FATTY ACID AUXOTROPHY IN *DROSOPHILA* LARVAE LACKING SREBP

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by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

June, 2006

ACKNOWLEDGEMENTS

After I finished my rotation in the Brown and Goldstein lab, I emailed Mike and Joe thanking them and saying that "It was the most I have ever learned from anyone in a two-month period". They have now been teaching me how to do science for four years.

It is unfortunately impossible to quantify emotion, but I hope the preceding anecdote puts into perspective the respect and gratitude I feel towards them. They have created the best possible scientific environment for a student. I cannot think of a better way to become a scientist than to present your data to them every week (and to occasionally have it ripped to shreds by Joe). They have shown me the importance of imagination, hard work and of never settling for mediocrity. They have shown me not only the importance of being thoughtful, but also that thought without action is meaningless. I thank them for setting the bar for the rest of my career.

All my work has been done in Rob Rawson's lab, and I have worked under his direct supervision. Rob is one of those rare supervisors who gives you all the freedom you want, but will still go out of his way whenever you need help. Obviously, this is the best combination a graduate student could ask for. He showed me the nitty-gritty of fly genetics and was a constant source of encouragement, ideas, good humor, and the occasional free pizza. Working with him has been one of the most enjoyable aspects of my time here.

Krista Matthews, a graduate student in the lab, has been a critical part of this effort. She originally helped me with the *dSREBP* screen and we have collaborated on a number of the later experiments in this study. Beyond being a collaborator, she is a good friend. Perhaps most importantly, she is the conduit through which her mom's cooking occasionally finds its

way into the lab. Irina Dobrosotskaya, a former postdoc, was a great source of technical expertise, general advice and constantly inspired me with her work ethic. Bilal Amarneh and Carol Lah helped with plasmid construction and transgenesis. Peter Espenshade, also a former postdoc in the department, was my rotation mentor and was patient enough to teach me all the basic lab techniques even though I had never run a protein gel before I met him.

The work presented in this dissertation could not possibly have been completed without the help of some superb technicians- Edward Tambe-Ebot, Joe Lockridge and Phuong Pham. Edward, in particular, has worked closely with me for the past two years. He has been a tremendous help in the extensive fly breeding involved in generating transgenic flies. The nutritional rescue experiments that are the most critical part of this work would not have been completed without him. He has scored approximately half of the 40,000 or so fly wings that it took to generate Table 4-5. Phuong maintains all our stocks and saves us all more than a day's work every week.

Through the years, I have had help from almost everyone in the Molecular Genetics department. I am particularly thankful to Young Ah-Moon and Scott Clark for giving me a crash course in fatty acid analyses when I needed quick results.

Outside the department, Jaemyoung Suh and Jon Graff have been extremely generous in letting me use the resources in their laboratory. Jae has also been a great person to discuss experiments with and has given me unfettered access to his collection of enhancer trap lines.

My committee members- Sandy Hofmann, Jon Graff and John Abrams have always kept me on track and given me a clear picture of what I need to accomplish.

I have to thank Deepak Srivastava for his help and advice. I worked in his lab for a year, and he could not have been more supportive when I realized that I wanted to do my graduate work in a different field.

Finally, I reserve my deepest gratitude for the three most important people in my life- my parents, Vidya and Sudhakar, and my wife Neha. My mom is one of the most strong-willed people I know, and her ability to keep going in the face of seemingly insurmountable odds is one of my greatest sources of inspiration. My dad is one of the most curious people I know, and has taught me to never take life so seriously that I stop enjoying it. Neha is the hardest person for me to write about. She is so intimately involved in every aspect of my life that there is no one thing I can credit her with. I don't know what I would do without her.

FATTY ACID AUXOTROPHY IN *DROSOPHILA* LARVAE LACKING SREBP

Publication No. _____

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The University of Texas Southwestern Medical Center at Dallas, 2006

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A rapid increase in size is a major characteristic of larval development in *Drosophila melanogaster*. Such growth presumably requires the concomitant production of membrane lipids and is also accompanied by a significant accumulation of neutral lipid stores. Growing larvae must accumulate fatty acids to permit the synthesis of these lipids. Interestingly, wild type *Drosophila* can grow in the complete absence of exogenous fatty acids.

This dissertation reports the finding that a lipogenic transcription factor, dSREBP (*Drosophila* Sterol Regulatory Element Binding Protein), is essential for the maintenance of this prototrophy. Drosophila larvae lacking dSREBP demonstrate a profound growth deficit in the second instar and die before reaching third instar. This is accompanied by transcriptional deficits in fatty acid synthetic genes. The growth deficit and lethality can be reversed by supplementing the culture medium with fatty acids. The most effective fatty acid, oleate, rescues 80 percent of *dSREBP* mutants to adulthood. Thus, a lack of dSREBP renders larvae auxotrophic for fatty acids. A reporter system demonstrates that dSREBP is active in tissues known to be involved in lipid metabolism- the fat body, oenocytes and anterior midgut. Finally, as expected of an end-product inhibited metabolic pathway, dSREBP activity can be suppressed by dietary supplementation with lipids. Thus, the dSREBP pathway coordinates endogenous synthesis with the dietary provision of exogenous lipids.

These results establish *Drosophila* as a viable model for the genetic study of the SREBP pathway and provide the first evidence that, at an organismal level, the essential role of the pathway is the accumulation of lipids. The auxotrophic mutants and other reagents described here should be useful tools for further study of the SREBP pathway in particular and fatty acid metabolism in general.

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PRIOR PUBLICATIONS

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2) **Kunte A**, Ivey K, Yamagishi C, Garg V, Yamagishi H and Srivastava D. A common cis-acting sequence in the DiGeorge critical region regulates bi-directional transcription of UFD1L and CDC45L. *Mech Dev.* 2001 Oct;108(1-2):81-92.

3) **Kunte A**, Misra V, Paranjape R, Mansukhani N, Padbidri V, Gonjari S, Kakrani V, Thakar M and Mehendale S. HIV seroprevalence & awareness about AIDS among pregnant women in rural areas of Pune district, Maharashtra, India. *Indian J Med Res.* 1999 Oct;110:115-22.

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P-element – A class of transposon that is largely restricted to *Drosophila*. Engineered P-elements that are not capable of self-transposition are used as tools in mutagenesis.

piggyBac – A separate class of transposons that is less species-restricted and has a different insertion site preference. These elements have also been engineered for mutagenesis.

Balancer – A multiply inverted chromosome that is incapable of recombining with its homolog. Balancers are usually homozygous lethal and can therefore be used to maintain heterozygous stocks of lethal mutations.

Binary or GAL4/UAS system – A two component reporter/misexpression system. One stock is called the 'driver' and carried a transgene encoding yeast GAL4 under control of a promoter of interest or a genomic enhancer. The other stock is called the 'responder' and carries a transgene with a reporter or gene-of-interest under control of the yeast upstream activating sequence (UAS) (Brand and Perrimon, 1993).

Imprecise Excision Screen – A P-element is mobilized. Flies that have lost the P-element are then screened for a deletion of the genomic region surrounding the insertions site.

CHAPTER ONE
Introduction

Lipids are a fundamental building block of all life forms, with the possible exception of some viruses. Membrane lipids, the major constituent of cellular membranes, are responsible for defining cellular boundaries and, in eukaryotes, for the formation of sub-cellular compartments. Storage lipids serve as the principal forms of stored energy in many organisms. Other lipids, although present in relatively small quantities, play crucial roles as enzyme cofactors, electron carriers, light-absorbing pigments, hydrophobic anchors, emulsifying agents, hormones and intracellular messengers (Nelson and Cox, 2004). On the other hand, disorders of lipid metabolism are central features of atherosclerotic disease and the metabolic syndrome, two of the main causes of morbidity in the Western world. Unraveling the mechanisms that control the synthesis, absorption and transport of lipids is thus critical to both the understanding of biology as well as the prevention of disease.

THE SREBP PATHWAY- MODEL SYSTEMS

Over the past 12 years, the SREBP pathway has emerged as an important regulator of lipid homeostasis in a variety of organisms. The effector of the pathway is a membrane-bound transcription factor called SREBP (Sterol Regulatory Element Binding Protein). In general, SREBP activity increases cellular lipid levels by *de novo* synthesis (via transcriptional upregulation of biosynthetic enzymes) or uptake (via transcriptional activation of cell surface receptors). The activity of SREBP is, in turn, regulated by membrane lipid levels via end-product mediated feedback inhibition. All SREBPs that have been studied share a common

topology. They have a cytosolic transcription factor domain that is rendered inactive by being anchored to endoplasmic reticulum (ER) membranes via transmembrane helices. Activation requires release of the transcription factor, which occurs by proteolysis. Wherever it has been studied, regulation of proteolysis has emerged as a key step in end-product mediated feedback inhibition of the SREBP pathway. A summary of the current state of knowledge about the SREBP pathway, and its role in metabolism, can be broadly divided into four categories based on the model systems used for its study.

Mammalian SREBPs

SREBPs were discovered in mammalian tissue culture cells (HeLa) (Briggs et al., 1993; Wang et al., 1993) and adipocytes (Tontonoz et al., 1993). Studies in mammalian cell lines have led the way in deciphering SREBP pathway function and regulation.

Pathway components and function

There are two *SREBP* genes in mammals, *SREBP-1* and *SREBP-2*. The *SREBP-1* gene codes for SREBP-1a and SREBP-1c, which are splice isoforms and preferentially activate genes involved in the *de novo* synthesis of unsaturated fatty acids, triacylglycerols and phospholipids. The *SREBP-2* gene produces the SREBP-2 protein, which preferentially activates genes involved in both the *de novo* synthesis as well as the cellular uptake of cholesterol (Horton et al., 2002; Pai et al., 1998). The mammalian SREBP pathway thus controls both membrane as well as storage lipid levels.

The mammalian SREBP pathway consists of a number of other components that are essential to either cleavage of SREBP or suppression thereof. The functions of these proteins-

Scap, Insig, Site-1 protease and Site-2 protease are described in greater detail in the following section.

Regulation of SREBP activity

Regulation of cleavage (*see Figure 1-1*): As mentioned above, the SREBPs are synthesized as inactive precursors bound to membranes of the ER and nuclear envelope (Brown and Goldstein, 1997). The precursor consists of three distinct domains: 1) an amino-terminal, cytoplasmic bHLH-ZIP transcription factor domain, 2) two transmembrane domains connected by a short (approximately 30 amino acids) luminal loop, and 3) a carboxy-terminal domain, termed the regulatory domain, which is necessary for regulated activation of the precursor. The mechanism of cleavage and regulation has been most extensively studied for mammalian SREBP-2, the activity of which is regulated by cellular cholesterol levels.

The membrane-bound SREBP-2 precursors exist as complexes with a polytopic membrane protein called Scap (SREBP cleavage activating protein) (Sakai et al., 1997). When cells are sterol depleted, the SREBP-2:Scap complex is recruited into COPII coated vesicles via which it exits the ER and is trafficked to the Golgi apparatus (DeBose-Boyd et al., 1999; Espenshade et al., 2002). This trafficking event renders SREBP susceptible to cleavage by a Golgi resident serine protease called Site-1 protease (S1P)(Sakai et al., 1998). This cleavage, which occurs within the luminal loop (Duncan et al., 1997), separates the two transmembrane domains. The amino terminal fragment, termed the intermediate fragment, can now be cleaved by a second protease- an integral membrane metalloprotease termed Site-2 protease (S2P) (Rawson et al., 1997). The second cleavage occurs within the first transmembrane domain, three residues from its cytoplasmic boundary (Duncan et al., 1998;

Sakai et al., 1996). This releases the transcription factor domain from membrane attachment, so that it can now translocate to the nucleus and activate gene transcription.

