; Wingrove and O'Farrell, 1999). As expected, supplementing larval food with 100-125mM L-NAME beginning at 1 day AEL resulted in loss of DAF2-DA fluorescence in the PG (data not shown), and delayed
pupariation (Figure 3.6S-D). Specifically, 66% of 100mM and 80% of 125mM L-NAME fed larvae remained as third instar larvae at day 7, while at day 10, 7% of 100mM and 54% of 125mM L-NAME fed larvae were still observed in the food (Figure 3.6SD).

Larvae fed with food supplemented with 125mM D-NAME, which is an inactive stereoisomer of L-NAME, pupariated on day 5, as did the control animals (Figure 3.6S-D). Thus, similar effects on developmental timing were achieved using both genetic and chemical approaches to block NO production.

Importantly, NOS-depleted larvae were the same size as wild type at day 5 AEL, at which time they should normally pupariate (Figure 3.6E-I, data not shown). However, the mutant larvae did not stop feeding, and continued to grow reaching a maximum size and weight at approximately 10 days AEL (Figure 3.6C, 6D, 6EII-III). In other studies where ecdysone production was compromised by alternative means, metamorphosis could be rescued by placing ecdysone in the food of third instar larvae (Bialecki et al., 2002; Ono et al., 2006). This was also the case for NOS-RNAi larvae. Approximately 50% of \textit{phm-GAL4>NOS-RNAiIR-X} larvae initiated metamorphosis the following day when their food was supplemented with ecdysone on the fifth day AEL (Figure 3.6E-IV, and data not shown).

An interesting aspect of the \textit{phm-GAL4>NOS-RNAiIR-X} phenotype is that the ring glands in these larvae grew to become as much as 6 times the size of their wild-type counterparts, and turn red (Figure 3.6E-III (arrow), 6F-II, S6A-II, S6B-II). The ring glands of NOS-RNAiNIG and NOS-RNAiIR-II larvae grew to an intermediate size without turning red (Figure 3.6SA-III, 6SB-III, 6SC, and data not shown), while NOS-RNAiVDRC larval ring glands were not noticeably enlarged or red (data not shown). Once again, these effects are in accord with the relative effectiveness of these lines on NOS protein knockdown. Measurement of
cell numbers and sizes (data not shown) showed that the increase in PG size is due to growth in cell (and nucleus) size and not number. These autonomous effects on ring gland size are consistent with well documented studies showing that cellular exposure to NO slows down cell growth and proliferation (Gibbs and Truman, 1998; Kuzin et al., 1996; Peunova and Enikolopov, 1995). In this case, blocking NO production in the PG appears to allow continued polytenization and cell growth.
Figure 3.6 - NOS is required for the onset of pupariation

Developmental profiles for (A) wild-type and (B) phm-GAL4>NOS-RNAiIr-X animals followed for 21 days. Line colors (see inlay code in A) depict the relative numbers of larvae, pupae, flies and dead animals on each day. Results are the average of triplicate data sets. Error bars depict standard deviation. C) Length measurement of NOS-RNAi larvae. D) Weight measurement of NOS-RNAi larvae. E) Panels I and II show typical wild-type and NOS-RNAiIr-X larvae at 5 days AEL. Panel III shows a 10 day old NOS-RNAiIr-X larva, and panel IV a 7 day old NOS-RNAiIr-X pupa following ecdysone feeding beginning on day 5. White arrowheads indicate red ring glands visible through the cuticle III and IV. F) Brain-ring gland complexes of 5 day old wild-type (I) and 10 day old NOS-RNAiIr-X (II) larvae. Ring glands are circled by dashed lines. Comparative phenotypes and responses of all 4 NOS-RNAi knock-down lines, and animals fed with L-NAME, are shown in Figure S6.

3.3.8. NOS-RNAi larval overgrowth is not due to the growth of imaginal tissues

As observed with ring gland size, the increased larval size in NOS-RNAi induced larvae could be due to a general increase in the growth of other tissues, either due to increased cell number or size (Gibbs and Truman, 1998; Kuzin et al., 1996). As a first step in addressing this, we measured the surface areas of imaginal discs, brains, salivary glands and fat bodies of NOS-RNAiIr-X larvae at 10 days AEL, which is when the majority of delayed larvae have reached their maximum weight and size.

Surprisingly, the brain, CNS and all imaginal discs in NOS-RNAi larvae were the same size as those observed in wild-type 5 day old larvae, with the same approximate numbers of cells (Figure 6SB, 6SE). Thus, the increased size of NOS-RNAi larvae is not due to a general increase in the growth of all tissues. However, the polytenized tissues we observed did show obvious
increases in relative size. The salivary glands of NOS-RNAi^{IR-X} larvae were on average 1.4 times the size of their wild-type counterparts, and fat bodies nearly 2 times their normal size (Figure 3.6SF). In all cases of tissue enlargement, the numbers of cells remained constant, but cellular diameters increased. An important distinction worth noting is that, while salivary gland nuclei diameters increased with age, those of fat body cells did not. Thus, the continued growth of larval tissues appears to vary in terms of genetic and cytoplasmic expansion. Taken together, though, it appears that tissues fated to give rise to adult structures are either prevented from continued growth, or fail to receive a stimulatory signal, whereas the polyploidal larval tissues observed show some form of growth. Nevertheless, none of these increases in size compared with the autonomously induced 6-fold increase in PG size.

3.3.9. *Reduction in NO results in a dramatic over-accumulation of lipids*

The fat body is the invertebrate equivalent to mammalian adipose tissue and is the major reservoir of lipids in the fly. Given the expansion in fat body size seen in NOS-RNAi flies, and the relatively high percentage of body weight contributed by the fat body, we assessed how much of the increase in larval body size and weight was due to increased lipid content.

Lipids in fat-body cells are stored in vacuoles referred to as lipid-droplets, with their size, number and lipid occupancy proportional to total larval lipid content. Consistent with the observed increase in fat body size, the number of lipid droplets increased approximately six-fold, droplet volumes increased at least two-fold, and empty, poorly stained droplets were no longer observed in NOS-RNAi fat bodies (Figure 3.7B). Remarkably, the amount of triacylglycerol, which is representative of overall lipid content, was fourteen-fold higher in NOSRNAi^{IR-X} 10 day old larvae as compared to wild-type 5-day-old larvae while, at the same time points, overall
protein levels showed no significant change (Figure 3.7D). Thus, it appears that much of the increased weight and size of NOS-RNAi\textsuperscript{IR-X} larvae is due to increased storage of fat.

**Figure 3.7 - Triacylglyceride and protein levels in larval fat cells.**

A) Wild-type fat body. Intracellular lipids are stained by Oil Red O (Red). Note that some cells contain vesicles devoid of lipids (black arrowhead). Dashed white lines outline the border of an individual fat cell.

B) NOS-RNAi\textsuperscript{IR-X} fat cells are larger and full of lipid droplets. C) Average number of lipid droplets per fat cell (Red bars). D) Levels of total triacylglyceride larvae (Red bar) and total protein (Green bar) per larvae in wild-type and NOS-RNAi\textsuperscript{IR-X} larvae. Error bars depict standard deviation.
3.3.10. The NOS-RNAi phenotype can be phenocopied by E75 and DHR3

We reasoned that if NO inhibits E75 protein activity in vivo, increasing expression of E75 may overwhelm the repressive action of NOS, leading to larval phenotypes similar to those observed in larvae with reduced NO levels. Likewise, the same phenotypic outcome should be achievable by knocking down the levels of DHR3. To increase levels of E75, we made use of a UAS-E75A transgene under control of the phm-GAL4 driver. As predicted, larvae over-expressing E75A in the PG failed to pupariate on time and continued to feed and grow (Figure 3.8C). The same effect was achieved by reducing DHR3 (Figure 3.8D). Conversely, decreasing the levels of E75 (Figure 3.8E), or increasing the levels of DHR3, yielded small larvae that appeared not to develop beyond the second instar (Figure 3.8F). A similar effect was achieved by driving expression of constitutively active mouse macrophage NOS in the PG (Figure S8AI, S8AII). The similarity in phenotypes between E75 down-regulation and DHR3 and NOS up-regulation, and conversely between E75 up-regulation and DHR3 and NOS down-regulation, are consistent with their opposing roles in larval development (White et al., 1997).

Another way to test whether the effects of NOS are being mediated via E75, and in turn DHR3, is to see if mutations in the latter genes can enhance or suppress the NOS-RNAi phenotype. Again, we used PG-specific drivers to restrict the deleterious effects of these manipulations to the ring gland. As expected, reducing DHR3 or over-expressing E75 in the NOS-RNAi background enhanced the failure to pupariate and to generate the large-larva phenotype (Figure 3.8G-H and data not shown). Conversely, the DHR3 over-expression phenotype was epistatic to the NOS RNAi phenotype, producing small larvae (Figure 8I) as seen with DHR3 upregulation alone. Taken together, our analyses of single- and double-transgenic combinations with NOS, E75 and DHR3 mutants are consistent with our model in which E75
interferes with DHR3-mediated target gene activation, but in the presence of NO, these negative effects are neutralized.

![Figure 3.8 - NOS, DHR3, and E75 are required in the ring gland for normal development](image)

**Figure 3.8 - NOS, DHR3, and E75 are required in the ring gland for normal development**

A) 5 day old wild-type larva. B-I) 10 day old by phm-GAL4 driven B) NOS-RNAi^{IR-X} C) UAS-E75A, D) DHR3-RNAi, E) E75-RNAi, F), UAS-DHR3, G) NOS-RNAi^{IR-X}; DHR3-RNAi, H) NOS-RNAi^{IR-X}; UASE75A and I) NOS-RNAi^{IR-X}; UAS-DHR3 larvae. Bar = 1 mm. The larval phenotype of NOS gain of function PGs is shown in Figure S8A. A diagram of the PG enervation and response pathway, and conserved vertebrate pathways, is shown in Figure S8B.
3.4. Discussion

3.4.1. NOS controls the timing of ecdysone production via E75

Although the effects of NO on cells and tissues are numerous, and many of the mechanisms well documented, this is the first demonstration of a direct effect on gene expression in vivo. Our studies in vitro, in cultured cells and now in vivo, are consistent with a direct interaction between NO and the coordinately bound heme group within the E75 LBD. Although this seems the most likely mode of action in vivo, we cannot, however, exclude the possibility that NOS and/or NO also act on E75-heme via redox, as NO has a reducing effect within cells, and a switch from oxidized to reduced environments has a similar effect on E75 heme structure and function as the binding of NO (Marvin et al., 2009; Pardee et al., 2009; Reinking et al., 2005). Whether this is also true in vivo is yet to be determined.

In vitro, both NO and reducing conditions displace one of the E75 LBD-heme coordinate bonds, which leads to inability of the E75 LBD to bind helix 12 of the DHR3 LBD. Other E75 interactions could also be affected by NO binding, such as the ability of E75 to bind corepressors, as observed with the E75 orthologues Rev-erb and (Pardee et al., 2009). The presence of E75 isoforms in both the cytoplasm and nucleus (Figure 3.1), in a seemingly regulated fashion, suggests the likelihood of additional types of interactions and consequences.

