Analysis of the cell-specific expression of the serpent gene and its role as a cell-fate switch in Drosophila melanogaster

Jennell Marie Miller
University of Nevada, Las Vegas

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University of Nevada, Las Vegas

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Examination Committee Chair

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Examination Committee Member

Examination Committee Member

Graduate College Faculty Representative
ABSTRACT

Analysis of the cell-specific expression of the serpent gene and its role as a cell-fate switch in Drosophila melanogaster

by

Jennell Marie Miller

Dr. Deborah K. Hoshizaki, Ph.D., Examination Committee Chair
Associate Professor of Biological Sciences
University of Nevada, Las Vegas

A universal problem in the development of complex organisms is programming totipotent cells into specialized cell types. I have focused on the Drosophila fat body and the characterization of the positional information required for the adoption of a fat-cell identity by mesodermal cells. Central to this study is serpent, a transcription factor gene, both necessary and sufficient for fat-cell development.

I characterized the patterns of activity for regulatory regions that control serpent expression within the fat body. The fat body is composed of three morphological domains (lateral fat body, ventral commissure, and dorsal fat cell projections) each of which requires serpent activity. Using these enhancer-regions in reporter-gene constructs, I traced the development of the dorsal fat-cell projections, the ventralmost lateral fat body, and a portion of the ventral commissure, and demonstrated that particular groups of fat-cell clusters give rise to these domains.
In parallel, I determined, through mutant and misexpression genetic analysis, that the transcription factor genes, *Abdominal B* and *tinman*, are critical for *serpent* activation within the dorsal fat-cell cluster. Putative binding sites for these factors are present within the regulatory regions of *serpent*. Differing cues activate *serpent* in distinct embryonic regions, and I discuss the possibility that these factors are also important for the establishment of genetic differences among fat cells, and thus, functional differentiation of the fat body. Additionally, I genetically tested *Ultrabithorax*, another transcription factor gene with putative binding sites within one *serpent*-enhancer region, for its ability to regulate *serpent* expression. I determined that *Ultrabithorax* is not involved in fat-cell specification, but is required for the proper morphological development of the fat body.

I have also tested the proposal that within the lateral mesoderm, *serpent* is a cell-type switch between fat and somatic gonadal precursor cells. I find that *serpent* does not serve as a switch between these two cell choices. However, I provide evidence for a similar cell-fate switch, in the dorsal mesoderm, between dorsal fat cells and circular visceral muscle precursors. This switch relies on both *serpent* and the homeotic transcription factor, *Abdominal B*. 

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TABLE OF CONTENTS

ABSTRACT ............................................................................................................................iii

LIST OF ABBREVIATIONS................................................................................................vii

LIST OF FIGURES ..................................................................................................................x

ACKNOWLEDGEMENTS..................................................................................................xiii

CHAPTER 1  INTRODUCTION ...........................................................................................1
  A Key Problem in Development ....................................................................................1
  Overview of Project ......................................................................................................1
  Advantages of Utilizing the Drosophila Fat Body as a Model System ......................3
  Formation of the Mesoderm .......................................................................................4
  Fat-Cell Formation .....................................................................................................6
  The Role of serpent in Fat-Cell Development ..........................................................11
  Molecular Mechanism of SERPENT Binding ............................................................13
  Models of Cell-Fate Acquisition ............................................................................14
  References ..................................................................................................................18

CHAPTER 2 IDENTIFICATION OF FAT-CELL ENHANCER REGIONS
  IN DROSOPHILA MELANOGASTER .................................................................24
  Prologue .....................................................................................................................25
  Abstract .....................................................................................................................26
  Introduction ...............................................................................................................27
  Results .......................................................................................................................30
  Discussion ..................................................................................................................39
  Experimental Procedures .......................................................................................42
  Acknowledgements .................................................................................................45
  References ................................................................................................................45
  Figures .......................................................................................................................51

CHAPTER 3 SERPENT, A GATA-LIKE TRANSCRIPTION FACTOR
  GENE, INDUCES FAT-CELL DEVELOPMENT IN
  DROSOPHILA MELANOGASTER .................................................................66
  Prologue .....................................................................................................................67
  Summary ....................................................................................................................68
  Introduction ...............................................................................................................69
  Materials and Methods .......................................................................................72
  Results .......................................................................................................................74
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABD-B</td>
<td>ABDOMINAL B protein</td>
</tr>
<tr>
<td>AbdB</td>
<td>Abdominal B gene</td>
</tr>
<tr>
<td>Adh</td>
<td>alcohol dehydrogenase gene</td>
</tr>
<tr>
<td>bap</td>
<td>bagpipe gene</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cli</td>
<td>clift gene</td>
</tr>
<tr>
<td>DFC</td>
<td>dorsal fat cell</td>
</tr>
<tr>
<td>DPP</td>
<td>DECAPENTAPLEGIC protein</td>
</tr>
<tr>
<td>dpp</td>
<td>decapentaplegic gene</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<td>EVE</td>
<td>EVEN-SKIPPED protein</td>
</tr>
<tr>
<td>eve</td>
<td>even-skipped gene</td>
</tr>
<tr>
<td>GAL4</td>
<td>yeast transcriptional activator, GAL4</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>IDGF3</td>
<td>imaginal disc growth factor 3 gene</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
</tbody>
</table>
kb  kilobase
mM  milliMolar
NaCl sodium chloride
nau  nautilus gene
NBT  4 - Nitro Blue Tetrazolium chloride
PCR polymerase chain reaction
PNR  PANNIER protein
pnr  pannier gene
PS  parasegment
R  deoxyribonucleic acid bases, A or G
RNA ribonucleic acid
S59  slouch gene
SGP somatic gonadal precursor
SLP SLOPPY-PAIRED protein
slp  sloppy-paired gene
SRP SERPENT protein
srp  serpent gene
TIN TINMAN protein
tin  tinman gene
TGFβ Transforming Growth Factor β
Tml Tropomyosin 1 gene
Tris Tris (hydroxymethyl) aminomethane
*twi*  twist gene

UAS  Upstream Activating Sequence

*Ubx*  *Ultrabithorax* gene

UBX  ULTRABITHORAX protein

*W*  deoxyribonucleic acid bases, A or T

*X*-phosphate  5-bromo-4-chloro-3-indolyl-phosphate sodium salt

*y*  yellow gene

*w*  white gene
LIST OF FIGURES

CHAPTER 1

Figure 1-1 Schematic representation of the ectodermal and mesodermal layers of stage-9 and stage-11 embryos ................................................................. 23

CHAPTER 2

Figure 1 Fat-cell development .................................................................................... 51
Figure 2 Map of genomic srp and sequence of A7.1ES ........................................... 53
Figure 3 A7.1ES is active in a subset of fat-body cells ........................................... 55
Figure 4 The A7.1ES-lacZ reporter recapitulates a portion of the srp expression pattern ........................................................................ 56
Figure 5 The ventral commissure is composed of two fat-cell bridges ................. 58
Figure 6 The A7.1EB-lacZ reporter is expressed in the posterior of the embryo .......... 59
Figure 7 The dorsal cell cluster is located between en stripes 13 and 14 ............... 61
Figure 8 Misexpression of Ubx leads to a morphological alteration of the dorsal fat-cell projection ................................................................. 62
Figure 9 Misexpression of Ubx leads to morphological alterations of the ventral commissure ................................................................. 63
Figure 10 Specification of the dorsal cell cluster requires the formation of the dorsal mesoderm ................................................................. 64
CHAPTER 3

Figure 1  Ectopic srp expression in a twist-GAL4; UAS-srp embryo .....................91

Figure 2  Ectopic expression of srp leads to expansion of the fat body ...................92

Figure 3  Development of endogenous fat cells is unaffected by misexpression of srp in the mesoderm .................................................................93

Figure 4  Late stages of gonad formation are disrupted by misexpression of srp ..............................................................95

Figure 5  Ectopic expression of srp leads to loss of late cli expression in the SGPs, but not early cli expression in the mesoderm .........................................97

Figure 6  Ectopic expression of srp leads to loss of visceral muscle .................................................................98

Figure 7  Ectopic expression of srp disrupts nau and S59 expression patterns ......................99

Figure 8  The somatic musculature fails to form when srp is ectopically expressed throughout the mesoderm ..................................................100

CHAPTER 4

Figure 1  Schematic summary of the locations of the precursors to the fat-body, visceral muscle, and somatic gonads in a stage-12 embryo ............................................118

Figure 2  AbdB is necessary for the formation of the DFC cluster and the DFC projections .................................................................119

Figure 3  AbdB is required for A7.1EB-lacZ activity ..................................................120

Figure 4  Misexpression of AbdB results in the serial duplication of the DFC cluster .................................................................121

Figure 5  AbdB negatively controls VMc precursor cell formation ................................122

Figure 6  Misexpression of AbdB does not induce ectopic SGP formation ..................123

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Figure 4-A  P-element mediated transformation and generation of a homozygous stock

CHAPTER 5

Figure 5-1  Schematic diagram of the equivalent precursor model

Figure 5-2  Schematic diagram of the overlapping subclones of enhancer region A7.1EB

Figure 5-3  An unidentified nuclear factor forms a complex with fragment D of the serpent dorsal fat-cell enhancer

APPENDIX 1

Figure A1-1  Schematic diagram of the UAS/GAL4 gene-misexpression system in Drosophila

xii

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CHAPTER 1

INTRODUCTION

A Key Problem in Development

The various cell types composing any given multicellular organism differ significantly from the fertilized egg from which they have arisen. Through the process of development, the generic cells resulting from the division of a zygote will become specified, proliferate, and differentiate into specific cell types. Some differentiating cells will participate in cell-cell interactions, which can provide further modification of a cell's developmental potential. Eventually, specific cell types function either as individuals (e.g., blood cells) or as part of multicellular tissues and organs. Programming the progeny cells of a fertilized egg into these multitudes of specialized cell types is a universal problem in the development of complex organisms. During the course of embryogenesis, it is thought that complex genetic regulatory networks and intercellular interactions progressively restrict the developmental potential of each mesoderm cell and its derivatives; however, these complex networks are not yet completely understood (Lawrence, 1992).

Overview of Project

To begin to understand the regulatory pathway leading to the development of one specific cell type, fat cells, this project has taken advantage of the GATA-transcription-
factor gene *serpent* (*srp*) and its central role in fat-cell and fat-body development in *Drosophila melanogaster* (Sam, et al., 1996; Rehorn et al., 1996; Chapter 3). Because *srp* is both necessary and sufficient for fat-cell formation, understanding how *srp* is initially activated and subsequently regulated is critical to explaining how non-specific mesodermal cells are directed to adopt fat-cell fates. In brief, I have determined that the positional information provided by the transcription factors ABDOMINAL-B and TINMAN is genetically necessary (Chapters 2 and 4) for *srp* expression within the cells of the dorsal fat-cell (DFC) cluster (see Fat-Cell Formation, below and Chapters 2 and 4). I have located putative binding sites for these trans-acting factors within the DFC-specific regulatory region of *srp*, which I have characterized (Chapter 2) and, I have begun to test the functional significance of these factors, *in vivo* (Chapter 4). In addition to investigating the regulation of *srp*, I have also tested its proposed role as a cell-fate switch within lateral and dorsal mesoderm-cell types (Chapter 3 and Chapter 4).

Riechmann and co-workers (1998; reviewed in Chapter 3) have proposed that *srp* functions as a cell-fate switch directing the choice between fat cells and somatic gonadal precursor (SGP) cells in the lateral mesoderm. I have determined, however, that because misexpressed *srp* does not inhibit or ‘switch off’ the specification of SGP cells as predicted by Riechmann’s (1998) model, *srp* cannot mediate a cell-fate choice between these two cell types (Chapter 3). Although *srp* does not serve in the choice between SGP and fat cells, I have identified a role for *srp* as part of a developmental switch mediating the choice between precursors to dorsal fat cells and circular visceral muscles, both of which arise within the dorsal mesoderm (VMc; Chapter 4). I demonstrate that the choice
between these two cell types relies upon both srp and the homeotic transcription factor-encoding gene, *Abdominal B* (*AbdB*: Chapter 4). I find that when *srp* is misexpressed, the cells that would normally form VM\(_c\) precursors develop as dorsal fat cells (Chapter 3). Furthermore, an absence of *srp* and *AbdB* permits the formation of VM\(_c\) precursors from cells that normally form dorsal fat cells (Chapter 4). This straightforward relationship between DFC and VM\(_c\) cells, based on the presence or absence of *srp* and *AbdB* is not yet evident between fat cells and any other cell type. Thus, this finding emphasizes an early developmental similarity between DFC and VM\(_c\) cells. I suggest that the pathways restricting the developmental potential of the DFC and VM\(_c\) precursors are initially very similar, if not identical. The similar genetic requirements of these two cell types (Chapter 4) suggests that precursor fat cells and precursors VM\(_c\) cells might be more closely related to one another in terms of the developmental pathways establishing their genetic ‘history’ than they are to other cell types.

**Advantages of Utilizing the *Drosophila* Fat Body as a Model System**

There are several advantages to using the development of the *Drosophila melanogaster* fat body for study of cell-type acquisition and cell-fate choices in the mesoderm. First, the fat-cell lineage has been well characterized (Hoshizaki et al., 1994; Riechmann et al., 1998), and cell markers for precursor fat cells and mature fat-body cells have been established (Hoshizaki et al., 1994). Second, the stereotyped, metamERICally repeating spatial arrangement of the precursor fat cells (described below and reviewed in
Chapter 2), suggests a responsiveness of fat-cell identity genes to positional information (Rizki and Rizki, 1978). Finally, the complex arrangement of the precursor fat cells has the potential for providing insight into the developmental mechanisms that can produce groups of genetically homologous cells that reside in more than one positional domain (Chapter 2). Because the fat body arises from groups of metameric precursors that arise from cells within non-homologous positions of the mesoderm (reviewed in Riechmann et al., 1998; Chapter 2; and see Fat-Cell Formation below), uncovering the positional cues for each fat-cell group should eventually provide insight into (1) the activation of cell-type determining genes requiring multiple enhancers and (2) the development of tissues deriving from precursors that arise in spatially non-homologous positions. Furthermore, the *Drosophila* fat body is a dynamic and essential tissue that participates in multiple biochemical functions, including fat storage and production, storage of proteins and carbohydrates, and production of antibacterial peptides, (reviewed in Chapter 2). Understanding the development of the fat body could lead to an increased understanding of the functional differentiation of this organ (Chapter 2) and any genetic and/or developmental conservation that might exist between the *Drosophila* fat body and homologous tissues in other insects and higher organisms.

**Formation of the Mesoderm**

The *Drosophila* embryonic mesoderm gives rise to fat body, blood cells, gonadal sheath cells, and somatic, visceral, and cardiac musculature (Hartenstein and Jan, 1992; Hoshizaki et al., 1994; Technau, 1987). I have focused here on the fat body and the
acquisition of fat-cell fate among mesodermal cells. As the mesodermal gastrulation process begins at stage 6 of embryonic development (stages are those of Campos-Ortega and Hartenstein, 1997) the presumptive mesoderm is composed of ectodermal cells that are located in the ventral-third portion of the blastoderm embryo (Campos-Ortega and Hartenstein, 1997). These cells express high levels of the maternal protein Dorsal, a NF-κB-related morphogen (Ip et al., 1991) that is required to activate two zygotic transcription factor-encoding genes, twist and snail (Boulay et al., 1987; Ip et al., 1992; Thissel et al., 1988). twist and snail are necessary for the formation of the mesoderm (Grau et al., 1984; Simpson, 1983). Although presumptive mesoderm cells are unspecified (Beer et al., 1987), they are not identical to one another. Prior to establishment of the presumptive mesoderm the pair-rule genes (e.g., even-skipped and sloppy-paired) establish intrinsic differences that are reiterated along the anterior/posterior axis among blastoderm cells (Lawrence, 1992). These differences are maintained within the presumptive mesoderm cells as they cells invaginate at gastrulation (Azpiazu et al., 1996; Bate and Baylies, 1996).

At stage 8 of embryonic development, the wide band of presumptive mesoderm cells begins to invaginate into the interior of the embryo, forming a furrow along the ventral midline (the ventral furrow; Leptin and Grunewald, 1990). During the process of invagination, ectodermal cells begin an ectoderm-to-mesenchyme transition. However, as demonstrated by transplantation experiments, these cells are not yet restricted to any particular mesodermal type and, thus, are considered unspecified (Beer et al., 1987). Once the presumptive mesoderm cells have invaginated completely, the ventral furrow closes to form the mesodermal tube (i.e., germ band; Campos-Ortega and Hartenstein, 1997). The
cells of this internalized mass divide mitotically in the first of three postblastodermal mitoses. During this division, the cells dissociate from one another and abandon all remaining ectodermal characteristics (Campos-Ortega and Hartenstein, 1997). At stage 9, the cells elongate and flatten, spreading out dorsally to form a cellular monolayer under the ectoderm; this layer is the mesoderm. These cells then undergo the second post-blastoderm mitosis, while still maintaining a monolayer (Campos-Ortega and Hartenstein, 1997).

By stage 10, the sheet of mesodermal cells has migrated to its dorsalmost limit, and shortly thereafter, the first morphological subdivision of these cells occurs coincident with the third postblastodermal mitosis: the dorsal half of the mesoderm segregates into an inner and outer mesodermal layer, the somatopleura and splanchnopleura, respectively (Borkowski et al., 1995; Campos-Ortega and Hartenstein, 1997). It was originally maintained, presumably based on histological analysis, that fat cells arise from the somatopleura, which gives rise to the somatic and cardiac musculature (Campos-Ortega and Hartenstein, 1997). However, Hoshizaki and co-workers (1994) demonstrate that the splanchnopleura, which gives rise to the circular visceral muscle precursors (VMc), also gives rise to the fat-body precursors. Because dorsal fat cells and VMc cells might share an early lineage-restriction pathway (Chapter 4), the evidence that fat cells and VMc cells arise from the same early mesodermal sublayer is significant.

Fat-Cell Formation

Prior to the advent of molecular techniques to mark individual cells, histological studies suggested that fat cells arise in the mesoderm after stage 13 (Poulson, 1950).
However, using the P-element enhancer-trap line P[29D], Hoshizaki and co-workers (1994) traced a portion of the fat-cell lineage, demonstrating that the nine bilateral clusters, present at stage 10/11, represent the specified progenitors to the fat cells. P[29D] is the only domain-specific marker for fat-cell progenitors; markers for the progenitors of the cells that populate the remaining fat body domains (e.g., the dorsal fat projections and ventral commissure) have yet to be identified.

In a subsequent study, Sam and co-workers (1996) detected SRP protein within the aforementioned nine bilateral fat-cell clusters and determined through the analysis of \( srp \) mutants that \( srp \) is necessary for fat-cell differentiation among these cells and for general fat-body development. Rehorm and co-workers (1996) also concurrently reported that \( srp \) is necessary for fat-body development. Because \( srp \) activity results in the differentiation of fat cells from progenitor cells (Sam et al., 1996), the appearance of \( srp \) establishes these cells as the ‘precursors’ to fat cells. Riechmann and co-workers (1998) also described additional \( srp \)-expressing cell clusters (primary fat-cell precursors, secondary fat-cell precursors, and the dorsal fat-cell cluster; see Chapters 2 and 3) that, with the previously described nine bilateral clusters, appear to represent the complete set of precursor fat cells (see Fig. 1 in Chapter 2). \( srp \) is not only the earliest marker for all precursor fat cells, but based on several lines of evidence (reviewed in Chapters 2 and 3), \( srp \) is also a cell-type-determining gene (Chapter 3).

In the process of cell-type determination, mesodermal cells are gradually and sequentially distinguished from one another as their developmental potential is increasingly restricted (Lawrence, 1992; Frasch, 1999; reviewed in Frasch and Nguyen, 1999). According to one model of cell-type acquisition, early patterning molecules (i.e.,
combinations of intrinsic regulatory factors and extrinsic patterning cues impinged upon mesodermal cells by overlying ectoderm) define dorsal/ventral and anterior/posterior positions among cells of the mesoderm (Reviewed in Lee and Frasch, 2000). These cues are thought to provide a cell with a unique ‘address’ that is associated with the competency to develop into a particular cell type (reviewed in Bate and Baylies, 1996; and see Models of Cell-Fate Acquisition, below). I have investigated, in this project, whether the *Drosophila* fat-body precursors form in response to positional cues provided at specific positions.