When cell membranes are sterol replete, Scap undergoes a conformational change (Brown et al., 2002). Likely as a result of this, the SREBP-2:Scap complex binds to ER resident proteins termed Insigs (Yabe et al., 2002; Yang et al., 2002). This sterol dependent interaction between Scap and Insig prevents budding of the SREBP-2:Scap complex from the ER and thus prevents SREBP cleavage (Nohturfft et al., 2000). Recent studies indicate that the sterol-sensing mechanism involves the direct binding of cholesterol to Scap (Radhakrishnan et al., 2004). There are also likely alternate mechanisms, other than direct binding to Scap, by which lipids regulate SREBP cleavage. This is because oxysterols, which do not bind Scap or cause it to change conformation, also suppress SREBP cleavage (Adams et al., 2004). Another layer of complexity to the regulation of SREBP cleavage was noted in the study of SREBP-1 in human embryonic kidney cells (HEK293). In these cells, in addition to sterols, polyunsaturated fatty acids can also suppress SREBP-1 cleavage. In fact, persistent suppression of cleavage by sterols requires the presence of a polyunsaturated fatty acid (Hannah et al., 2000).

Regulation of mRNA stability: This mechanism of regulation was suggested by Xu et al (Xu et al., 2001). These investigators found that, in rat primary hepatocytes, polyunsaturated fatty acids reduce the half life of SREBP-1 mRNA.

Transcriptional regulation: The SREBPs are subject to a number of regulatory influences at the level of transcription. The SREBPs are transcriptional targets of themselves. This may have some feed-forward effect on the pathway, especially in the case of *SREBP-1c* (Liang et

al., 2002; Yang et al., 2001). *SREBP-1c* is a target of signaling by the oxysterol receptor, LXR. This regulatory mechanism results in an increase in fatty acid synthesis when sterol levels increase and is likely the basis of hypertriglyceridemia observed with high cholesterol diets (Repa et al., 2000). *SREBP-1c* is also the target of insulin signaling, leading to the increase in fatty acid synthesis seen after refeeding starved animals with high carbohydrate diets (Shimomura et al., 1999).

The mammalian SREBP pathway *in vivo*

In addition to individual cells, the mammalian SREBPs have been shown to play important roles in the maintenance of whole body lipid homeostasis. The pathway plays a role in the ability of the liver to co-ordinate its synthetic activity with the dietary availability of lipids. This is exemplified by the response of hepatic SREBP-2 cleavage to dietary cholesterol surplus or depletion (Shimomura et al., 1997a). SREBPs in the liver also respond to the nutritional status of the body as a whole by interacting with other nutrient sensitive signaling systems such as insulin and the oxysterol receptor (LXR).

Studies in intact animals have revealed a number of differences/nuances to the regulation of the SREBP pathway that are not detectable in cultured cells, 1) The relative abundance of SREBP-1a vs 1c are reversed in the liver vs cultured cells. SREBP-1c is the predominant isoform in the liver. SREBP-1a, which has a stronger ability to drive cholesterol synthesis, is the predominant isoform in cultured cell lines, even if they are originally derived from liver (Shimomura et al., 1997b); 2) There exists a liver-specific isoform of Insig-2, which is negatively regulated by insulin signaling (Yabe et al., 2003a). Although is hard to precisely compare the relative levels of the two proteins, this phenomenon may create a

situation in fasting mice where Insig-1 levels are low and Insig-2 levels are high. This is opposite to the Insig-1/Insig-2 ratio in cultured cells; 3) Unlike the situation in most cultured cells, SREBP-1 and SREBP-2 appear to be regulated differentially in the liver. a) When wild type mice are fed a cholesterol rich diet, cleavage of SREBP-2 is much more sensitive to suppression than that of SREBP-1 (Engelking et al., 2005). b) When mice are subjected to a fasting/refeeding protocol, the levels of both the precursor as well as the nuclear form of SREBP-1 show a decrease followed by an increase that overshoots the basal level. This overshoot phenomenon requires SCAP (Matsuda et al., 2001) and can be blocked by Insig-1 (Engelking et al., 2004). It does not, however, occur for SREBP-2. Specific stimulation of SREBP-1c transcription by insulin can explain the difference at the level of the precursor. However, it is not yet clear how a situation arises where SREBP-1 cleavage can be stimulated without stimulating SREBP-2; 4) Spermatogenic cells in rat testes produce an alternative SREBP transcript that encodes only the transcription factor domain (Wang et al., 2002).

Clearly, then, study of the SREBP pathway in intact animals has the capacity to unveil additional and important information about the functions of the SREBP pathway, its regulation and its role in physiology.

Drosophila SREBP

The *Drosophila* SREBP ortholog (dSREBP) was initially isolated fortuitously from the mbn-2 hemolymph cell line (Theopold et al., 1996). Subsequent studies have been

performed in Schneider S2 cells (Schneider, 1972), an embryonic cell line that exhibits gene expression patterns similar to larval fat body and hemocytes (Cherbas and Cherbas, 1997).

Pathway components and function

The *Drosophila* genome contains a single *SREBP* gene, which produces a single protein called dSREBP. There also exist *Drosophila* homologs of Scap, S1P and S2P (called dSCAP, dS1P and dS2P, respectively) (Seegmiller et al., 2002). Seegmiller et al also showed that dSREBP is indeed cleaved in a manner that permits the N-terminal cytosolic domain to enter the nucleus. Analogous to the mammalian pathway, this cleavage is dependent on dSCAP and is sensitive to mutations known to abolish Site-1 and Site-2 cleavage. There does not, however, appear to be a clear *Drosophila* homolog of the Insig proteins (Rawson, 2003).

In S2 cells, dSREBP regulates a number genes involved in *de novo* fatty acid synthesis- acetyl CoA carboxylase (ACC), acetyl CoA synthase (ACS) and fatty acid synthase (FAS). A change in dSREBP activity does not, however, affect genes in the sterol biosynthetic pathway- hydroxymethylglutaryl CoA reductase (HMGCR) or hydroxymethylglutaryl CoA synthase (HMGS) (Dobrosotskaya, 2003; Seegmiller et al., 2002). This is not surprising in light of the fact that insects, being sterol auxotrophs, have no *de novo* synthesis to regulate (Clark, 1959). When dSREBP mRNA was eliminated by RNAi, the rate of fatty acid synthesis fell four-fold and S2 cells eventually died (Seegmiller, 2002). Further studies revealed that the main role of dSREBP dependent fatty acid synthesis is likely to generate precursors for the production of phospholipids (Dobrosotskaya et al., 2002).

Regulation of SREBP activity

Regulation of cleavage: Analogous to the mammalian system, cleavage of dSREBP is regulated by end-product mediated feedback inhibition. Therefore, it is not affected by sterols and responds to the addition of a fatty acid (palmitate, C16:0) to the culture medium (Seegmiller et al., 2002). The specificity of the fatty acid requirement provided the clue that led to a hypothesis about the identity of the actual regulating molecule (Dobrosotskaya et al., 2002). It was shown that palmitate serves two purposes once added to the medium; 1) It serves as a substrate for the synthesis of sphingolipids that are eventually broken down to yield phosphoethanolamine; 2) It is required for an additional non-specific function, which is likely the synthesis of diacylglycerol. This hypothesis is supported by the observation that, in the presence of exogenous ethanolamine, other fatty acids can satisfy the additional requirement. The end result of these two processes is an increase in the synthesis of phospholipids, predominantly phosphatidylethanolamine (PE). PE is the major phospholipid in *Drosophila* membranes and is thus likely the molecule that regulates dSREBP cleavage, analogous to cholesterol in mammalian cells. Whether this is achieved by direct binding of PE to SCAP, or by another mechanism, is an open question.

Transcriptional regulation: The dSREBP mRNA level falls more than two-fold when dSREBP cleavage is eliminated by RNAi against dSCAP, suggesting that *dSREBP* is a transcriptional target of itself. It is not known whether, analogous to the mammalian pathway, other transcriptional inputs impinge on *dSREBP*. It should be noted, however, that these inputs were uncovered either *in vivo* or in primary culture. Neither of these approaches has been used to study the dSREBP pathway.

C. elegans SREBP

The *C. elegans* SREBP homolog, called lpd-1, was discovered based on its sequence similarity to the mammalian protein.

Pathway function

Using promoter fusions, McKay et al (McKay et al., 2003) showed that *lpd-1* is expressed in enterocytes (multi-functional cells that serve as the fat storage site in worms). They further showed that worms lacking *lpd-1* function arrest at larval stages, have depressed transcription of fatty acid biosynthetic genes, and do not stain with Nile-red (a fluorescent vital dye that binds lipids). Kniazeva et al (Kniazeva et al., 2004) have suggested that *lpd-1* regulates elongases involved in the biosynthesis of mono-methyl branched chain fatty acids (mmBCFAs). Synthesis of mmBCFAs, though required for larval development, is not the only essential function of *lpd-1*. This is because *lpd-1* mutants cannot be rescued by mmBCFA supplementation alone. Whether sterol biosynthetic genes are affected by lack of *lpd-1* has not been tested. Given the fact that *C. elegans* are sterol auxotrophs, it is likely that *lpd-1* does not regulate these genes.

Regulation of the pathway

Based on upregulation of *lpd-1* promoter activity in worms lacking an elongase, Kniazeva et al have suggested that *lpd-1* transcription responds to the levels of mmBCFAs. Whether the lpd-1 protein requires cleavage for its actions and whether cleavage is regulated has not yet been studied.

Yeast SREBP

The yeast SREBP pathway was also identified based on sequence similarities to components of the mammalian pathway. Interestingly, the pathway seems to be present in fission yeast (*Schizosaccharomyces pombe*) but not in baker's yeast (*Saccharomyces cerevisiae*) (Hughes et al., 2005).

Pathway components and functions

The *S. pombe* genome encodes two SREBP homologs, Sre1 and Sre2. Sre1 is predicted to have the characteristic SREBP topology whereas Sre2 lacks the C-terminal cytosolic domain. Of these two proteins, Sre1 has been shown to be an SREBP ortholog. There is a *S. pombe* protein homologous to Scap, called Scp1. While an Insig homolog (Ins1) exists, this protein does not appear to be required for Sre1 regulation. Thus, either Ins1 is not an ortholog or there are protein/s that can substitute for it. Though Sre1 is indeed cleaved, the responsible proteases or their cleavage sites have not yet been identified.

Like mammals, *S. pombe* is capable of *de novo* sterol synthesis and the chief membrane sterol is ergosterol. Accordingly, Sre1 regulates genes involved in ergosterol synthesis, albeit only oxygen-requiring enzymes in the final stages of the biosynthetic pathway. Sre1 does not affect transcription of fatty acid synthase. An important discovery made by Hughes et al was that Sre1 also regulates a number of enzymes involved in oxygen sequestration and other oxygen dependent reactions. As a result, Sre1 is required for continued growth in hypoxic conditions.

Regulation of Sre1 activity

Regulation of cleavage: Mechanistically, Sre1 cleavage appears to be regulated by sterol levels similar to the regulation seen in mammalian cells. Hughes et al suggest an interesting

model for the physiological relevance of this regulation. Based on their data regarding 1) Sre1 target genes involved in oxygen sequestration, 2) the requirement of Sre1 for hypoxic growth, 3) the requirement of oxygen for sterol synthesis, and 4) data suggestive of feedback regulation by sterols, they suggest that the *S. pombe* SREBP pathway uses oxygen-dependent sterol synthesis as an indirect measure of oxygen availability and responds by titrating the levels of transcripts required for adaptation to hypoxia.

Transcriptional regulation: Like the other cases where this has been studied, Sre1 seems to be an activator of its own transcription.