In the PG, the NO effect on E75 frees DHR3 to activate expression of FTZ-F1, which in turn, leads eventually to ecdysone synthesis. Although this NR cascade is generally described as linear, it appears to be a feed forward loop that is largely circadian gated. This can be seen in the daily nocturnal peaks of ecdysone production seen over the course of insect larval development (Truman, 2005). Although less regular in amplitude and timing in Drosophila, these circadian peaks are much more so in more primitive insect species (Steel and Vafopoulou, 2006).
3.4.2. *Ring gland size is not the key determinant of metamorphosis timing*

In several recent studies, it was shown that manipulation of ring gland growth and size, via PG-autonomous genetic manipulation of insulin response genes, could accelerate or postpone the timing of pupariation (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). A conclusion often made from these studies is that ring gland size is a key determinant of when pupariation will occur (Mirth et al., 2005). Our results, however, suggest that ring gland size *per se* is not the primary determinant of metamorphosis initiation. We observed up to a six-fold increase over normal ring gland size with no triggering of metamorphosis. Similarly, altering PG size by manipulation of Dmyc or Cyclin D expression could also increase PG size without triggering premature ecdysone production (Colombani et al., 2005). Furthermore, metamorphosis can be induced prematurely by overexpression of Raf or down-regulation of DHR4 without affecting PG size (Caldwell et al., 2005; King-Jones and Thummel, 2005). These seemingly contradictory results are likely due to overlaps of the insulin and NO response pathways.

The remarkable endoreduplication and growth of the PG due to loss of NO, and markedly reduced size upon ectopic macNOS induction (not shown), is noteworthy and consistent with previous studies showing that NO has negative effects on cell growth and division [reviewed in Contestabile (2008), and Villalobo (2006)]. This activity has also had important implications and use in the control of oncogenic growth.

3.4.3. *NOS activation and ecdysone production are controlled by dual neuronal inputs*

Achievement of critical weight at the end of the 3rd larval instar coincides with a
reduction of circulating levels of insulin-like peptides (ILPs) (Brogiolo et al., 2001; Rulifson et al., 2002) and, consequently, production of prothoracicotropic hormone (PTTH) peptide by specific neurons located within the larval brain (McBrayer et al., 2007). The axons of these PTTH-producing neurons end at the surface of the PG, which is where PTTH is released (see Figure 3.S8B) and where binding of the secreted PTTH peptide to Torso receptor results in intracellular signaling (Rewitz, 2009). These signals include the activation Ras/Raf, MEK/Erk, and PKA, as well as PLC and PKC, and their various downstream effects (Rewitz, 2009; Rybczynski et al., 2001; Rybczynski and Gilbert, 2006). Key among these responses is the production of Calmodulin and cytoplasmic Ca++ influx (Chen et al., 2001; Meller et al., 1988; Rybczynski and Gilbert, 2003), both of which are absolutely required for dNOS enzyme activity (Gribovskaja et al., 2005; Muller and Bicker, 1994; Ray et al., 2007; Regulski and Tully, 1995; Stevens-Truss et al., 1997). Hence, we propose that PTTH acts in large part through NOS activation.

Importantly, PTTH release is also under circadian control [reviewed in Taghert and Shafer (2006), and Truman and Riddiford (1974)]. The PTTH neurons fasciculate with those of the lateral circadian clock neurons, which prevent PTTH release at night (McBrayer et al., 2007; Siegmund and Korge, 2001; Steel and Vafopoulou, 2006). This ensures that pupariation begins during the day, when attraction to light may also serve to attract larvae away from the decomposing food source, and toward a safer location for metamorphosis and eclosion. Coincidentally, as described above, the mammalian clock is regulated, in part, by the DHR3 and E75 orthologues RORα and Rev-erbβ (Guillaumond et al., 2005; Preitner et al., 2002), suggesting that DHR3 and E75 may also act in the Drosophila clock neurons under NO control, thereby placing these molecules at multiple levels within this neuroendocrine pathway. Indeed,
they may act once again within ecdysone-responding tissues downstream of the ecdysone receptor.

3.4.4. The role of heme

In addition to neuronally controlled PTTH and circadian gating, several other inputs likely contribute to E75/DHR3 mediated ecdysone production. One of the most likely is the E75 component, heme, which is required for E75 stability, NO binding and has a major role in all metabolic processes. In addition to its role in O₂ and CO₂ delivery and removal, heme is also a component of many metabolic enzymes, including those required to produce and metabolize lipids, steroid hormones, ATP and reactive oxygen species. Notably, both NOS and the enzyme that catalyzes heme breakdown, Heme Oxygenase, also require heme in their catalytic centres (Cui et al., 2008; Marletta, 1993; Zhang et al., 2004). The breakdown of heme by Heme Oxygenase is the major source of CO, which is another yet to be tested potential regulator of E75 activity in vivo.

Interestingly, some blood sucking insects, such as Rhodnius, require a blood meal before circadian ecdysone production, molting and growth can proceed (Wigglesworth, 1940). Female mosquitoes also require a blood meal to produce ecdysone and complete oogenesis (Hagedorn et al., 1975; Wigglesworth, 1940). Heme may be one of the essential cues provided by these blood meals. Heme levels have also been shown to vary in humans diurnally and to control circadian rhythm (Rogers et al., 2008). Indeed, Rev-erbs regulate heme synthesis in cultured cells via PGC1α regulation (Wu et al., 2009). The red ring glands that we observe in NOS knockdown larvae may therefore be the result of heme overaccumulation due to disruption of this homeostatic process. Thus, heme may serve as a major systemic timer that both reflects and controls metabolic activity, acting in part via E75 and its vertebrate counterparts.
3.4.5. NO signaling and lipid metabolism

Loss of NO production in the PG had major effects on lipid uptake and storage, leading to a nearly twenty-fold increase in larval lipid content, which is already relatively high at this time to support the enormous energy needs required by metamorphosis. This further increase in lipid content coincided with increased size, occupancy and numbers of lipid vesicles in fat body cells. Consistent with this observation, a similar increase in fat body lipid content has been achieved by specifically disrupting the ecdysone response pathway within fat body cells (Colombani et al., 2005).

This NO-based control of lipid metabolism is likely to be conserved in vertebrates, as triple-NOS-knockout mice that survive gestation are morbidly obese, with side-effects such as diabetes, hypertension and atherosclerosis that result in mortality prior to 10 months of age (Tsutsui et al., 2006). Conversely, NO upregulation via arginine supplementation promotes lipolysis, fatty acid oxidation, mitochondria biogenesis, glucose metabolism and lifespan [reviewed in Jobgen et al. (2006), Li and Stocker (2009), and Mustafa et al. (2009)].

In insects, pulses of ecdysone production during molts and upon achievement of critical weight coincide with the cessation of feeding. This negative effect on feeding, together with the negative effects of ecdysone on fat deposition, are likely the major reasons that premature NOS activation, DHR3 upregulation and E75 downregulation all result in small larvae that never reach critical weight. The reciprocal extended eating and fat deposition phenotype caused by failure to produce ecdysone may equate on many levels to processes underlying obesity and diabetes in humans. Indeed, these processes may be somehow conserved as ecdysone has been shown to decrease feeding, cholesterol synthesis and weight gain in mammals (Kizelsztein et al., 2009), and ecdysone is being used by some body builders and athletes to burn fat and increase muscle
mass [reviewed in Lafont and Dinan (2003)]. Given this ability to switch fat cell activity from lipid storage to lipid mobilization, a better understanding of the signals and mechanisms underlying these NO- and ecdysone-mediated effects should provide new insights and possible uses in disorders such as obesity, metabolic syndrome and diabetes.

3.4.6. Other conserved roles for NO and Rev-erbs in vertebrates

In vertebrates, the coordination of circadian and metabolic activities is controlled by the hypothalamus, which is subdivided into a number of nuclei that control various aspects of diurnal behavioral and metabolic functions. One of these centers is the Suprachiasmatic nucleus (SCN), which uses optic input to control the central circadian clock (Bob and Fedor-Freybergh, 2008, Kalsbeek et al., 2007). Rev-erbs also play a key role in this process (Preitner et al., 2002) and, as referenced above, are also regulated by NO. As SCN clock function is also affected by NOS and NO modulating compounds, these may be acting via the Rev-erbs.

The SCN, in turn, dictates the circadian output of the other hypothalamic nuclei. These other nuclei control metabolism, body temperature, appetite, thirst and blood pressure. For example, the production of cortisol in the adrenal gland prior to awakening is initiated by release of the peptide hormones vasopressin and corticotropin releasing hormone form the paraventricular nucleus of the hypothalamus, and then adrenocorticotropic hormone (ACTH) from the pituitary (Figure 3.9B). Rhythmic production of Thyroid Hormone in the thyroid gland, and steroid hormones in the gonads, is also controlled by the hypothalamus-pituitary axis. An increase in the frequency and intensity of these signals leads to the onset of puberty (Sisk and Foster, 2004) which, like metamorphosis, represents a transition from growth to reproductive phases of the life cycle. As with PTTH signaling in Drosophila, this transition is also under the control of a peptide hormone [kisspeptin; reviewed in Oakley et al (2009), and Tena-Sempere
which activates the same intracellular signaling pathways in the hypothalamus as seen in
the PG (including cytoplasmic Ca\(^+\) and calmodulin accumulation). Importantly, all of the tissues
involved in the onset of puberty, including the SCN and VPN nuclei of the hypothalamus, as
well as the anterior pituitary and the adrenal cortex, express NOS gene isoforms, and have also
been shown to produce, and in most cases require, NO production (Rettori et al., 2009).

Interestingly, when the hypothalamic nucleus that expresses SF1, the vertebrate
orthologue of FTZ-F1, is ablated, animals exhibit a similar phenotype to larvae with NOS,
DHR3 or FTZ-F1 depleted in the ring glands They eat voraciously and become obese
(Hasegawa, 2008; King and Frohman, 1985; King and Smith, 1985; Majdic et al., 2002). As
noted above, the same occurs in NOS mutant mice.

As with metamorphosis in flies, weight appears to be a major contributor to the timing of
sexual maturation in humans. For example, prior to entering puberty, children tend to accumulate
additional ‘baby fat’, and increasing pre-teen obesity in recent times has caused a consequential
lowering of puberty onset age (Ahmed et al., 2009). Fertility also requires a minimal supply of
available lipids in order to proceed (O’Sullivan, 2009). In short, RORs, Rev-erbs and NOS may
function at the core of many or most diurnal, lunar and seasonal rhythms, matching feeding
behavior, growth, reproduction and senescence to food availability, day length and temperature
within various environmental niches. Disruption of these functions may be at the core of many
human metabolic, sleep, stress, immune, and hypertension disorders, all of which are controlled
by the hypothalamus and NOS function [reviewed in Bose et al. (2009)]. Thus, further
elucidation of the roles of RORs, Rev-erbs and NOS proteins in endocrine tissues should provide
significant new insight into how these circadian and metabolic processes are linked and
coordinated.
3.5. Experimental Procedures

3.5.1. Drosophila Stocks

Wild-type flies used were Oregon-R. phantom-GAL4, UAS-mCD::GFP flies were provided by L. Riddiford, Amnc651-GAL4 by M. Stern, hs-macrophage NOS2 (hs-NOS) by G. Enikolopov, and TubP-Gal4 by the Bloomington Stock Center. UAS-NOS-RNAiIR lines were generated by I. Roberts, UAS-DHR3, hs-DHR3, and hs-E75B lines by C. Thummel, UAS-DHR3-RNAi and UAS-NOS-RNAiNIG by the National Institute of Genetics in Japan, and UAS-E75A-RNAi and UAS-NOS-RNAiVDRC by the Vienna Drosophila RNAi Center.