*srp* activation, and subsequent development of fat-cells occurs within defined positions in the mesoderm. Precursor fat cells arise as groups of clusters that emerge within various domains of patterning information. Some of these clusters metamERICALLY repeat (Riechmann et al., 1998). The three major groups of precursor fat cells (see Fig.1 in Chapter 2), described below in the context of influencing patterning cues, are: a) Primary Fat-Cell Clusters; b) Secondary Fat-Cell Clusters; and c) The Dorsal Fat-Cell Cluster.

a) Primary Fat-Cell Clusters

The primary fat-cell clusters emerge within the lateral mesoderm in the cell-intrinsic *even-skipped* (eve-) expression domain (Azpiazu et al., 1996; Riechmann et al., 1997). EVE, a homeodomain transcription factor, is first detected within the nuclei of cells occurring in a series of seven transverse stripes at the cellular blastoderm stage (Frasch and Levine, 1987). After gastrulation, seven more stripes of eve expression appear, resulting in a total of 14 evenly spaced stripes that persist until germ band elongation, dividing each parasegment (Fig.1-1 and Frasch and Levine, 1987). In eve mutants, three of the six primary fat-cell clusters are absent, thus, eve is required for the formation some
primary fat-cell clusters and, therefore, for normal lateral fat-body development (Riechmann et al., 1997). The development of the three remaining primary fat-cell clusters in the eve-mutant embryo is likely to depend, not on eve, but on fushi tarazu (ftz), a transcription factor-encoding gene that is expressed in alternating stripes with eve (Frasch and Levine, 1987). This idea, however, remains to be tested. Additional regulatory cues necessary to restrict srp expression to particular cells along the dorsal/ventral axis within the eve-expression domain have yet to be identified.

b) Secondary Fat-Cell Clusters

The two sets of secondary fat-cell clusters arise shortly after the emergence of the primary fat-cell clusters (Hoshizaki et al., 1994; Riechmann et al., 1998). The ventral secondary fat-cell clusters arise in PS 3-12 in the sloppy-paired (slp)-expression domain (a 14-stripe pattern, with each slp stripe located posterior to an eve stripe; Fig. 1-1 and Lawrence, 1992; Riechmann et al., 1998). slp encodes a forkhead domain transcription factor that negatively regulates eve activity (Hacker et al., 1992). Thus, based on the requirement of some primary fat-cell clusters for eve, the ventral secondary fat-cell clusters arise in a domain of positional information that would seem incompatible with fat-cell development. Using a srp enhancer-region/reporter-gene construct to mark the ventral secondary fat cells, I demonstrate that the ventral secondary fat-cell clusters in PS 3-5 give rise to the ventral commissure (Chapter 2). The lateral secondary fat-cell clusters arise in a position posterior to the primary clusters in PS 4-12 in the eve-expression domain but may extend into the slp-expression domain (Riechmann et al., 1998). The lack of lateral secondary fat-cell-specific markers has made it difficult to study the contribution of these clusters to the mature fat body, however, based upon their position, they most
likely contribute to the lateral fat body. Because these clusters appear to straddle the eve- and slp-expression domains, it is unclear to which anterior/posterior positional cue they respond. Additional patterning cues to restrict srp expression within the ventral and lateral secondary fat-cell clusters along the dorsal/ventral axis remain to be identified.

c) Dorsal Fat-Cell (DFC) Cluster

I have focused much of this study on the dorsal fat-cell (DFC) cluster, which is a non-repeating cluster that arises in PS 13 and part of PS 14. The DFC cluster spans eve- and slp-expression domains (Chapter 2). Along the dorsal/ventral axis, the DFC cluster depends on formation of the dorsal mesoderm and the activities of decapentaplegic (dpp) and dpp-dependent tinman (tin; Chapter 2). dpp encodes a TGF-β superfamily morphogen that is expressed throughout the dorsal ectoderm beginning at the blastoderm stage (St Johnston and Gelbart, 1987). Ectodermally expressed dpp, in addition to its role in patterning the dorsal epidermis, is also thought to participate inductively in mesodermal patterning through maintenance of the second phase of tin expression within the mesoderm (Fig. 1-1 and Frasch, 1995). tin encodes a NK2-homeobox transcription factor (reviewed in Harvey, 1996), which is necessary for the formation of the dorsal mesoderm (Azpiazu and Frasch, 1993). Using a srp enhancer-region/reporter gene construct to mark DFC cluster, I demonstrate that this particular cluster of cells populates the DFC projection (Chapter 2). I also demonstrate that the DFC cluster depends on dpp-dependant tin (Chapter 2). The DFC cluster is not formed throughout the tin-expression domain and, instead, is limited to PS 13. This anterior/posterior restriction is due to the presence of the Bithorax Complex transcription factor, ABDOMINAL-B, (ABD-B); AbdB is both
necessary and sufficient for \textit{srp} expression in the dorsal mesodermal cells (i.e., cells within the \textit{tin} domain; Chapter 4).

In sum, precursor fat cell clusters can be categorized into three major groups: Primary and Secondary and Dorsal Fat-Cell Clusters, and these groups lie within distinct domains of patterning information.

The Role of \textit{serpent} in Fat-Cell Development

\textit{serpent (srp)} is a key gene in the specification and maintenance of the fat-cell lineage (Sam et al., 1996; Rehorn et al., 1996). \textit{srp} encodes the GATA transcription factor SERPENT (SRP; also known as dGATAb), which is expressed in all cells of the fat-cell lineage, as well as in other cell types (Abel et al., 1993; Rehorn et al., 1996; Sam et al., 1996). Currently, the identified \textit{Drosophila} GATA factor-encoding genes, in addition to \textit{srp} (Abel et al., 1993), are \textit{grain (grn; Brown and Castelli-Gair Hombria, 2000), pannier (pnr; Ramain et al., 1993) and GATAe (FlyBase accession number FBgn0038391). In vertebrates and invertebrates, GATA factors are important to both cell specification and differentiation (Lin et al., 1995; Ramain et al., 1993; Winick et al., 1993). SRP was initially identified as a transcriptional activator of \textit{alcohol dehydrogenase (Adh; Abel et al., 1993)}, a fat-cell terminal differentiation gene (Hoshizaki et al., 1994), that is first detected at stage 15 in fat-body cells. Initially named, 'Box-A binding factor' (ABF), SRP protein binds a portion of \textit{Adh}’s regulatory region known as the ‘Box-A’ element in the transcriptional activation of \textit{Adh} (Abel et al., 1993). Recently, it has also been demonstrated that \textit{srp} is required in larvae for the fat-cell specific expression of Cecropin.

Prior to its role in Adh and CecA1 activation, srp plays a central role in the establishment of the fat-cell lineage, (Sam, et al., 1996; Rehorn et al., 1996; Chapter 3). First detected in fat cells at embryonic stage 10-11, srp is the earliest-expressed fat-cell gene (Sam et al., 1996); it is expressed throughout maturation of the embryonic fat body (Sam et al., 1996) and it is also detected in the larval fat body (Petersen et al., 1999). srp is necessary for the differentiation of cells that give rise to the lateral fat body, the dorsal fat-cell projections, and the ventral commissure (Rehorn et al., 1996; Sam et al., 1996). In the absence of srp activity, P[29D]-marked fat-cell progenitors are established but do not proliferate and differentiate (Chapter 3), and no terminal differentiation markers are detected at later stages (Sam et al., 1996). Within the lateral portion of the germband of srp-mutant embryos, apoptotic cells are detected, suggesting that loss of srp activity in srp-mutant embryos blocks differentiation of these cells (Sam et al., 1996). Loss of srp in lateral fat-cell precursors does not 'switch off' a fat-cell fate, such that another fate can be achieved. Instead, primary and lateral secondary precursor fat cells undergo cell death when srp activity is absent (Sam et al., 1996).

In addition to being necessary for fat-cell development, ectopically expressed srp is sufficient to induce the fat-cell program in cells normally committed to other cell fates (Chapter 3). Use of the UAS/GAL4 targeting system of Brand and Perrimon (1993; see Appendix 1) to misexpress srp throughout the mesoderm results in widespread fat-cell production and an expansion of the fat body as evidenced by ectopic expression of
terminal differentiation fat-cell markers, Adh and DCgl (type 1V collagen), and IDGF3 (imaginal disc growth factor 3; Chapter 3). The srp misexpression experiments served as a means to test srp's role as a cell-fate switch. The ectopic fat cells formed in the srp-misexpression embryos develop at the expense of VMc precursors and certain body-wall muscle precursors. Ectopic srp expression does not block specification of the somatic gonadal precursor (SGP) cells, but does disrupt the ability of the embryo to form a wild-type gonad (Chapter 3). This evidence supports the proposed role of srp in the differentiation of fat cells (Sam et al., 1996) but does not support the role of srp proposed by Riechmann and co-workers as a cell-fate switch within the lateral mesoderm between SGP cells and primary fat cell clusters.

Molecular Mechanism of SERPENT Binding

The role of srp in fat cell specification lies within its ability to regulate (activate/repress) downstream targets that act as the effectors of fat-cell development and maintenance. SRP protein is classified as a member of the GATA family of transcription factors based upon the ability of SRP to bind to the DNA sequence, TGATAA (Abel et al., 1993). This binding sequence is consistent with the mammalian GATA family consensus DNA sequence WGATAR (Orkin, 1992). In general, the zinc-finger proteins are a large and diverse group in which the DNA-binding region contains projections (fingers) with Cys and/or His residues folding around a zinc ion (reviewed in Iuchi, 2001). The srp gene is unusual in that it codes for a single Cys-4 zinc-finger domain (Abel et al., 1993). Most mammalian GATA factors have at least two zinc fingers and both are necessary for
specific and stable DNA binding, although the carboxyl-terminal is most critical in DNA binding (Yang and Evans, 1992).

In addition to its necessity for fat-body development, srp activity has been detected within, and determined to be necessary for the development of, amnioserosa cells, anterior and posterior midgut primordia, and both classes of hemocytes: crystal cells and plasmatocytes/macrophages (Rehorn et al., 1996; Sam et al., 1996; Lebestky et al., 2000). How srp is involved in the specification of these different cell types might depend on srp co-factors. pannier (pnr; another Drosophila GATA factor), for example, can play differing roles depending on whether or not U-shaped (Ush), a GATA factor co-factor is bound to PNR's N-terminal zinc finger (Fossett et al., 2001). srp is unlike pnr in that it only encodes a single zinc finger. However, a survey of the srp genomic sequence shows an ORF within the third intron of the gene that putatively encodes an N-terminal zinc finger with 96% homology to that of pnr (Fossett et al., 2001). This finding raises the possibility that USH protein might interact with an alternatively spliced isoform of SRP during hematopoiesis (Fossett et al., 2001).

Models of Cell-Fate Acquisition

Various models have been developed to explain how cells achieve their unique fates. Current general models, which I have explored as candidate models to explain the specification of fat cells are: a) Combinatorial Positional Cues Model; b) "Differentiate or Die" model; c) Asymmetric Cell Division Model; and, d) Lateral Inhibition Model. These models are not exclusive of one another and likely lie within part of the progression of cell specification for some cell types.
a) Combinatorial Positional Cue Model

It is thought that a number of molecules that act both intrinsically to and inductively upon mesodermal cells to participate in the specification of individual cell types through the ultimate activation of cell-type determining genes (see also Fat-Cell Formation, above). The overlapping expression patterns of the genes encoding these molecules establish the metameric arrangement of cell-type primordia in the dorsal, lateral, and ventral mesoderm (see figure 1-1; Dunin-Borkowski et al., 1995; Lawrence et al., 1995; Azpiazu et al., 1996). These position-providing genes can be divided into two groups. The first group is responsible for patterning the mesoderm along the dorsal/ventral axis and consists primarily of two genes, *decapentaplegic (dpp)* and *tinman (tin)*; Fig. 1-1 and reviewed in Bodmer and Frasch, 1999; and see c) Dorsal Fat-Cell (DFC) Cluster, above, and Chapters 2 and 4 for reviews of the relationship between *dpp* and *tin*). *dpp* inductively, and *tin*, intrinsically, are required for the specification the dorsal mesoderm, which gives rise to the heart, visceral muscles, dorsal somatic muscles and dorsal fat body (Azpiazu and Frasch, 1993).

The second group is responsible for patterning the mesoderm along the anterior/posterior axis; these genes subdivide the mesoderm into segmental and subsegmental units. Members of this group include *wingless (wg; inductive)* and *sloppy paired (slp; intrinsic)*, which are required for the formation of the heart and somatic muscles but not for specifying circular visceral muscles. *hedgehog (hh; inductive)* and *even-skipped (eve; intrinsic)* are required for the formation of the circular visceral muscles and some primary fat-cell clusters but not for the formation of the heart and somatic muscles. The *Bithorax*
Complex genes are also members of this anterior/posterior patterning group (reviewed in Bodmer and Frasch, 1999).

The expression patterns of the genes from the first and second group intersect in every parasegment (reviewed in Bodmer and Frasch, 1999). Genes that are expressed at defined dorsal/ventral and anterior/posterior locations in the mesoderm do so in response to activation or repression by dorsal/ventral genes and anterior/posterior genes (groups 1 and 2, above). These genes control the cell-type specification of the cells in which they are expressed. The combinatorial model most likely explains the stereotyped emergence of all precursor fat cells.

b) Mammalian Gonad Development Model

Mammalian gonad development exemplifies a developmental model in which, initially, two intermingling pools of cells exist, each competent to give rise to a particular cell type. Positional cues direct one pool of cells to thrive and differentiate into a specific cell type, while the presence of negative (repressive) cues or absence of positive (activating) cues for the other pool results in a failure to differentiate and cell death. In mammals, gonads of both sexes develop initially (Hughes, 2001). In females, the Müllner duct cells differentiate into the oviduct; the Wolffian duct degenerates (Higgins et al., 1989). Meanwhile, in males, the Wolffian duct cells develop into the vas deferens because production of Müllerian inhibiting substance in the testis represses differentiation of Müllerian duct cells (Higgins et al., 1989). The “differentiate or die” model is a possible model to describe the relationship between somatic gonadal precursors and fat-cell precursors, which develop in analogous positions in different parasegments within the lateral mesoderm (Chapter 3).
c) Asymmetric Cell-Division Model

According to this model, cytoplasm is distributed asymmetrically between the two daughter cells of a single common precursor. The two daughter cells acquire differing cytoplasmic determinants (e.g., mRNA for transcription factors that are responsible for dissimilar gene activity in their nuclei) and these cytoplasmic factors bring about different patterns of development (Muller, 1996). In the ectoderm, asymmetric cell divisions are responsible for creating cell diversity among the daughter cells of neuroblasts (neuron precursors) in the development of the Drosophila nervous system (reviewed in Jan and Jan, 2001). Carmena and co-workers (Carmena et al., 1998) have demonstrated that, within the mesoderm, differentiation of some embryonic muscle progenitors relies on asymmetric cell divisions. Whether or not fat-cell precursors undergo asymmetric cell divisions is not known; as markers for functional differentiation of fat cells become available, it will be possible to test this idea.

d) Lateral Inhibition Model

This model stresses the importance of "local cell-cell interactions in mediating precise cell-fate decisions" (reviewed in Rooke and Xu, 1998). The lateral inhibition model of cell specification is based, in large part, on studies of the EGF receptor gene Notch, which plays a significant role in Drosophila neurogenesis. In this process, groups of cells in defined positions of the ectoderm or epidermis acquire the potential to become neural. However, only one cell in the group is specified to adopt a neural fate. During specification, this cell inhibits all surrounding cells from acquiring a neural fate. This process is termed 'lateral inhibition'. In the case of Drosophila neurogenesis, the Notch gene encodes the receptor for lateral inhibition and Delta is the membrane-bound ligand
(reviewed in Corbin, 1991). Corbin and co-workers (Corbin et al., 1991) have discovered a role for Notch in the mesoderm in the specification of certain somatic muscle cells. With these data in mind, the lateral-inhibition model was initially considered as a means to explain the cell-type choice I have described between DFC cells and VMc cells. However, based upon our current understanding of fat-cell and visceral mesoderm precursor development in the dorsal mesoderm, Notch likely does not play a role in this cell-fate choice. Future studies could test whether Notch plays a role in fat-cell formation through the examination of Notch-mutant embryos for aberrations in dorsal fat-cell development.

References


Figure 1-1  Schematic representation of the ectodermal and mesodermal layers of sections of a stage 9 and stage 11 embryo. (Adapted from Riechmann et al., 1998 and Bodmer and Frasch, 2001)

See text for details. Abbreviations: TINMAN, TIN; DECAPENTAPLEGIC, DPP; SLOPPY PAIRED, SLP; EVEN-SKIPPED, EVE; ABDOMINAL B, ABD-B; circular visceral muscle precursors, VMc; primary fat-cell precursor, 1°, lateral secondary fat-cell precursor, L2°; ventral secondary fat-cell precursor v2°.
CHAPTER 2

IDENTIFICATION OF FAT-CELL ENHANCER REGIONS IN

DROSOPHILA MELANOGASTER

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Prologue

Within this chapter, I describe the enhancer regions of *serpent* (*srp*) A7.1ES and A7.1EB (which is embedded in A7.1ES), which are active in specific regions of the fat body. Prior to this work, there were no discreet markers for specific fat-body domains. The previously characterized fat-cell markers are *serpent* (*srp*), *alcohol dehydrogenase* (*Adh*), *type IV collagen* (*DCgl*), and P[29D]. *srp* is expressed in all fat-cell precursors and continues to be expressed throughout fat-cell development (Sam et al., 1996; Riechmann et al., 1998). *Adh* and *DCgl* are expressed in all fat-body cells (Hoshizaki et al., 1994), and the P-element enhancer-trap construct, P[29D], marks nine bilateral clusters of progenitor fat cells giving rise to a portion of the lateral fat body (Hoshizaki et al., 1994). The A7.1ES and A7.1EB enhancer regions are responsible for *srp* activity in distinct regions of the fat body, and thus, A7.1 EB and A7.1 ES enhancer region/reporter gene constructs are domain-specific, fat-cell markers. Using these constructs I have traced, in detail, the developmental lineages of the cells giving rise to the dorsal fat-cell projections, the ventralmost lateral fat body, and the ventral commissure.

The A7.1 EB construct characterized in this chapter has been critical in testing genetic regulatory factors of *srp* important explicitly for the specification of the dorsal fat-cell cluster and the formation of the dorsal fat-cell projection (see also Chapter 4). This construct has allowed me to confirm the identity of cells suspected to be dorsal fat-cell clusters in misexpression experiments (Chapter 4). Additionally, sequence analysis for the A7.1EB regulatory region has revealed putative binding sites for transactivators of *srp* e.g., *tinman* and *Abdominal B*, which are required for *srp* activity only in the dorsal fat-cell cluster. Other cues important for the activity of *srp* in the other fat-cell clusters remain
to be determined. Identification of independent enhancers of srp that depend on differing cues for activation led to the hypothesis that the functional differentiation detected in the larval fat body might also be established, early in precursor fat-cell formation, by the various genes that activate srp. I anticipate that A7.1ES will serve as a discreet marker for the ventral secondary precursor clusters in future experiments of this type.


Abstract

transcription factor gene that is sufficient to induce fat-cell formation. This enhancer region drives expression in specific groups of precursor-cell clusters, which we show give rise to defined regions of the mature embryonic fat body. We present evidence that \textit{srp} expression in different precursor fat cells is controlled by independent \textit{cis}-acting regulatory regions, and we have tested the role of \textit{trans}-acting factors in the specification of some of these cells. We suggest that the different positional cues regulating \textit{srp} expression, and therefore general fat-cell specification, might also be involved in the functional specialization of fat cells. This may be a common mechanism in insects to explain the origin of biochemically distinct regions of the larval/adult fat body.

\section*{Introduction}

The insect fat body is a dynamic tissue that participates in multiple biochemical functions, including energy storage, intermediary metabolism, detoxification, communication and the immune response. The insect fat cell is the main site for storage and production of proteins, lipids and carbohydrates. It produces a variety of stage-specific, amino acid-storage proteins, including calliphorin in blowflies (\textit{Calliphora}) and drosophilin and hexamerins (e.g. the larval serum proteins) in \textit{Drosophila} (Keeley, 1985 and references therein). The fat body is also the primary biosynthetic site of diacylglycerol and the major insect sugar, trehalose. Both diacylglycerol and trehalose are energy-storage molecules that are key to the survival of the animal and central for energy-intensive behaviors such as long-distance migration (e.g., \textit{Locusta migratoria}; Becker \textit{et al.}, 1996; Vroemen \textit{et al.}, 1998). Fat-body cells produce a number of other significant proteins.
including vitellogenins for oocyte maturation in *Drosophila* and mosquitoes (*Aedes aegypti*) as well as diapause proteins and hemoglobin in midge fly larvae (*Chironomus thummi*; reviewed in Keeley, 1985). The fat body has been compared to the vertebrate liver in its role in trehalose biosynthesis and release and through its response to adipokinetic signaling, which is analogous to the hormone (insulin)-mediated synthesis and release of glucose by the liver (Becker *et al.*, 1996). Interestingly, the fat body of the desert ant (*Cataglyphis niger*) is the major synthetic site of hydrocarbons involved in communication among colony members (Soroker and Hefetz, 2000). The fat body also plays a central role in the insect innate immune response (reviewed in Hoffmann *et al.*, 1996; Engstrom, 1999).