A number of points can be made based on the preceding discussion. 1) There is striking conservation in the SREBP pathway among various model systems. Though certain components or subsets of function may be absent in one or the other system, the core logic of the pathway is conserved (ie. end-product mediated feedback inhibition). 2) Each invertebrate model system has illuminated a new, and different, aspect of SREBP function or regulation. While it remains to be determined how many of these discoveries find application in mammals, they clearly provide food for thought and experiment. 3) Comparison between model systems reveals certain correlations that may have evolutionary implications, a) regulation of the pathway by membrane lipids (unknown for *C. elegans*), b) the occurrence of *de novo* sterol synthesis in organisms with two SREBP genes, and c) regulation by sterols in organisms that have Insig-like proteins.

In conclusion, study of the SREBP pathway in invertebrate model systems has been, and is likely to continue being, a fruitful undertaking towards the understanding of lipid biology.

FORWARD GENETICS IN THE STUDY OF THE SREBP PATHWAY

Previous successes

Mutagenesis and selection schemes using Chinese hamster ovary (CHO) cells have been used with remarkable success in the study of the SREBP pathway. Somatic cell genetics has been used to screen for one of two phenotypes- cholesterol auxotrophy leading to amphotericin resistance, or inability to repress synthesis leading to 25-hydroxycholesterol resistance (Goldstein et al., 2002). Study of cells with the former phenotype led to the identification of Site-1 protease and Site-2 protease by complementation with cDNA or genomic libraries, respectively (Rawson et al., 1997; Sakai et al., 1998). Dominant mutants from the second class were used to generate cDNA libraries from which the causative mutation could then be identified. This led to the discovery of SCAP and the definition of its sterol sensing domain (Hua et al., 1996). Thus, of the five proteins considered core components of the SREBP pathway, three were isolated using mutagenesis and genetic selection.

Limitations of mammalian genetic models

Despite its tremendous success, somatic cell genetics suffers from the inescapable drawbacks of a tissue culture system. Tissue-restricted events cannot be explored (eg. the

liver specific differential regulation of SREBP-1c and -2). Interactions that occur only at the level of organ systems cannot be dissected (eg. the compensatory upregulation of adipose tissue fatty acid synthesis in liver specific Scap knockouts (Kuriyama et al., 2005)). Given the new discoveries that can be made by study of the pathway *in vivo* (see pg. 5), a genetically tractable whole-animal model system is highly desirable.

Mice can and have been used for reverse genetic approaches – to explore the role of known genes or candidate interactions. The complementary approach of large scale mutagenesis and selection/screening is impractical however, especially given the non-visible phenotypes to be expected.

Drosophila as a candidate genetic model system

The use of *Drosophila* as a tool in genetic screens does not require extensive introduction. Perhaps the best example is the fact that a single screen (Nusslein-Volhard and Wieschaus, 1980) can spawn more than 25 years of research and lead to the discovery of multiple developmental pathways that are used in all metazoans studied.

In addition to its use in developmental biology, *Drosophila* has been successfully used as a model for the study of physiology. Examples include the study of circadian rhythmicity (Williams and Sehgal, 2001), olfaction (Vosshall, 2000), memory (Margulies et al., 2005), innate immunity (Hultmark, 2003), and even sleep (Greenspan et al., 2001). Furthermore, pathological processes such as neurodegeneration (Celotto and Palladino, 2005) and tumor metastasis (Pagliarini and Xu, 2003) have been modeled successfully in *Drosophila*.

The potential for use of *Drosophila* in the study of lipid metabolism has not historically received significant attention. The groundwork for such analyses has been laid by the classical studies of Sang (Sang, 1956) and Church and Robertson (Church and Robertson, 1966), which established the nutritional requirements and growth characteristics of *Drosophila*, respectively. A number of investigators have analyzed the fatty acid synthetic capabilities of *Drosophila* (de Renobales and Blomquist, 1984; Keith, 1967a) and studied environmental (Keith, 1967b) or genetic (Geer et al., 1979) influences on the process. However, concerted efforts to isolate genes/mutations that affect the processes of lipid synthesis or storage have not been reported until recently (Gronke et al., 2003; Gronke et al., 2005; Hader T, 2003). The reason for this phenomenon is likely the difficulty in creating 'screenable' lipid synthesis/storage phenotypes and the inability to create sensitized backgrounds by using reverse genetics.

The last 10-15 years have seen the development of a number of reverse genetic techniques (Adams and Sekelsky, 2002), the ability to generate mosaic animals (Xu and Rubin, 1993) and reporter/transgenesis systems that enable the analysis and manipulation of gene expression *in situ* in live animals (Brand and Perrimon, 1993; Duffy, 2002). These developments should significantly ameliorate the problems mentioned above. In addition to these developments, there are some specific advantages to using *Drosophila* as the model for the genetic exploration of the SREBP pathway at the whole organism level: 1) Previous tissue culture studies have validated that important mechanistic aspects of dSREBP cleavage and its regulation are conserved with mammals., 2) The existence of a well-worked out tissue culture system opens up the possibility of moving rapidly between systems in order to

confirm or extend findings., 3) Protocols exist for the large scale separation of organs, if needed (Zweidler and Cohen, 1971).

PROJECT GOAL

The goal of this project was to initiate the genetic study of the SREBP pathway in *Drosophila*. Before exploratory forward genetic approaches could be adopted, it was necessary to obtain a basic understanding of the importance of the pathway (i.e. one has to have a phenotype to screen for or against). Previous studies in the tissue culture system provided excellent clues, but the relevance of these findings to whole-animal physiology was unknown. Furthermore, it was necessary to develop methods to manipulate and monitor pathway activity *in vivo*. Finally, it was necessary to develop sensitized genetic backgrounds that could be used in screens.

Towards fulfilling these requirements, I aimed to understand the essential role of the SREBP pathway in *Drosophila* physiology. It was presumed that successfully achieving this goal would, as a by-product, generate the conditions and reagents needed for further studies. Simply put, I attempted to answer the question 'Why do flies need SREBP?'

To answer this big question, I asked a series of sub-questions, 1) What are the functional consequences of a lack of SREBP?, 2) Can these consequences be ameliorated by lipid supplementation?, 3) Where in the animal is SREBP most active?, and 4) Does the pathway respond to environmental stimuli?

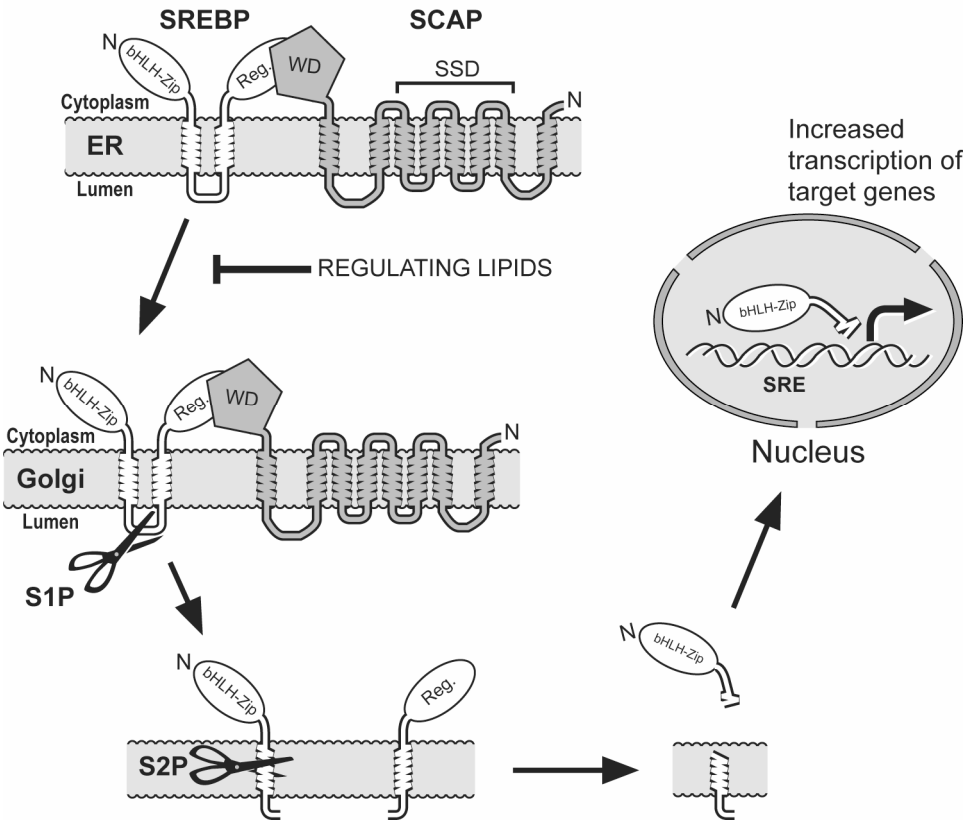
The results in this dissertation show that loss of *dSREBP* results in a transcriptional deficit of the genes of fatty acid synthesis, reduced fatty acid content, and larval lethality.

Lethality is suppressed by supplementing the diet with fatty acids. In wild type larvae, dietary supplementation suppresses the cleavage of dSREBP and the accumulation of its target genes. These results provide evidence, at an organismal level, that the essential function of SREBP in flies is the maintenance of fatty acid biosynthesis. They further suggest that the pathway enables animals to balance *de novo* fatty acid biosynthesis with dietary input, thus enabling efficient resource allocation for rapid growth.

Figure 1-1

Schematic depiction of the mechanism of SREBP cleavage and its regulation by membrane lipids. This graphic was obtained from Dr. Robert Rawson and modified.

Figure 1-1


SREBP
SCAP
N
bHLH-Zip
Reg.
WD
SSD
Cytoplasm
ER
Lumen
N
REGULATING LIPIDS
Increased
transcription of
target genes
N
bHLH-Zip
SRE
Nucleus
N
bHLH-Zip
Reg.
WD
Cytoplasm
Golgi
Lumen
N
S1P
N
bHLH-Zip
Reg.
S2P
N
bHLH-Zip

CHAPTER TWO

LIPID METABOLISM IN *DROSOPHILA*

Nutritional requirements

Sterols- Drosophila, like all insects, are sterol auxotrophs and have an obligatory requirement for sterols in the diet.

Fatty Acids- There are no essential fatty acids. The studies of Sang (Sang, 1956) have shown that *Drosophila* can be cultured in the absence of all lipids save cholesterol.

Synthetic capacity

Studies on partially purified preparations of *Drosophila* fatty acid synthase (FAS) revealed some differences from mammalian FAS (de Renobales and Blomquist, 1984). Unlike the mammalian enzyme, whose predominant product is palmitate (C16:0) (Aprahamian et al., 1982), the *Drosophila* enzyme synthesizes significant quantities of 14 and 18 carbon fatty acids (myristate and stearate, respectively). The chain length distribution of FAS products can be altered *in vitro* by ionic strength and the malonyl CoA/acetyl CoA ratio. Fractionation of lipid classes revealed that most of the C14 fatty acids are present in neutral lipids: the di- and triglycerides.

Steady state measurements in a number of studies (de Renobales and Blomquist, 1984; Geer et al., 1979; Keith, 1966; Keith, 1967b; Teague et al., 1986) have revealed that the chief fatty acids present in *Drosophila* are myristate (C14:0, approximately 15-20%), palmitate (C16:0, approximately 15-20%), palmitoleate (C16:1, approximately 19-25%) and

oleate (C18:1, approximately 19-25%). Other fatty acids, each comprising <10% of the total are laurate (C12:0), stearate (C18:0) and linoleate (C18:2). The detection of linolenate (C18:3) is variable, with only one study reporting values above trace levels (1.6%). Longer chain fatty acids with higher degrees of unsaturation, such as arachidonate (C20:4), have not been reported in these studies.