3.5.2. Whole mount in situ hybridization and immunohistochemistry

Larval in situ hybridization and immunohistochemistry were performed according to (Lecuyer et al., 2008) with minor modifications. Third instar Oregon-R larvae were fixed in PBS with 4% paraformaldehyde and 7% (v/v) saturated picric acid (Benveniste et al., 1998). Primary antibodies used were rabbit anti-E75A (1:100) (Hill et al., 1993), mouse anti-E75B MAb10E11 (1:20) (Schubiger and Truman, 2000), rabbit anti-DHR3 (1:100) (Lam et al., 1997), mouse anti-dNOS1 MAb 6/57 (1:400) (Regulski et al., 2004), rabbit anti-βFTZ-F1(1:10,000) (Murata et al., 1996), and rabbit anti-GFP Ab6556 (Abcam; 1:1000). Horseradish peroxidase-conjugated secondary antibodies (1:1000; Jackson Immunoresearch) were incubated overnight at 4°C followed by detection with either Tyramide-Alexa488 (Invitrogen) or Tyramide-Cy3 (Perkin Elmer). For in situ hybridization, DIG-UTP (Billas et al.) labeled antisense βFTZ-F1 RNA probes were transcribed from a FTZ-F1 cDNA using primers containing T7 promoter overhangs. Primers were designed to generate short, multiple RNA probes to increase signal strength.
3.5.3. *DHR3 ligand sensor activity*

Staged larvae (≥ 18 hours BPF) carrying hs-GAL4-DHR3-LBD; UAS-EGFPnls (Palanker et al., 2006) and either hs-NOS, hs-E75B, or hs-E75B/hs-NOS transgenes were heat-treated at 37ºC for 35 minutes. Before dissection, larvae were allowed to recover at 25ºC for 9 hours before GFP expression was visualized.

3.5.4. *Ectopic expression of DHR3, E75B, NOS2 and Organ Culture*

Larvae carrying heat inducible transgenes for DHR3, E75B and/or NOS were heat-treated on sealed, yeasted, apple-juice agar plates submerged in a 37ºC water bath for 40 minutes. Before dissection, larvae were allow to recover at 25ºC for either 20 minutes, for tissue culture experiments, or for 60 minutes, for whole mount in situ hybridization or immunohistochemistry.

For organ culture, dissected larval CNS and ring glands were cultured for 2 hours at 25ºC in Schneider’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), and 100 units/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (Emery et al., 1997; Gibbs and Truman, 1998). For NO application, the NO donor DETA-NO (Sigma Aldrich) was included in the culture media at a final concentration of 100 µM. Dissected larvae were incubated for 2 hours at 37ºC, then processed for RNA *in situ* hybridization and immunohistochemistry.

3.5.5. *Ecdysone feeding*

Five day old *phm*-GAL4; NOS-RNAiIR-X larvae were fed either yeast paste containing 3.3% ethanol, or yeast paste supplemented with 0.33 mg/mL 20-hydroxyecdysone in 3.3% ethanol (Sigma-Aldrich) and larvae were score daily for the appearance of pupae (Bialecki et al., 2002; Ono et al., 2006).
3.5.6. Developmental staging

Eggs were collected in yeasted apple-juice plates for 8 hours at 25°C, transferred into boxes containing standard food supplemented with 0.05% bromophenol blue, and allowed to develop to the third instar at 25°C. Four day AEL wandering larvae with dark blue guts were collected and transferred into smaller boxes containing standard food supplemented with 0.05% bromophenol blue, and their developmental profiles recorded over 21 days at 25°C. For each cross, experiments were performed in triplicate.

3.5.7. Triacylglyceride and Protein Assays

For each sample, 10 wandering third instar larvae were homogenized in 250 l icecold PBS. Samples were heated at 70°C for 5 minutes to inactivate endogenous enzymes, then centrifuged at room temperature for 5 minutes at 14,000 rpm. Supernatant was collected and stored at -20°C until needed, or used immediately. For determining total protein content, 5 l of supernatant was added to a 96-well flat bottom tissue culture plate (Sardtedt) along with 200 l of Bio-Rad Protein reagent assay (Cat.# 500-0006). For determining total triacylglyceride (TAG) levels, a LiquidColor Triglyceride kit (StandBio Laboratories) was used. 30 l of supernatant or standard along with 120 l of TAG reagent was loaded into each well. Once all samples were loaded, the 96-well plates were incubated at 37°C for 5 minutes, and readings at 500 nm (for TAG levels) and 595 nm (for protein levels) were immediately taken in a spectrophotometer. Triplicates of each sample were loaded onto the same plate and the average readings determined. Three independent TAG and protein assays were performed per genotype.

3.5.8. Lipid Staining and Analysis

For analysis of lipid droplets in the larval fat cells and larval organs, Oil Red O staining was performed as described (Gutierrez et al., 2007), with minor modifications. Samples were fixed,
stained and rinsed in 1.5 ml eppendorf tubes, and DAPI (1:1000, Molecular Probes) added as a counterstain to the 1:1 glycerol:PBS mounting media prior to mounting. Samples were visualized by DIC microscopy. Each cell was photographed as a series of 1 m optical sections using a Leica DMRA2 upright stereo microscope with a 100X (NA 1.3) oil objective. The number of lipids per fat cell was manually counted from z-stacks using the Point Counter tool from Openlab 3.1.7 software (Improvision).

3.5.9. **Protein extracts and Western blot Analysis**

Protein extracts for Western blot analyses were prepared from ring glands of 5 day old third instar wandering larvae. 50 wild-type, NOS-RNAi<sup>VDRc</sup> and NOS-RNAi<sup>NIG</sup> larvae, and 20 NOS-RNAi<sup>IR-X</sup> larvae were dissected in PBS. As samples were being dissected, they were immediately added to 10 µl cold protein loading buffer (5M urea, 0.125M Tris (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.1% Bromophenol blue (Van Buskirk and Schupbach, 2002). The samples were boiled, pelleted and either used immediately or stored at -20ºC until needed. 5 l of the lysate was loaded per lane onto an 8% SDS-PAGE gel. The blot was incubated with anti-DNOS1 primary MAb 6/57 (1:2000) (Regulski et al., 2004) at 4ºC overnight, and then goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Thermo Scientific, 1:10,000) for 1 hour at room temperature. Bands were visualized with Supersignal West Dura Extended Duration Substrate (Thermo Scientific) and quantified using Adobe Photoshop CS2.

3.5.10. **DAF2-DA Staining**

Ring glands were dissected in PBS and incubated with 10 µM 4,5-diaminofluorescein diacetate (DAF2-DA, Sigma-Aldrich) for 1 hour at 25ºC and unfixed samples were either immediately visualized or were fixed for 20 min in 1X PBS containing 5% paraformaldehyde and 0.2%
TritonX-100 for later analysis. Stained tissues were imaged using a Leica DMRA2 upright
steromicroscope running Openlab 3.1.7 imaging software (Improvision).

3.5.11. L-NAME Treatments

Eggs were collected in yeasted apple-juice plates for 8 hours at 25°C on day 0. A fixed number
of first instar larvae were collected the following morning and transferred onto tissue culture
plates (150X20mm, Sarstedt) containing 25ml standard food supplemented with 0.05%
bromophenol blue and either 0mM, 100mM or 125mM NG-nitro-L-arginine methyl ester (L-
NAME) or 125mM NG-nitro-D-arginine methyl ester (D-NAME). Plates were covered with
Kimwipes (23.1X16.8cm, Wypall) and sealed with elastic. Larvae were allowed to develop at
25°C, but were transferred onto new chemical supplemented food every 2 days in order to ensure
the stability of the chemical was not compromised at 25°C. The number of larvae, pupa, adults
and dead larvae were counted daily.

3.5.12. Larval Weight and Length Measurements

Beginning at 80 to 110 hr AEL, larvae were weighed and measured each day over 11 days.
Larvae were weighed in groups of ten, and then placed in a drop of water, allowed to extend and
measurements taken with a millimeter ruler.

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Figure 3.9 (Supplemental). NOS knockdowns using RNAi and L-NAME elicit similar phenotypes with variable penetrance.

A) Brains and ring glands of I) wild-type, II) NOS-RNAi\textsuperscript{IR}, and III) NOS-RNAi\textsuperscript{NIG} larvae. Ring glands are indicated by dashed lines. B) Ratios of ring gland to brain size (Red bars) and percentages of larvae that fail to pupate (Green bar) in different NOS-RNAi lines driven by the phm-GAL4 driver. Error bars depict standard deviation. n.d. = not determined. C) Wing, eye and leg imaginal discs dissected from 5 day old wild-type and 10 day old NOS-RNAi third instar larvae. Red color is due to the presence of Oil Red O, which was used for co-staining of lipids in fat body. D) Salivary glands and associated fat body from 5 day old wild-type and 10 day old NOS-RNAi third instar larvae. NOS-RNAi salivary glands and cells are ~1.4 fold larger than WT. NOS-RNAi cells are also rounder (arrow) than their wild-type counterparts (arrowhead). The black dotted circle indicates a single salivary gland cell. Tissues were stained with the lipid-dye Oil Red O (Red). E) Graphical representation of the relative number of L-NAME fed larvae still present at 7 days (Green bars) and 10 days (Red bars) AEL. Larvae fail to pupate in a L-NAME concentration-dependent manner. In 125mM L-NAME containing food, 80% and 54% of animals are still larvae at 7 and 10 days AEL, respectively, while in 100mM L-NAME food, 66% and 7% of animals are larvae at 7 and 10 days AEL. In contrast, no larvae were detected at day 7 when fed either normal food, or food containing the inactive LNAME stereoisomer, D-NAME.
Figure 3.10 (Supplemental). NOS as part of a conserved neuroendocrine pathway.

A) Activated NOS expression in the PG yields small larvae. Mouse macrophage NOS was expressed using the phm-GAL4 driver and UAS-GAL4 promoter. Panel I shows a wildtype 5 day old larva and panel II, a 5 day old phm-GAL4 >UAS-NOS\textsuperscript{mac} larva. The mouth hooks in panels III and IV show that the small NOS\textsuperscript{mac} larva are third instar. B) Diagrammatic comparison of Ecdysone synthesis induction in the \textit{Drosophila} prothoracic gland (PG, left) and Cortisol induction in the mammalian adrenal cortex (AC, right), as a consequence of intracellular cascades initiated by PTTH and ACTH in \textit{Drosophila} and vertebrates respectively. Both PTTH and ACTH transduce their signals via intracellular cAMP, Ras/raf and Ca++/Calmodulin induction. Thus, steroid synthesis might be regulated by NOS-dependent inhibition of Rev-erb activity in the mammalian brain and responding steroidogenic tissues. PTTH, prothoracicotropic hormone; ACTH, adrenocorticotropic hormone; PVN, paraventricular nucleus; SCN, Suprachiasmatic nucleus.