The fat body is one of several tissues produced by the embryonic mesoderm (Hartenstein & Jan, 1992; Hoshizaki *et al.*, 1994; Technau, 1987). In general, the primordia for each tissue type lie in defined positions determined by the anterior-posterior and dorsal-ventral patterning of the mesoderm. In *Drosophila*, pair-rule genes such as *even-skipped* help to establish intrinsic differences between mesodermal cells in a metameric pattern along the anterior-posterior axis (Azpiazu *et al.*, 1996), while along the dorsal-ventral axis, *decapentaplegic* (*dpp*) expression in the dorsal embryonic ectoderm specifies the dorsal mesoderm through the maintenance of *tinman* (*tin*) expression (Frasch, 1995; Staehling-Hampton *et al.*, 1994). The establishment and maintenance of morphologically distinct cell types are controlled by homeotic genes that determine segment identity through the regional activation of target genes necessary for cell specification (Weatherbee & Carroll, 1999; Weatherbee, 1998). These patterning systems
provide mesodermal cells with unique addresses that serve as positional cues to establish distinct populations of cells.

The fat-body precursors are organized in a metameric pattern. Within a given range of parasegments, they are located in defined dorsal-ventral positions (reviewed in Riechmann et al., 1997). It is likely that the specification of precursor fat cells requires the integration of different anterior-posterior and dorsal-ventral positional cues and homeotic gene information. The maturation of the fat body requires the coordinated migration of cells from the fat-cell clusters to their final positions, where they are organized into a functional organ composed of three morphological domains: the lateral fat body, the dorsal fat-cell projections and the ventral fat-cell commissure (Campos-Ortega & Hartenstein, 1997; Hoshizaki et al., 1994; Riechmann et al., 1998). A variety of studies have suggested that the fat body is also divided into different regions based on the diverse biochemical functions of this tissue (reviewed in Haunerland & Shirk, 1995).

We describe here, a 2.1 kb enhancer region from the *serpent* (*srp*) promoter that drives expression in a subset of fat cells. The *srp* gene is one of three known *D. melanogaster* genes belonging to the GATA transcription-factor family (Brown & Castelli-Gair Hombria, 2000; Lin et al., 1995; Ramain et al., 1993; Winick et al., 1993) and was initially identified as a transcriptional activator of the *alcohol dehydrogenase* (*Adh*) gene in fat cells (Abel et al., 1993). SRP protein is present at embryonic stage 10/11 (embryonic stages are those of Campos-Ortega & Hartenstein, 1997) in all fat-cell precursors (Sam et al., 1996) and is the earliest known gene expressed in the fat-cell lineage (Abel et al., 1993; Rehorn et al., 1996; Sam et al., 1996). Genetic analysis has revealed that *srp* is
necessary for the maintenance of the fat-cell lineage (Sam et al., 1996) and is sufficient to induce fat-cell formation within the mesoderm (Hayes et al., 2001). Thus, the activation of \( srp \) is a likely step in fat-cell specification. Because precursor fat cells arise from stereotypic anterior-posterior and dorsal-ventral positions within the mesoderm, it is possible that a combination of positional information directs \( srp \) expression through different enhancers located within the \( srp \) regulatory region.

The \( srp \) regulatory/promoter region spans at least 8kb, based on the location of \( srp \) regulatory mutations (Rehorn et al., 1996). Sequences within this region were identified as an \textit{in vitro} target of the homeodomain transcription factor, Ultrabithorax (UBX) and contain putative UBX response elements (Mastick et al., 1995). We describe enhancer activity that is associated with this region. By using this enhancer region, we have traced the origin of specific morphological regions of the fat body and tested factors that might \textit{trans}-activate \( srp \). We discuss the possibility that the positional information that directs the specification of precursor fat cells is also responsible for the eventual biochemical differences found in the larval fat body.

Results

Developmental studies of the embryonic fat body demonstrate that fat cells originate from specific precursor-cell clusters that lie in the lateral, ventral, and dorsal mesoderm (Fig. 1; Hoshizaki et al., 1994; Hoshizaki et al., 1995; Riechmann et al., 1998). At stage 10/11, precursor fat cells lie in the lateral mesoderm in a metamerically repeating pattern (Fig. 1A). These cells make up the primary cell clusters and are organized as serially...
duplicated clusters positioned in the *even-skipped* domain of parasegments (PS) 4-9 (Fig. 1A,B). These cell clusters are likely to populate most of the lateral fat body (Hoshizaki *et al.*, 1994; Riechmann *et al.*, 1997). Within the dorsal mesoderm of PS 13 lies a large cluster of cells (Fig. 1A,B); this particular group of cells is likely to make up the dorsal fat-cell projections (Fig. 1E,F; Riechmann *et al.*, 1998). At stage 11, two secondary sets of cell clusters are identified that are serially duplicated along the anterior-posterior axis in the lateral and ventral mesoderm (Fig. 1C,D; Riechmann *et al.*, 1998). Each morphological region of the fat body is thought to arise from spatially distinct precursor-cell clusters, but the lack of cell markers for specific precursor fat cells has made it difficult to confirm this idea.

**Putative UBX Response Elements Are Associated With Enhancer Activity in a Subset of *srp*-Expressing Cells**

We have completed a genomic walk of the *srp* locus and have positioned the *srp* gene within this region (Fig. 2A and see Rehorn *et al.*, 1996). Within this walk is the A7.1ES fragment, which contains three putative UBX protein-binding sites (Fig. 2B; Mastick *et al.*, 1995). We have tested this region for enhancer activity *in vivo*. A7.1ES was subcloned into the P-element vector pCaSpeR-hs43-*lacZ* and transgenic animals were generated. The activity of the A7.1ES-*lacZ* reporter was studied in whole-mount embryos. We found that the A7.1ES-*lacZ* reporter was active in a subset of *srp*-expressing cells, including cells that make up specific regions of the fat body (Figs. 3,4).
The wild-type expression pattern of SRP protein has been described in detail (Sam et al., 1996). Briefly, srp is expressed in fat-cell precursors and in the fat body. It is also expressed in several other tissues: the primordia of the anterior and posterior midgut; the cephalic mesoderm and hemocytes; the amnioserosa primordium and amnioserosa; and the lymph glands (see Fig. 2 in Sam et al., 1996). Within the fat body, we detected A7.1ES-lacZ reporter activity in only a subset of fat-body cells, the ventral- and posterior-most edge of the mature embryonic lateral fat body, the dorsal fat-cell projection and a portion of the ventral commissure (Fig. 3). Using A7.1ES-lacZ as a cell-lineage marker, we have traced the origins of the aforementioned groups of fat body cells (Figs. 4,5). A7.1ES-lacZ reporter activity was detected at stage 10 in a single cluster of cells located within the dorsal mesoderm of parasegment (PS) 13 (Fig. 4A). The cells of this cluster populate the fat-cell projections and the posterior-most cells of the lateral fat body and are described in more detail in the following section. By stage 11, the number of A7.1ES-lacZ-expressing cells in the aforementioned dorsal cell cluster has increased and we began to detect expression in cells located in the ventral region of the mesoderm (Fig. 4B). By stage 12, strong expression was detected in the ventral mesoderm in serially duplicated cell clusters composed of 4-6 cells located in PS 3-11 and in a smaller cluster in PS 12 (Fig. 4C). The ultimate fate of the small cluster in PS 12 is not known, because β-galactosidase activity ceased to be detected in its cells after stage 13. By stage 14, the cell clusters in PS3-11 fused to form a 1-2 cell-wide row that defines the ventral edge of the lateral fat body (Fig. 4G,H). These cell clusters correspond to the secondary ventral fat-cell clusters.
The ventral secondary cell clusters in PS 3-5 also contribute to the ventral commissure as further described in detail below (Riechmann et al., 1998). At stage 16, A7.1ES was active in a portion of the ventral commissure that makes up the posterior bridge, including the bilateral horns, but was not active in the anterior bridge (Fig. 4I). The ventral commissure lies in the anterior region of the embryo and eventually spans the ventral midline (Fig 5). It consists of at least two fat-cell bridges that arise from cells of the secondary ventral cell clusters in PS 3-5. The anterior bridge is derived from cells in PS 3/4 and is located more internally than the posterior bridge (Fig. 5C). The posterior bridge is derived from cells in PS 5 and is associated with the ventral most cells of the lateral fat body (Fig. 5C). Bilateral horns of fat cells extend from the posterior bridge and cross the anterior bridge (Fig. 5).

The A7.1ES-lacZ reporter also was active in the cephalic mesoderm and in the prohemocytes as they migrate from the cephalic mesoderm (Fig. 4B). Reporter gene activity, however, was absent in the majority of haemocytes by stage 14. A7.1ES-lacZ reporter expression also was detected in the amnioserosa, but only in the posterior-most cells (Figs. 4D,E). At stage-12, we detected novel expression of A7.1ES-lacZ in cells that arose from the caudal pole (Fig. 4C). These cells subsequently appeared as rows of cells ensheathing the midgut (Fig. 4F). Based on the morphology and position of these cells (Campos-Ortega & Hartenstein, 1997), it is likely that A7.1ES is active in the longitudinal visceral muscle fibres and their precursors.
Expression of srp in the Dorsal Fat-Cell Cluster Is Controlled Separately from the Remaining Fat-Body Cells

A7.1ES has strong activity in posterior srp-expressing cells (i.e., the posterior amnioserosa and the dorsal cell cluster). We have restricted this posterior enhancer activity to the distal portion of A7.1ES, designated A7.1EB (Fig. 1A). Transgenic lines carrying the A7.1EB-lacZ reporter exhibited activity only in the posterior fat cells and their precursors and in the posterior amnioserosa cells (Fig. 6). Lying partially beneath the posterior precursor fat cells are srp-expressing cells that make up the posterior midgut primordium. The A7.1EB enhancer is not active in these cells. The simple expression pattern driven by A7.1EB has allowed us to carry out a detailed examination of the origin of the posterior fat cells, i.e. the dorsal fat-cell projections and the posterior-lateral fat body, without the difficulties presented by the underlying SRP-expressing posterior midgut primordium. Using A7.1EB-lacZ, we mapped the dorsal cell cluster in the mesoderm relative to the engrailed (en) stripes in the ectoderm (Fig. 7). The majority of the dorsal cell cluster lies between en stripes 13 and 14, which mark the anterior portions of the parasegments in the ectoderm (Lawrence, 1992). A few dorsal cells lie beneath the en stripes, but are not positioned beyond the boundaries of these two stripes.

Using the A7.1EB-lacZ reporter, we detected strong expression at stage 11 in the dorsal cell cluster of PS 13 and in a single cell located immediately ventral to this large cluster (Fig. 6A,B). By early stage 12, the single cell formed a small cluster of approximately 4-6 cells. Although this cell cluster is located in the ventral mesoderm, its
behaviour at later stages revealed that it is distinct from the secondary ventral cell clusters. During germband retraction, the bilateral ventral PS 13 cell cluster fused across the ventral midline (Fig. 6D) and later contributed to the posterior-most cells of the lateral fat body (Fig. 6J). At late stage 12, the dorsal cell cluster began to separate, and by stage 13, two distinct subgroups of cells can be identified (Fig. 6E,G). The dorsal-most subgroup forms the dorsal fat-cell projection and the other subgroup coalesces with the fused ventral cluster to form the posterior region of the lateral fat body (Fig. 6L).

UBX Affects the Morphology of the Fat Body

The precursor fat-cell clusters are located in defined positions within the specific segments of the mesoderm. Because UBX-target sequences map to A7.1EB (Fig. 2), we tested whether the Ubx gene might play a role as a transcriptional regulator of srp. Because Ubx is expressed in the mesoderm of PS 6-12 but not of PS 13 (Akam & Martinez-Arias, 1985; White & Wilcox, 1985), Ubx might function to repress formation of a dorsal cell cluster in anterior segments. We examined the fat-cell phenotypes caused by both the loss-of-function and mis-expression of Ubx. The mis-expression of Ubx throughout the mesoderm was achieved by employing the GAL4/UAS targeted gene expression system of Brand and Perrimon (1993).

We found that Ubx does not have a role in the specification of the dorsal cell cluster. Loss of Ubx function did not induce ectopic dorsal cell clusters in the anterior segments nor did mis-expression of Ubx throughout the mesoderm (including PS13) repress dorsal fat-cell specification (data not shown). We note, however, that mis-expression of Ubx in PS 13 leads to morphological alterations of the dorsal fat-cell projections. The fat-cell
projection is normally 6-8 cells in width at its broadest region. In the Ubx mis-expression embryos, the dorsal fat-cell projections extended properly in the anterior direction, but they were composed of only a single row of cells (Fig. 8B). This change in cell number was also reflected in the number of the A7.1EB-lacZ positive cells (data not shown). On the other hand, the loss of Ubx occasionally caused a slight reduction in the number of cells present in the projections (Fig. 8C). Taken together these data suggest that Ubx does not play a direct role in the trans-activation of srp, but that it is involved in determining segment identity and can affect the differentiation of cells that contribute to the dorsal fat-cell projections.

Surprisingly, Ubx had a dramatic effect on the formation of the ventral commissure even though the precursors to the commissure lie in PS 4-5 and not in the UBX domain. It has been previously shown that loss of Ubx activity results in strong derepression of srp within the secondary ventral cell clusters in PS 6-12 (Fig. 9B and see Fig. 4 in (Mastick et al., 1995). We have followed up on this observation and have found that in Ubx mutants, a third bridge arises from PS 6 (Fig. 9E). Thus, it appears that UBX might be directly or indirectly involved in repressing the formation of fat-cell bridges. To further test this involvement, we asked whether UBX is sufficient to suppress the formation of the endogenous ventral commissure by employing the GAL4/UAS system of Brand and Perrimon (1993). In twi-GAL4;UAS-Ubx embryos, we found that both bridges of the ventral commissure were absent (Fig. 9F). These data suggest that Ubx is involved in suppressing the differentiation of the commissure in posterior segments through its role as
a segment identity gene. Ubx might repress a hierarchy of genes that control whether the fat-cell bridges will arise from the ventralmost cells of the lateral fat body.

**Role of tin and dpp in the Specification of the Dorsal Cell Clusters**

The dorsal cell cluster lies in the dorsal mesoderm. We hypothesize that only cells within the dorsal mesoderm are competent to acquire a dorsal fat-cell fate, and that srp, through the A7.1EB enhancer might be responsive to factors that establish the dorsal mesoderm. Key to the formation of the dorsal mesoderm is the NK homeodomain protein tin (Kim & Nirenberg, 1989). tin is expressed in three phases during embryonic development. In the first phase, it is expressed in a pan-mesodermal fashion. In the second phase, tin expression is lost within the mesoderm except in the dorsal region where it is maintained by the action of dpp in the overlying ectoderm. In the third phase, tin expression is restricted to and marks the precursor to the heart (Azpiazu & Frasch, 1993; Bodmer, 1993; Yin & Frasch, 1998).

Based on the different characterized roles for tin, we predicted that the second phase of tin expression is important for the eventual establishment of the dorsal cell cluster. Moore et al., (1998), however, suggest that early tin expression is important in the specification of fat cells based upon a comparison of the phenotypes associated with the loss of tin and dpp activity. This comparison can distinguish between the first and second phase of tin expression because the second phase, but not the first phase of tin expression, is dependent upon dpp. In tin mutants, in addition to the loss of visceral, heart and dorsal somatic muscle (Azpiazu & Frasch, 1993; Bodmer, 1993), there is a loss of some lateral fat cells
based upon the location and number of srp-expressing cells (see Fig. 4 in Moore et al., 1998). In a dpp mutant, more fat cells appear to be present than in a tin mutant, although because of the gross developmental defects associated with the loss of dpp, it is difficult to determine whether any fat cells are absent (see Fig 4 in Moore et al., 1998). Based upon this comparison, it was suggested that early tin is involved in the specification of fat cells (Moore et al., 1998).

We have re-examined tin's role in fat-cell development using the A7.1EB-lacZ reporter gene as a cell marker for the dorsal cell cluster and the dorsal fat-cell projections. The reporter gene was introduced into both tin- and dpp-mutant backgrounds using standard genetic crosses. We found tin-mutant embryos had few, if any, cells in the dorsal cluster, and concomitantly, there was a loss of the dorsal fat-cell projections (and the posterior-most cells of the lateral fat body) (Fig. 10C,D). To determine whether this loss is due to the first or second phase of tin expression, we examined dpp-mutant embryos. We found that reporter gene activity was nearly absent in the location of the dorsal cell cluster, and concomitantly the dorsal fat-cell projections were absent (Fig. 10F,G). These data suggest that specification of the dorsal cell cluster requires formation of the dorsal mesoderm and depends upon the second phase of tin expression. To further examine the role of dpp-dependent expression of tin, we examined twi-GAL4; A7.1EB-lacZ; UAS-dpp embryos where dpp was expressed throughout the mesoderm. In these experimental embryos, the dorsal cell cluster was expanded to fill most of PS 13, and the dorsal fat-cell projection was not formed (Fig. 10H-J).
Discussion

Over twenty years ago, Rizki and Rizki (1978) suggested that the larval fat body has a segmental origin. Recent studies have firmly established the segmental nature of the precursor fat cells and have identified different classes or groups of metamerically repeating cell clusters that contribute to the embryonic fat body (Abel et al., 1993; Hoshizaki et al., 1994; Riechmann et al., 1998). In our efforts to understand the molecular mechanism underlying the commitment to and specification of a fat-cell fate, we have focused our attention on *srp*, a transcription factor gene involved in the specification (Hayes et al., 2001) and differentiation of fat cells (Sam et al., 1996). We present here, the first analysis of *srp* regulatory regions active in the secondary ventral cell clusters and the dorsal cell cluster. Because the precursor fat cells arise from unique anterior-posterior and dorsal-ventral positions within the mesoderm, it is likely that a combination of positional information and input from different homeotic genes are involved in the establishment and maintenance of fat cells that populate the different regions of the fat body. The capability of *srp* to induce fat-cell formation (Hayes et al., 2001) suggests that this patterning information is integrated at the *srp* locus through the use of the different *srp* fat-cell enhancers. We have examined *UBX* as a possible direct activator of *srp*, but find no evidence that *Ubx* plays a role in establishing the dorsal cell cluster or the secondary ventral cell clusters. Although *Ubx*-mutant embryos displayed morphologically altered fat bodies, *Ubx* does not appear to directly control *srp*’s activation or repression, rather *Ubx* appears to function in maintaining the segment-specific characteristics of fat-cell clusters.
In the dorsal-ventral axis, we find the dorsal cell cluster and secondary ventral cell clusters are sensitive to dorsal-ventral patterning cues. The persistent expression of tin in the dorsal region of the mesoderm subdivides the mesoderm. The secondary ventral cell clusters are located in the ventral mesoderm in PS 3-11, and thus lie outside this tin domain. The loss of tin activity has no effect on the specification of these cells (data not shown); however, the specification of these cells can be repressed by the expansion of the dorsal mesoderm into the ventral region by the mis-expression of dpp through the mesoderm (data not shown).

The dorsal cell cluster lies within the dorsal mesoderm. Loss of the dorsal mesoderm (through the loss of tin or dpp expression) results in the loss of the dorsal cell cluster; conversely, the extension of the dorsal mesoderm results in the expansion of the dorsal cell cluster. tin clearly plays a genetic role in the specification of the dorsal cell cluster. At this time, we cannot distinguish between a direct or indirect role of tin in this process. TIN might be a direct transcriptional activator of srp or TIN might indirectly activate srp through downstream factors intrinsic to the dorsal mesoderm. It is likely, however, that the activation of srp in the dorsal cell cluster will require both TIN and other dorsal mesoderm-intrinsic factors. We find that within the A7.1EB sequence there are TIN-binding sites at position 53 to 36, position 200 to 181 and position 257 to 274 that contain single base-pair differences from the consensus TIN binding site $^5[TCAAGTGG]_3^3$ (Gajewski et al., 1997). The presence of these sites raises the possibility that specification, at least on the dorsal-ventral axis, requires transcriptional activation of srp through the binding of TIN to A7.1EB. We hope that further dissection of the A7.1EB sequence will
help to identify relevant cis-acting sequences that play a role in integrating the combination of positional information cues that activate srp and specify the dorsal cell cluster.