There is no information about the tissue distribution of *de novo* fatty acid synthesis in *Drosophila*. Based on the fact that most lipid is stored in the fat body, which is also said to have synthetic functions analogous to the mammalian liver (Law and Wells, 1989), it is likely that the fat body is a major site of fatty acid biosynthesis. In some insects, there is experimental evidence of *de novo* fatty acid synthesis in the fat body (Beenakkers et al., 1985). Another tissue likely to have a significant amount of *de novo* fatty acid biosynthesis is the oenocytes. Oenocytes are segmentally repeated clusters of cells that synthesize cuticular hydrocarbons in a number of insects including *Drosophila* (Ferveur et al., 1997). The hydrocarbons are synthesized by elongation and decarboxylation of medium-chain fatty acids (Blomquist and Jackson, 1979; Jallon et al., 1997). Romer (Romer, 1980) observed avid uptake of radiolabeled acetate by oenocytes of the mealworm *Tenebrio molitor*. The more recent data of Fan *et al* showed that, in the cockroach *Blatella germanica*, enzymatically dissociated oenocytes are capable of synthesizing hydrocarbons without the exogenous addition of fatty acid substrates (Fan et al., 2003). Thus, it seems likely that *de novo* fatty acid synthesis occurs at a significant rate in this tissue.

Absorption of dietary lipids

Dietary triglycerides (TG) and phospholipids (PL) are hydrolyzed by lipases in the midgut. This process is aided by a high luminal pH in most insects. Insects do not have bile salts to aid in lipid emulsification, which is therefore accomplished by other means such as the use of lysophospholipids or fatty-acyl amino acid complexes. Absorption of fatty acids occurs as either free fatty acids or monoglycerides. Absorption of sterols also occurs in the midgut, either as free or esterified sterols.

In the midgut epithelium, the absorbed fatty acids are re-esterified by one of two pathways analogous to those in mammalian systems, 1) esterification of absorbed 2-monoacylglycerol, 2) acylation of glycerol-3-phosphate (phosphatidic acid pathway). The esterified lipids are then either stored or exported. Unlike mammalian systems, the chief export form is di-glyceride (DG) and not tri-glyceride (TG) (Canavoso et al., 2001).

Transport and storage

Knowledge about these processes is mostly extrapolated from studies in larger insects like the tobacco hornworm *Manduca sexta*. Like mammalian systems, lipids are transported through the circulatory system as lipoproteins. The main circulating lipoprotein is called lipophorin. Unlike mammals, the apolipoproteins are not synthesized *de novo* in the midgut epithelium. Rather, nascent lipophorin (synthesized in the fat body) is loaded with lipid at the cell surface (Canavoso and Wells, 2000). This process is aided by a docking receptor (lipophorin receptor) and an accessory protein termed the lipid transfer particle (LTP) (Canavoso and Wells, 2001).

The absorbed fatty acids are delivered for storage to the fat body via lipophorin where they are stored as tri-glycerides (TG).

A notable difference between lipoprotein metabolism in insects and mammals is that insect lipophorin is used as a reusable shuttle, rather than being synthesized during assembly and degraded during delivery. An exception is the delivery of lipids to the oocyte, where receptor-mediated endocytosis of a lipoprotein (vitellogenin) plays a role (Ziegler and Van Antwerpen, 2006). Additionally, recent discoveries have implied a role for receptor-mediated lipophorin endocytosis in locust (*Locusta migratoria*) fat body (Van Hoof et al., 2003).

Mobilization

During times of metabolic demand, such as flight, TG from the fat body are hydrolyzed to generate DG (not free fatty acids like mammals) that are then exported via lipophorin. This process can be stimulated by two hormones- adipokinetic hormone (AKH, a peptide) and octopamine (a catecholamine) (Arrese et al., 2001).

CHAPTER THREE
Methodology

Genetic strains

All marker mutations and balancer chromosomes are described and referenced by FlyBase (2003). Crosses were carried out at 25 ºC in vials containing freshly yeasted cornmeal-molasses agar (1 L of cornmeal-molasses medium contains 60 g cornmeal, 15 g dry yeast, 80 ml unsulphured molasses and 12 g agar) except where noted. Oregon-R flies served as wild type. P-element transposon insertion line KG03723 was obtained from the Bloomington *Drosophila* stock center. The transposon allele was allowed to freely recombine with wild type for three generations before being formally isogenized and tested for lethal and sterile phenotypes prior to use in the excision screen. PiggyBac transposon insertion line $dSREBP_{52}$ was provided by Ernst Wimmer (Horn et al., 2003). This chromosome also harbored an unrelated pupal lethal mutation that was revealed during the course of rescue experiments. This mutation was removed by recombination with wild type. The resulting $dSREBP^{52}$ allele was fully rescuable. The P{UAS-dSREBP}, P{UAS-dSREBP1-452} and P{*GAL4-dSREBPg*} transgene insertions are on the second chromosome. These stocks were created by standard germline transformation techniques using the Δ2-3 helper plasmid (Rubin and Spradling, 1982). GAL4 expressing lines 6487 and 6450 were obtained from the Bloomington *Drosophila* Stock Center. S_1106 was a gift of Ron Davis (Baylor, Houston). DcG-GAL4 was provided by Jon Graff and J. Suh (U.T. Southwestern).

Buffers

Buffer A contains 10 mM HEPES-NaOH (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, and 1 mM EGTA. Buffer F contains 125 mM Tris-HCl (pH 6.8), 8 M Urea, 5% SDS.

Monoclonal antibodies

IgG-3B2, against the amino-terminal domain of dSREBP was described previously (Seegmiller et al., 2002). IgG-611B-1 against acetylated tubulin was obtained from Sigma (St. Louis).

Plasmids

pP{UAST-dSREBP} - full length *dSREBP* cDNA was amplified by PCR with the addition of EcoR1-Xba1 linkers. The resulting fragment was digested and cloned into the EcoR1-Xba1 sites of pUAST (Brand and Perrimon, 1993). The coding sequence was then sequenced in its entirety.

pP{UAST-ndSREBP }- A fragment encoding a.a. 1-452 of dSREBP and flanked by EcoR1-Xba1 linkers was obtained by PCR amplification using full length dSREBP as a template. This fragment was then ligated into the EcoR1-Xba1 sites of pUAST. The coding sequence was then sequenced in its entirety.

pP{*dSREBPg*} - an 8.7 kb genomic fragment (containing the entire *dSREBP* gene, 2.9 kb upstream and 0.7 kb downstream) was amplified by PCR using the High-Fidelity PCR System (Roche). The forward primer used for amplification was 5'-

CGTCTAGACGCATGCTCCCAGAGATGGCACTTTGG –3’ and the reverse primer was 5’- GCTCTAGACACATGTCATCACTGTCAGCGGGATACC-3’. Xba1 linkers were added during amplification and the resultant fragment was ligated into Xba1 digested pCaSpeR-4 (Thummel and Pirotta, 1992) to obtain pP{*dSREBPg*}. The open reading frame was sequenced in its entirety.

pP{GAL4-*dSREBPg*} - restriction sites for Asc1 and Fse1 were inserted into pP{*dSREBPg*} at the beginning of the ORF (Asc1, inserted immediately after a.a.3) and immediately following the bHLH region (Fse1, inserted immediately preceding a.a. 362). The primers used for insertion of the Asc1 site were 5'- GCAGCATTCGCAATGGACACGGCGCGCCTGAACTTAATAGACGCT-3' and 5'-AGCGTCTATTAAGTTCAGGCGCGCCGTGTCCATTGCGAATGCTGC-3'. Primers used for insertion of the Fse1 site were 5'- GCGACGGCTCCAAGGTGAAGGCCGGCCTTCAGCTGGGCACTCGGC-3' and 5'-GCCGAGTGCCCAGCTGAAGGCCGGCCTTCACCTTGGAGCCGTCGC-3'. The sites were inserted individually into pP{*dSREBPg*}. A Nar1 (for the Asc1 site) or Nar1-Nhe1 fragment (for the Fse1 site) were excised out of the resultant vector and then subcloned together into Nar1-Nhe1 digested pP{*dSREBPg*}. The resultant vector pP{*dSREBPg*/AF} was sequenced in the regions that had been subject to PCR. In order to generate pP{GAL4-SREBP}, a cDNA fragment encoding a fusion of the GAL4 DNA binding domain fused to the VP16 transactivation domain was amplified by PCR from pMGstV (a gift from Thomas Sudhof, UT Southwestern). Asc1 and Fse1 linkers were added during amplification. This fragment was then ligated into Asc1/Fse1 digested pP{*dSREBPg*/AF}.

Generation of deletion mutants

Standard P element excision screens (Castrillon et al., 1993) were performed using KG03723. Briefly, insertion bearing chromosomes were crossed together with a chromosome carrying P{*ry+, Δ*2-3}(Robertson et al., 1988). In the next generation, the transposase was crossed out and *w-* derivatives of the insertion-bearing chromosomes were selected and used to establish balanced lines. Screening by PCR (for viable lines) or Southern blot analysis (for lethal lines) identified those events where excision resulted in loss of genomic DNA extending to the *dSREBP* ORF.

Mapping of deletions

Southern blots- Genomic DNA was prepared from balanced heterozygous flies carrying the candidate deletions. 10 fly equivalents of DNA were digested overnight with 50U EcoR1. 6 fly equivalents were loaded/lane on a 0.8% agarose gel. Wild type flies served as control. DNA was transferred to nylon membranes using standard Southern blot techniques. Probes were prepared by random-primed labeling using the Rediprime II kit (Amersham Biosciences) and purified using Probequant G50 columns (Amersham Biosciences). the primer sequences used to generate the probe fragments were the following:

Far Probe: 5'- CAAGTCCAAGGCCTCCAGTTTACTGAAGTGCCGC and

5'- CAGGACAATGGACAAACTGGGATTAGGCTGCCC.

Exon 1 probe: 5'- CCTTAGGCCCGAACTACTGCTCGTCATCCCTG

5'- CCACTGGTTTCCCGATCTGATTTCCGCG

Mapping the dSREBP^{189} breakpoint- The fragment was amplified using the Expand High Fidelity PCR kit (Roche). Sequencing of the fragment and comparison to the sequence in the genome database yielded the breakpoint. The primers used were:

5'- GGCCGCGCTGGAGAAAGGTCTTGAAGGG and

5'- CCCACTCTATGCCGCTCTATCGGGTGTGCG

Lethal phase assays

Embryos from *dSREBP^{189}* /TM3, *Actin-GFP, Ser* or *dSREBP^{52}*/TM3, *Actin-GFP, Ser* stocks or from a cross between the two were plated on 60 mm dishes (1 dish/time point) containing semi-defined medium (Backhaus, 1984) at a density of 20 mg embryos/plate. At the indicated time, all larvae were washed off the plates, separated from the food by floatation on 2-3 M NaCl, and scored based on fluorescence detection of actin-driven GFP. Survival of homozygotes is plotted as a percentage of the expected ratio of homozygotes to heterozygotes (0.5 = 100 %).

Whole Fly Lysis

15 adult males or third instar larvae of the indicated genotype were homogenized in buffer F supplemented with a cocktail of protease inhibitors (Seegmiller et al., 2002). Homogenates were cleared by centrifugation at 20,000g for 10 min. Supernatants were transferred and used for a measurement of the protein concentration. The indicated amount of the lysates were electrophoresed, transferred to a nitrocellulose membrane and probed with an anti-dSREBP antibody (3B2) at 2 μg/ml.

Transgenic rescue

dSREBP cDNA rescue - The various GAL4 drivers were first crossed into a *dSREBP* ^{189} background to generate w^{1118};P{w^{+},*GAL4*}/ P{w^{+},*GAL4*}; *dSREBP* ^{189} /*TM6B, Tb Hu e* flies (for homozygous viable transgene insertions) or w^{1118};P{w^{+},*GAL4*}/ *CyO; dSREBP* ^{189} /*TM6B, Tb Hu e* (for homozygous lethal transgene insertions). Similarly, the responder transgene was crossed into the *dSREBP*^{189} background in order to generate w^{1118};P{w^{+}, *UAS-dSREBP*}/ P{w^{+}; *UAS-dSREBP*}; *dSREBP*^{189} /*TM6B, Tb Hu e* (for homozygous viable transgene insertions) or w^{1118};*UAS-dSREBP/CyO; dSREBP* ^{189} / *TM6B, Tb Hu e* (for homozygous lethal transgene insertions). For rescue experiments, the driver and responder lines described above were crossed and the emergence of various classes of adults was scored using the *Hu* and *Cy* markers. The same strategy was used for rescue with the P{w^{+};*UAS-dSREBP 1-452*} transgene.