Supplemental Experimental Procedures

\textit{Imaging and Measurements of Larval Brain and Ring gland}

Larval brain and ring gland complexes were dissected in 1X PBS and fixed for 20 minutes in 1X PBS containing 5\% paraformaldehyde and 0.02\% Triton X-100. Fixed samples were rinse with 1X PBS and stained with DAPI (1:1000, Molecular Probes). Images of were captured with a 10X objective. The surface area of the larval \textit{Drosophila} brains and ring glands were measured using Volocity measuring tool from Volocity 2.5Improvision software.
**L-NAME Treatments**

Eggs were collected in yeasted apple-juice plates for 8 hours at 25°C on day 0. A fixed number of first instar larvae were collected the following morning and transferred onto tissue culture plates (150x20mm, Sarstedt) containing 25mL standard food supplemented with 0.05% bromophenol blue and either 0mM, 100mM or 125mM NG-nitro-L-arginine methyl ester (L-NAME) or 125mM NG-nitro-D-arginine methyl ester (D-NAME). Plates were covered with Kimwipes (23.1x16.8cm, Wypall) affixed with an elastic band. Larvae were allowed to develop at 25°C, but were transferred onto freshly prepared media every 2 days in order to ensure the stability of the chemical was not compromised at 25°C. The number of larvae, pupa, adults and dead larvae were counted daily.

**Imaging and Measurements of Drosophila Larval Organs**

*Drosophila* imaginal discs were stained with Oil Red O (see experimental procedure of main text) and imaged in a Leica DMRA2 upright stromicroscope using the Improvision Openlab 3.1.7 imaging software. Differential contrast (DIC) Images of the larval organs were captured with a 20X lens. The surface area of the larval *Drosophila* organs were measured using Volocity measuring tool from Volocity 2.5 Improvision software.
Chapter 4 – Discussion

4.1. General Summary

In this thesis I have described a novel tool for the visualization of the patterns of NR activity in Drosophila melanogaster. This tool was developed by producing transgenic Drosophila carrying heat-shock inducible chimeric fusions of the GAL4 DBD and the C-terminal LBD of each of the 18 Drosophila NRs, and was based on a previous study that demonstrated that a GAL4-DBD-RXR-LBD chimeric transgene was capable of reporting the patterns of ligand regulated receptor activity in vivo (Solomin et al., 1998).

Using the EcR ligand sensor, I was able to demonstrate that NR activity is ligand dependent. In addition, I have shown that its activity patterns can be expanded both spatially and temporally by exogenously provided ligand. Thus, I hypothesize that nuclear receptor ligand sensor activity patterns are likely to reflect the locations of cognate ligands and the tissues in which these receptors are normally active. I have successfully used the Drosophila NR ligand sensor system as a tool to catalogue the distinct spatio-temporal patterns of receptor activity for all 18 Drosophila NRs. Half of these receptors show dynamic patterns of activity during embryogenesis and larval development, and descriptions of these NR activity patterns have provided further insight into the spatio-temporal roles of each NR during Drosophila development.

The inability of nine of the eighteen ligand sensor constructs to activate reporter gene expression is consistent with several earlier studies that have demonstrated repressive functions for each of the nine corresponding NRs (Ayer et al., 1993; King-Jones et al., 2005; Pitman et al., 2002; White et al., 1997; Yu et al., 1994; Zelhof et al., 1995a; Zelhof et al., 1995b), suggesting
that the lack of NR activity in these lines is most likely due their function as dedicated repressors.

Results from the nine positively acting ligand sensors have indicated that certain tissues, including the amnioserosa, yolk, gut, trachea, and epidermis, are ‘hotspots’ of nuclear receptor activity as several receptors show overlapping patterns of activity in these tissues. In addition, certain receptors including DHR96 and FTZ-F1 exhibit highly restricted activity patterns whereas E78 shows a widespread pattern of activity. Interestingly, dERR displays a remarkable switch in activity during mid-embryogenesis, from strong widespread activation in the myoblasts to specific activation in the CNS.

Overall, these transgenic lines serve as valuable tools for the genetic and molecular dissection of the receptors they represent, the pathways they regulate and the upstream factors and cofactors that modulate their activity.

In addition, I have demonstrated for the first time that NOS controls the timing of ecdysone production via E75 and have provided evidence supporting a direct interaction between NO and the coordinately bound heme group within the E75 LBD. To do so I have demonstrated that E75, DHR3, and NOS are all expressed in the larval ring gland and that all three are required for the expression of FTZ-F1 and the resulting production of ecdysone.

By using both gain- and loss-of-function transgenes, tissues manipulated in culture, and a chemical inhibitor of NOS function, I have also demonstrated that NO acts directly on the *Drosophila* nuclear receptor E75 in the ring gland by reversing its ability to block DHR3 activity. Through genetic perturbation of this signaling pathway I have shown that NO signaling
through E75 is essential for both the timely onset of ecdysone production and metamorphosis, and in cell non-autonomous regulation of fat body lipid stores.

There exist a multitude of potential future avenues of investigation based on the experimental work that I have described here. In this chapter I will provide an overview of the open questions raised by my thesis and those experiments that I believe would be best suited to addressing these questions.

In addition, I will describe experimental strategies for optimizing the ligand sensor system for the identification of novel ligands for Drosophila orphan NRs, and will discuss potential models of the in vivo role of the interaction between E75 and NO in Drosophila development. This chapter is organized in two parts: the first part deals with the NR ligand sensor system, and the second with the regulation of E75 by NO.

4.2. Part 1 – The nuclear receptor ligand sensor assay

4.2.1. Overview

Although, in its current form, the Drosophila nuclear receptor ligand sensor assay presents a useful tool for the identification of the developmental timepoints during which NRs are active and the specific tissues that they are active in, further refinement would greatly expand the usefulness of this system in identifying both novel ligands for orphan receptors, and interaction partners that regulate NR function.

In this section I will describe several specific improvements that I believe have the ability to dramatically extend the usefulness and versatility of the ligand sensor system.
4.2.2. Further characterization of tissues displaying ligand sensor activity

In characterizing the patterns of *Drosophila* NR activity I employed LacZ and enhanced GFP reporter transgenes containing nuclear localization signal (NLS) tags. These tags have been indispensable in providing an easily detectable and inexpensive read-out of NR activity. In general, nuclear targeting of reporter proteins has a concentrating effect that results in a more easily discernible signal in comparison to reporters localized to the cytoplasm. Nuclear targeting does, however, have its drawbacks as it makes the positive identification of cell types harboring NR activity quite difficult. Specifically, the morphological details of individual cells which can be readily visualized through the use of membrane-bound or cytoplasmically distributed reporters, are not available with reporters targeted to the nucleus.

Therefore, combining nuclear and cytoplasmic reporters has the potential to provide a greater deal of morphological resolution towards the accurate assessment of the tissue specific distribution of receptor activity. Towards this goal, I have successfully used a combination of a nuclearly localized red fluorescent protein (RFP) reporter, UAS-DSRED\textsuperscript{NLS}, and a cytoplasmically distributed GFP reporter, UAS-tau-mGFP, in conjunction with various ligand sensor lines to help better identify the type of cells that display nuclear receptor activity during embryogenesis (Figure 4.1). Although signal levels tend to be reduced with cytoplasmically targeted reporters in comparison to their nuclearly targeted counterparts, due to the relatively large volume of the cytoplasm in comparison to that of the nucleus, the use of cytoplasmically localized reporters allows for the identification of cell type based on morphology and is useful for the positive identification of complex cell types in which ligand sensors are active. By using cytoplasmic ligand sensor reporters in conjunction with both cell and tissue specific morphological markers it would, in principle, be possible to more accurately identify the various cell types that show NR activity throughout *Drosophila* development.
Figure 4.1 - Detection of ligand sensor activity using membrane bound GFP in conjunction with nuclear RFP.

ERR ligand sensor activity in a small cluster of cells in the anterior region of a 16 hour AEL Drosophila embryo is shown using a combination of both a nuclear UAS-RFP reporter (red) and a membrane bound GFP reporter (green). White arrows indicate cells whose nuclei exhibit RFP expression and that exhibit GFP-expressing projections.
4.2.3. **Identification of novel nuclear receptor ligands: The utility of the Drosophila ligand sensor system for chemical screening**

Elucidation and description of the patterns of *Drosophila* nuclear receptor activity using the ligand sensor assay has provided a map of the endogenous patterns of receptor activation. Knowledge of these endogenous patterns of activity has provided not only deeper insight into the tissue-specific functions of NRs in development, it has also provided a ‘baseline’ for chemical screens aimed at identifying novel NR agonists and antagonists through either the expansion or restriction of activity patterns resulting from the application of libraries of small molecules.

As a first step, the EcR ligand sensor has proven indispensable as a positive control. As mentioned previously, when I began my studies into NR activity, EcR was the only receptor for which a ligand had been identified. Preliminary work with the EcR ligand sensor demonstrated that the spatio-temporal pattern of receptor activity during embryogenesis can be expanded dramatically through the application of 20-hydroxyecdysone. In addition, the level of GFP expression in response to the application of individual ecdysteroid agonists correlates with their binding affinities for EcR (data not shown, and Baker *et al.*, 2000). Specifically, I determined that the synthetic ecdysteroid Ponasterone A is capable of activating EcR to a greater extent than 20-hydroxyecdysone when applied at the same concentration (between $5.0 \times 10^{-8}$ M and $5.0 \times 10^{-6}$ M) to EcR ligand sensor embryos (data not shown). This is consistent with a previous study which has shown that the effective concentration for eliciting $50\%$ ($EC_{50}$) of maximal activation of the ecdysone receptor for Ponasterone A is less than half of that for 20-hydroxyecdysone (Baker *et al.*, 2000).

In addition, I demonstrated that it is possible to identify novel NR ligands through ligand treatment experiments involving the identification of CITCO as a synthetic agonist of DHR96 through a screen using known agonists of its human homologs (Palanker *et al.*, 2006). Therefore,
the screening for small molecule modifiers of NR ligand sensor activity in *Drosophila* embryos holds promise as an inexpensive and effective means towards the identification of novel NR ligands.

Several technical points regarding the limitations of ligand sensor based chemical screening must first, however, be considered. One is that the identification of agonists will be difficult in lines such as E78 that show nearly ubiquitous patterns of activity throughout embryogenesis. It may be possible to overcome this hurdle through precise quantification of the level of GFP expression by using high sensitivity fluorescence-based plate readers or high-throughput confocal imaging workstations. In this manner it may be possible to identify ligands that are capable of subtly modifying NR activity in lines that demonstrate widespread activity.

Another important consideration is that screening through large libraries of compounds requires a scaling-up from the small format ligand treatment methodology described in Chapter 2 to a high-throughput 96-well based assay. With respect to this point, I developed an effective method for the large scale permeabilization and chemical treatment of *Drosophila* NR ligand sensor embryos based upon the permeabilization protocol described by myself (Palanker *et al.*, 2006) and others (Strecker *et al.*, 1994) (data not shown).