Our analysis of srp regulatory regions reveals that different enhancers control srp expression in specific precursor-cell clusters and that these precursor cells give rise to fat cells that populate specific regions of the fat body. Additional factors intrinsic to the region of the mesoderm from which the precursors arise are likely to help define the overall morphology of the fat body. It has not escaped our attention that our observations also provide a model for the origin of functionally distinct regions in the fat body. The fat body is a diverse and extremely complex tissue involved in many metabolic processes; it is not a functionally homogenous tissue and some of its functions are restricted to cells lying within specific regions of this tissue (reviewed in Haunerland & Shirk, 1995). These so-called functional domains have been characterized mainly within the orders diptera and lepidoptera. For several Drosophila species, eye pigment biosynthesis is compartmentalized within the fat body. The tryptophan pyrrolase metabolite kynurenine is localized in the anterior region of the larval fat body, whereas the pteridine-precursor isoxanthopterin is restricted to the posterior regions (Rizki, 1961). Additionally, in Drosophila melanogaster cells in the posterior larval fat body store more protein granules than do the cells of the anterior fat body (Tysell & Butterworth, 1978). In the flesh fly (Sarcophaga peregrina), anterior fat body protein (AFP), which is similar to mammalian regucalcin (a calcium-binding liver protein), is almost exclusively expressed in the anterior regions of larval fat body (Nakajima & Natori, 2000). The fat bodies of the
Indianmeal moth (*Plodia interpunctella*) and the corn earworm (*Helicoverpa zea*) also compartmentalize the accumulation of storage proteins within distinct regions (reviewed in Haunerland & Shirk, 1995). Because specific precursor-cell clusters populate defined regions of the fat body, we suggest that the combinatorial positional cues regulating *srp* expression might also participate in prepatternning different biochemical functions of the embryonic fat body. These positional cues might regulate as-yet-unidentified genes in specific precursor fat cells to specify their biochemical capability and to define different functional domains within the fat body. Further experiments to precisely map functional domains within the embryonic fat body along with the characterization of the remaining enhancers of *srp* will be necessary to test this idea and establish the genetic hierarchy leading to fat-cell specification by *srp* and the specialization of fat-body cells.

**Experimental Procedures**

**Genomic Walk**

A cDNA clone of *srp* (a generous gift from T. Abel) was used to screen an EMBL-3 SP6/T7 *Drosophila melanogaster* genomic library (Clontech) using standard molecular procedures (Maniatis *et al.*, 1982). The clones were restriction mapped and aligned. Our map is in agreement with the published map of Rehorn and co-workers (Rehorn *et al.*, 1996).

**Drosophila Stocks**

The transgenic lines carrying reporter gene constructs of A7.1EB or A7.1ES were generated by standard microinjection techniques using pCaSpeR-hs43-*lacZ*, a vector.
specifically designed to test for enhancer and tissue-specific regulatory elements in transgenic flies (Thummel & Pirrotta, 1992). A7.1ES is a 1.2kb EcoRI-Sacl fragment and A7.1EB is an EcoRI-BamHI 0.47kb subclone of A7.1ES. The Ubx^{9-22} mutant has a 1587bp deletion that removes the final 1.4kb of the 50kb intron, the splice acceptor site of the 3' exon, and 48 codons of the homeobox, and has a sequence of 6 nucleotides inserted between the deletion breakpoints (Weinzierl et al., 1987). The tin^{346} mutant (generously provided by M. Frasch) has a 103 bp deletion of the transcription unit (Azpiazu & Frasch, 1993). dpp^{H48} is a loss of function allele (Andrew et al., 1997; Held & Heup, 1996; Mason et al., 1997). Ubx and dpp were ectopically expressed using the GAL4-UAS system derived from yeast (Brand & Perrimon, 1993). The stocks used in ectopic expression experiments were: twi-GAL4 (Baylies & Bate, 1996; Greig & Akam, 1993), UAS-Ubx (Akam, 1996), and UAS-dpp (Staehling-Hampton & Hoffmann, 1994). These three lines were provided by the Bloomington Stock Center. The twi-GAL4 driver contains the entire regulatory region of the twi promoter and directs ectopic expression in a pattern similar to that of the endogenous twi gene. twi-driven expression is first detected in the mesoderm and in the mesectoderm at gastrulation. Uniform expression persists in the mesoectoderm and in the mesoderm until early stage 10 and late stage 11, respectively. twi-driven expression also persists in a subset of muscle-cell progenitors until at least stage 12 (Baylies & Bate, 1996).
Immunohistochemical Staining and in situ Hybridization to Whole-Mount Embryos

The generation of SRP antibody and immunohistochemistry to whole-mount embryos has been previously described (Hoshizaki et al., 1994; Sam et al., 1996). Goat anti-rabbit IgG alkaline phosphatase conjugate, anti-β-galactosidase monoclonal antibody, and goat anti-mouse IgG alkaline phosphatase conjugate were obtained from Promega. The anti-\textit{engrailed} monoclonal antibody developed by C. Goodman was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Department of Biological Sciences, Iowa City, IA 52242) (Patel et al., 1989). Double labeling with anti-β-galactosidase and Adh anti-sense RNA was carried out as described by Lloyd and Sankonju (Lloyd & Sakonju, 1991). Adh anti-sense RNA was prepared from a 1.3kb region of Adh coding sequence subcloned from P13E-3 (Heberlein et al., 1985) and inserted into pGEM1 at the \textit{SalI-BamHI} sites (kindly provided by K. Hales). The RNA probe was synthesized using a digoxygenin-labeled uracil triphosphate as described by the manufacturer (Boehringer Mannheim). Chromogenic substrates were X-phosphate and NBT (Boehringer Mannheim).

Microscopy

Embryos were equilibrated in mounting solution (50% glycerol, 150mM NaCl, 10mM Tris-HCl pH 8.0). Microscopic analyses were performed on a Zeiss Axioplan2 microscope using Nomarski optics. Whole-mount embryos were photographed onto Kodak tungsten slide film with a 35mm camera attached to the microscope and digitized by scanning at 600dpi using a U-max S-12 scanner. Digital images of whole-mount
embryos were taken with a Kodak MDS120 digital camera attached to the microscope. Both types of images were assembled into figures using CorelDraw8 software.

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References


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Figure 1. Fat-cell development.

(A, C, E) Lateral views of whole-mount embryos immunostained for SRP protein and (B, D, F) corresponding schematic drawings highlighting precursor fat-cell clusters and fat body domains. (A, B) Stage-10 embryos. The dorsal cell cluster is located in the dorsal mesoderm of PS13 and the primary cell clusters are located in the lateral mesoderm of PS 4-9. (C, D) Stage-11/12 embryos. The secondary ventral cell clusters are located in the ventral mesoderm of PS 3-11. A second group of subsidiary precursor fat cells are located as small cell clusters immediately posterior to the primary cell clusters in PS 4-9 and in the equivalent position in PS 10-12. (E, F) Stage-16 embryos. The fat body is made up of
Figure 1. (*continued*)

three morphological domains: the dorsal fat-cell projections, which extend in the anterior
direction from the posterior-dorsal region of the lateral fat body; the lateral fat body
which spans the lateral region of the embryo; and the ventral commissure which extends
from the anterior-lateral fat body and spans the ventral midline.
Figure 2. Map of genomic srp and sequence of A7.1ES.
Figure 2. (continued)

(A) A schematic representation of the srp gene and a portion of its regulatory region. Genomic clones encompassing the srp walk are indicated as horizontal lines above the map and the location of the A7.1ES and A7.1EB fragments are shown below the map. Short arrows indicate the positions of the UBX response elements (UREs) previously identified in a yeast one-hybrid assay (Mastick et al., 1995). As mapped by Rehorn and coworkers (Rehorn et al., 1996), the two P-element inserts, srp " and srp "z, are shown as triangles below the map and the location of the srp transcript is indicated by an arrow. Restriction sites are B, BamHI; E, EcoRI; S, SalI; Ss, SstI; X, XbaI; Xh, XhoI. (B) Genomic sequence of A7.1ES (GenBank Accession Number: AF707404). Arrows indicate extended core sequences of the UREs and boxes indicate potential TIN-binding sites based on the published consensus TIN-binding site, 5'-TCCAAGTGG-3' (Gajewski et al., 1997). Underlined are the EcoRI site (5'-GAATTC-3') at position 1, the BamHI site (5'-TGCGCA-3') at position 464 and the SalI site (5'-GTCGAC-3') at position 1143. The A7.1ES sequence is defined as an EcoRI-SalI fragment (position 1-1148) and the A7.1EB sequence is defined as an EcoRI-BamHI fragment (position 1-469).
Figure 3. A7.1ES is active in a subset of fat-body cells.

(A) Wild-type, stage-15 embryo stained for A7.1ES-lacZ activity (brown) and Adh transcripts (blue). (B) Enlargement of (A). A7.1ES is active in the dorsal fat-cell projections (overline) and in the posterior-most and ventralmost cells (bracket) of the lateral fat body. A7.1ES is also active in portion of the ventral commissure (see figure 5 and text for more detail).
Figure 4. A7.1 ES-locZ reporter recapitulates a portion of the srr expression pattern.
Figure 4. (continued)

(A) Lateral view of a stage-10 embryo. Reporter activity is first detected in the cephalic mesoderm and in PS13 in a dorsal cluster of cells, which eventually gives rise to the dorsal fat-cell projection and the posterior-most lateral fat body. (B) Lateral view of a late stage-11 embryo. Prohaemocytes are detected as they migrate from the cephalic mesoderm (underline). Transient expression is detected in single cells in the ventral mesoderm (arrow) and later persists as 9 clusters of cells. (C) Lateral and (D) dorsal view of a stage-12 embryo. (C) Reporter gene activity persists in bilateral clusters of cells located in PS 3-11, which are the ventral secondary cell clusters, and is detected in a small bilateral cluster of cells in PS 12. (D) Another small bilateral cluster of cells is detected in the ventral mesoderm of PS 13 adjacent to the dorsal cell cluster. Reporter gene activity is detected in the amnioserosa cells abutted to the posterior edge of the retracting germ band (star in (D)) and the in large dorsal cell cluster in PS 13. Reporter gene activity is also detected in cells likely to be the precursors to the longitudinal visceral muscle fibers (bracket in (C)). (E) Dorsal and (F) lateral view of a late stage-12 embryo. Reporter gene activity is detected in the posterior amnioserosa cells (star in (E)) and in the putative precursor longitudinal visceral muscle cells (bracket in (F)). The ventral cell cluster in PS13 has fused across the midline (arrow in (E)) and the bilateral ventral cluster in PS 12 is absent. (G) Lateral view of a stage 14/15 embryo. Secondary ventral cell clusters have begun to fuse to form the ventral edge of the lateral fat body. (H) Lateral and (I) ventral and (J) dorsal view of early stage-16 embryos. Reporter gene activity is present (H) in the ventral (brackets) and posterior cells of the lateral fat body, (I) in one of the two bridges that form the ventral commissure (bracket) (see text and figure 5 for details) and (J) in the dorsal fat-cell projections (arrowheads) and posterior amnioserosa cells (star).
Figure 5. The ventral commissure is composed of two fat-cell bridges.  
(A) Ventral and (C,D) ventral-lateral views of stage-16 wild-type embryo immunostained for SRP protein. (B) Schematic of the ventral commissure. The ventral commissure is composed of fat cells that span the ventral midline. The anterior bridge of cells extends from the secondary ventral cell clusters in PS 3 and 4 and fuse across the ventral midline. The posterior bridge extends from cells in the bilateral ventral cell cluster in PS 5 and also fuse across the ventral midline. (C) The posterior bridge lies in a more peripheral position within the embryo than the anterior bridge and is a continuation of the ventralmost cells of the lateral fat body. (D) Extending from the posterior bridge are two horns that extend in the anterior direction and crossover the anterior bridge (Second horn is out of the plane of focus).
Figure 6. The A7.1EB-lacZ reporter is expressed in the posterior of the embryo

(A) Lateral and (B) dorsal view of a stage-11 embryo. Reporter gene activity is detected in the dorsal cell cluster of PS13 (square bracket) and in a small bilateral ventral cell cluster (arrow). (C) Lateral and (D) dorsal view of a stage-12 embryo. As the germ band retracts, the bilateral ventral clusters of cells in PS 13 fuse across the ventral midline.
Figure 6. (continued)

Reporter gene expression is also detected in the posterior-most amnioserosa cells (star in (C,D)) and in the dorsal cell cluster (bracket in (C,D)). (E) Lateral and (F) dorsal view of a late stage-12 embryo. As the germ band fully retracts, the dorsal cell cluster begins to separate into two subgroups of cells (square brackets, 1 and 2) and the ventral cluster becomes located below the dorsal cell cluster (arrow in (E)) and is out of the plane of focus in (F). Reporter gene expression is still detected in the posterior amnioserosa cells (star). (G) Lateral and (H) dorsal view of a stage-14 embryo. (I) Lateral and (J) dorsal view of a stage-16 embryo. A subgroup of cells from the dorsal cell cluster (bracket 1 from (G)) forms the dorsal fat-cell projection (bracket 1 in (I,J)) and the second subgroup (bracket 2 from (G)) along with the ventral cell cluster of PS 13 form the posterior-most portion of the lateral fat body.
Figure 7. The dorsal cell cluster is located between *en* stripes 13 and 14.

(A) Lateral and (C) dorsal view of a stage 11 A7.1EB-*lacZ* embryo immunostained for ENGRAILED and β-galactosidase protein. (B) is a higher magnification of (A). The dorsal cell cluster lies in the mesoderm beneath the ectodermal *en* stripes 13 and 14. Cells of the cluster lie within the boundaries of the stripes but do not extend beyond them.
Figure 8. Misexpression of Ubx leads to a morphological alteration of the dorsal fat-cell projection.

(A) Dorsal view of wild-type. (B) twi-GAL4; UAS-Ubx, and (C) Ubx mutant stage-16 embryos immunostained for SRP protein. (A) wild-type dorsal fat-cell projection (arrow) 
(B) Misexpression of Ubx reduces the width of the projections (arrow) to 1-2 cells compared to the normal 6-8-cell width and causes the loss of the lymph glands. (C) Loss of Ubx has little or no effect on the number of cells contributing to the projection (arrow). The lymph glands have hypertrophied (arrowhead) as previously described by Mastick et al., (Mastick et al., 1995).
Figure 9. Misexpression of *Ubx* leads to morphological alterations of the ventral commissure.

(A) Ventral-lateral view of stage-13 wild-type. (B) *Ubx* mutant and (C) *twi-GAL4;UAS-Ubx* embryo immunostained for SRP protein. (B) Loss of *Ubx* results in the derepression of *srp* in the ventral cell clusters in PS 6-12 while (C) misexpression of UBX throughout the mesoderm has little or no effect on *srp* expression in the ventral cell clusters. (D) Ventral view of stage-16 wild-type. (E) *Ubx* mutant and (F) *twi-GAL4;UAS-Ubx* embryos immunostained for SRP protein. (E) Loss of *Ubx* results in the formation of an ectopic fat-cell bridge within PS 6 (bracket) while (F) misexpression of *Ubx* throughout the mesoderm represses the formation of the ventral commissure.
Figure 10. Specification of the dorsal cell cluster requires the formation of the dorsal mesoderm.

Lateral views of (A,B) A7.1EB-lacZ wild-type and (C,D) A7.1EB-lacZ; tin-mutant
embryos immunostained for β-galactosidase. (C) In a tin-mutant embryo at stage-12, the dorsal cell cluster consists of a few cells as revealed by the loss of lacZ-expressing cells and results in (D) the loss of the fat-cell projections (and posterior-most cells of the lateral fat body). (A1) and (C1) are higher magnification of (A) and (C), respectively. Dorsal view of (E) A7.1EB-lacZ wild-type and (F) dpp; A7.1EB-lacZ embryos. (F) A dpp-mutant embryo (approximately stage-12) has reduced number of cells in the dorsal cell cluster. The PS 13 ventral cell cluster appears unaffected, and the wild-type fusion of these two cell clusters takes place across the ventral midline. (G) A dpp-mutant embryo (approximate stage-16) exhibits little to no reporter gene activity. Dorsal view of (H) stage-12 and (I) stage-15 twi-GAL4; UAS-dpp; A7.1EB-lacZ embryos immunostained for β-galactosidase. Misexpression of dpp causes the ventral expansion of the dorsal fat-cell cluster to occupy most of PS 13. (J) Lateral view of (I).
CHAPTER 3

SERPENT, A GATA-LIKE TRANSCRIPTION FACTOR GENE, INDUCES FAT-CELL DEVELOPMENT IN DROSOPHILA MELANOGASTER

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Prologue

Chapter 3 is a published paper co-authored by Steven A. Hayes, Jennell M. Miller and Deborah K. Hoshizaki. Within this work, post-doctoral fellow Hayes demonstrates that misexpressed *serpent* (*srp*) activity results in ectopic fat-cell production throughout the mesoderm. The ectopic fat cells formed develop at the expense of visceral muscle precursors and cause a disruption in the development in other tissues, such as somatic musculature and somatic gonadal sheath precursors (SGP) cells.

Prior to this study, Riechmann and co-workers (1998) had proposed that *srp* functions in a switch mechanism that allows equivalent cells to make a choice between primary fat cells and SGP cell fates. This model is based on the observation that primary fat-cell precursors and SGPs develop in the equivalent dorsal/lateral and anterior/posterior position within the parasegment; cells in the appropriate position within parasegments 4-9 develop into primary fat cells, whereas equivalently positioned cells in parasegments 10-12 develop into SGPs (Riechmann et al., 1998; Moore et al., 1998). The observation that fat-cells appear to replace SGP clusters in *srp* mutants supports this model (Riechmann et al., 1998). However, if *srp* serves as switch between fat cells and SGP cells in the lateral mesoderm, the predicted outcome of *srp* misexpression is a failure in SGP-cell specification. Dr. Hayes's study provided me with an excellent opportunity to test this model.

I found that misexpression of *srp* does not disable the specification of SGP cells (this chapter) but, it does repress another gene, *clift*, that is necessary for the coalescence of the cells given rise to by the SGP cells. Thus, *srp* disrupts the formation of the gonads but not their specification. An interesting observation in this study was that visceral muscle
precursors were completely obliterated in \textit{srp} misexpression embryos, suggesting that \textit{srp} blocks the specification of these cells. Based upon these data, it is plausible that a switch mechanism might exist between visceral muscle precursors and dorsal fat-cells, and in Chapter 4 I describe the results of testing these ideas.


Summary

The GATA-like transcription factor gene \textit{serpent} is necessary for embryonic fat-cell differentiation in \textit{Drosophila} (Sam et al., \textit{Mechanisms of Development} 60, 197-205,1996) and has been proposed to function in a cell-fate choice between fat-cell and somatic gonadal precursors (Moore et al., \textit{Development} 125, 837-44,1998; Riechmann et al., \textit{Development} 124, 2915-22,1998). Here, we report that deregulated expression of \textit{serpent} in the mesoderm induces the formation of ectopic fat cells and prevents the migration and coalescence of the somatic gonadal precursors. The ectopic fat cells do not arise from hyperproliferation of the primary fat-cell clusters but they do associate with endogenous fat cells to form a fat body that is expanded in both the dorsoventral and anteroposterior axes.

Misexpression of \textit{serpent} also affects the differentiation of muscle cells. Few body-wall muscle precursors are specified and there is a loss of most body-wall muscle fibers. The precursors to the visceral mesoderm are also absent and concomitantly the visceral muscle is absent. We suggest that the ectopic fat cells might originate from cells that have the potential, but do not normally, differentiate into fat cells or from cells that have

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acquired a fat-cell fate. In light of our results, we discuss the role of *serpent* in fat-cell specification and in cell-fate choices.

**Introduction**

During the course of embryogenesis the potential of cells becomes increasingly restricted as they are programmed to adopt a specific cell fate or lineage. The subdivision of the *Drosophila* embryo by anteroposterior and dorsoventral patterning genes plays a critical role in providing cells with a progressively restricted identity (Azpiazu et al., 1996; Frasch, 1995; Riechmann et al., 1997; Staehling-Hampton et al., 1994). Although it is well established that positional information helps to guide cell-fate choices, little is known about the molecular mechanisms that control the final steps of cell specification and the irreversible commitment (or determination) of cells to differentiate as a specialized cell type.

Fat body, visceral and somatic muscle, heart (dorsal) muscle, hemocytes, gonad-sheath cells (gonadal mesoderm), and peritracheal cells arise from the mesoderm (Hartenstein and Jan, 1992; Hoshizaki et al., 1994; Technau, 1987). In *Drosophila*, the subdivision of the mesoderm provides positional cues for individual cell fates. For example, in the dorsoventral axis, *decapentaplegic (dpp)*-dependent activation of *tinman (tin)* is necessary for the specification of the dorsal mesoderm and consequently the precursors to visceral muscle (Frasch, 1995; Staehling-Hampton et al., 1994). In the anteroposterior axis, pair-rule genes such as *even-skipped (eve)* and *sloppy-paired (slp)* subdivide the mesoderm into metamerically repeating units and aid in defining
mesodermal sub-populations such as the precursors to fat cells and gonadal mesoderm (Azpiazu et al., 1996; Riechmann et al., 1997).