Rescue with genomic construct- Males of the genotype w^{1118}/ : P{w^{+}; *dSREBP-g*}/Cyo*: dSREBP*^{189}/TM6B *Tb,Hu* were crossed to females of the genotype *w-/w-: Sp/CyO: dSREBP*^{189}/TM6B *Tb,Hu*. Progeny of the cross were scored for homozygosity at the endogenous *dSREBP* locus using the *Hu* marker and for presence of the rescue transgene using the w^{+} marker that is present on pCasper-4. Emerging progeny were counted daily so that the median developmental time could be calculated.

Analysis of fatty acid composition

Embryos were collected and plated on 60mm dishes containing 9 ml of semi-defined medium (Backhaus, 1984). Larvae of the desired genotype were collected from plates between 37-41 hours after egg laying. 400-450 larvae were pooled for each sample and three samples were analyzed for each genotype. The larvae were homogenized in 200 µl Buffer A supplemented with a cocktail of protease inhibitors. 150 µl of the lysates were extracted with Folch reagent (2:1 chloroform:methanol) (Folch et al., 1957), after the addition of 40 µg of pentadecanoic acid (C15:0) as an internal standard. Samples were transesterified according to the method of Lepage and Roy (Lepage and Roy, 1986). Fatty acid methyl esters were separated by gas chromatography using a Hewlett Packard 6890 Series GC System. The identity of the fatty acid methyl esters was determined by comparing retention times with 37 methylated fatty acid standards (Supelco 37 Component FAME Mix). Fatty acids in each sample were quantified by comparison to pentadecanoic acid. Pentadecanoic acid was not detected in samples processed without this addition. The remaining 50 µl of each homogenate was centrifuged at 20,000 g for 10 min. Protein was measured from the supernatants using a BCA protein assay kit (Pierce).

Nutritional rescue of *dSREBP* mutants

Preparation of medium - The relevant compound was added in a solid form (w/vol), with constant stirring, to molten cornmeal-molasses-agar. When the powder appeared dispersed, the medium was aliquoted into vials at 9 ml/vial, stored at 4 °C and used within one week. Soybean lipid extract was purchased from Avanti Polar Lipids. Na C12:0, Na C14:0, Na

C16:0, Na C18:0, Na C18:1 and tripalmitin were purchased from Sigma Aldrich. Na C16:1 was prepared from C16:1 (Sigma Aldrich) as described (Hannah et al., 2000).

Rescue of mutants - Embryos were collected overnight from a *dSREBP* ^{189}*/TM3,Act-GFP,Ser* stock. Embryos were brushed off the plates, washed extensively with PBS-Tween and water and then added to preweighed tubes containing PBS. The tubes were then reweighed in order to obtain a suspension of embryos of known weight/volume. The suspension was agitated by gentle vortexing, the necessary volume was pipetted out and added to vials containing the desired culture medium. We added 1 mg embryos per vial except as noted in the Table legends. Flies were allowed to develop and emerging adults were scored until they stopped emerging (approximately day 18 after plating). In order to calculate the percent rescue, the observed ratio of homozygotes to heterozygotes was divided by the expected ratio (0.5).

The embryo collection and pipetting method was first validated to ensure that the volume of suspension pipetted had a linear relationship to the number of embryos (Figure 3-1). Day to day variation was also tested and found to be approximately 10-15%.

Quantitative analysis of transcripts

Embryos were collected for 2 hours and plated as described above. Larvae were allowed to develop 37-41 hours. Heterozygous (+/-) larvae were scored based on fluorescence detection of balancer chromosome-encoded GFP. Larvae not expressing GFP were scored as homozygous (-/-). Total RNA was prepared from approximately 100 first instar larvae for each genotype examined using RNA-Stat 60 (Tel-Test, Inc) according to the manufacturer's

instructions. cDNA was prepared by using the Superscript First-Strand Synthesis kit (Invitrogen). Real-time quantitative TaqMan PCR analysis (Heid et al., 1996) was performed using primers as described previously (Dobrosotskaya et al., 2002), except that 20 ng of cDNA was used per reaction and primers for *CG6295* were (5'-ATCTCTGGCTCGCACTTCAAC, 5'- GGAGGACCAGCCGTGGATA). Expression of dRP49 (5' – CCCACCGGATTCAAGAAGTTC, 5' - AAACGCGGTTCTGCATGAG) was used as an internal standard for normalization. The relative amounts of all mRNAs were calculated using the Comparative C_T method and standard deviation of $\Delta\Delta C_T$ and the range were calculated as described in User Bulletin #2 (PE Applied Biosystems).

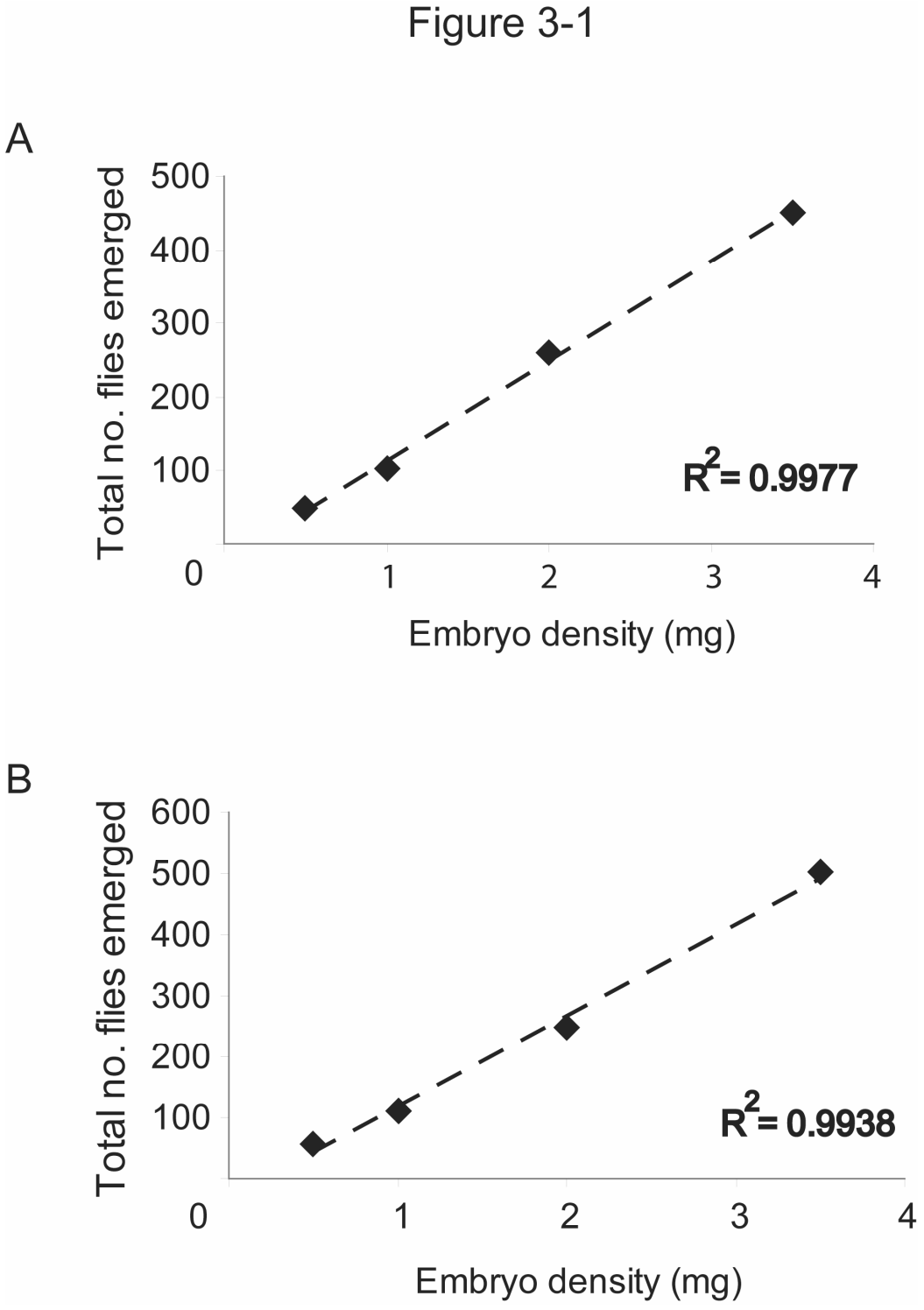
Microscopy

Fluorescence images were obtained using a Leica MZ16FA fluorescence microscope equipped with an Evolution MP digital camera (Media Cybernetics) and In Focus software (Meyer Instruments, Houston, TX). GFP fluorescence was visualized using a GFP2(+) filter set for MZ16 FA, 480/40, 510nm and images were captured using ImagePro software.

Figure 3-1

Embryos were collected overnight from a *dSREBP*^{189} stock. The indicated weight of embryos was then seeded in culture vials using the method described on pg 30. For each data point, 5 vials were seeded. The emerging flies were counted and the number of emerging heterozygous flies was used as an indirect measure of the number of embryos actually seeded into the vials. The number of flies emerging is linear with embryo weights. The result implies that it is possible to generate a homogenous embryo suspension such that the volume seeded is linear with the number of embryos. **A**) and **B**) are replicate sets of embryo suspensions prepared in parallel.

Figure 3-1


A
Total no. flies emerged
500
400
300
200
100
0
1
2
3
4
Embryo density (mg)
R^2= 0.9977
B
Total no. flies emerged
600
500
400
300
200
100
0
1
2
3
4
Embryo density (mg)
R^2= 0.9938

CHAPTER FOUR
Results

Mutant alleles of *dSREBP*

To obtain flies harboring deletions in the *dSREBP* open reading frame (ORF), I used transposase-mediated P element excision (Robertson et al., 1988). I screened 1200 independent excisant lines by a combination of Southern blotting, for homozygous viable lines, and PCR analysis for homozygous lethal lines. Among the homozygous lethal lines, I identified ten lines with imprecise excisions extending into dSREBP. After further screening by PCR, one line (designated $dSREBP^{189}$), was selected for further study. Figure 4-1A depicts the Southern blot screening strategy that led to the initial identification of this allele. Genomic DNA from balanced heterozygous excisants was digested with EcoR1 and used for Southern blotting with two probes, one at the far end of the EcoR1 fragment and one in Exon 1 of *dSREBP*. In wild type flies, both probes identify an identical 10 kb fragment. In flies heterozygous for $dSREBP^{189}$, an additional 8 kb fragment was detected by the far probe, but not by the Exon 1 probe (Figure 4-1B). This suggested the presence of an internally contained 2 kb deletion that included Exon 1 of *dSREBP*. In order to map the lesion down to the nucleotide level, PCR across the breakpoint junction was attempted (Figure 4-1A). Sequencing of the resultant fragment revealed a 2.5 kb deletion that originates at the 5' end of the site of P element insertion. It removes all transposon sequences, and extends 697 nucleotides into the *dSREBP* ORF, up to amino acid 233 in exon 3. The next in-frame start codon is at amino acid 265, immediately preceding the DNA binding domain.

Under standard culture conditions, balanced stocks of *dSREBP*^{189} yielded few homozygous adults (0-4% of expected). Even under optimal cultures grown at low larval density, only up to 10-20 % of homozygotes survived to adulthood. Using timed cultures, it was determined that any 'escaper' adults are developmentally delayed, emerging 6-10 days after their heterozygous siblings (Figure 4-2).