I validated this method using the EcR ligand sensor in conjunction with Ponasterone A as a positive control (data not shown) and showed that the ligand sensor embryos are amenable to high throughput chemical screening using 96-well glass-bottom microplates. Similar screens have been described in both cell culture and vertebrate based systems (Baker *et al.*, 2000). However, a combination of the power of *Drosophila* genetics, the relative ease with which large numbers of precisely staged *Drosophila* embryos can be collected, and the relatively high degree of homology between fly nuclear receptors and their vertebrate counterparts make *Drosophila* an...
ideal organism in which to conduct screens for potential agonists and antagonists. Therefore, by using this approach in conjunction with a high-throughput microscopy platform, it would be possible to screen through large libraries of small molecules in order to identify novel compounds capable of modulating NR activity.

4.2.4. The identification of endogenous nuclear receptor ligands

With the exception of gas exchange, the *Drosophila* embryo is essentially a sealed vessel during the course of embryogenesis. As a consequence, any ligands required for NR activation must be either maternally deposited and released in a regulated fashion, or synthesized endogenously from existing precursor substrates during embryogenesis.

Considering that any ligands required for NR activity are contained within the embryo, it is plausible that endogenous NR ligands could be isolated by producing concentrated, lipophilic extracts from precisely staged *Drosophila* embryos at developmental timepoints during which particular NR’s are active. These extracts could be tested on various ligand sensor lines for their ability to modulate NR activity, and individual ligands could subsequently be identified through several rounds of fractionation and activity screening against specific NR ligand sensor lines. This approach holds tremendous promise for the identification of novel, *bona fide*, endogenous NR ligands.

4.2.5. Identification of an endogenous ligand for the nuclear receptor ERR

The NR ERR is an excellent example of a receptor for which the identification of a cognate ligand(s) would prove directly beneficial to human health. In humans, ERR has been shown to play a critical role in both breast cancer (Stein and McDonnell, 2006) and energy homeostasis (Giguere, 2008), however it has yet to be associated with a natural ligand and is thus classified as an orphan receptor (Giguere, 2008). The identification of agonists and/or
antagonists capable of modulating ERR function has the potential to provide novel tools for the
treatment of breast cancer and disease states resulting from imbalances in energy homeostasis
such as diabetes, obesity, and cardiovascular disease. In addition, novel ERR ligands could serve
as probes for dissecting the precise role of ERR in cancer and cellular energy regulation.

My study into ERR ligand sensor activity during embryogenesis has revealed that, in
contrast to vertebrate ERR, *Drosophila* ERR does not function as a constitutive activator *in vivo*. Rather, dERR activation exhibits dynamic spatio-temporal changes that most likely arise as a
result of precise modulation by protein co-factors and/or ligands. Overall, there exists a high
degree of amino acid conservation in the LBD between dERR and its human homolog. However,
dERR activity, unlike that of its human homolog, is not subject to regulation by the ERR
antagonists diethylstilbestrol (DES), tamoxifen, or its metabolite, 4-hydroxytamoxifen (Ostberg
et al., 2003). A recent study has reported that mutation of three amino acid residues in the dERR
LBD, selected on the basis of homology modeling, served to ‘humanize’ dERR by enabling
ligand-dependent suppression of its transcriptional activity in response to binding of either DES
or OHT (Ostberg et al., 2003).

Thus, by using both the wild-type and mutated forms of the dERR LBD, the *Drosophila*
NR ligand sensor system has the potential to serve as a useful tool in the identification of novel
small molecule ERR agonists and/or antagonists and to provide a better understanding of the
unique functional features of both *Drosophila* and human ERR.

4.2.6. *Investigation into the role of Bisphenol A (BPA) as an ERR antagonist*

Recent evidence has demonstrated that the persistent and prevalent man-made
environmental pollutant, bisphenol-A (BPA), is a potent endocrine disruptor that elicits its
effects, at least in part, through ERR (Matsushima et al., 2008; Takayanagi, 2006). One of the
most well understood toxicological features of BPA is that it has been shown to trigger premature onset of puberty (Roy et al., 2009). It has recently been demonstrated that BPA binds strongly to human ERRγ (KD = 5.5 nM), with high selectivity over the estrogen receptor (ER) (Okada et al., 2008). In addition, BPA preserves the ERRγ’s basal constitutive activity, and protects the selective ER modulator, 4-hydroxytamoxifen, from deactivating of ERRγ (Okada et al., 2008).

In order to better understand the toxicological role of BPA in ERR signaling, ERR ligand sensor embryos could be used to test the function of BPA in modulating receptor activity. Changes in pattern activity resulting from BPA application could be followed up by protein binding assays to confirm whether BPA interacts directly with dERR and, if so, to better understand the binding kinetics and the physiological dynamics of the ERR-BPA interaction.

4.2.7. Towards the identification of genes involved in ligand sensor activity

One major limitation of the ligand sensor assay is that it is impossible to simultaneously manipulate transgenes using the GAL4/UAS system without affecting UAS-based reporter gene expression. Considering the widespread use of the GAL4/UAS system as a means for transgene manipulation in Drosophila, this presents a significant limitation to the NR ligand sensor system. In particular, tissue specific genetic manipulation using either UAS-based ectopic overexpression lines or the large library of UAS-based RNAi knockdown fly lines made available recently through two independent public stock centres (Vienna Drosophila RNAi Centre and the National Institute of Genetics, Japan) would allow one to test the requirement of any given gene for the activity of each of the 18 Drosophila NR’s. Such an analysis is not only feasible but also amenable to a high throughput format.
Therefore, in order to extend the usefulness of the ligand sensor system towards the identification of genes required for NR activity, a novel ligand sensor system must be developed. Towards this goal, the dual binary bacterial LexA system could serve as a suitable substitute for the GAL4/UAS system in the ligand sensor assay. The LexA system functions in much the same way as the GAL4/UAS system in that bacterial LexA encodes a DNA binding protein that binds specifically to a LexA operator (LexAOp) sequence, and is thus capable of regulating the expression of genes immediately downstream (Brent and Ptashne, 1981).

Moreover, the bacterial LexA binary expression system has recently begun to be employed as an alternative to the GAL4/UAS system in *Drosophila* genetics (Brent and Ptashne, 1981; Diegelmann et al., 2008; Lai, 2006; Viktorinova and Wimmer, 2007). Although the GAL4/UAS system has been shown to be slightly more effective at activating gene expression than the LexA system, LexA based transgene expression is comparable to that of GAL4/UAS (Viktorinova and Wimmer, 2007). In addition, several LexA regulated reporters in *Drosophila* have been established (Brent and Ptashne, 1981; Diegelmann et al., 2008; Lai, 2006).

Therefore, by exchanging the GAL4 DNA-binding domain with the LexA DNA-binding domain for all of the NR ligand sensor constructs it would be possible to establish transgenic fly lines compatible with transgene manipulation in parallel using the GAL4/UAS system. If employed in combination with a LexA-Op regulated reporter transgene, these LexA based NR ligand sensor lines should, in principle, recapitulate the patterns of activity seen with the GAL4 DBD containing ligand sensors.

4.2.8. Identification of genes required for NR ligand sensor activity

A large number of genes that are involved in either directly or indirectly regulating NR activity likely exist, and manipulation of the expression of these genes has the potential to reveal
their involvement in NR function through detectable changes in NR activity. These genes may be involved in biosynthetic pathways required for the synthesis and/or breakdown of endogenous ligands, in detoxification pathways responsible for the chemical conversion of exogenous ligands and/or toxins, or may themselves be coregulatory proteins required for NR signaling. This latter group includes both coactivators and corepressors required for NR signaling and the genes that regulate them. Specific examples include the previously identified *Drosophila* NR coregulators such as BONUS (Beckstead et al., 2001), Blimp-1 (Agawa et al., 2007), Rigor mortis (Gates et al., 2004), and Moses (Baker et al., 2007).

4.2.9. *Cytochrome P450 genes as regulators of NR activity*

CYP450 enzymes are responsible for catalyzing a diverse range of small-molecule chemical modifications including aromatic and aliphatic oxidation, hydroxylation, dealkylation, epoxidation, oxidative deamination, desulfuration, and dehalogenation (Agosin, 1985; Lewis, 1996). As a consequence, CYP450’s play a central role in the metabolism of both endogenous compounds such as steroids, fatty acids, and prostaglandins, and of xenobiotics such as drugs, carcinogens, and environmental pollutants (Feyereisen, 1999; Gonzales, 1996; Maurel, 1996; Ronis, 1996; Schuler, 1996; Scott, 1999). Considering their importance in small molecule cellular metabolism, the CYP450 gene family serves as an excellent starting point from which to begin identifying genes mediating the production of endogenous NR ligands. To date, 90 CYP450 genes have been identified in the *Drosophila* genome (Tijet et al., 2001). UAS-based ectopic overexpression or knockdown by RNAi lines available for any of these 90 CYP450 genes (National Institute of Genetics, Japan; Vienna *Drosophila* RNAi Centre, Austria; Bloomington *Drosophila* Stock Centre, Indiana; independent research laboratories) could be used in combination with LexA based NR ligand sensor lines in order to identify which of them is involved in the production and/or breakdown of ligands for particular NR’s.
As demonstrated previously and in this thesis, mutations in the Halloween-family genes result in a dramatic decrease in embryonic EcR ligand sensor activity (Palanker et al., 2006). Therefore, if effective, RNAi based knockdown of these genes should also result in a corresponding decrease in EcR activity. Thus, a suitable proof of concept screen would involve testing whether GAL4/UAS based RNAi knockdown of individual genes coding for enzymes in the ecdysone biosynthetic pathway is capable of decreasing EcR ligand sensor activity.

4.2.10. Visualization of NR mediated transcriptional repression

Despite the fact that NR mediated transcriptional repression has been shown to play a fundamental role in the regulation of gene expression throughout development (Ayer et al., 1993; King-Jones et al., 2005; Pitman et al., 2002; White et al., 1997; Yu et al., 1994; Zelhof et al., 1995a; Zelhof et al., 1995b), relatively little progress has been made towards the visualization of gene repression in real time. In this thesis I have demonstrated that half of the eighteen NR’s in Drosophila likely act as transcriptional repressors. However, as explained in Chapter 2, the ligand sensor assay is not capable of providing a visual read-out of the patterns of NR mediated repression during development.

Thus, the generation of novel tools for visualizing dynamic changes in NR mediated transcriptional repression would provide a better understanding of its roles during development. To do so, however, requires the development of a novel reporter system.

A method has been established in which transcriptional repression mediated by the Giant repressor has been visualized in Drosophila embryos (Nibu and Levine, 2001). This approach is limited by the fact that embryos must be fixed in order to visualize the effects of Giant repression on gene expression, and thus it is not possible to monitor repression in vivo. Despite this limitation, this approach has demonstrated that transgenes carrying promoter elements that direct
reporter gene expression can be repressed by the binding of a GAL4-repressor fusion proteins at UAS sites upstream of the regulatory region of a core promoter.

By extension, an NR ligand sensor repressor screen could be produced based on a combination of the existing NR ligand sensor fusions and a reporter based in part on the one described for the Giant repressor. This approach, however, would require the production of both a repressor transgene and a novel ligand sensor reporter transgene.