Although numerous genes responsible for anteroposterior and dorsoventral patterning of the mesoderm have been identified, only a few genes (e.g. tin, bap) have been shown to respond to this patterning information and to regulate the commitment of cells to a single developmental pathway. The gene serpent (srp) responds to patterning information (Azpiazu et al., 1996) and is a candidate target gene for specifying fat cells and triggering fat-cell differentiation (Sam et al., 1996; Rehorn et al., 1996). Fat cells are derived from several spatially distinct clusters of cells that coalesce into a single-cell thick fat body. The fat body is morphologically made up of three domains: the lateral fat body, the dorsal fat-cell projection and the ventral collar (Hoshizaki et al., 1994; Moore et al., 1998; Riechmann et al., 1998). The bulk of the fat body is organized into a lateral fat body that forms a bilateral ribbon of cells interrupted by the protrusion of internal organs. Extending from the posterior of the lateral fat body and lying upon the dorsal side of the embryo are the dorsal fat-cell projections that form oblong protrusions of fat cells flanking the dorsal vessel (Hoshizaki et al., 1994; Riechmann et al., 1998). The third morphological domain is the ventral collar that extends from the anterior region of the lateral fat body and spans the ventral midline (Campos-Ortega and Hartenstein, 1997; Miller and Hoshizaki, unpublished). Each morphological domain arises from spatially distinct clusters of cells. The lateral fat body arises from primary fat-cell clusters located in the eve domain of parasegment (PS) 4-9 and from two secondary sets of fat-cell clusters. One set arises immediately posterior to the primary fat-cell clusters and the other set arises in a position
ventral to the primary clusters in the *slp* domain in PS 3-12. The dorsal fat-cell projections arise from a large cluster of cells in PS 13 (Riechmann et al., 1998).

*srp* is an essential gene for fat-cell development. *srp* is one of three *D. melanogaster* genes belonging to the GATA transcription factor family and was initially identified as a transcriptional activator of the *alcohol dehydrogenase (Adh)* gene in fat cells (Lin et al., 1995; Ramain et al., 1993; Winick et al., 1993; Abel et al., 1993). Analysis of *srp* expression during embryogenesis reveals the presence of SRP protein at stage 10/11 in all the fat-cell precursors (Sam et al., 1996). *srp* is the earliest expressed fat-cell gene and is likely to play a role in fat-cell specification or differentiation (Abel et al., 1993; Rehorn et al., 1996; Sam et al., 1996). Based on mutational analysis, *srp* is necessary for maintaining a fat-cell identity in the lateral fat body (Sam et al., 1996). In transcript- and protein-expressing *srp* null mutants, *srp* cells are readily detected in the position of the fat-cell precursors but later undergo apoptosis (Sam et al., 1996). We find that loss of *srp* function also does not prevent early wild-type expression of an enhancer-trap line that marks the precursor fat cells that give rise to the lateral fat body (Sam et al., 1996). Thus, *srp* appears not to be necessary for specification of the lateral fat body, but it is necessary for the maintenance of fat-cell differentiation.

Based on several lines of evidence, however, Riechmann et al., (1998) and Moore et al., (1998) have suggested *srp* also controls the fate of a common precursor in PS 4-12. The presence of *srp* in the common precursor in PS4-9 specifies the primary fat-cell clusters while the absence of *srp* activity in the common precursor in PS10-12 allows specification of the somatic gonadal precursors (SGPs). A prediction of the common
precursor model is that srp is capable of inducing fat-cell formation and suppressing SGP specification. In this report, we have directly tested srp's role by forcing expression of srp in the mesoderm. We show that expression of srp throughout the mesoderm results in the production of ectopic fat cells. These excess fat cells do not arise from hyperproliferation of pre-existing precursor fat cells and thus srp is able to induce fat cells from cells that normally would not give rise to fat cells. While on the surface these results are in favor of the common precursor model, we find that forced expression of srp does not affect the specification of the SGPs; rather, the SGPs fail to migrate and coalesce to form the gonads. We present an alternative model for the choice between precursor fat cells and SGPs and discuss the role of srp in fat-cell and SGP specification.

Materials and Methods

Fly Stocks

The twist-GAL4, 24B-GAL4, and 69B-GAL4 stocks were provided by the Drosophila Stock Center, Bloomington, Indiana. The twist-GAL4 driver contains the entire regulatory region of the twist (twi) promoter and drives expression in a pattern similar to that of the endogenous twi gene. twi-driven expression is first detected in the mesoderm and in the mesectoderm at gastrulation. Uniform expression persists in the mesoectoderm and in the mesoderm until early stage 10 and late stage 11, respectively. twi-driven expression also persists in a subset of muscle progenitor cells until at least stage 12 (Baylies and Bate, 1996). The 24B-GAL4 line drives expression in the mesoderm at stage 11 and later in embryonic muscle (Brand and Perrimon, 1993). The 69B-GAL4 line directs expression in
the ectoderm starting at stage 9 (Brand and Perrimon, 1993; Castelli-Gair et al., 1994; Staehling-Hampton et al., 1994). UAS-srp flies were generated by P-element mediated germline transformation of the pUAST-srp plasmid into w1118 embryos.

**Plasmids**

pUAST-srp was assembled by subcloning the entire srp coding sequence into pUAST (Brand and Perrimon, 1993). The srp cDNA clone pABF (Abel et al., 1993) does not contain the complete 5'-end. To construct a full-length clone, the 5' end of srp was amplified from genomic DNA using the polymerase chain reaction (PCR). Primers were designed based on the full-length cDNA sequence from GENBANK (accession #Y07662) and included an engineered EcoRI site at the 5' end. The PCR product was isolated after restriction enzyme digestion by EcoRI and NotI; the incomplete srp cDNA was cut at the endogenous NotI site and SpeI site of the polylinker and isolated. These two fragments were then ligated into pUAST that had been previously digested with EcoRI and XbaI.

The clift plasmid, pBlue-eyaII (Bonini et al., 1993), was a generous gift from S. DiNardo. Robert Storti generously provided Tropomyosin I (TmI) (Bautch et al., 1982) and S59A (Dohrman et al., 1990) contained in pBluescript KS+.

**In situ Hybridization to Whole-Mount Embryos**

*In situ* hybridization was carried out as described previously (Hoshizaki et al., 1994). Antisense RNA probes for Adh, DCg1, nau and the DNA probe for 412 have been previously described (Hoshizaki et al. 1994; Tan et al. 1996). Digoxigenin substituted cli, S59 and TmI antisense RNA probes were synthesized *in vitro*. Following hybridization to fixed embryos, transcripts were detected using alkaline phosphatase-conjugated anti-
digoxigenin antibody (Boehringer-Mannheim), followed by the addition of chromogenic substrates, X-phosphate and NBT (Boehringer-Mannheim).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (Hoshizaki et al., 1994). Mouse anti-β-galactosidase and anti-mouse alkaline phosphatase were obtained from Promega. Anti-serpent antiserum was raised against the DNA-binding domain of srp fused to GST protein (Pharmacia) and affinity purified (Sam et al., 1996). Alkaline phosphatase was detected using X-phosphate and NBT (Boehringer-Mannheim).

**Microscopy**

For microscopic analysis, embryos were equilibrated in mounting solution (50% glycerol, 150mM NaCl, 10mM Tris-HCl pH 8.0). Whole-mounted embryos were imaged on the Zeiss Axioplan2 microscope using Nomarski optics and documented using either a Kodak MDS120 digital camera or a 35mm camera attached to the microscope. Slides and prints were scanned at 1600dpi and 600dpi, respectively with a U-Max S-12 scanner. CorelDraw 8 software was used to prepare figures from digital images.

**Results**

**srp Can Promote Widespread Fat-Cell Development**

To test whether srp is sufficient to induce fat-cell development, we employed the GAL4 targeted expression system described by Brand and Perrimon (Brand and Perrimon, 1993). twist-GAL4 (Baylies and Bate, 1996) and UAS-srp constructs were used to express srp throughout the mesoderm beginning at gastrulation. In stage 10-11 embryos, srp
expression is readily detected in the primary fat-cell clusters in PS 4-9 and in the secondary fat-cell clusters in PS 10-12 (Fig. 1A). In twist-GAL4;UAS-srp embryos, srp expression is expanded into a continuous band of cells throughout the mesoderm (Fig. 1B). Several molecular cell markers were used to monitor the effects of ectopic srp expression on the segregation of mesodermal derivatives. Two genes, alcohol dehydrogenase (Adh) and Drosophila collagen gene 1 (DCg1) were used to detect the fat body. Both Adh and DCg1 are embryonic terminal fat-cell differentiation markers. Adh expression is first detected during embryogenesis at stage 14 in the anterior wall of the midgut and the atrium of the posterior spiracle, and in the late precursor fat cells at stage 15 (Hoshizaki et al., 1994). By stage 16, high levels of Adh transcripts are detected in the lateral fat body and lower levels are found in the dorsal fat-cell projections (Fig. 2A) (Hoshizaki et al., 1994). Within the developing fat cells, the temporal expression pattern of DCg1 is similar to Adh although DCg1 transcripts are easily detected at equal levels in both the dorsal fat-cell projections and the lateral fat body by stage 16 (Fig. 2C,E). DCg1 is also expressed in the hemocytes (Hoshizaki, 1994; references therein). In twist-GAL4;UAS-srp embryos, the premature expression of srp throughout the mesoderm does not induce premature expression of Adh. As in wild-type embryos, Adh transcripts were first detected in the fat cells of experimental embryos at stage 15. The Adh-positive fat cells however, occupied most of the lateral mesoderm and formed an expanded lateral fat body (Fig. 2B).

Because srp is a direct transcriptional activator of the Adh promoter in transient transfection assays (Abel et al., 1993), the increase in Adh-expressing cells might be the
result of ectopic transactivation of Adh by srp rather than a reflection of the activation of the entire fat-cell genetic program. To test this possibility, we examined the expression pattern of a second fat-cell marker, DCg/l. In embryos carrying twist-GAL4;UAS-srp, the temporal pattern of DCg/l expression was also normal. DCg/l transcripts were first detected in hemocytes and then in fat cells at stage 15. The number of DCg/l-expressing hemocytes was comparable to that of wild type. However, similar to the results obtained with Adh, there was an increase in the number of DCg/l-expressing fat cells (Fig. 2D). The DCg/l-expressing fat cells formed an expanded lateral fat body and dorsal fat-cell projections were expanded and fused with the lateral fat body in the posterior region of the embryo (Fig. 2D,F). An increase in lateral fat body cells was also detected in experimental embryos using a third fat-cell marker, imaginal disc growth factor 3 gene, IDGF3 (Kawamura et al., 1999) (data not shown). Similar results, albeit less extreme, were obtained using a second mesodermal GAL4 driver, 24B, that is first active in the presumptive mesoderm (data not shown) (Brand and Perrimon, 1993). These data demonstrate that ectopic expression of srp in the mesoderm leads to an increase in cells composing the lateral fat body and dorsal fat-cell projections.

To determine whether mesoderm-specific components are necessary for srp to promote fat-cell development, we ectopically expressed srp in the ectoderm starting at stage 9 using the 68B-GAL4 driver (Castelli-Gair et al., 1994). We found that ectopic expression of srp in the ectoderm is not sufficient to induce either Adh or DCg/l in the ectoderm, nor does it alter the normal development or morphology of the lateral fat body.
and dorsal fat-cell projections (data not shown). These data suggest that \textit{srp} requires mesodermal factors for induction of fat cells.

**The Increase in Fat Cells Is Not Due to Hyperproliferation of the Endogenous Fat-Cell Lineage**

At least two distinct mechanisms could account for the expansion of the fat body in the \textit{twist-GAL4;UAS-srp} embryos. On one hand, the misexpression of \textit{srp} could alter cell fates within the mesoderm by recruiting cells into the fat-cell developmental pathway. Alternatively, premature and ectopic expression of \textit{srp} in the mesoderm could cause hyperproliferation of endogenous precursor fat cells. To distinguish between these possibilities, we employed the P-element enhancer-trap line, P[29D], as a \textit{lacZ} reporter gene for the primary fat-cell clusters in PS 4-9 and in the secondary fat-cell clusters in PS 10-12 (Fig. 3A,C,E) (Hoshizaki et al., 1994). Early expression of P[29D] is independent of \textit{srp} (Sam et al., 1996). By marking precursor fat cell with the P[29D] \textit{lacZ} reporter we could test whether expression of \textit{srp} throughout the mesoderm causes hyperproliferation of endogenous fat cells. We found \textit{lacZ} expression was not significantly altered in \textit{twist-GAL4;UAS-srp} embryos. Specifically, the number and organization of the fat cells marked by P[29D] appeared normal (Fig. 3B). Thus, the increase in fat cells is likely not to be due to hyperproliferation of endogenous fat-cell lineage although we have not eliminated the possibility that the ectopic fat cells originate from the secondary fat-cell clusters not marked by P[29D].
Misexpression of *srp* Disrupts Gonad Formation

The forced expression of *srp* in the mesoderm results in the production of ectopic fat cells. Because it is not likely that the endogenous fat-cell lineage undergoes hyperproliferation, we suggest that *srp* might be capable of inducing fat-cell development in cells that normally would not contribute to the fat body. Such a capability has been proposed for *srp* in the developmental choice model between somatic gonadal precursors (SGPs) and precursor fat cells (Moore et al., 1998; Riechmann et al., 1998). If *srp* activity can direct a fat-cell fate upon a common precursor that otherwise would be specified as SGPs, then it follows that ectopic expression of *srp* should repress the specification of SGPs in PS 10-12 and these cells should be replaced by fat cells.

To test for *srp*’s role in the repression of SGP specification, we examined *twist*-GAL4;UAS-*srp* embryos using 412 as a cell marker for the SGPs and the gonadal mesoderm (Brookman et al., 1992). In wild-type stage-12 embryos, 412 is expressed in PS10-12 in the SGPs and in cell clusters in PS 2-9 and PS 14 (Fig. 4A). During germband retraction, expression of 412 declines but persists in the SGPs (PS 10-12) (Brookman et al., 1992). It is unclear which cell lineage(s) the PS 2-9 and PS 14 clusters of cells represent (Tan et al., 1996). However, by over-staining for 412 transcripts we can detect late 412 expression in the dorsal-most cells of the lateral fat body (Hoshizaki and Hayes, unpublished). At stage 13, the SGPs migrate and coalesce to form the gonadal mesoderm cells which will eventually ensheath the germ cells (Fig. 4C,E). At stage 16, 412 transcripts are still detected in the gonadal mesoderm (Fig. 4G,I) (Brookman et al., 1992).
In stage-12 twist-GAL4;UAS-srp embryos the expression of 412 is similar to that observed in wild-type embryos (compare Figures 4A and 4B). The SGPs are specified and initial association of pole cells with gonadal precursors appears normal (data not shown). The first alteration in the development of the gonadal mesoderm is detected at stage 13 when the SGPs fail to migrate and coalesce (Fig. 4D,F). The number of 412-expressing cells gradually declines in the experimental embryos and the few remaining 412-expressing cells are dispersed in the posterior region of the embryo (Fig. 4H,J). Misexpression of srp in the mesoderm does not affect the formation of the SGPs, however it does disrupt the ability of the SGPs to migrate and coalesce into a gonad.

To confirm the effects of srp on SGP and gonad formation, we examined twist-GAL4;UAS-srp embryos for expression of a second gonadal mesoderm cell maker, clift (cli) (Boyle et al., 1997). cli is expressed throughout the mesoderm but by early stage 11 is lost in most mesodermal cells. During late stage 11, cli expression is detected in SGPs and in lateral muscle precursors as well as in the ectoderm (Boyle et al., 1997). Based on mutational analysis of a transcript-producing cli mutant, cli \textsuperscript{fle}, cli is necessary for maintenance of SGPs and their migration and coalescence into a gonad (Boyle et al., 1997).

In twist-GAL4;UAS-srp embryos, misexpression of srp does not affect early cli expression in the mesoderm (Fig. 5B). However, in older embryos, cli transcripts are not detected in either the SGPs or in the precursors to the lateral muscles although cli expression is still detected in the ectoderm (see Fig 5D). Because cli is necessary for the migration and coalescence of the SGPs, the inability of the SGPs to form a mature gonad...
is most likely due to the loss of \textit{cli} expression in these cells. We suggest that in the experimental embryos, \textit{srp} does not prevent the specification of the SGPs but can block the differentiation of the SGPs by repressing \textit{cli} expression in these cells.

\textbf{Ectopic Fat Cells Are Correlated With the Loss of Muscle Cells}

To test whether other lineages are affected by misexpression of \textit{srp}, we examined \textit{twist-GAL4;UAS-srp} embryos for heart and visceral muscle precursors. To mark heart precursors, we employed the homeobox gene \textit{tinman (tin)} (Bodmer, 1993). \textit{tin} is involved in the specification of the dorsal mesoderm and in the formation of heart muscle precursors (Azpiazu and Frasch, 1993). In embryos carrying \textit{twist-GAL4;UAS-srp}, heart precursors develop normally to form the heart (data not shown). Thus, ectopic expression of \textit{srp} does not affect heart development.

We used the \textit{bagpipe (bap)} gene to mark the visceral mesoderm, which gives rise to visceral muscle precursors. The visceral mesoderm is made up of 10 metameric clusters of cells located in the dorsal mesoderm (Fig. 6A). \textit{bap} is expressed in these cells and is necessary for the formation of the visceral muscle (Azpiazu and Frasch, 1993). We found that \textit{twist-GAL4;UAS-srp} embryos lacked the \textit{bap}-expressing cells (Fig. 6B) and exhibited a \textit{bap}-like phenotype, in which the midgut failed to undergo its normal constrictions (Fig. 6D) (Azpiazu and Frasch, 1993). The loss of \textit{bap}-expressing cells in the experimental embryos might reflect a cell-fate change that allows the replacement of visceral muscle precursors by fat-cell precursors (see discussion).
Finally, we investigated the effect of srp misexpression on somatic or body wall muscle. The absence of cli-expressing lateral (body wall) muscle precursors suggests that these muscles might be absent in the experimental embryos (see below). The body-wall muscle is derived from the lateral region of the slp domain that exhibits the highest levels of twist expression (Borkowski et al., 1995). We used nautilus (nau) and S59 as cell markers for a subset of the founder or precursor cells for body-wall muscle (Dohrmann et al., 1990; Michelson et al., 1990; Paterson et al., 1991) and tropomyosin I (Tm1) gene as a marker for body-wall muscle fibers (Bautch et al., 1982). nau is first active at stage 10 and is expressed in a dynamic pattern (Michelson et al., 1990; Paterson et al., 1991). nau expression is detected in cells flanking the ventral midline and later in lateral and dorsal-lateral cell clusters. S59 expression is initially detected in a single, large mesodermal cell in a segmentally repeating pattern. At late stage-11 these cells give rise to two founder muscle cells and in each segment a second cluster of four S59-expressing cells appears that also contributes to precursor muscles (Dohrmann et al., 1990). In embryos carrying twist-GAL4;UAS-srp, the early nau and S59 pattern of expression is disrupted and various nau- and S59-expressing cells are absent (Fig. 7).

To determine whether there is a general effect on body-wall muscle, twist-GAL4; UAS-srp embryos were stained for the body-wall muscle marker Tm1. Consistent with the loss of nau- and S59-expressing cells, we found a dramatic loss of muscle fibers (Fig. 8B,D). The few remaining muscle fibers were mono- and bi-nucleated and their number and location varied from embryo to embryo. These remaining muscle fibers might reflect an incomplete loss of the founder muscle cells and/or myoblasts. It is possible that the
remaining fibers are due to loss of bap expression, which leads to visceral mesoderm precursors assuming a somatic muscle identity (Azpiazu and Frasch, 1993). We conclude that the ectopic expression of srp in the mesoderm can disrupt body-wall muscle differentiation.

Discussion

srp has several roles in the developing fat cell. srp is a direct transcriptional activator of Adh in the fat body (Abel et al., 1993) and is involved in the larval fat-cell specific expression of Cecropin A1 (Petersen et al., 1999). Based on genetic studies, using mis-sense and non-sense srp mutants, srp is also necessary for maintaining the fat-cell lineage (Sam et al., 1996 and see figure 6 in Rehorn et al., 1996). Recently, Moore et al. (1998) and Riechmann et al. (1998) have suggested that the primary fat-cell clusters in PS 4-9 and the SGPs in PS 10-12 are derived from homologous metameric clusters of cells. Central to determining the fate of these cells is srp; srp activity specifies a fat-cell fate in the precursor cells that otherwise would adopt an SGP fate.

Here, we have further examined the role of srp in fat-cell development and its possible role in this developmental switch. We find that srp, in addition to maintaining the fat-cell lineage, is sufficient to promote fat-cell development. We favor the idea that production of ectopic fat cells is not due to the hyperproliferation of the endogenous fat cells but rather is the result of changes in either cell fate or proliferation of potential fat cells. The capability of srp to generate fat cells is also dependent upon other factors because premature expression of srp does not result in premature expression of fat-cell genes nor does misexpression of srp in the ectoderm result in ectopic fat cells. It is likely that the
preservation of the correct temporal sequence reflects the dependence of srp upon temporally restricted mesoderm factors and/or whether a cell has previously acquired the potential to develop as a fat cell.