In addition to disrupting the *dSREBP* gene, the *dSREBP*^{189} deletion removes the first exon of the adjacent gene, *GyC76C*. In order to confirm that lethality of *dSREBP*^{189}/*dSREBP*^{189} animals results from disruption of *dSREBP* and not from disruption of *Gyc76C*, I performed P element mediated germline transformation (Rubin and Spradling, 1982). For the rescue construct, I used a fragment of genomic DNA including the entire *dSREBP* gene but no other coding sequences (P{*dSREBPg*}; Figure 1A). When introduced into *dSREBP*^{189}/*dSREBP*^{189} flies as a single copy on the second chromosome, two independent insertions of this construct completely rescued lethality and restored normal rates of development (Table 4-1). Furthermore, these rescued flies can be maintained as stocks that are homozygous for *dSREBP*^{189}. Thus, absence of dSREBP is lethal to flies prior to adulthood.

A piggyBac transposon insertion in *dSREBP* (located 3 bp into exon 1, Figure 4-3A) was obtained from Dr. Ernst Wimmer. This allele, designated *dSREBP*^{52} (Horn et al., 2003), is also substantially lethal when homozygous, as are the two alleles *in trans*. As measured by real-time RT-PCR using primers specific for exon 8 (outside the deletion), transcription of *dSREBP* was profoundly deficient in both the insertion and deletion mutants (Figure 4-3B). In first instar *dSREBP*^{52}/*dSREBP*^{52} or *dSREBP*^{52}/*dSREBP*^{189} larvae, transcripts were detected

at less than 5% of the wild type level while in *dSREBP*^{189}/*dSREBP*^{189} larvae, transcripts are consistently detected at less than 0.5% of wild type levels.

Figure 4-3C shows an immunoblot of lysates of adult male flies using an antibody directed against the NH_2-terminal domain of dSREBP. In wild type males (lane 1) or in *dSREBP*^{189}/+ males (lane 2), the precursor form of dSREBP is readily detected. In the rare 'escaper' *dSREBP*^{189}/*dSREBP*^{189} flies, no dSREBP precursor is detected (lane 3). In *dSREBP*^{52}/*dSREBP*^{189} males, low levels of dSREBP protein are detectable (lane 4). The presence of detectable dSREBP transcripts and protein in *dSREBP*^{52}/*dSREBP*^{189} mutants indicates that some functional transcription occurs from the insertion allele. Therefore, at the level of protein production, *dSREBP*^{189} is a null allele and *dSREBP*^{52} is strongly hypomorphic.

Since lethality of *dSREBP*^{189} homozygotes is solely the result of a lack of dSREBP (i.e. there is no contribution from *Gyc76C*), I henceforth refer to animals carrying this allele simply as *dSREBP* mutants. For experiments where the endpoint is lethality (or lack thereof), I display data using the null *dSREBP*^{189} allele. For experiments exploring phenotypes not addressed by the transgenic rescue experiments, I display data from the strongly hypomorphic *dSREBP*^{52}/*dSREBP*^{189} allele combination in order to rule out any contribution to these phenotypes from *Gyc76C*.

dSREBP **mutants fail to progress through second instar**

Figure 4-4A shows the lethal phase for the *dSREBP* mutants. Between 1.5 and 3 days after egg laying, corresponding approximately to the first two larval instars, the frequency of

dSREBP homozygotes in the population is near the expected for both alleles, with $dSREBP^{189}/dSREBP^{189}$ larvae showing a somewhat lower frequency than $dSREBP^{52}/dSREBP^{52}$. By 3-3.5 days, the frequency of homozygotes diminishes considerably. By 4-4.5 days almost no homozygotes are observed. Thus, flies lacking *dSREBP* die predominantly at the time when they should have become third instar larvae. $dSREBP^{52}/dSREBP^{189}$ larvae die at this same larval stage (Figure 4-4B), indicating that death at this time point occurs solely owing to mutations in *dSREBP*. These results establish the lethal phase due to a zygotic lack of dSREBP. It is possible that lethality will occur earlier if there is also a maternal lack of dSREBP. Given that there is no detectable dSREBP protein in the first 12 hours of embryogenesis (not shown), it is unlikely that maternal loading of dSREBP itself permits survival till second instar. It is possible, however, that the level of maternally loaded lipids in the egg will be different with a maternal lack of dSREBP, and this may lead to earlier lethality.

Comparison of $dSREBP^{189}/dSREBP^{189}$ larvae to their $dSREBP^{189}/+$ siblings showed that failure to reach third instar in homozygotes correlated with a profound growth defect during the second larval instar (Figure 4-5). The few homozygotes that do progress to third instar (as determined by anterior spiracle morphology) are typically undersized (not shown). I observed comparable results with $dSREBP^{52}/dSREBP^{52}$ and $dSREBP^{52}/dSREBP^{189}$ larvae (not shown).

It had previously been shown that when dSREBP activity in *Drosophila* S2 cells was diminished by RNAi treatment, *de novo* synthesis of fatty acids fell four-fold (Seegmiller et al., 2002). This study also identified acetyl coenzyme A (Ac CoA) carboxylase (ACC), Ac

CoA synthase (ACS), and fatty acid synthase (FAS) genes as highly regulated dSREBP targets. *dSREBP* mutant larvae of each genotype showed deficits in the transcription of these genes in first instar, prior to the onset of growth arrest. Figure 4-6A shows data from $dSREBP^{52}/dSREBP^{189}$ larvae as compared to wild type. Additionally, in other experiments, I had identified a gene of undetermined function, *CG6295*, as a potential dSREBP target. Its transcription was also deficient in mutant larvae (Figure 4-6A).

I measured the fatty acid content of first instar larvae and found that *dSREBP* mutants contained significantly less total fatty acid than wild type or heterozygous larvae (Figure 4-6B). However, the relative abundance of various fatty acid species did not differ significantly among these animals (Figure 4-6C, Table 4-2).

Lethality rescued by dietary supplementation

We hypothesized that the lethality of *dSREBP* mutants might result from a lipid deficiency secondary to the transcriptional deficit of genes needed for lipid synthesis such as FAS. To test this, we supplemented fly culture media with a number of different lipids and evaluated their ability to rescue *dSREBP* mutants. The survival of $dSREBP^{189}/dSREBP^{189}$ flies to adulthood was markedly improved in the presence of soy lipids (also called 'lecithin') and increased with increasing concentration (Figure 4-7A). The homozygous adults that emerged from supplemented cultures were indistinguishable in mass from their heterozygous siblings (Figure 4-7B), and were morphologically normal (Figure 4-7C). The results for $dSREBP^{189}/dSREBP^{189}$ null mutants are shown since they afford a more rigorous test of the ability of soy lipid supplementation to supplant all the essential functions of dSREBP than do

the hypomorphic *dSREBP*^{52} mutants. *dSREBP*^{52}/*dSREBP*^{189} animals show similarly increased survival on supplemented medium (Appendix- Figure S1). Supplementation of the larval diet with soy lipids thus restores nearly normal growth to *dSREBP* mutants.

Expression required in fat body and midgut

The piggyBac transposon in the *dSREBP*^{52} allele encodes a GAL4 enhancer trap. When used to drive a UAS-GFP construct, it permitted determination of where *dSREBP* is transcribed. In larvae, the *dSREBP* promoter is active in fat body, midgut, and oenocytes (Appendix- Figure S2). However, dSREBP requires transport and cleavage to produce active transcription factor from the membrane-bound precursor. To determine in which tissue(s) dSREBP is not only expressed but active, I designed a reporter system to follow dSREBP cleavage in living animals. I replaced the transcription factor domain of the genomic rescue construct (pP{*dSREBPg*}) with GAL4-VP16 to make pP{*GAL4-dSREBPg*}(Figure 4-8A). This construct is transcribed under control of the native *dSREBP* promoter(s) and the resulting chimeric protein (GAL4-dSREBP) is subject to the same physiologically-regulated proteolytic processing as wild type dSREBP (Figure 4-8B). When used to drive expression of a dSREBP cDNA encoding only the transcription factor domain, GAL4-dSREBP afforded complete rescue of *dSREBP* mutants (Table 4-3). This result suggests that the spatiotemporal expression pattern of this construct closely recapitulates that of endogenous dSREBP.

I then used this construct to drive expression of a UAS-GFP reporter in transgenic animals (Figure 4-9). No green fluorescence was seen in animals harboring either the GAL4-

dSREBP or the reporter alone (Appendix- Figure S3). Thus, green fluorescence is an indicator of where dSREBP is expressed and cleaved in these animals.

Flies harboring both the P{*GAL4-dSREBPg*}driver and the P{UAS-GFP} responder transgenes in a wild type background showed activity throughout larval development (Figure 4-9A). Substantial activity was seen in fat body, midgut and in oenocytes of larvae (Figure 4-9B,D) and in the corpus allatum of the ring gland (Figure 4-9C).

To explore further the tissues in which activity of dSREBP is required, I placed a full length dSREBP cDNA under control of the yeast UAS. Experiments were performed using various GAL4 enhancer trap and promoter fusion lines to drive its expression in spatially restricted domains during larval life. Table 4-4 correlates the expression of these various drivers (in tissues where dSREBP is active) with their ability to rescue $dSREBP^{189}$ mutants. The expression pattern of the GAL4-dSREBP driver is included as a reference for the domains in which SREBP activity is normally detected. The S_1106 driver, which rescues *dSREBP* null animals, is expressed only in the midgut and fat body. These are therefore the tissues where dSREBP activity is sufficient. Activity in the oenocytes and ring gland is not required for survival. Between the fat body and midgut, we further attempted to dissect whether expression in either is necessary. For this, we used the DcG-GAL4 and the 6450 drivers. The DcG-GAL4 driver, which rescues only weakly, is expressed strongly in the fat body but not in any part of the gut. Therefore, expression in the gut is necessary for full viability. The 6450 driver, which does not rescue at all, is expressed strongly in the gut but not in the fat body. Therefore, expression in the fat body is necessary for viability. We

conclude that dSREBP carries out its essential functions in the fat body and region/s of the midgut.

Failure of 6450 and DcG-GAL4 to rescue did not simply result from weak expression of GAL4. A UAS-GFP reporter transgene revealed strong fluorescence in the posterior midgut and fat body, respectively, with these non-rescuing drivers (Appendix- Figure S4).

Supplementation suppresses cleavage of dSREBP

If soy lipids provide an end-product of dSREBP activation, then that product should suppress cleavage of dSREBP in a manner analogous to cholesterol in mammalian cells. In the presence of increasing concentrations of soy lipids, I observed diminished accumulation of the nuclear form of dSREBP (Figure 4-10A). Suppression was also observed with the GAL4-*dSREBPg*/UAS-GFP reporter system. Larvae reared on unsupplemented medium showed much greater fluorescence relative to siblings reared on medium supplemented with 9% soy lipids (Figure 4-10B, left panels).

This difference results from proteolytic regulation of dSREBP activity. By contrast to the membrane-bound product of the P{GAL4-dSREBPg}construct, the GAL4 in the *dSREBP*^{52} enhancer trap line is expressed as a soluble protein that does not require cleavage for transcriptional activity. When this insertion was used to drive UAS-GFP, strong fluorescence was observed in fat body and midgut on both unsupplemented and 9% soy lipids media (Figure 10B, right panels).