A schematic of a tripartite transgenic system for visualizing NR repression *in vivo* is shown in Figure 4.2 below. This system employs the aforementioned heat shock-inducible GAL4 DBD-NR LBD fusions that, when expressed in response to heat shock, are capable of repressing the expression of a constitutively expressed LexA DBD/repressor fusion in specific tissues through binding to tandem UAS sites flanking the Act5C promoter. Under non-heat shock conditions, the LexA DBD/repressor fusion would bind to tandem LexA operator sites flanking a constitutively active promoter such as the Actin 5C (Act5C) promoter (Chung and Keller, 1990), thus preventing the expression of a downstream EGFP_{NLS} reporter. EGFP_{NLS} expression would thus serve as a visual read-out of NR mediated repression.

One important technical point is that the repressor should ideally be capable of repressing reporter gene expression in all embryonic and larval tissues throughout development, and should have a high turnover rate in order to allow for a rapid response to decreases in its expression mediated by an NR ligand sensor repressor fusion. Repressor turnover could be promoted through the addition of protein degradation sequences such as the PEST motif which targets proteins for degradation (Corish and Tyler-Smith, 1999).
Overall, the system described above could greatly extend the usefulness of the ligand sensor assay and would serve to help further understand the dynamics of nuclear receptor based repression.

**Figure 4.2 - A Genetic System for Visualizing NR Ligand Sensor Repression in vivo**

Under non-heat shock conditions (A), GAL4 DBD -NR LBD ligand sensor fusions are not expressed, allowing the expression of a LexA DBD – Repressor fusion from an Actin5C promoter. Once translated, this LexA DBD – Repressor fusion protein binds to LexA Operator sequences flanking an Actin5C promoter on a reporter transgene thus preventing the expression of EGFP<sub>NLS</sub>. Upon heat shock induction (B. heat shock), GAL4 DBD -NR LBD ligand sensor fusions are expressed and, subsequent to translation, bind to UAS sites flanking an Actin5C promoter thereby preventing the expression of a LexA DBD – Repressor fusion from an Actin5C promoter. As a result, LexA Operator sequences are not bound by the LexA DBD – Repressor fusion and the Actin5C promoter drives the expression of the EGFP<sub>NLS</sub> reporter.
4.3. Part 2 – The Regulation of E75 by NO

4.3.1. Overview

Consistent with both in vitro studies and with studies in cultured cells, I have demonstrated that there exists a direct in vivo interaction between NO and heme-bound E75, and that this interaction influences the repressive function of E75 during Drosophila development.

The following represents a summary of my findings and a set of further experiments that would help to reinforce the validity of my results.

4.3.2. Does E75 function as a redox sensor?

NO is known to elicit an oxidizing effect within cells, and a switch from reducing to oxidizing environments has a similar effect on E75 heme structure and function to the binding of NO (Marvin et al., 2009; Pardee et al., 2009; Reinking et al., 2005). In particular, it has been shown in vitro that both NO and oxidizing conditions displace one of the E75 LBD-heme coordinate bonds, preventing the E75 LBD from binding to helix 12 of the DHR3 LBD (Reinking et al., 2005). This displacement has been shown to be triggered by the oxidation of the heme iron centre from the ferrous (Fe$^{2+}$) to the ferric (Fe$^{3+}$) state (Reinking et al., 2005). My in vivo results with E75/NO support this hypothesis and suggest that E75 is also sensitive to oxidation in vivo. However, although the regulation of E75 by direct binding to NO seems the most likely mode of action I cannot exclude the possibility that NO acts on heme-bound E75 via redox. In order to resolve this issue it would be useful to test whether NO-independent manipulation of cellular redox potential within the PG has an effect on E75 based repression. Several groups have demonstrated that cellular reducing potential can be manipulated by altering either cellular nicotinamide adenine dinucleotide phosphate (NADPH/NADP) ratios (Dioum et al., 2002) or cellular hydrogen peroxide (H$_2$O$_2$) levels (Grover et al., 2009). Using either of these
approaches, it would be possible to manipulate redox potential specifically in the PG and to determine whether changes in redox elicit the same effect on E75 function as do changes in NO. If redox potential regulates E75 function in a similar manner to NO, then one would predict that E75 would function as a repressor at high NADPH/NADP ratios or low H₂O₂ concentrations. Conversely, the repressive function of E75 would be prevented at low NADPH/NADP ratios or high H₂O₂ concentrations.

As a further step towards understanding the role of redox in E75 signaling, the novel genetically-encoded redox sensor roGFP (Cannon and James Remington, 2009) could be employed in conjunction with the GAL4/UAS system in order to visualize dynamic changes in redox potential in the PG. If used in combination with E75 and/or redox manipulation, the roGFP reporter could provide novel insights into the role of redox in E75 signaling.

4.3.3. Identification of additional NO-dependent E75 interactors

As mentioned, both NO-binding and reducing conditions result in a physical rearrangement in the E75 LBD, preventing it from binding to DHR3 (Reinking et al., 2005). Recent evidence has also shown that NO binding regulates the ability of the human E75 orthologues Rev-erbα and Rev-erbβ to interact with the NR corepressors, RIP140 and NCOR (Pardee et al., 2009). This finding suggests that NO most likely regulates the physical interaction between E75 and corepressor complexes in Drosophila as well.

Consistent with a role in regulating ecdysone biosynthesis, I have demonstrated that NO, E75A, E75B, DHR3, and FTZ-F1 are all expressed in the ring gland. One important point is that E75A and E75B both show dynamic patterns of localization between the nucleus and cytoplasm in the larval PG, suggesting that additional types of interactions and modes of regulation likely exist. One such possible mode of regulation of E75 function is through binding of coregulator
proteins. Although E75-interacting corepressors have not yet been identified in *Drosophila*, their identification would help to further establish *Drosophila* as a model system in which to study the E75/Rev-Erb signaling pathway. The identification of E75 corepressors would require the use of either forward genetic screens or protein interactor screens aimed at identifying genes involved in mediating E75 repression. One such approach could involve the use of the DHR3 ligand sensor as a read-out of E75 repression in conjunction with RNAi based screening as described in part one of this Chapter. Once identified, E75 interactors could be tested for an NO dependency of E75 binding, thereby helping to further elucidate the role of NO in E75 signaling.

4.3.4. *Visualizing the cyclical dynamics of the ecdysone signaling hierarchy.*

Although the NR signaling cascade involving Ecdysone, EcR, E75, DHR3, and FTZ-F1 is generally described as linear, my results suggest that it is a feed forward loop that is largely circadian gated.

Recent quantification of the ecdysone titre over the course of third instar larval development has clearly demonstrated that there exist several small ecdysone peaks that progressively increase, in a cyclical manner, over the course of the third larval instar (Warren et al., 2006). As a consequence, one important outstanding question is how these subtle cyclical fluctuations in the ecdysteroid titre function through E75 and DHR3 to prepare the animal for the large pulse of ecdysone that triggers the transition from the larval to the pupal stage. In this thesis I have demonstrated that the interaction between E75 and NO is a critical determinant in the onset of puparium formation. However, further determination of the molecular and temporal dynamics of how the E75-NO interaction both responds to and influences these ecdysteroid peaks would provide a deeper understanding of how this interaction regulates ecdysone biosynthesis, cell growth, and developmental timing. To do so would require imaging methods
capable of allowing for the direct visualization of E75, DHR3, and NOS protein, and ftz-F1 RNA in the ring gland.

The advent of novel homologous recombination-based approaches for the production of tagged versions of genes in an endogenous genomic regulatory context (Ejsmont et al., 2009; Huang et al., 2009a; Huang et al., 2009b; Venken et al., 2009; Venken et al., 2008) has made the development of tagged versions of endogenous E75, DHR3, NOS, and FTZ-F1 possible. If used in conjunction with cutting edge microscopic techniques such as Selective Plane Illumination Microscopy (Huisken et al., 2004; Keller et al., 2008; Reynaud et al., 2008; Verveer et al., 2007), these tools could allow for the dynamic, in vivo visualization of the EcR, E75, NO, DHR3, FTZF1 signaling cascade.

4.3.5. The role of E75 as a heme sensor

Several lines of evidence have provided substantial support for a model of E75-heme binding in which heme is an obligate component of E75. Two of the most compelling of these are the fact that E75 stability is dependent upon heme binding, and that heme does not readily dissociate once bound to heme (Reinking et al., 2005). Thus, a logical extension to my study into NO regulated E75 function would be to investigate the role of E75 as a heme sensor. One way in which to do so is to examine how perturbations in heme levels affect the stability and function of E75 in vivo.

Heme biosynthesis is known to proceed through eight enzymatic steps (Panek and O'Brian, 2002) and all of the genes encoding these corresponding enzymes are conserved in Drosophila. The enzyme responsible for both the first and the rate limiting step in the heme biosynthetic pathway in animals is δ-aminolevulinate synthase (ALAS), a homodimeric enzyme that is localized to the mitochondrial matrix and which catalyzes the formation of δ-
aminolevulinic acid by condensation of glycine and succinyl-CoA (Ferreira, 1995; Kappas, 1995; May et al., 1986; Ponka, 1997). A Drosophila homolog of the vertebrate ALAS gene has been identified and its spatio-temporal pattern of expression during development has been described (Ruiz de Mena et al., 1999). In addition, the enzyme responsible for the rate limiting step in heme catabolism, Heme Oxygenase, has also been identified in Drosophila (Zhang et al., 2004).

As mentioned previously, heme serves as a prosthetic group in a wide range of hemoproteins [reviewed in Gilles-Gonzalez and Gonzalez (2005)]. Notably, both Heme oxygenase and NOS also require heme in their catalytic centers (Cui et al., 2008; Marletta, 1993; Zhang et al., 2004).

Importantly, UAS-based RNAi knockdown lines against ALAS have been produced and are available for public distribution (NIG, Japan), and GAL4-based ectopic overexpression and RNAi knockdown transgenic fly lines have been described for Drosophila Heme oxygenase, demonstrating that it plays an essential role in the development of adult tissues (Cui et al., 2008).

Thus, tools which allow for the tissue specific perturbation of heme levels in vivo are available. Using these tools in conjunction with either ubiquitous or PG-specific GAL4 drivers it should be possible to investigate how perturbations in heme levels affect both the stability and function of E75 as a first step to studying the mechanisms controlling heme synthesis and its coordination with E75 function. This could be accomplished by using protein immunohistochemistry as a read-out of E75 stability and FTZ-F1 in situ hybridization as a read-out of E75 functionality in the PG in a manner similar to that described in this thesis.
One question that also remains is whether heme is synthesized cell-autonomously or whether it is synthesized in a certain cell type(s) and transported to other sites for uptake and incorporation. Considering the high level of CYP450 enzyme expression and function in the prothoracic gland, energetic economy would argue that heme be synthesized and regulated locally, although this remains to be verified experimentally.

Intriguingly, it has been shown that the expression of both ALAS and Heme oxygenase is subject to circadian regulation in adult flies (Ceriani et al., 2002). It would be interesting to determine whether this also holds true in the *Drosophila* larval prothoracic gland where it is likely that the synthesis of heme is closely coordinated with the expression of the heme-containing ecdysteroid biosynthetic enzymes.