Is srp Involved In a Developmental Choice Between Fat Cells and SGPs?

A model has been suggested by the work of the Leptin and Lehmann groups that predicts that within PS 4-12 of the lateral mesoderm there reside metamerically repeating precursors that have the potential to give rise to either precursor fat cells or SGPs. Based on mutational studies, segmentation and dorsoventral patterning genes control srp activity within the common precursors (Riechmann et al., 1998; Moore et al., 1998). In PS 4-9 srp is active in the common precursor while in PS10-12 the presence of abdominal-A represses srp in the common precursor. Double mutant analysis of abdA and srp confirms that abdA’s role in SGP formation is solely to repress srp (Moore et al., 1998). The choice between fat-cell precursors and SGPs is dependent upon srp, where srp activity induces fat-cell specification while blocking SGP specification. The ground or default state of the common precursor is a somatic gonadal mesoderm cell fate. Thus, in the absence of srp the SGPs are specified (Riechmann et al., 1998; Moore et al., 1998).

We have tested srp for some of its proposed activities in this cell-fate switch model. We find that srp, indeed, is capable of inducing fat-cell formation. srp can also disrupt gonad formation and appears to do so by preventing cli expression in the SGPs. cli function is necessary for 412 expression and for the migration and coalesce of the SGPs to form the gonads but not for the specification of the SGPs (Boyle et al., 1997). In the srp
misexpression embryos, early expression of \textit{cli} and \textit{412} is normal; both wild-type pan-mesoderm expression of \textit{cli} and the metameric repeating expression of \textit{412} are readily detected. The normal decline of \textit{412}-expression takes place in PS 2-9 and \textit{412}-expression persists in the SGPs (PS10-12). The two most notable effects of misexpression of \textit{srp} are the absence of \textit{cli} expression in the SGPs and the inability of the SGPs to migrate and coalesce to form the gonads. It is likely that the defect in gonad formation is due to loss of \textit{cli} expression specifically in the SGPs. We also note that loss of \textit{cli} expression in the SGP does not affect the \textit{412}-specific SGP expression. It is likely that the early pan-mesoderm expression of \textit{cli} is responsible for \textit{412} expression.

We find that \textit{srp} has only one of its two predicted activities required for the common precursor model. \textit{srp} is capable of inducing fat-cell formation but is not able to repress SGP specification. To reconcile these results, we suggest that the precursors to the SGPs and the fat cells are derived from independent groups of cells and that positional cues within each parasegment specifies which group will differentiate and be maintained. This model, in general terms, is analogous to the interaction between the Wolffian and Müllerian ducts. In early mammalian development, before gonadal differentiation both ducts are present. In females the Müllerian duct develops into the oviduct and the Wolffian duct degenerates, while in males the production of Müllerian inhibition substance in the testes causes the degeneration of Müllerian ducts and the Wolffian duct develops into the vas deferens (Higgins et al., 1989).

In this alternative model, the absence of \textit{srp} activity in PS 10-12 would allow the SGPs to be specified and differentiate while the fat-cell precursors would not be maintained. We
have previously demonstrated that *srp* is necessary for the maintenance of the fat-cell lineage (Sam et al., 1996). In PS 4-12 *srp* activity would allow the proliferation and maintenance of the precursor fat cells and the SGPs would not differentiate. We report here that *srp* has the capability to block SGP differentiation by repressing *cli* expression.

**srp Can Disrupt Muscle Differentiation**

The misexpression of *srp* in the mesoderm also results in the loss of body wall and visceral muscle. The body wall muscle consist of a syncytium that is derived from a single founder muscle cell that serves as a focal point for fusion with the surrounding myoblasts (Knirr et al., 1999; Rushton et al., 1995). We used *nau* and *S59* to mark some of the founder muscle cells and find that misexpression of *srp* results in the loss of both *nau*-expressing and *S59*-expressing founder muscle cells and consequently loss of body wall muscles. Based on genetic analysis, *nau* is a non-essential gene that is not necessary for the formation of muscle precursors. However, a distinct subset of muscle fibers is missing in individuals lacking *nau* activity (Keller et al., 1998). The misexpression of *srp* results in a severe muscle defect where most muscle fibers and myoblasts are absent. It is likely that this is due to the absence of the founder muscle cells. Whether the loss of myoblasts is a secondary effect caused by the loss of founder muscle cells or is a direct consequence of *srp* expression is not known.

The misexpression of *srp* also results in the loss of the visceral muscle. This loss is due to the absence of the precursor cells as marked by *bap*-expression in stage-10 embryos. Because *bap* is the earliest known marker for the visceral muscle precursors, we cannot distinguish between loss of *bap* expression from loss of the precursor cells. We favor the
idea, however, that *srp* prevents the specification of the precursors to the visceral mesoderm prior to *bap* expression.

It is clear that *srp* is capable of disrupting the establishment and differentiation of specific mesodermal derivatives and inducing fat cell formation. The origin of the ectopic fat cells however is unknown. The fat body is derived from cells that originate in distinct positional addresses. In the lateral mesoderm the primary fat-cell clusters and the SGPs represent metamERICally repeating clusters. We suggest that the precursors to the primary fat-cell clusters and the SGPs coexist in each parasegment and misexpression of *srp* results in the formation of ectopic fat cells by allowing the differentiation of precursor fat cell in PS 10-12.

In the dorsal mesoderm in PS 2-13 the precursors to the visceral muscle and the dorsal fat cell projections might represent homologous metamERIC repeating cell clusters (Riechmann et al., 1998; Miller and Hoshizaki, unpublished results). Misexpression of *srp* also leads to the complete absence of visceral muscle precursors. We suggest that a common precursor might exist between the precursors to visceral muscle and dorsal fat-cell projection and *srp* might control a developmental switch between these two cell types. Alternatively, the precursors to visceral muscle and dorsal fat-cell projection might coexist in each parasegment (PS2-13) and *srp* determines which cell type will flourish. In either model, the misexpression of *srp* would result in ectopic fat cells. In the common precursor model, misexpression of *srp* would drive the common precursors in PS 2-12 to a fat-cell fate and in the alternative model, misexpression would allow the coexisting
precursor fat cells in PS 2-12 to differentiate. Experiments to further test these ideas and to
identify factors that cooperate with srp in fat-cell development are in progress.

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Figure 1. Ectopic srp expression in a twist-GAL4;UAS-srp embryo.

(A,B) Lateral view of stage-11 embryos stained for srp mRNA. (A) Wild-type embryo. srp is expressed in the primary fat-cell clusters in PS 4-9 and in the secondary fat-cell clusters in PS 10-12. (B) twist-GAL4;UAS-srp embryo. srp is expressed throughout the mesoderm.
Figure 2. Ectopic expression of srp leads to expansion of the fat body.

(A,B) Stage-16 embryos stained for Adh mRNA. (A) Lateral view of a wild-type embryo. The lateral fat body consists of a ribbon-like band of cells with multiple disruptions or holes. (B) Lateral view of a twist-GAL4;UAS-srp embryo. The lateral fat body is expanded in both the dorsal/ventral and anterior/posterior axis. (C-F) Stage-16 embryos stained for DCG1 mRNA. (C) Lateral view of a wild-type embryo. DCG1 is expressed in the lateral fat body (arrowhead bracket), and also in the hemocytes (open arrowhead). (D) Lateral view of a twist-GAL4;UAS-srp embryo. The lateral fat body is expanded (arrowhead bracket). (E,F) Higher magnification and dorsal view of (C,D) respectively. (E) The two dorsal fat-cell projections are clearly visible. (F) The dorsal fat-cell projections are fused with the lateral fat body (arrows), and cover the posterior...
Figure 3. Development of the endogenous fat cells are unaffected by misexpression of srp in the mesoderm.

(A,B) Lateral view of stage-11 embryos stained for lacZ mRNA. (A) P[29D] embryo containing a progenitor fat-cell enhancer trap. Reporter gene expression marks the primary precursor fat-cell clusters in PS 4-9 and also marks secondary precursor fat-cell clusters in PS 10-12 that give rise to fat cells that surround the gonads (arrows). (B) twist-GAL4;UAS-srp/ P[29D] embryo. Reporter gene expression in the endogenous fat-cell clusters is normal. (C,D) Lateral view of stage-12 embryos stained for β-galactosidase protein. (C) P[29D] embryo. Reporter gene expression marks the developing endogenous fat cells. (D) twist-GAL4;UAS-srp/P[29D] embryo. Development of the endogenous fat cells is normal. (E,F) Lateral view of stage-16
embryos stained for β-galactosidase protein. (E) P[29D] embryo. Fat cells derived from
the endogenous fat cells are visible, as well as the ectodermal stripe pattern of the gp150
enhancer (open arrowhead) (Tian and Zinn, 1994). (F) twist-GAL4;UAS-srp/
P[29D]embryo. Fat cells derived from the endogenous fat cells develop normally.
Figure 4. Late stages of gonad formation are disrupted by misexpression of srp.
Figure 4. (continued)

(A,B) Lateral view of stage-12 embryos stained for 412 mRNA. (A) Wild-type embryo. 412 is expressed in clusters of cells in PS 2-12. (B) twist-GAL4;UAS-srp embryo. Expression of 412 is wild type. (C-F) Lateral view of stage-13 embryos. (C) Wild-type embryo. 412 transcripts persist in PS10-12 where the SGPs have coalesced into a precursor gonad. (D) twist-GAL4;UAS-srp embryo. 412-positive cells fail to coalesce and remain scattered in the posterior region. (E,F) higher magnification of (C,D) respectively. (G-J) Lateral views of stage-16 embryos. (G) Wild-type embryo. 412 expression marks the mature gonad. (H) twist-GAL4;UAS-srp embryo. Few 412-positive cells are detected and are scattered throughout PS10-12. (I,J) Higher magnification of (G,H) respectively.
Figure 5. Ectopic expression of srp leads to loss of late cli expression in the SGPs but not early cli expression in the mesoderm.

Lateral view of stage-10/11 embryos (A,B) and stage-12 embryos (C,D) stained for cli mRNA. (A) Wild-type and (B) twist-GAL4;UAS-srp embryos. Early cli expression in the mesoderm is normal. (C) Wild-type embryo. cli expression is detected in the ectoderm, lateral muscle precursors and in the SGPs (stars) and (B) twist-GAL4;UAS-srp embryo. cli expression is absent in the SGPs and lateral muscle precursors.
Figure 6. Ectopic expression of srp leads to loss of visceral muscle.

(A,B) Lateral view of stage-10/11 embryos stained for bap mRNA. (A) Wild-type embryo. bap is expressed in the precursors to visceral muscle. (B) twist-GAL4;UAS-srp embryo. bap-expressing cells are absent. (C,D) Dorsal view of stage-16 unstained embryos. (C) Wild-type embryo. The midgut has undergone its normal constrictions. (D) twist-GAL4;UAS-srp embryo. The midgut constrictions are absent, resulting in a bap-like mutant phenotype.
Figure 7. Ectopic expression of *srp* disrupts *nau* and *S59* expression patterns.

(A,B) and (C,D) Lateral view of stage-11 embryos stained for *nau* and *S59* mRNA, respectively. (A,C) Wild-type embryos. (A) *nau* is expressed in ventral, lateral and dorsal lateral groups cells that represent a subset of the muscle founder cells. (C) At late stage-11, *S59* is expressed in two metamerically repeating founder muscle cells and in a cluster of four *S59*-expressing cells that also contributes to precursor muscles. (B,D) twist-GAL4;UAS-*srp* embryos. (B) The initial *nau* expression pattern is disrupted, and by late stage-11 variable cell clusters are missing. (D) *S59* expression is also disrupted and there is a loss of *S59*-expressing cells.
Figure 8. The somatic musculature fails to form when *srp* is ectopically expressed throughout the mesoderm.

(A-D) Lateral views of stage-16 embryos stained for *Tml* mRNA. (A) Wild-type embryo. *Tml* is expressed in the somatic body-wall fibers. (B) *twist*-GAL4;UAS-*srp* embryo. Most somatic muscle fibers are missing. (C,D) Higher magnification of (A,B) respectively.
CHAPTER 4

THE ROLE OF SERPENT AND ABDOMINAL-B IN DORSAL FAT AND VISCERAL MUSCLE FORMATION IN DROSOPHILA MELANOGASTER

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Abstract

The precursors to the *Drosophila* fat body are arrayed in a complex, but stereotypic pattern within the mesoderm of the developing embryo. The specific positions, from which different groups of precursor fat cells arise, reflect intrinsic differences among mesodermal cells. These differences are determined, in part, by various patterning cues localized both along the anterior/posterior and dorsal/ventral axes. Differentiation and maintenance of precursor fat cells are dependent upon the expression of *serpent*, a GATA transcription factor gene that is sufficient to induce fat-cell formation. *serpent's* initial expression in the mesoderm is controlled by a variety of dorsal/ventral and anterior/posterior cues. Within the lateral region of the posterior mesoderm, the homeotic selector gene *Abdominal B* represses *srp*. Here we present evidence that within the dorsal region of the posterior mesoderm, *AbdB* is critical for activation of *serpent* and the subsequent specification of the precursor dorsal fat cells. Furthermore, within the dorsal mesoderm *Abdominal B* and *serpent* can independently repress circular visceral muscle formation. We suggest that, in the appropriate context, *Abdominal B* and *serpent* might participate in a molecular switch between visceral muscle and fat-cell specification.

Introduction

The *Drosophila* embryonic fat body is derived from groups of cells arrayed within various mesodermal domains that collectively span most of the anterior/posterior and dorsal/ventral extent of the mesoderm. Through cell differentiation and proliferation
(and possibly cell recruitment), precursor cells and their derivatives populate the three morphological fat body domains (Hoshizaki et al., 1994; Miller et al., 2002; Riechmann et al., 1998). The largest domain is the lateral fat body, which spans almost the entire length of the embryo. The lateral fat body is composed of a single layer of cells that form a bilateral ribbon lying between the somatic musculature and the gut. Fat cells that extend from the lateral fat body form the second and third domains. The second domain is the ventral commissure, which is represented by extensions of fat cells in the anterior region that form bridges connecting the two lateral halves of the fat body across the ventral midline. The third domain of fat cells, the bilateral dorsal fat projections, extends from the posterior-most, dorsal region of the lateral fat body and is composed of cells that extend along either side of the dorsal vessel (Campos-Ortega and Hartenstein, 1997; Hoshizaki et al., 1994; Miller et al., 2002; Riechmann et al., 1998).

Based upon their parasegmental positions and temporal emergence, it is likely that different arrays of cues are responsible for the initial specification of individual precursor fat-cell clusters (Fig. 1 and Hoshizaki et al., 1994; Riechmann et al., 1998). Within the lateral mesoderm, the primary fat-cell clusters occupy positions in six consecutive parasegments (PS 4-9). These clusters are located in the anterior compartments and three of which have been shown to depend on even-skipped (eve; Lee and Frasch, 2000; Riechmann et al., 1998). The lateral secondary fat-cell clusters arise in nine parasegments (PS 4-12) posterior to the primary clusters and straddle portions of the eve and sloppy-paired (slp) expression domains (Riechmann et al.,
The primary and lateral secondary fat-cell clusters contribute to the lateral fat body (Hoshizaki et al., 1994; Riechmann et al., 1998). Nine ventral secondary fat-cell clusters are located in the ventral region of the mesoderm within the slp expression domain of PS 3-11 (Riechmann et al., 1998). The ventral secondary fat-cells clusters in PS 3-5 give rise to the ventral commissure (Campos-Ortega and Hartenstein, 1997; Rizki and Rizki, 1978; Miller et al., 2001), whereas the ventral secondary fat-cells clusters in PS 6-13 contribute to the ventralmost cells of the lateral fat body (Miller et al., 2002). The dorsal fat-cell (DFC) cluster, which is located within PS 13 and part of PS 14, gives rise to the dorsal fat projections and to the posteriormost cells of the lateral fat body (Hoshizaki et al., 1994; Miller et al., 2002; Riechmann et al., 1998).

Key to the specification, differentiation, and maintenance of Drosophila fat cells is the activity of the GATA transcription factor gene, serpent (srp). Members of the GATA transcription family are conserved in their roles in cell-fate specification and differentiation (Gajewski et al., 1999; Harigae et al., 1998; Ramain et al., 1993; Winick et al., 1993). Although srp is expressed in derivatives of all three germ layers, within the trunk mesoderm srp is expressed in precursor fat cells and is the earliest identified fat-cell gene (Abel et al., 1993; Rehorn et al., 1996; Sam et al., 1996). srp is a key regulator of fat-cell development; loss of srp results in the loss of all fat cells (Rehorn et al., 1996; Sam et al., 1996) whereas misexpression of srp in the mesoderm is sufficient to induce ectopic fat cells and to disrupt the specification/differentiation of the somatic and visceral muscle (Hayes et al., 2001). These lines of evidence suggest that srp operates as a cell-type determining gene within the mesoderm.
It is likely that the confinement of \textit{srp} activity is regulated by earlier expressed patterning cues. We expect \textit{srp} to be a target of multiple transcriptional regulators that together provide compound positional cues associated with, and according to, the stereotypic location of individual fat-cell clusters. Along the anterior/posterior axis, the presence or absence of groups of fat-cell clusters varies according to segment (or parasegment) identity. It is likely that the Bithorax Complex genes, as regulators of segment identity (Lawrence, 1985; Hooper, 1986; Bate, 1990; Michelson, 1994), might influence whether fat cells are specified. A number of target genes for various homeotic genes have been identified. In most cases, however, these target genes are involved in the final steps of cell or tissues differentiation and are removed from the initial cell specification steps. \textit{srp}, a cell-specifying gene has been identified as an \textit{in vitro} target of the homeotic transcription factor Ultrabithorax (UBX) (Mastick et al., 1995). However, based on genetic analysis, \textit{Ubx} appears to be required for proper morphological development of two fat body domains but not for fat-cell specification (Miller et al., 2001). In this report, we identify a role for the Bithorax Complex gene, \textit{Abdominal B (AbdB)}, in the specification of fat cells. We present evidence that, within the dorsal mesoderm, \textit{AbdB} is necessary for genetically activating \textit{srp} and establishing a fat-cell identity in PS 13. Furthermore, we find that \textit{AbdB} and \textit{serpent} have redundant roles in repressing circular visceral muscle (VM$_2$) precursor cells. Based on these data, we suggest that dorsal fat and circular visceral musculature initially share a common lineage, and that \textit{AbdB} and \textit{srp} provide a switch between the two cell fates.
Materials and Methods

Fly Stocks

yw1118 served as the wild-type stock. The AbdBD16-, srp-, and bap- mutant stocks were obtained from the Bloomington Stock Center. Ectopic expression was achieved with the GAL4-UAS system (Brand and Perrimon, 1993) using twi-GAL4 (Greig and Akam, 1993) and UAS-AbdB (Castelli-Gair et al., 1994), both of which were obtained from the Bloomington Stock Center. The transgenic line, A7.1EB-lacZ is active in the DFC cluster, a small ventral cluster in PS 13, and within the posterior amnioserosa (Miller et al., 2002). P-element enhancer-trap line P[29D]-lacZ is active in the primary fat-cell clusters in PS 4-9 and the secondary fat-cell clusters in PS 4-12 that give rise to the fat cells that surround the gonads (Hoshizaki et al., 1994). Combinations of mutations and transgenic stocks were generated through standard genetic crosses.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Hoshizaki et al., 1994). Anti-β-galactosidase, goat anti-mouse IgG alkaline phosphatase conjugate, and goat anti-rabbit IgG alkaline phosphatase conjugate were obtained from Promega. Anti-SERPENT rabbit antisera were raised against GST-SRP, which includes the GATA Zn finger motif, and were affinity purified as previously described (Sam et al., 1996). Chromogenic substrates were X-phosphate and NBT (Boehringer Mannheim).
**In situ Hybridization to Whole-Mount Embryos**

*In situ* hybridization using the 412 DNA probe and bap RNA probe was carried out as previously described (Hoshizaki et al., 1994). bap antisense RNA was transcribed *in vitro* from a 1.5kb bap cDNA (a generous gift of M. Frasch).

**Microscopy**

Embryos were equilibrated in mounting solution (50% glycerol, 150mM NaCl, 10mM Tris-HCl pH 8.0). Whole-mount embryos were imaged on a Zeiss Axioplan2 microscope using nomarski optics and documented with a Kodak MDS digital camera. CorelDraw 8 software was used to prepare figures from digital images.