Suppression of dSREBP cleavage should result in reduced accumulation of target gene transcripts. The abundance of transcripts was thus compared in wild type first instar

larvae cultured in the presence or absence of 9 % soy lipids. In larvae reared on supplemented medium, transcript abundance for dSREBP, ACC, ACS, and FAS was reduced as compared to larvae on unsupplemented medium (Figure 4-11). By contrast, transcripts for SCAP (a gene also unaffected in the *dSREBP* mutant animals (Figure 4-6A)) were unchanged. In $dSREBP^{189}/dSREBP^{189}$ or $dSREBP^{52}/dSREBP^{189}$ larvae, the low levels of these transcripts were not further reduced in the presence of soy lipids (Appendix –Figure S6). This indicates that the transcriptional changes depend on dSREBP. Finally, transcription of these genes in mutant larvae was not restored to wild type levels following lipid supplementation, indicating that the reduced abundance of these transcripts in *dSREBP* mutant animals is not a secondary consequence of end product depletion.

dSREBP mutants are fatty acid auxotrophs

Soy lipid extract is largely comprised of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol in approximately equal proportions. The concentration of this required for rescue of *dSREBP* mutants was strikingly high (maximal at 9 %). Since hydrolysis of phospholipids produces free fatty acids, we tested whether any of the major fatty acids (those comprising ≥ 1 % of the total fatty acid of wild type flies) could restore growth (Table 4-5). The only such species not tested was C18:2 owing to its susceptibility to oxidation. The fatty acids all rescued $dSREBP^{189}$ homozygous animals at much lower concentrations than did soy lipids (predominantly phospholipids) or synthetic triglyceride. For example, medium supplemented with 0.15 % C18:1 afforded 80% survival of homozygotes compared to less than 60% survival on 9 % soy lipids. Comparable results were observed with

dSREBP^{52}/*dSREBP*^{189} larvae (Appendix- Table S1). Similar to the results with lecithin, fatty acid supplementation can suppress dSREBP activity in wild type larvae. This is shown using the GAL4-*dSREBPg*/UAS-GFP system (Appendix- Figure S7). Suppression occurs at concentrations that rescue *dSREBP* null animals.

The data do not permit us to account for the differing efficiencies with which the various fatty acids rescue mutant larvae. This may reflect differences in the delivery of the various fatty acid species or in their metabolic fates when supplied exogenously (Keith, 1967b).

The role of dSREBP in adult *Drosophila melanogaster*

Given the critical requirement for dSREBP in larval growth, the bulk of my studies were aimed at understanding the requirement for dSREBP at that stage. However, dSREBP is also expressed in the adult stages of the life cycle (Theopold et al., 1996). To determine if dSREBP activity is required in adult flies, I generated flies that only lack dSREBP during adult life. For this, I employed the P{Switch} system to control expression of dSREBP in a temporal manner. In this variation of the GAL4 enhancer trap system, the yeast GAL4 transcription factor is fused with a progesterone receptor (PR) ligand binding domain. The transcription of genes under the control of the yeast upstream activating sequence (UAS) then depends on the presence of PR ligands such as RU486 (Roman et al., 2001).

We used P{Switch} line $S_1$106 (Roman et al., 2001) to drive expression of dSREBP in *dSREBP*^{189}/*dSREBP*^{189} larvae. These animals survived to pupariation and emerged as adults only in the presence of RU486, (Table 4-6) confirming the requirement of dSREBP for

larval development. No dSREBP is detectable in the rescued adults in the absence of RU486, (Figure 4-12), demonstrating that the protocol can indeed generate adults that are *dSREBP* null. When kept in the absence of RU-486, the rescued homozygous adults survive and have a median life span that is approximately one-third that of their heterozygous counterparts or of wild type flies (Figure 4-12B). Table 4-7 shows the results of a small scale analysis of the fertility of dSREBP null adults. When scored for fertility in the first five days of life, females lacking dSREBP show somewhat diminished fertility whereas males appear normal. If these flies are aged however, both males and females show a dramatic depression of fertility compared to heterozygous controls. The absolute requirement for dSREBP during larval life is thus slightly relaxed during adulthood, though dSREBP is clearly required for normal longevity and reproductive lifespan.

Given the relaxed requirement for dSREBP during adulthood, I determined the spatial pattern of dSREBP activation during adulthood. Differences were apparent between larval and adult patterns of expression in fat body and anterior midgut. In larvae, these tissues were major sites of dSREBP activity (Figure 4-9B, D). Adults showed weak to no activity in fat body (Figure 4-13A). No activity was seen in the anterior midgut in adults, though activity was observed in discrete patches of the posterior midgut (Figure 4-13D). Oenocytes continued to show intense activity in adults (Figure 4-13A, B). In males, the ejaculatory bulb also showed intense fluorescence (Figure 4-13D) as did the proventriculus (cardia) in both sexes (Figure 4-13C and data not shown (males)).

Table 4-1. Rescue by Genomic Construct

	No Transgene		Transgene Present	
Line	*dSREBP* +/-	*dSREBP* -/-	*dSREBP* +/-	*dSREBP* -/-
Number of Adults Eclosed				
G_6-1	257	0	561	355
G_6-2	152	0	374	225
Days to Median Eclosion				
G_6-1	15	n/a	15	15
G_6-2	16	n/a	16	17

Adult progeny from a cross of *w*^{1118}; Sp/CyO; *dSREBP*^{189}/*TM6, Tb, Hu e* **X** *w*^{1118}; P{*dSREBPg*}/CyO; *dSREBP*^{189}/ *TM6, Tb, Hu e* were scored for the presence of *dSREBP*^{189} homozygotes. Two independent transgene insertions on the second chromosome (G_6-1 & -2) were examined and both rescued mutants to adulthood ($p<0.005$ compared to flies lacking the transgene). The reason for the somewhat greater than expected recovery of homozygotes among the transgenic flies is unknown. Rescued flies develop at the same rate as control flies. Median eclosion is defined as the day by which half of the total progeny had emerged as adults. No homozygous 'escapers' emerged in this experiment. The transgene thus restored normal rates of development to *dSREBP*^{189}/*dSREBP*^{189} flies.

Table 4-2. Fatty Acid Composition of First Instar Larvae

		Wild Type*		*189/+*		*52/+*		*189/189*		*52/52*		*52/189**	
Percent of total Fatty Acid by weight	C12:0	1.0	(0.52)	0.5	(0.50)	1.4	(0.16)	0	(0)	0.0	(0)	0	(0)
	C14:0	15.7	(0.74)	15.2	(0.60)	16.0	(1.60)	17.0	(0.97)	15.7	(0.29)	16.1	(0.54)
	C16:0	23.1	(0.80)	20.5	(0.88)	20.3	(1.95)	22.6	(1.85)	21.6	(0.54)	22.0	(0.60)
	C16:1	24.9	(0.81)	28.8	(1.57)	27.3	(2.68)	27.3	(2.96)	28.1	(0.84)	28.3	(1.15)
	C18:0	5.0	(0.33)	4.6	(0.27)	4.2	(0.33)	5.9	(0.62)	3.7	(1.85)	6.1	(0.20)
	C18:1	27.7	(0.86)	28.6	(1.49)	27.8	(2.64)	26.1	(2.38)	28.8	(0.87)	27.6	(1.45)
	C18:2	2.5	(0.09)	1.9	(0.94)	2.7	(0.23)	1.2	(1.18)	2.0	(1.03)	0	(0)

The data displayed graphically in Fig. 4-6C (*) are again presented here to facilitate comparison to the results from other genotypes. The numbers in parentheses indicate the standard error of the calculated percentage.

Table 4-3. Rescue by expressing the dSREBP transcription factor domain under control of GAL4-dSREBPg

	Responder *OR* Driver		Responder *AND* Driver	
Line	*dSREBP* +/-	*dSREBP* -/-	*dSREBP* +/-	*dSREBP* -/-
1	28	0	36	18
2	34	0	40	22
3	44	3	20	9
4	29	0	49	26

Adult progeny from a cross between w^{1118}; P{GAL4-*dSREBPg*}/*CyO*; $dSREBP^{189}$/*TM6 Tb Hu e* and w^{1118}; P{UAS-ndSREBP}/*CyO*; $dSREBP^{189}$/*TM6 Tb Hu e* were scored for the presence for $dSREBP^{189}$ homozygotes. Lines 1-4 represent 4 different insertions of the P{UAS-ndSREBP} transgene on the 2nd chromosome. Expected numbers of homozygous adults are seen only when both driver and responder constructs are present. Thus, when used as a rescue driver, GAL4-*dSREBPg* is transcribed and cleaved in an appropriate spatiotemporal pattern. This experiment is a particularly stringent test of this assertion, because expression of the transcription factor domain without membrane attachment is toxic unless precisely controlled (personal observations).

Table 4-4. Domains of GAL4 Expression that Rescue *dSREBP* Mutants

	Rescue (%)		Midgut	Fat Body	Oenocytes	Ring Gland	Other
P{GAL4-*dSREBPg*}	Yes	(108)	+	+	+	+	-
$S_1$106	Yes	(91)	+	+	-	-	-
DcG-GAL4	Weak	(8)	-	+	-	-	+
6450	No	(0)	+	-	-	-	+

Multiple lines harboring GAL4 drivers with distinct patterns of tissue expression were used to drive UAS-dSREBP in a *dSREBP^{189}/TM6 Tb, Hu, e* background. The ability of each GAL4 driver to rescue *dSREBP^{189}* homozygous larvae to adulthood was determined as described in *Experimental Procedures*. The number in parentheses is the emergence of homozygotes as the percentage of expected. The number of flies on which this percentage is based is as follows: P{GAL4-*dSREBPg*}, n=88**;** $S_1$106, n=464; 6487, n=457; DcG-GAL4, n=242; 6450, n=217. The expression pattern of each driver was then determined by crossing the GAL4 driver lines with a line harboring a UAS-GFP reporter. The presence (+) or absence (-) of GFP fluorescence in the five domains where dSREBP is active in wild type larvae (see Figure 4-9) was determined by dissection of late first, second, and third instar larvae.

Table 4-5. Rescue of *dSREBP*^{189} Homozygotes by Dietary Supplementation

Compound	Percent rescue	(SEM)	Conc. g%	No. of Trials
No additions	4.2	(0.5)	n/a	171
C12:0	23.5	(3.9)	0.075	10
Tripalmitin	28.7	(11.7)	2	7
C16:1	27.3	(3.7)	0.15	12
C16:0	51.5	(4.3)	0.6	52
Soy lipids	53.1	(3.9)	9	50
C18:0	56.9	(14.0)	1.2	5
C14:0	67.5	(9.2)	0.075	9
C18:1	80.3	(11.9)	0.15	9

Multiple concentrations were tested for each compound (Soy lipids- 1, 3 and 9 %; Tripalmitin- 0.6, 2 and 6 %; sodium salts of fatty acids 0.075, 0.15, 0.3 and 0.6 %). For C18:0, an additional experiment with 1.2 % was performed. The table reports the maximal rescue obtained with each compound tested and the concentration at which this rescue was obtained. At concentrations lower or higher than shown, rescue was less robust (except for 18:0, for which the highest concentration tested produced maximum rescue). The standard error of the mean is shown in parentheses. For data from *dSREBP*^{52}*/dSREBP*^{189} transheterozygotes, see Appendix-Table S1. The mean number of animals in each trial = 53.

Table 4-6. Rescue of *dSREBP*^{189} Homozygotes using the P{Switch} System

	0 μM RU486				45 μM RU486			
	Driver Alone		Responder *AND* Driver		Driver Alone		Responder *AND* Driver	
dSREBP	+/-	-/-	+/-	-/-	+/-	-/-	+/-	-/-
UAS-dSREBP Line A	35	0	62	0	49	0	92	48
UAS-dSREBP Line B	59	0	110	0	87	0	127	52

w^{1118}; $S_1$106/$S_1$106; *dSREBP*^{189}*/TM6, Tb Hu* virgins were crossed to *w*^{1118}; *UAS-dSREBP/CyO; dSREBP*^{189}*/TM6, Tb Hu* males in vials of media containing the indicated concentration of RU486 and raised at 25°C until adults had emerged. Adult progeny were scored as heterozygous (+/-) or homozygous (-/-) at the *dSREBP* locus. Rescue is only observed in the flies harboring both transgenes in the presence of RU486.