As previously mentioned, blood sucking insects including *Rhodnius* and female mosquitos require a blood meal in order for normal development and the circadian production of ecdysone (Hagedorn et al., 1975; Wigglesworth, 1940). One potential candidate signal for triggering the underlying blood meal-dependent developmental signaling pathways is heme. In humans, heme levels vary diurnally and control circadian rhythm (Rogers et al., 2008) and, in fact, most of the proteins in the circadian clock have been shown to bind heme (Rogers et al., 2008) supporting a role for heme as an important developmental signal.

Thus, heme likely serves as a major systemic timer that is both responsive to and capable of regulating metabolic activity, and that acts, at least in part, through E75 and its vertebrate counterparts. It is tempting to speculate that the red ring glands that I observed in NOS knockdown larvae, which contain dramatically elevated levels of heme (data not shown), may be reflective of a disruption in this homeostatic process that ultimately manifests itself as an overaccumulation of heme. Consistent with this hypothesis, it has recently been shown that the
human orthologues of E75, the Rev-erbs, regulate heme synthesis in cultured cells and, when knocked down, lead to heme overproduction (Wu et al., 2009). However, further analysis of the underlying molecular defect that results in reddening of the ring gland is required in order to validate this hypothesis.

4.3.6. E75/DHR3 and the circadian regulation of Drosophila metamorphosis

Over the course of the past several decades, an appreciable amount of work has gone into the elucidation of the nature and function of the circadian rhythm system in insects. It has since become clear that metamorphosis in holometabolous insects is under circadian regulation (Mirth et al., 2005). In particular, if the signaling peptide prothoracicotropic hormone (PTTH) is not released within a specific temporal gating period then larvae will not pupariate and will wait instead until the next day when this circadian gate opens again. Although the exact nature of this gating mechanism is not fully understood, it is known that as critical weight is achieved at the end of the 3rd larval instar, the production of both insulin-like peptides (ILPs) by insulin producing cells in the brain and juvenile hormone (JH) in the corpora allata of the ring gland decrease (Brogiolo et al., 2001; Rulifson et al., 2002; Tatar et al., 2001).

A recent study has demonstrated that the neurons that innervate the PG and that are responsible for the production and secretion of PTTH participate in an overlapping dendritic field with the principal circadian rhythm generating neurons in the larval brain (Siegmund and Korge, 2001). These circadian rhythm neurons express the neuropeptide pigment dispersing factor (PDF) that has been proposed to play a role in coupling circadian outputs to downstream neurons in order to coordinate rhythmic outputs (Taghert and Shafer, 2006). Thus, the identification of a direct, physical interaction between the circadian pacemaker cells and the
PTTH secreting neurons that innervate the PG has raised the question of whether the circadian rhythm neurons participate directly in the circadian release of PTTH.

It has been shown that PTTH transcriptional periodicity is indeed regulated by circadian pacemaker neurons and that it also correlates with the ecdysteroid titre (McBrayer et al., 2007). Based on these findings the authors speculate that periodic fluctuations in PTTH transcription precede increased bursts of PTTH release that ultimately signal to the PG and determine the temporal progression of transcriptional responses during the third larval-instar by stimulating small, periodic increases in the basal ecdysteroid titre. In addition, they proposed that the actual timing switches are the minor pulses of PTTH observed, and subsequent small ecdysteroid peaks that occur prior to the major rise in ecdysteroid titre that initiates metamorphosis. Their proposal that critical weight responds to small changes in ecdysteroid titres is consistent with the fact that small increases in basal ecdysteroid levels resulting from the manipulation of insulin signaling in the PG results in a decrease in critical weight and a resulting precocious onset of metamorphosis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005).

Intracellular signaling may not, however, represent the only means for the circadian regulation of PG function. It is known that a core component of the circadian clock, Period, is expressed in the pupal ring gland (Saez and Young, 1988). In addition, an elegant study has shown that Period expression in the PG of Drosophila pupae oscillates with a circadian rhythmicity (Emery et al., 1997). This finding points to a possible role for the circadian clock in the larval PG as well and raises the intriguing possibility that the function of the PG, and in particular ecdysteroid biosynthesis, may be subject to direct regulation by cell-autonomous function of the circadian clock.
In this thesis I have shown that in the PG, the NO effect on E75 frees DHR3 to activate expression of *Ftz-F1*, that in turn, leads to the eventual synthesis of ecdysone. Although this NR cascade is generally described as linear, it appears to be a feed-forward loop that is largely circadian gated. Taken together, it is interesting to speculate that E75 and DHR3, like their vertebrate counterparts, may somehow be involved in influencing metamorphosis through their interplay with the molecular determinants of circadian rhythmicity. Considering that the vertebrate homologs of E75 and DHR3, Rev-erb and ROR, respectively, are both expressed in a circadian manner and are critical regulators of circadian rhythm (Emery and Reppert, 2004; Guillaumond et al., 2005; Jetten, 2009; Preitner et al., 2002; Storch et al., 2002), it would be interesting to see how these genes interact, both genetically and physically, with Period in the PG. To do so would require the identification and use of several candidate target genes whose expression can be used as an output of the circadian regulation by Period, E75, and/or DHR3. The *Drosophila* gene *Cycle* may serve as a suitable candidate as it is the orthologue of the vertebrate circadian regulator BMAL1 whose expression has been shown to be directly regulated by both Rev-erb and ROR (Preitner et al., 2002). It would, therefore, be interesting to determine whether E75 and DHR3 play a similar role in regulating *Cycle* expression in the *Drosophila* PG. It is also important to consider and test the possibility that DHR3 and E75 may play a role in regulating circadian rhythms in the *Drosophila* clock neurons, and that this is also under NO control.

4.3.7. *Transduction of the PTTH signaling cascade*

Regarding the regulation of metamorphosis by E75, one outstanding question that remains is whether PTTH signaling regulates the function of E75.
Although the intracellular cascade induced by PTTH has not yet been fully elucidated, the receptor tyrosine kinase Torso has been identified as a *bona fide* PTTH receptor in the PG, and signals through a downstream signaling pathway that involves RAS, RAF, and ERK (Rewitz et al., 2009). In addition, the PTTH signaling cascade in the PG is also known to include the activation of MEK, PKA, PLC and PKC and their various downstream effectors (Rybczynski et al., 2001; Rybczynski and Gilbert, 2006). Key among these responses is the production of Calmodulin and the cytoplasmic influx of Calcium (Ca\(^{2+}\)) from intracellular stores (Meller et al., 1988; Rybczynski and Gilbert, 2003) both of which are essential components for *Drosophila* NOS enzymatic activity (Regulski and Tully, 1995; Gribovskaja et al., 2005; Muller and Bicker, 1994; Ray et al., 2007; Regulski and Tully, 1995; Stevens-Truss et al., 1997). Thus, it is conceivable that PTTH may regulate E75 function indirectly through its ability to modulate NOS function through alterations in intracellular Ca\(^{2+}\) levels.

Importantly, Ca\(^{2+}\) been shown to play a critical role in insect ecdysteroidogenesis. In *Manduca sexta*, both Ca\(^{2+}\) and cAMP appear to be required for PTTH-stimulated ecdysone release (Gilbert LI, 1996:60-107), whereas similar experiments carried out in *Drosophila* have implicated Ca\(^{2+}\) but not cAMP in ecdysone release (Henrich, 1995). Recent evidence has demonstrated a requirement for the inositol 1,4,5-trisphosphate (IP3) receptor (ITPR), an intracellular Ca\(^{2+}\) channel that couples cell membrane receptor signaling, via the second messenger IP3, to Ca\(^{2+}\) signal transduction in ecdysteroidogenesis (Venkatesh and Hasan, 1997). Moreover, the high level of both ITPR and Calmodulin expression in the PG and the strikingly similar ecdysone-dependent extension of the larval period seen in both PTTH and ITPR mutants (Andruss, 1997; Venkatesh and Hasan, 1997) have provided further evidence that Ca\(^{2+}\) signaling in the *Drosophila* PG is required for ecdysteroidogenesis.
Considering that I have shown that NOS is required in the ring gland for both ecdysone biosynthesis and the timely onset of metamorphosis, and that previous work has demonstrated that the function of *Drosophila* NOS is dependent upon the binding of Ca\(^{2+}\) via Calmodulin (Regulski and Tully, 1995), a logical step in further understanding the role of E75/NOS in the PG would be to determine whether Ca\(^{2+}\) levels are responsive to PTTH signaling, and whether, in turn, NOS function is sensitive to intracellular Ca\(^{2+}\) levels. To do so would require the ability to manipulate PTTH levels and to simultaneously monitor the levels of both NO production by NOS, and intracellular Ca\(^{2+}\) levels independently of one another.

As previously mentioned, circadian expression of the Period gene has been monitored for several days in cultured brain-ring gland complexes of *Drosophila* pupae (Emery et al., 1997), suggesting that it is possible to culture larval brain-ring gland complexes while monitoring dynamic changes at the cellular level, in real time. In addition, the use of ectopically administered PTTH protein has been used to trigger Ca\(^{2+}\) mobilization from intracellular stores in both *Manduca sexta* and *Bombyx mori* PGs (Dedos et al., 2005; Fellner et al., 2005).

Thus, it should be possible to test the ability of recombinant PTTH to activate NOS in the *Drosophila* PG through elevations in intracellular Ca\(^{2+}\) levels by visualizing NO production and fluctuations in Ca\(^{2+}\) levels *in vivo* through the application of DAF2-DA and DETA-NO to cultured *Drosophila* brain-ring gland complexes. This approach could be taken a step further in testing the effects of PTTH application on E75 function. To do so would simply require monitoring the effects of recombinant PTTH application on FTZ-F1 expression in combinations of ectopic DHR3 and E75 expressing transgenic lines as described in this thesis.

Overall, this approach would provide a better understanding of the signaling events that underlie ecdysone biosynthesis in the PG and visualize their dynamics in real time *in vivo.*
4.3.8. Ring gland size is not the key determinant of metamorphosis timing

Recently, several reports have demonstrated that the timing of pupariation can be accelerated or postponed through the manipulation of ring gland growth and size (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2009). These studies have relied upon the PG-specific manipulation of insulin responsive genes, implicating insulin in the regulation of ecdysteroid signaling and developmental timing, and have led to the conclusion that ring gland size is a key determinant in the timing of pupariation onset (Mirth et al., 2005; Mirth and Riddiford, 2007). This conclusion, however, is not in accordance with my results that clearly demonstrate that PG-specific NOS knockdown results in a six-fold increase in ring gland size without a corresponding acceleration in the onset of pupariation. My results, therefore, are inconsistent with the hypothesis that ring gland size itself is the primary underlying determinant of metamorphosis initiation.

In line with my findings, two recent studies have reported that perturbation of either the sumoylation gene Smt3 or the proposed transcriptional regulator, Molting defective (Mld), result in increases in the size of the Drosophila PG that correlate with defects in both ecdysone synthesis and pupariation and ultimately lead to an extension of the larval stage and a corresponding increase in organismal size (Neubueser et al., 2005; Talamillo et al., 2008). PG size can be increased without triggering premature ecdysone production by manipulating the levels of Dmyc or Cyclin D expression in the PG (Colombani et al., 2005).