**Results and Discussion**

**The Homeotic Gene AbdB Is Both Necessary and Sufficient for Specification of Dorsal Fat Cells**

The dorsal fat-cell (DFC) projections represent one of the three major domains of the fat body. We recently described, in detail, the origin and development of the DFC projection (Miller et al., 2002). Briefly, the DFC projections arise from a bilateral cluster of cells first detected at stage 10 (stages are those of Campos-Ortega and Hartenstein, 1997) within the dorsal mesoderm (Miller et al., 2002; Riechmann et al., 1998). The DFC cluster spans parasegment 13 and part of parasegment 14, from *engrailed* (*en*) stripe 13 to stripe 14 (Riechmann et al., 1998; see Fig. 7 in Miller et al., 2001). During germband retraction, each DFC cluster separates along the dorsal/ventral axis into two subgroups of cells. Eventually the cells of the dorsal DFC
subgroup migrate anteriorward, crossing parasegmental boundaries, to form the bilateral DFC projections, which flank the dorsal vessel (Miller et al., 2002). The cells of the ventral DFC subgroup, along with a small cluster of ventral cells in PS 13, form the posterior-most portion of the lateral fat body (Miller et al., 2002).

The DFC cluster arises within the mesodermal expression domain of the morphogenetic (m) isoform of the homeotic gene *AbdB* (Boulet et al., 1991; Casanova et al., 1986). *AbdB* is expressed at its highest levels in PS 13, and is present in declining levels in PS 11-12 (Delorenzi and Bienz, 1990). To test for a role for *AbdB* in DFC specification, we examined *AbdB*-mutant embryos for alterations in *srp* activity. In *AbdB* mutants, the DFC clusters were absent and consequently, the DFC projections were not present in older embryos (Fig. 2). However, it was difficult to resolve the absence of the posterior cells of the lateral fat body, which are derived from the ventral subgroup of the DFC cluster. To confirm the loss of these cells, we examined *AbdB*-mutant embryos carrying, A7.1EB*-lacZ*, a DFC reporter that we have previously characterized (Miller et al., 2002). In the A7.1EB*-lacZ*; *AbdB* embryos, *lacZ* activity was absent in the posterior-most cells of the lateral fat body as well as in the DFC cluster, the small ventral cluster, and the DFC projections, (Fig. 3). These data suggest that *AbdB* is necessary for the specification of the DFC cluster and the activation of *srp*.

To further examine the role of *AbdB* in the specification of the DFC cluster, we used the GAL4-UAS targeting system of Brand and Perrimon (1993). We generated *twi*-GAL4; UAS-*AbdB* embryos to express ABD-B protein throughout the mesoderm.
In these embryos, the primary and secondary fat cells were absent, and the only srp-expressing cell clusters present were those serially duplicated in the dorsal mesoderm in the anterior regions of PS 2-12 (Fig. 4A). These ectopic srp-expressing cells did not collectively span the entire parasegment but appeared to be confined to the eve-expression domain of parasegments 2-12. Based on their size and location in the dorsal mesoderm, the ectopic srp-expressing cell clusters were not likely to be composed of primary or ventral secondary precursor fat cells. Furthermore, we do not detect the ventral commissure bridges, which are derived from the ventral secondary fat cell precursors in PS 3-5 (Fig. 4 E, F). However, because srp is expressed in all fat cells, we confirmed the identity of the ectopic srp-expressing cells using the A7.1ES enhancer. We examined twi-GAL4; UAS-AbdB embryos carrying the A7.1EB-lacZ reporter. In the experimental embryos, lacZ activity recapitulated the ectopic srp-expression pattern, thus confirming our hypothesis that the ectopic cells correspond to serially duplicated DFC cluster, (Fig. 4 D-F). These data suggest that AbdB is sufficient to activate srp and induce DFC formation in the dorsal mesoderm. As the twi-GAL4; UAS-AbdB embryos mature, the ectopic cells merge to form a superficial lateral fat body (Fig. 4 C,D).

**Role of AbdB as a Developmental Switch in the Dorsal Mesoderm**

In twi-GAL4; UAS-AbdB embryos, the ectopic fat-cell clusters are present in the dorsal mesoderm in PS 2-12 in a pattern reminiscent of the precursors to the circular visceral muscle (VM_c) cells. The VM_c precursors are derived from the dorsal
mesoderm within the anterior compartment of PS 2-12 and are marked by *bagpipe* 
(*bap*) expression (Azpiazu and Frasch, 1993; San Martin et al., 2001). The *bap* gene is 
expressed in, and is necessary for the formation of, eleven clusters of VM_{c} precursor 
cells (Azpiazu and Frasch, 1993). These clusters give rise to the circular visceral 
muscles that surround the gut (Azpiazu and Frasch, 1993) and are necessary for the 
sterotypic constrictions that model the gut in mature embryos (Bate, 1993).

We noted that *twi-GAL4; UAS-AbdB* embryos had a distended, unconstricted gut 
(data not shown) similar to that detected in a *bap* loss-of-function mutant embryo 
(Azpiazu and Frasch, 1993). Based upon this observation, we suspected that the VM_{c} 
precursors were disrupted in the *twi-GAL4; UAS-AbdB* embryos. Furthermore, 
according to their location, the ectopic precursor DFC clusters might have replaced 
VM_{c} precursor cells. To test this idea, we examined *twi-GAL4; UAS-AbdB* and 
*AbdB*-mutant embryos for alterations in VM_{c} precursor cells using *bap* as a cell marker (Fig. 
5). In the *twi-GAL4; UAS-AbdB* embryos, *bap* expression was absent (Fig. 5B) 
whereas in *AbdB*-mutant embryos, an ectopic cluster of VM_{c} precursor cells is 
detected in PS 13 (Fig. 5C). These data suggest that *AbdB*’s normal role is to 
simultaneously induce DFC formation and repress VM_{c} formation in PS 13. Thus, in 
anterior segments, the absence or reduced levels of ABD-B permits the formation of 
VM_{c} cells.

Our data also suggest that a developmental relationship might exist between VM_{c} 
precursors and DFC precursors. The apparent developmental switch between DFC and
VM₀c precursors in the dorsal mesoderm is reminiscent of the proposed developmental choice that might take place in the lateral mesoderm between primary fat-cell precursor clusters and somatic gonadal precursors (SGP) (Moore et al., 1998; however see Discussion in Hayes et al., 2001 for an alternative model). This proposed developmental choice between fat and SGP cell fates relies on srp activity to both block a SGP-cell fate and induce a fat-cell fate within a pool of equivalent cells capable of differentiating into either cell type.

Likewise, in the dorsal mesoderm, srp might also serve as a developmental switch between DFC and VM₀c precursor cell fates where srp activity simultaneously blocks VM₀c and induces DFC formation. We have previously shown that misexpression of srp leads to repression of VM₀c formation (Hayes et al., 2001), and in this report we demonstrate that, in the dorsal mesoderm AbdB-dependent srp expression induces a DFC fate. To confirm the role of srp as a repressor of VM₀c precursor cells, we examined srp mutants for ectopic VM₀c cells. In srp loss-of-function mutants, ectopic bap clusters were not detected (data not shown). These data suggest that, an additional gene is expressed in PS 13 that functions redundantly to srp in repressing bap.

The absence of ectopic bap-expressing cells in PS 13 of srp mutants might be due to ABD-B. Within the dorsal mesoderm, Abd-B might promote DFC specification (through srp activation) and repress VM₀c precursor specification. Therefore, the absence of VM₀c cells in PS 13 is due to the presence of both AbdB and srp. Because misexpression of AbdB automatically leads to ectopic expression of srp, it is difficult
to demonstrate that \textit{AbdB} can independently repress VM$_c$ formation. To explicitly test whether \textit{AbdB} can repress \textit{bap}, we shall examined \textit{twi-GAL4; UAS-AbdB; srp}-mutant embryos, which lack \textit{srp} activity and in which \textit{AbdB} is misexpressed throughout the mesoderm. If, \textit{AbdB} also represses \textit{bap}, then we would predict loss of the circular visceral mesoderm cells. If \textit{bap} activity is absent, then \textit{AbdB} can repress VM$_c$ precursor formation. We would conclude from such data that within the dorsal mesoderm, \textit{AbdB} is sufficient and necessary for the activation of \textit{srp} and that \textit{srp} and \textit{AbdB} can individually repress VM$_c$ formation.

\textbf{Role of \textit{AbdB} in the Lateral Mesoderm}

The \textit{AbdB} misexpression experiments also reveal a role for \textit{AbdB} in the lateral mesoderm. In contrast to the capacity of \textit{AbdB} to activate \textit{srp} and induce DFC formation in the dorsal mesoderm, \textit{AbdB} represses formation of the primary and secondary precursor fat cells in the lateral mesoderm. This observation is consistent with the proposed role of \textit{AbdB} as a repressor of precursor fat-cell formation in the lateral mesoderm of PS 12 (Moore et al., 1998). In wild-type embryos, somatic gonadal precursor (SGP) clusters form within PS 10-12 in a position equivalent to that of the primary fat-cell clusters in PS 4-9. The loss of \textit{AbdB} activity results in the replacement of the SGP cluster in PS 12 with a \textit{jfp}-expressing fat-cell cluster (Moore et al., 1997). To formally test \textit{AbdB}'s role as a general repressor of fat-cell specification in the lateral mesoderm, we examined \textit{twi-GAL4; UAS-AbdB} embryos carrying enhancer trap P[29D], a cell marker for primary fat-cell clusters (Hoshizaki et al., 1994). Because, in \textit{srp}-mutant embryos, P[29D]-expressing cells are present but do
not differentiate, it is likely that P[29D] reflects the expression pattern of a fat-cell
gene that lies upstream of srp in the specification of fat cells (Sam et al., 1996).

In twi-GAL4; P[29D]-lacZ; UAS-AbdB embryos, lacZ activity was detected in the 6-7
cells of each primary fat-cell cluster. These cells, however, did not differentiate, and thus
did not contribute derivative cells to the fat body (data not shown). We also examined twi-
GAL4; UAS-AbdB embryos for alterations in the formation of SGP cells using the SGP
marker 412 (Brookman et al., 1992). Although AbdB is required for SGP formation in PS
12 (Boyle and DiNardo, 1995; Brookman et al., 1992), misexpression of AbdB did not
induce ectopic SGP development (Fig. 6). These data suggest that although AbdB is
sufficient to repress srp expression in the lateral mesoderm, AbdB is not sufficient to
repress the initial specification of primary fat-cell clusters, and it does not induce SGP
specification per se. These data are consistent with srp's proposed role in the maintenance
of the lateral fat body (Hayes et al., 2001; Sam et al., 1996).

**srp Restriction Is Important to the Specification of the Mesoderm**

**srp** has a critical role in fat-cell development and in establishing the correct
architecture of the specified mesoderm. Based on misexpression experiments, srp can
induce ectopic fat-cell development and disrupt the specification of VMc cells and the
differentiation of somatic muscle cells and SGP cells (this report; Hayes et al., 2001).
Thus, the spatially restricted induction of srp expression is necessary for the proper
specification of the mesoderm. In this report, we have genetically identified AbdB as a
positive regulator of srp in the dorsal mesoderm for the specification and development
of dorsal fat cells, whereas in the lateral mesoderm AbdB is a negative regulator of srp thereby repressing fat-cell development. The opposing responses of srp to AbdB are likely to reflect the fundamental patterning differences that exist between the dorsal and lateral mesoderm.

The dorsal mesoderm, unlike the lateral mesoderm, is defined by the activity of decapentaplegic (dpp)-maintained tinman (tin). tin is an NK homeodomain protein gene that is expressed in three phases during embryonic development (Kim and Nirenberg, 1989). dpp is required to inductively maintain the second phase of tin expression, which is restricted to, and required for, the formation of the dorsal mesoderm (Frasch, 1995). Recently, we reported that dpp-dependent tin expression is necessary and sufficient for the formation of the DFC cluster along the dorsal/ventral axis within PS 13 (Miller et al., 2001). AbdB and dpp-dependent tin might operate synergistically to directly activate srp and consequently induce fat-cell development. It is also possible that establishment of the dorsal mesoderm results in the activation of yet-unidentified cascades of genes important for AbdB-mediated activation of srp. Experiments are currently underway to determine whether ABDB and/or TIN directly bind within the srp enhancer region.

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References


Figure 1. Schematic summary of the locations of the precursors to the fat body, visceral muscle, and somatic gonads within a stage-12 embryo.
Figure 2. *AbdB* is necessary for the formation of the DFC cluster and the DFC projections.

(A,C,E) wild-type and (B,D,F) *AbdB*-mutant embryos stained immunohistochemically for SERPENT protein. (A,B) Lateral view of stage-13 embryos. (A) In a wild-type embryo, the DFC cluster (dashed circle) forms in PS 13. (B) In an *AbdB*-mutant embryo, the DFC cluster is absent. (C,D) Lateral and (E, F) dorsal views of stage-16 embryos. (C) In a wild-type embryo, the DFC projection (dashed outline) extends from the posteriormost lateral fat body. (E) Same embryo as in (C), the bilateral DFC projections are bracketed. (D, F) The DFC projections are absent in a stage-16 *AbdB*-mutant embryo.

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Figure 3. Formation of the DFC cluster requires *AbdB*

(A, C, E) A7.1EB-*lacZ* wild-type and (B, D, F) A7.1EB-*lacZ*; *AbdB*-mutant embryos immunostained for β-galactosidase. (B, D) The DFC cluster is reduced to a few cells in a stage 13/14 A7.1EB-*lacZ*; *AbdB*-mutant embryo as revealed by the loss of *lacZ*-expressing cells. (C) Dorsal view of (A); (D) Dorsal view of (B). The loss of *AbdB* results in the complete loss of the DFC projection in (F) a stage-15 embryo.
Figure 4. Misexpression of AbdB results in the serial duplication of the DFC cluster. (A, B, C) twi-GAL4; UAS-AbdB embryos immunostained for SERPENT protein and (D, E, F) twi-GAL4; UAS-AbdB; A7.1EB-lacZ embryos immunostained for β-galactosidase. (A) in a stage-13 embryo, the DFC cluster is present in PS 13 and ectopic srp-expressing clusters are present in the posterior compartment of PS 2-12 within the dorsal mesoderm. Primary and secondary fat-cell clusters are not detected (for wild-type comparison, see Fig. 1 in Miller et al., 2002). (D) A7.1EB-lacZ activity recapitulates the ectopic srp-expression pattern. The ectopic clusters give rise to a superficial lateral fat body in (B, E) stage-16 embryos. (C, F) ventral views of (B, E) respectively. Ventral commissures are not detected in AbdB misexpression embryos (for wild-type comparison, see Fig. 9 in Miller et al., 2002)
Figure 5. \textit{AbdB} negatively controls VM, precursor-cell formation.

(A-C) Lateral view of stage-10 embryos stained for \textit{bagpipe} (\textit{bap}) mRNA. (A) wild-type embryo. \textit{bap} transcripts are detected within 11 cell clusters located in PS 2-12 and within the proctodeum (open arrowhead). (B) Within \textit{twi-GAL4}; UAS-\textit{AbdB} embryos, no \textit{bap} transcripts are detected; proctodeum-specific \textit{bap} transcripts are unaffected. (C) Within \textit{AbdB}-mutant embryos, an ectopic cluster \textit{bap} cluster is detected in PS 13, in addition to the 12 endogenous \textit{bap}-expressing clusters.

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Figure 6. Misexpression of AbdB does not induce ectopic SGP formation.

Lateral views of (A, C, E) wild-type and (B, D, F) twi-GAL4; UAS-AbdB embryos stained for 412 mRNA. (A, B) Stage-11 embryos (C, D) Stage-14 embryos (E, F) Stage-16 embryos. In the development of the gonad, there is no detectable difference between wild-type embryos and embryos in which AbdB is misexpressed throughout the mesoderm.
Addendum

**Functional Relevance of the Putative ABDOMINAL-B**

**Binding Sites Located Within the Dorsal Fat-cell**

*serpent Enhancer A7.1 EB*

An important approach to determining the regulatory cascades necessary for fat-cell development is the characterization of regulatory elements and the corresponding transactivators of the *serpent* (*srp*) gene. I have demonstrated, genetically, that the homeotic transcription factor gene *Abdominal B* (*AbdB*) and the NK-class homeobox transcription factor gene *tinman* (*tin*) are, together, necessary and sufficient for *srp* activation and formation of dorsal fat-cell cluster (Chapter 4). These findings led to a tentative model (see CHAPTER 5 and Fig. 5-1 therein) for dorsal fat-cell formation, circular visceral muscle formation, and regulatory switch between these two cell types.

This model relies on the presence of ABD-B binding sites within the regulatory regions of *srp* and *bagpipe* (*bap*). In this model and in accordance with the genetic data, *AbdB* serves as a direct activator of *srp* and direct repressor of *bap* (Chapter 4). Thus, ABD-B simultaneously activates *srp* and represses *bap* in the dorsal mesoderm, and ABD-B can also repress *srp* in the lateral mesoderm. Furthermore, *srp* can also repress *bap* (Hayes et al., 2001; Chapter 3). If this model is correct, it is also possible that yet unidentified factors (in addition to ABD-B) bind to the regulatory regions of *srp* and *bap* control whether *AbdB* will serve as an activator or repressor.

The consensus binding site for ABD-B is $^5$[TTTATGGC]$^3$ (Ekker et al., 1992). There are three putative binding sites for ABD-B protein within the enhancer region.
A7.1EB of srp localized to positions 258 to 251, 271 to 278, and 278 to 285, designated A, B, and C respectively. Additionally, based upon the TIN binding site $^{5'}$[TCAAGTGG]$^{3'}$ found within the heart enhancer of the Drosophila mef2 gene (Gajewski et al., 1997), a putative TIN binding site containing a G/C substitution is present at position 257 to 264. In order to determine whether these ABD-B and TIN binding sites are functionally relevant for the regulation of the srp gene, it is necessary to mutagenize the binding sites individually within the A7.1EB enhancer and re-test the modified enhancer in vivo. I began this line of experimentation using PCR to replace the putative ABD-B binding site B with the restriction site NotI. This construct was micro-injected into $\gamma w$ embryos and survivors were mated in a pairwise fashion (Fig. 4-A; and see Methods). Red-eyed offspring were made homozygous and tested for the presence of lacZ activity. In no stocks was there any defect in the activity of lacZ in the formation of the dorsal fat-cell cluster. These findings indicate that ABD-B-binding site B, alone is not critical for binding of ABD-B and subsequent activation of srp. In spite of this negative finding, I suspect that AbdB does indeed directly activate srp. It is likely that either A and C individually, or all three binding sites are important to this process, and that the loss of one will be overcome or masked by the binding at the other two sites for the activation of srp. Future studies remain to test the remaining ABD-B and TIN binding sites.
Methods

Microinjection of Drosophila Embryos

The following protocol was adapted for the Hoshizaki Lab by Jennell M. Miller and James Bogenberger from the protocols described by Kierhart et al., 2000; Spradling, 1986 and suggestions from the laboratories of Dr. E. Ferguson and Dr. R. Storti

Materials and Solutions

Agar Block: Blocks for aligning embryos can be made by melting down used egg lay plates or by using fresh egg-lay plate media. A few drops of food color (McCormick and Co., Inc.) is thoroughly stirred into the hot media as the final step before pouring the media into a shallow, level container. The agar block should be poured about 3-4 mm thick. The food coloring provides background contrast to aid in viewing the embryos under the dissecting microscope.

Drying chamber: Spread out a thin layer of “Drierite” (anhydrous calcium sulfate) along the bottom of small (sandwich size) airtight container.

Egg-lay plates: Mix 500 ml of apple juice with 500 ml of dH2O in a big plastic beaker. Heat in microwave for 5 min. Stir in 27.5 g of agar and 109 g of table sugar and reheat for 5 min. Make yeast slurry with 22.5 g of instant yeast and stir in while heating occasionally. Stir in 10 ml of Cal-Tech. acid mix. (Acid mix can be lethal to flies at high concentrations, so make sure it is thoroughly mixed in.)

Glue: Cut Approx. 1 meter of Scotch 3M transparent mailing tape into small pieces and stuff into a 100 ml screw cap glass jar containing 50 ml of heptane. Shake vigorously on a mechanical shaker for about 1 hr. Discard the pieces of tape and transfer liquid to 15 ml falcon tubes. Centrifuge at maximum speed for 10 min. The supernatant is the glue. Transfer the supernatant to a fresh screw cap glass jar for storage.

Injection Buffer: 2.5 ml 1M KCl
50 µL 1M NaH2PO4
Fill with dH2O to 500 ml

Lodge: Center a 150 x 15 mm plastic petri dish on a plastic tri-tip 1 L beaker and trace the circumference of the opening onto the petri
Needle Breaker: Break off approximately one inch of glass tube from the dispensing end of a Pasteur pipette. Glue the tube to a clean glass slide along one of the long edges. Permount works well as glue. Allow the Permount to dry completely before using the needle breaker.

Recovery Plates: 150 x 15 mm glass petris – line the circumference of the plate with 2 kimwipes, folded several times lengthwise. When ready for use, moisten the kimwipes with sterile water to provide humidity to the recovering embryos.