Taken together, these results support my finding that ring gland size itself is not the critical determinant of organismal size, rather that the way in which the underlying molecular determinants overlap and interact with the ecdysone biosynthetic pathway are the key determinants in developmental timing through ecdysone production and release.
Overall, very little is known about how ecdysteroid biosynthesis in the PG is regulated, and only a few transcriptional regulators have so far been identified for this pathway. These include βFTZ-F1 (Parvy et al., 2005) that, as previously mentioned, is involved in the transcriptional regulation of Dib and Phm (Ono et al., 2006; Parvy et al., 2005), Without children (Woc) (Warren et al., 2001; Wismar et al., 2000), that controls the conversion from cholesterol to 7dC, and Molting defective (Mld) (Neubueser et al., 2005), that is involved in the regulation of the ecdysone biosynthetic enzyme Spook. In addition, a handful of other genes in Drosophila exist for which a role in the regulation of ecdysone titres has been implicated, but for which the mechanism of participation in steroidogenesis is poorly understood. These include lethal(3)dre4 (dre4) (Sliter, 1992), Inositol 1,4,5,-tris-phosphate receptor (ITPR) (Venkatesh and Hasan, 1997), Giant ring gland (Grg) (Klose, 1980), Lethal giant larvae (Lgl) (Hadorn, 1937), ecdysoneless (ecd) (Henrich et al., 1987), defective in the avoidance of repellants (dare) (Freeman et al., 1999), Smt3 (Talamillo et al., 2008), Giant (Henrich et al., 1987).

Thus, the size of the ring gland itself may only provide a superficial clue as to how a diverse range of underlying molecular determinants function in timing pupariation through the synthesis and release of ecdysone. Further understanding of the mechanisms by which these factors regulate both ring gland size and function will help to elucidate the nature of the regulatory pathways that control ecdysone synthesis, organismal size, and developmental timing.

4.3.9. Cell non-autonomous roles of NO production in the prothoracic gland

Although the cell autonomous role of NO in triggering growth arrest has been described in detail (Kuzin et al., 1996; Peunova and Enikolopov, 1995; Peunova et al., 2001) and is consistent with my finding that NOS knockdown results in a dramatic overgrowth of the third
instar larval PG, the cell-non autonomous overgrowth of the fat body seen with NOS knockdown in the PG cannot be explained as easily.

The dramatic increase in the number and size of fat body lipid droplets, and triacylglycerol levels with no accompanying increase in protein levels in the fat bodies of NOS knockdown larvae in comparison to their wild-type counterparts indicates that the increased weight and size of these animals is due almost exclusively to the increased storage of lipid in the fat body. In addition, the fact that none of the diploid tissues destined to give rise to adult tissues demonstrate a change in either cell size or number in PG NOS knockdown larvae is consistent with the requirement of both insulin-like peptides (ILPs) and ecdysone for the continued growth of imaginal disks beyond that normally achieved during larval development (Mirth et al., 2005; Henrich et al., 1987). Taken together with the fact that the growth of larval tissues is normally repressed by ecdysone (Baehrecke, 2000; Cakouros et al., 2004a; Cakouros et al., 2004b; Jiang et al., 1997; Rusten et al., 2004) the continued growth of larval tissues in PG NOS knockdown animals is likely due to delayed ecdysone production, a hypothesis that is consistent with my finding that ecdysone feeding can rescue NOS knockdown larvae. In addition, the delayed pupariation and increased larval size of animals in which either E75 has been ectopically overexpressed or DHR3 has been knocked down in the PG, also support the conclusion that defects in the ecdysone signaling pathway in the PG result in defects in the proper regulation of organismal size. Although I cannot rule out the possibility that there may be ecdysone-independent effects of PG NOS function on lipid accumulation by the larval fat body.

The cell non-autonomous regulation of the growth of the larval fat body by PG NOS knockdown suggests that a circulating factor produced by the PG regulates fat body lipid storage. Although by all accounts ecdysone fulfills the criteria for this factor, it is possible that the
interaction of the PG with physically interacting cells through NO signaling also plays a role in regulating fat body lipid stores.

One possibility is that the PG may regulate the function of the closely associated corpora cardiaca (CC) cells that send long projections deep into the PG (Lee and Park, 2004). The CC is known to be responsible for the production and secretion of Adipo-kinetic hormone (AKH), the Drosophila homolog of human Glucagon, which is a critical regulator of larval fat body homeostasis (Lee and Park, 2004; Isabel et al., 2005; Kim and Rulifson, 2004). AKH signals through its receptor, AKHR, in the fat body to trigger the conversion of stored lipids to hemolymph circulating trehalose (Gronke et al., 2007). Of importance here is that flies in which CC cells have been ablated show defects in trehalose mobilization from the fat body through the breakdown of stored lipids (Isabel et al., 2005; Lee and Park, 2004), a phenotype consistent with PG NOS knockdown.

Considering that NO is capable of freely diffusing between cells and thus functioning as an intercellular signaling molecule it would be interesting to test whether NOS activity in the PG is somehow sensed by cells of the CC. Specifically, determination of whether the cell non-autonomous function of NO produced in the PG on CC cells underlies the fat body lipid hyperaccumulation phenotype that I have described could provide a possible alternative explanation for the cell non-autonomous effects of PG NOS knockdown. One piece of evidence supporting this alternative hypothesis is that soluble Guanylate Cyclase (sGC), a well defined intercellular target of NO signaling that catalyzes the formation of cGMP in response to NO binding (Bicker, 1998; Boon et al., 2005; Boon and Marletta, 2005; Wildemann and Bicker, 1999) is expressed at high levels in the CC of the Drosophila third instar larval ring gland (Wildemann and Bicker, 1999). sGC has also been shown to play an important NO-dependent
role in the development of the *Drosophila* visual system (Gibbs et al., 2001; Gibbs and Truman, 1998).

In order to determine whether NO produced in the PG signals through sGC in the CC to regulate fat body lipid storage it would be informative to first determine whether sGC knockdown in the CC phenocopies PG NOS knockdown. Towards this end, both a CC-specific GAL4 driver (AKH-GAL4) and sGC–specific RNAi lines have been described (Ellinger-Ziegelbauer et al., 1994; VDRC, Vienna; NIG, Japan) allowing for this analysis to be achieved with relative ease.

Interestingly, a complex physical overlap between the axons of the insulin producing cells (IPCs) of the *Drosophila* brain and the cellular projections of CC cells is strikingly similar to the organization and function of the vertebrate pancreas (Kim and Rulifson, 2004). Moreover, insulin signaling in the *Drosophila* PG is known to play a critical role in developmental timing and the determination of organismal size (Colombani et al., 2005; Mirth et al., 2009). Taken together, these studies underscore the importance of *Drosophila* as a model system for the study of endocrine signaling.

It is becoming increasingly clear that a complex, subtle, and fundamental interplay between the cells of the larval ring gland, circadian pacemaker cells, and insulin producing cells serves to precisely integrate nutritional, circadian and developmental cues. Considering that the antagonistic activities of glucagon and insulin control metabolism in mammals, and that disruption of this balance ultimately results in the onset of diabetes, studies in *Drosophila* show great promise towards providing a greater understanding of the mechanisms that underlie the pathogenesis of this disease.
4.3.10. Conserved neuroendocrine pathways in vertebrates

In vertebrates, Rev-erb’s have been shown to play an important role in the regulation of circadian rhythms through their function in the hypothalamus (Preitner et al., 2002). The hypothalamus is a central component of the mammalian brain that is made up of a collection of distinct neuronal clusters or nuclei. Each of these nuclei are responsive to a range of inputs including neuronal, hormonal, blood borne, and olfactory signals, and serve to both integrate and relay these signals through the synthesis and secretion of neurohormones. These hypothalamic neurohormones are responsible for controlling development, growth, body temperature, hunger, metabolism, circadian rhythms, reproduction, and homeostasis, and include vasopressin, oxytocin, somatostatin, growth hormone releasing hormone (GHRH), dopamine, thyrotropin releasing hormone (TRH), Gonadotropin releasing hormone (GnRH), and corticotrophin releasing hormone (CRH) [reviewed in Szarek et al. (2010)]. Thus, the hypothalamus functions as a central regulator in a wide range of developmental and homeostatic processes.

The specific region of the hypothalamus in which Rev-erb’s are known to play an important role is the suprachiasmatic nucleus (SCN) (Duez and Staels, 2009). The SCN serves as a central “master clock” that integrates incoming light information from the eye and synchronizes overall physiology to the day/night cycle by signaling to other hypothalamic nuclei and the pituitary gland (Bob and Fedor-Freybergh, 2008; Duez and Staels, 2009; Kalsbeek et al., 2007). As mentioned previously, and in line with a role for NOS in the regulation of E75 and Rev-Erb’s, it is well established that NOS controls reproduction by regulating hypothalamic GnRH secretion and the preovulatory leutinizing hormone surge in pituitary gonadotropes (Bhat et al., 1995; Brann et al., 1997; Ceccatelli et al., 1993; Dawson et al., 1991; Garrel et al., 1998; Kishimoto et al., 1996; Klein et al., 1998; Lee et al., 1999; Lozach et al., 1998; McCann et al.,
Thus, NOS function in both *Drosophila* and vertebrates participates in hormonal signaling pathways that regulate metabolism and development.

In addition to a conservation in NOS function, FTZ-F1 and its vertebrate ortholog SF-1 have both been shown to regulate the development of steroidogenic tissues including the ring gland and the hypothalamo-pituitary axis (Fayard et al., 2004; Parvy et al., 2005). Of great interest is the fact that SF-1 regulates the expression of nNOS in pituitary gonadotropes (Wei et al., 2002), implicating its role in a negative regulatory feedback circuit that may exist in the *Drosophila* PG as well.

Moreover, ablation of the hypothalamic nucleus in which SF1 is expressed produces animals that display a dramatic increase in feeding and a massive accumulation of fat (Hasegawa, 2008; King and Frohman, 1985; King and Smith, 1985; Majdic et al., 2002). Interestingly, mice lacking insulin receptor substrate-2, a critical component of the insulin signal transduction pathway, in the hypothalamus also exhibit a dramatic increase in both food intake and body fat deposition in addition to a major impairment of reproduction (Lin et al., 2004). These phenotypes are strikingly similar to those seen in larvae in which either DHR3 or NOS have been knocked down, or E75 has been ectopically expressed in the PG.

Thus NOS, FTZ-F1, and insulin signaling play similar roles in regulating growth and development in the *Drosophila* ring gland and the hypothalamus and, taken together, these results suggest that the endocrine signaling pathways that regulate organismal growth and development in both flies and vertebrates are likely conserved.

In summary, the *Drosophila* neuroendocrine signaling axis, which includes the CC, CA, PG, and IPCs, shows remarkable similarity in terms of its structure and underlying molecular
architecture to that of the vertebrate hypothalamus. In particular, studies in *Drosophila* have the potential to shed light on the function of RORs, Rev-erbs and NOS in the endocrine regulation of vertebrate development, growth, reproduction, circadian rhythm, and disease. Further elucidation of the roles of RORs, Rev-erbs and NOS has the potential to provide significant new insights into how these processes are coordinated on both the molecular and organismal level.
References


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