10X Embo Wash: (500 ml)
35 g NaCl
2 ml Triton X-100 (0.04%)
Bring to 500 ml with dH₂O

Miscellaneous Materials Required

- Halocarbon oil 95
- Halocarbon oil 27
- Squirt bottles for 50% bleach, EMBO wash, and dH₂O
- 1 L bottle of dH₂O
- Transfer pipettes
- Dissecting needles
- Tweezers
- Cell strainers
- Razor blades
- Soft paint brushes

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Preparation for Harvesting

Note: Egg-lay plates are stored at 4° but must equilibrate to room temperature before changing, both on injection and non-injection days.
The experimental stock should be expanded to 15-25 synchronous bottles and older flies discarded. Young flies must be transferred to fresh bottles as soon as they eclose. After 4-6 days these young flies are placed in a lodge with a fresh egg-lay plate and a fresh yeast smear. Flies should not be added to the lodge in excess of the amount that can cover the egg-lay plate. Flies should be allowed to feed for 1-2 days while in the lodge. It is very important to change plates (adding fresh yeast) every day when not injecting. When injecting, plates will be changed every hour to obtain synchronous early-stage embryos. (Yeast is applied as small dots instead of a smear on injection days, so that the embryos are not trapped in a blob of yeast.) This process should be initiated 2 hrs before beginning the injection procedure so that the flies can adjust to the constant interruptions. It is important not to disturb the flies during the 1 hr egg-lay interval.

Preparation of Embryos

Note: Once the chorion is removed embryos are surrounded only by the vitelline membrane, making them extremely susceptible to damage.
1. Remove all adults from egg-lay plates using forceps.
2. Squirt 1X EMBO WASH onto plates to loosen embryos.
3. With a soft bristled brush gently loosen and transfer embryos to strainer basket.
4. Submerge the embryos in 50% bleach for nearly 2 min. to remove the chorion. (DO NOT treat with bleach for more than 2 min.) Rinse immediately with dH2O until odors of bleach can no longer be detected.
5. With a moist brush transfer embryos to a colored agar block for alignment. The embryos should be placed along the middle of the agar block, leaving adequate space for ease of alignment and transfer.
6. Use a dissecting needle to align embryos under the lowest feasible light setting with the dissecting microscope. The micropyle marks the anterior of the embryo.
7. Apply a single layer of glue with a pipet, in a strip along the edge of a glass slide. Hold slide at a 45° angle to allow the glue to dry. The surface tension will result in a thicker layer. (If the glue is allowed to spread out over the slide it may be too thin to secure the embryos.) Set aside to let glue dry. Use the prepared slide to pick up the aligned embryos off the agar block. The glue-covered slide should just come into contact with the agar block, as any pressure will easily damage the chorion-free embryos.
8. Place the slide in a drying chamber for 3-5 min. to desiccate the embryos. Drying times must be optimized for each injection sequence according to changes in humidity, temperature and barometric pressure.
9. After drying, place a thin layer of halocarbon oil 95 over the embryos.
Injection Procedure Using the Narishige IM-300 Microinjextor

1. Turn on nitrogen gas to and regulate to 100 PSI – do not exceed 100 PSI
2. Set the Narishige Microinjector as follows:
   - Power – on
   - Pressure Mode
     - P: Fill > 102 PSI
     - P: Inject 17.0 PSI
     - P: Balance .5 PSI
     - P: Hold 23.1 PSI
   - Time Mode
     - T: CLR Manual 0s
     - T: CLRH 0.01s
     - T: Fill Manual 0s
     - T: Inject 0.03s
   - Count Mode
     - C: CLR 1
     - C: CLRH 1
     - C: Fill 1
     - C: Inject 1
3. Secure needle to the Narishige injection wand
4. Attach wand to micromanipulator
5. Under low power (compound microscope) focus on the needle breaker slide
6. Focus the needle using the micromanipulator controls
7. Break needle by gently moving the stage such that the pipette breaks the tip of the needle
8. Clear debris from the needle by pressing CLR a few times
9. Place vent and bain
10. Place a drop (5μl) of experimental DNA plus helper DNA in a larger drop of mineral oil on a siliconized glass cover-slip attached to a slide
11. Focus on DNA
12. Focus needle
13. Insert needle into DNA drop and press FILL
14. If needle doesn’t hold the solution, then there isn’t a proper seal between the needle and the wand
15. Place your slide with the decorionated embryos on the stage of the scope with their posterior ends facing towards the needle. Focus the needle and the embryos under low, then high power. DO NOT USE HIGH LIGHT! Use a very low light setting, as heat from the lamp will kill the embryos.
16. Using the stage controls, push the posterior of the embryo into the needle. The needle should only enter 1/10th of the embryo. If you are using fast green in the
DNA solution, you will see the flow of solution into the embryo.

17. After attempting to inject all embryos on a slide, transfer the slide to a dissecting scope. Use a clean needle to eliminate all uninjected embryos.

18. Leaking cytoplasm or blebs should be brushed away from the posteriors of the injected embryos with a needle.

19. Remove most of the Halocarbon 95 oil and replace it with Halocarbon 27 oil.

20. Moisten the kim wipes inside a recovery chamber with a little distilled water.

21. Place slide inside of recovery chamber and allow the embryos to recover at 25°C

References


replace one ABD-B-binding site with a restriction site

Figure 4-A. P-element mediated transformation and generation of a homozygous stock.
CHAPTER 5

GENERAL DISCUSSION

Conclusions

This study has provided evidence for the cell-specific activation of a cell-type determining gene by earlier expressed patterning cues. I have isolated positional cues that are genetically responsible for the cell-specific activation of *serpent (srp)*, a gene that is necessary and sufficient for the initiation of the fat-cell program in embryonic *Drosophila melanogaster*. The fat body has several morphological domains, all of which require *srp* for development. However, prior to this work there have been no specific cell markers for the cells giving rise to the individual fat-body domains. I have characterized cell markers for the ventralmost lateral fat body and the dorsal fat-cell (DFC) projections. I have demonstrated that *srp* is independently activated in distinct fat body domains and have used these *srp* enhancer region-reporter gene constructs (A7.1ES-*lacZ* and A7.1EB-*lacZ*) to trace the development of the two aforementioned fat body domains. I utilized these constructs as markers for fat-cell domains to test the roles of various genes in fat-cell specification in both mutant and misexpression experiments.

Although the A7.1ES enhancer region was originally isolated as a target of ULTRABITHORAX (UBX) *in vitro* (Mastick et al., 1995), I have determined through mutant and misexpression analysis that *Ubx* does not play a role in fat-cell specification and instead is involved in the morphological development of the fat-body. Therefore, *Ubx*
is not part of the model of early fat-cell specification. On the other hand, misexpression and mutant analysis of \textit{tinman (tin)} and \textit{Abdominal B (AbdB)} have clearly demonstrated that these two transcription factor genes are necessary and sufficient for dorsal fat-cell development. Therefore, \textit{AbdB} and \textit{tin}, at their position of intersection within the dorsal mesoderm of PS 13, provide the positional cues for the activation of \textit{srp} and, consequently, dorsal fat-cell formation.

Like the DFC cluster, circular visceral muscle (VM) precursor clusters also arise in the TIN-expression domain (in PS 2-12). Although VM precursor clusters and the DFC cluster develop in the equivalent dorsal-ventral position within different parasegments they have not previously been studied as related cell types. An unexpected finding of this work is that \textit{AbdB} and \textit{srp}, which are critically and simultaneously involved in dorsal fat-cell development, are also both independently capable of repressing VM cell formation. I have concluded that, within a single precursor cell, a switch mechanism might exist that mediates the choice between DFC and VM cell fates; and, this switch depends upon \textit{AbdB} and \textit{srp} (Figure 5-1). In 1940, C. H. Waddington predicted the theory of 'switch' genes. Switch genes direct a cell to follow one developmental pathway through the activation of an appropriate cascade of downstream genes. Following the switch, any alternative pathways, which the cell was previously capable of initiating would no longer be available (reviewed in Gilbert, 1991). The genetic evidence suggests that \textit{srp} and \textit{AbdB} might function as such switch genes.

I have compared this possible switch between dorsal fat cells and visceral muscle cells to a switch previously described by Reichmann and co-workers (1998) between primary fat cell clusters and somatic gonadal precursors in the lateral mesoderm. I have formally
tested Riechmann's (1998) proposed switch, genetically, by examining *srp* misexpression embryos and have found that *srp* does not block specification of SGPs, however coalescence of the mature gonad is disrupted. Therefore, I have concluded that unlike the relationship between the DFC cluster and VM\(_C\) cells, the distinction between lateral fat cells and gonadal cells is not mediated wholly or in part by *srp* according to a straightforward switch mechanism.

Further Testing of the Proposed Switch
Mechanism Between Dorsal Fat-Cell and Visceral Muscle-Cell Fates

Dorsal fat cells and visceral muscle precursors might develop in their respective parasegments, initially by following an identical developmental fate-restriction pathway prior to specification. This is to say that, according to the equivalent precursor model (Fig. 5-1), prior to activation of the dorsal fat-cell switch DFC precursors and VM\(_C\) precursors are genetically indistinguishable, and both cell types are dependent upon *tin* in the dorsal/ventral axis. In PS 13, at the intersection of *tin* with *AbdB* and *srp*, a fat-cell fate promoting cascade is initiated and the VM\(_C\) cell-fate promoting cascade is blocked. In this model, *srp* (which promotes fat-cell development) could be directly or indirectly regulated by *AbdB* and *tin*, and such binding sites for these factors would be present in the *srp* enhancer sequences.

Putative *AbdB* and *tin* binding sites are present in the regulatory region of *srp* and remain to be tested. In parasegments 2-12 where *srp* and high levels of *AbdB* are absent, the fat-cell fate-promoting cascade is not initiated whereas the VM\(_C\) cell-fate promoting
cascade is initiated. And according to the model, bagpipe (bap; which promotes VM$_c$ development) would also be directly or indirectly positively regulated by tin and negatively regulated by AbdB. Thus, in PS 13 dorsal mesoderm, AbdB must serve as both an activator of srp and repressor of bap. In anterior parasgements, AbdB serves as a repressor of srp in the lateral mesoderm. It is likely that ABD-B accomplishes this dual role either in working with cofactors or by functioning through other downstream transacting factors.

An alternative explanation for the observed 'switch' in cell types might be that the PS 2-13 dorsal clusters are actually pools of intermingling but distinct precursor cells. In this model, the presence of ABD-B and SRP yields dorsal fat-cell differentiation for the appropriate set of cells within the cluster, while those cells that could have given rise to circular visceral muscle perish. The activation of BAP, on the other hand, yields VM$_c$ differentiation, while those cells within the cluster that could have given rise to dorsal fat cells perish. This 'differentiate or die' model suggests that the split between the two cell types occurs earlier in development than is suggested by the equivalent precursor model.

To distinguish between these two possibilities, it would be necessary to construct transgenes that link the regulatory regions of srp and bap to distinct reporter genes. By confocal microscopic detection of the two transgenes' activity with different fluorescently labeled antibodies/antisense mRNA, it should be possible to determine if bap and srp are activated in the same or different cells. The equivalent precursor model predicts that ABD-B is responsible for repressing bap expression. For this model to be correct, and if ABD-B's action is direct, ABD-B binding sites would exist within the regulatory region of bap. By cloning the regulatory regions of the bap gene (as has already been done for srp)
one can examine the sequence for the published optimal binding site of ABD-B. Furthermore, by mutating the found ABD-B binding site and linking the regulatory region to a marker gene, one can determine whether or not the site is functional \emph{in vivo}. Since, ABD-B is a repressor of \emph{bap} in the equivalent precursor model, I would predict a derepression of \emph{bap}-reporter gene activity in PS 13 in the experimental embryos. Furthermore, one could determine whether there is an equivalent precursor or a pool of mixed cells by doubly labeling embryos for the activities of \emph{srp} and the \emph{bap} driven transgene using fluorescent label and confocal microscopy.

\textbf{Testing Putative Binding Sites Within \emph{srp} Enhancer Sequences}

To test whether ABD-B and TIN directly bind to the DFC enhancer region through their putative binding sites, it is possible to conduct electrophoretic mobility shift assays (EMSA). The enhancer fragment A7.1 EB has been divided into six subclones, each approximately 80 bp in length (Fig. 5-2). I have already conducted a pilot EMSA experiment with nuclear extract from stage 9-12 embryos using purified A7.1EB subclone D, which contains putative ABD-B and TIN binding sites, as the probe. The results of this preliminary study indicate that the D fragment of A7.1 EB is capable of forming a complex with yet unidentified nuclear proteins (Fig. 5-3) that could potentially represent TIN and ABD-B. It would be possible to ascertain whether the complex forming on the D fragment of A7.1 EB indeed contain TIN and AbdB-B proteins though antibody supershift EMSA with anti-TIN and anti-Abd-B antibodies. Use of EMSA methodologies will also
be important for testing binding of factors important to srp activation in other regions of the fat body.

Defining Functional Differences Between Morphological Regions of the Fat Body

Morphological regions of the fat body have been described, and I have suggested that early genetic differences in precursor fat-cell clusters might be responsible for functional differences in the fat body regions (Miller et al., 2001). It remains to be determined whether or not early patterning events create fundamental differences among groups of fat-cell clusters. In order to establish whether such fundamental differences exist, one could perform a subtractive analysis of mRNAs expressed in individual precursor fat-cell-cluster groups that takes advantage of the enhancer elements A7.1 EB and ES. This experiment would require linking each of these elements to a cloned copy of Green Fluorescent Protein (GFP; from jelly fish) and creating transgenic flies. In the A7.1ES-GFP and A7.1EB-GFP carrying transgenic flies, GFP would only be expressed in the ES or EB domains, respectively. With these constructs, it would then be possible to purify embryonic cells through fluorescence-activated cell sorting (FACS), enriching for specific fat-body domain cells. To determine whether a difference in transcriptional activity exists between the GFP-expressing domains and the remainder of the fat body, cell purification would be followed by subtractive mRNA screening between mRNAs isolated from fat cells of specific domains and from the complete isolated fat body. Although a number of studies using purified cells and continuous cells lines have provided valuable vertebrate information, there have been only a few attempts to isolate and study pure populations of Drosophila cells (reviewed in Kierhardt, 2000). Mahowald and coworkers have shown
that highly enriched populations of pole cells (germ-line precursors; Allis et al., 1977) and neuroblasts (Furst and Mahowald, 1985) can be obtained in a reasonable quantity from embryos. Bernstein and coworkers (1978) and Storti and coworkers (1978) have described procedures for the isolation of myoblasts in quantity. Provided the GFP signal in transgenic cells is bright enough, FACS technology should make obtaining purified cell lines even more practical.

Identification of Evolutionarily Conserved Sequence Elements

To completely understand srp activation within all of the cells in which srp is expressed, it will be necessary to locate the remaining enhancers of srp. A methodology used to identify functional enhancer elements the identification of conserved sequences in the upstream regions of orthologous genes in species that have been separated by moderate spans of evolutionary time (reviewed in Xu et al., 1998). This approach is based on the premise that the two species chosen are far enough removed evolutionarily so that by the neutral theory of evolutionary change, all non-critical sequences will have been subjected to randomization (reviewed in Xu et al., 1998). Previous comparisons of *Drosophila melanogaster* and *Drosophila virilis* genes reveal that conserved coding regions have diverged approximately 10%, whereas nonfunctional sequences within introns and 5' sequences upstream of functional conserved enhancers retain a high degree of sequence similarity (Xu et al., 1998). These types of studies could be important not only for finding additional regulatory regions of srp but also for comparative evolutionary studies of the fat body among *Drosophila* species and subspecies.
References


Figure 5-1 Schematic diagram of the equivalent precursor model.
Figure 5-2. Schematic diagram of the overlapping subclones of enhancer region A7.1-EB.

A7.1-EB has been subdivided into six fragments, each of which have been subcloned.

Subclone C contains one putative ABD-B binding site and Subclone D contains one putative TIN binding site and two putative ABD-B binding sites.
Figure 5-3. An unidentified nuclear factor forms a complex with fragment D of the *serpent* dorsal fat-cell enhancer.

Electrophoretic mobility shift assay (EMSA) with purified nuclear extract obtained from midstage 9 to midstage 12 embryos. P-labeled fragment D was the probe. Labeled probe alone was run in lane 1. In lane 2, labeled probe was incubated with nuclear extract, and a DNA/protein complex is readily detected. Unlabeled fragment D served as an effective competitor to labeled fragment D in lane 3. Unlabeled fragment F, also, from the *serpent* dorsal fat-cell enhancer was an ineffective competitor to labeled fragment D as shown in lane 4.
APPENDIX 1

UAS/GAL4 Gene Misexpression System

Misexpression experiments can be applied, in vivo, as a means of investigating the developmental role of any cloned gene. The misexpression of important patterning genes is often lethal; and the misexpression of cell-type identity genes is also lethal in that it usually grossly disrupts, or occurs at the expense of, other cell types. Therefore, it is generally not possible to construct stable misexpression or gain-of-function stocks. To overcome this obstacle, Brand and Perrimon (1993) have developed a bipartite misexpression system in Drosophila that takes advantage of the yeast (Saccharomyces cerevisiae) transcriptional activator GAL4 and its cognate binding sequence, the Upstream Activating Sequence (UAS; see figure A1-1). With this system, stable stocks can be maintained that contain half of the genetic components needed to misexpress any cloned gene. For the experiments conducted within this project, all UAS and GAL4 lines were obtained from the Bloomington Stock Center (Indiana).

In the UAS/GAL4 system of Brand and Perrimon (1993), the GAL4 encoding sequence is linked to the regulatory sequence of a cell- or tissue-specific gene. For experiments conducted within this dissertation project, it was necessary to misexpress cell-type specific genes as well as region-restricted mesodermal genes. The promoter of the twist (twi) gene is an ideal driver for early pan-mesodermal misexpression of genes because twi,
which encodes a mesodermal transcription factor important for the establishment of the mesoderm, is expressed in every cell of the mesoderm. Akam (1996) generated the *twi*-GAL4 stock, in which the GAL4-encoding sequence was linked to the *twi* promotor to create a *twi*-GAL4 construct that was then inserted into the genome by micro-injection of embryos and P-element mediated transformation. In the *twi*-GAL4 transgenic flies, *twi*-GAL4 is present in every cell, but GAL4 is only produced in those cells where *twi* is activate (all mesodermal cells).

The gene-to-be misexpressed is linked to UAS, a yeast sequence to which GAL4 binds in the activation of the transcription of various yeast genes involved in the metabolism of galactose. To create UAS stocks, a DNA construct in which a cloned copy of the gene-to-be misexpressed is linked to UAS and this construct is integrated into the *Drosophila* genome through microinjection and P-element mediated transformation. In these transgenic embryos, although the UAS construct is present in every cell of the body, UAS is not transactivated and consequently the linked gene is not expressed in those cells where GAL4 is not misexpressed. By crossing the promotor-GAL4 stock with the UAS-gene (to-be-misexpressed) stock, (e.g., *twi*-GAL4 x UAS-*s*p) the gene linked to UAS is expressed as directed by the promotor linked to GAL4 in the resulting progeny.
Figure A1-1. Schematic representation of the UAS/GAL4 system in Drosophila.

This system requires two Drosophila stocks for the misexpression of a single gene. The first stock contains a transgenic insertion such that the GAL4 gene is linked to the promoter region of a known gene that is expressed within a particular cell or tissue type (e.g., hypothetical tissue x). The second transgenic stock is created such that the cognate
Figure A1-1 (continued)

binding site of GAL4 protein. UAS is linked upstream of a gene to be misexpressed. When the two stocks are crossed, the offspring produce GAL4 protein only within tissue-x cells, because within these cells, the promoter region derived from the tissue-x specific gene is transactivated.
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VITA

Graduate College
University of Nevada, Las Vegas

Jennell Marie Miller

Local Address:
2120 Ramrod Av
#1524
Henderson, NV 89014

Home Address:
Same as Above

Degrees:
Bachelor of Arts, 1996
California State University, San Bernardino
San Bernardino, CA

Special Honors and Awards:
- GREAT Award, Graduate College, UNLV Summer 2001
- Summer Session Scholarship, Graduate College, UNLV Summer 2000
- Terry Evans Award, DBS, UNLV Spring 1999
- Graduate Student Association Research Award, UNLV Fall 1999
- Barrick Fellowship, Graduate College, UNLV 1999-2000
- Regents Award Program Fellowship, UNLV Spring 1998
- Graduate Student Association Travel Award Spring 1998
- Graduate Student Association Research Award Fall 1998
- Department of Biological Sciences Travel Award Fall 1998

Publications:


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Dissertation Examination Committee:
Chairman, Dr. Deborah K. Hoshizaki, Ph.D.
Committee Member, Dr. Daniel Thompson, Ph.D.
Committee Member, Dr. Stephen Roberts, Ph.D.
Committee Member, Dr. Elizabeth Eldon, Ph.D.
Graduate Faculty Representative, Dr. Bryan Spangelo, Ph.D.