Table 4-7. *dSREBP* null adults display early senescence

		Percent fertile	
	Genotype	**at 0-5 days old**	**after 11-16 days old**
Males	*dSREBP* -/-	85	33
	dSREBP +/-	95	89
Females	*dSREBP* -/-	55	0
	dSREBP +/-	100	89

0-1 day old rescued flies, or their heterozygous siblings that emerged the same day, were crossed 5 flies of the opposite sex. 20 flies of each genotype were tested. The test flies were given new partners every three days. Fertility was scored as the presence of larvae in the vials. Fertility was scored at 0-5 days old and once the flies reached an age of 11-16 days.

Figure 4-1

(**A**) **Molecular characterization of the *dSREBP*^{189} allele**: The *dSREBP* gene is indicated in light grey, with exons indicated by arrows pointing in the direction of transcription. The open reading frame is indicated by a heavy line (ORF). The grey triangle indicates the site of insertion of KG03723 (grey triangle), the P element mobilized to generate *dSREBP*^{189} (filled bar). The deletion extends 697 bp into the ORF. The horizontal black bars indicate the 'far probe' and 'exon 1 probe' referred to in Figure 4-1B. The filled black triangles indicate the primers used to amplify the junction fragment that was then sequenced to determine the exact location of the breakpoints. The genomic fragment used for transgenic rescue of the mutants is identified as such.

(**B**) **Southern blots representing the screening strategy that led to the identification of *dSREBP*^{189}**: Genomic DNA from balanced heterozygous flies carrying candidate deletions was digested with EcoR1, electrophoresed and transferred by Southern blotting to nylon membranes. The membranes were then initially probed with DNA probes corresponding to the 'far probe' region depicted in Figure 4-1A. Wild type DNA (Lane 1) produced a single 10kb fragment with this probe. Genomic rearrangement between the two EcoR1 sites results in the production of an additional band (Lane 2-6). When the same blot was then probed with the exon 1 probe, the additional 8 kb band in line 189 (Lane 2) was no longer detectable, suggesting a 2 kb deletion internal to the EcoR1 fragment that extended into the *dSREBP* gene. This was confirmed by using PCR to amplify and sequence the junction fragment. The primers used are depicted in Figure 4-1A. The other lines (Lane 3-6) likely represent lesions that do not extend into *dSREBP* exons and were thus of no interest for this study.

Figure 4-1

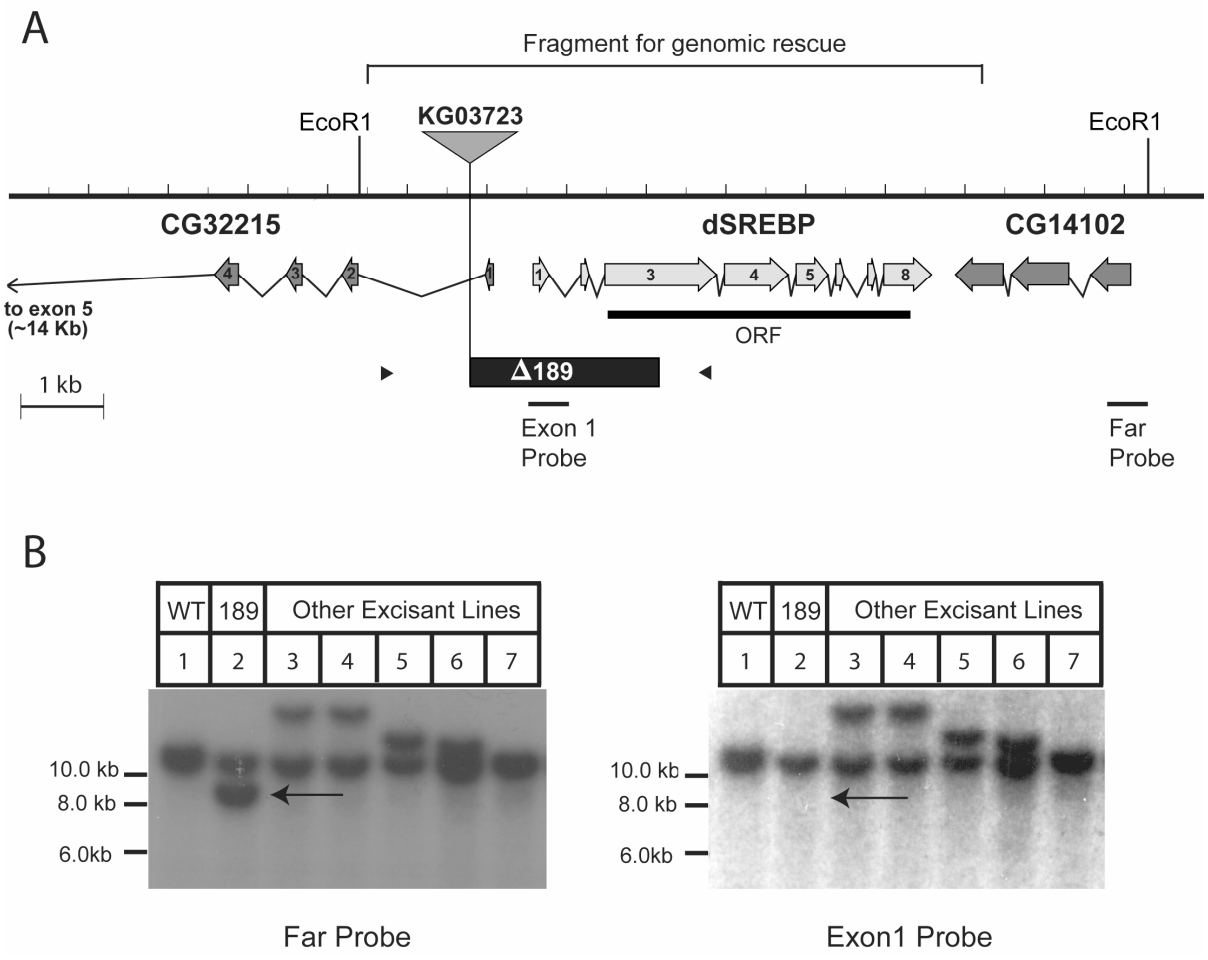

A
Fragment for genomic rescue
EcoR1
KG03723
EcoR1
CG32215
dSREBP
CG14102
to exon 5
(~14 Kb)
ORF
Δ189
1 kb
Exon 1
Probe
Far
Probe
B
WT
189
Other Excisant Lines
1
2
3
4
5
6
7
10.0 kb
8.0 kb
6.0kb
Far Probe
Exon1 Probe

Figure 4-2

Developmental delay of the *dSREBP^{189}* mutants: Embryos from a balanced heterozygous *dSREBP^{189}/TM3, Actin-GFP* culture were collected and seeded in culture bottles with cornmeal-molasses agar medium at 40 mg embryos/bottle. Emerging adults were counted daily from day 10 after egg laying. Any homozygotes that emerge are delayed compared to their heterozygous siblings. Peak emergence from parallel wild type cultures coincided with that of the heterozygotes (not shown).

Figure 4-2

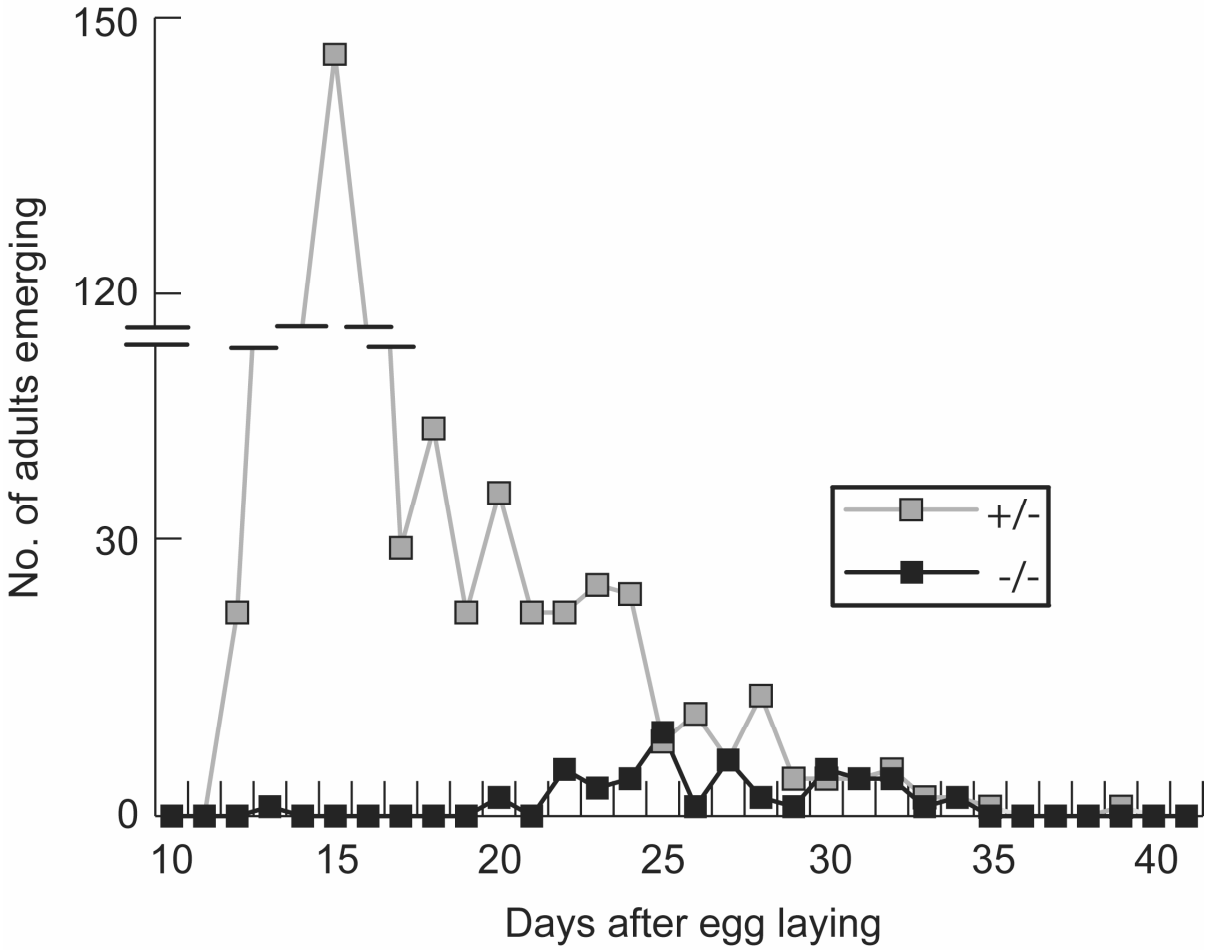

150
120
30
0
No. of adults emerging
10
15
20
25
30
35
40
Days after egg laying
+/-
-/-

Figure 4-3

(**A**) **The *dSREBP*^{52} insertion allele**: The *dSREBP* gene is indicated in light grey, with exons indicated by arrows pointing in the direction of transcription. The open reading frame is indicated by a heavy line (ORF). *dSREBP*^{52} (white triangle) is a piggyBac transposon insertion at bp 3 of exon 1 that disrupts *dSREBP* expression (Horn et al., 2003). The *dSREBP*^{189} deletion is shown again here for reference.

(**B**) **Quantitative analysis of dSREBP transcripts**: Transcripts were measured in wild type versus mutant first instar larvae of the genotype indicated. The number above each bar indicates the relative abundance of dSREBP transcripts as compared to wild type (=1).

(**C**) **Immunoblot analysis of whole fly lysates**: Whole fly lysates of the genotype indicated were electrophoresed (30 μg/lane). The blot was probed with monoclonal antibody against the NH_2-terminal fragment of dSREBP (upper panel). The membrane was then stripped and re-probed with anti-tubulin antibody as a loading control (lower panel).

Figure 4-3

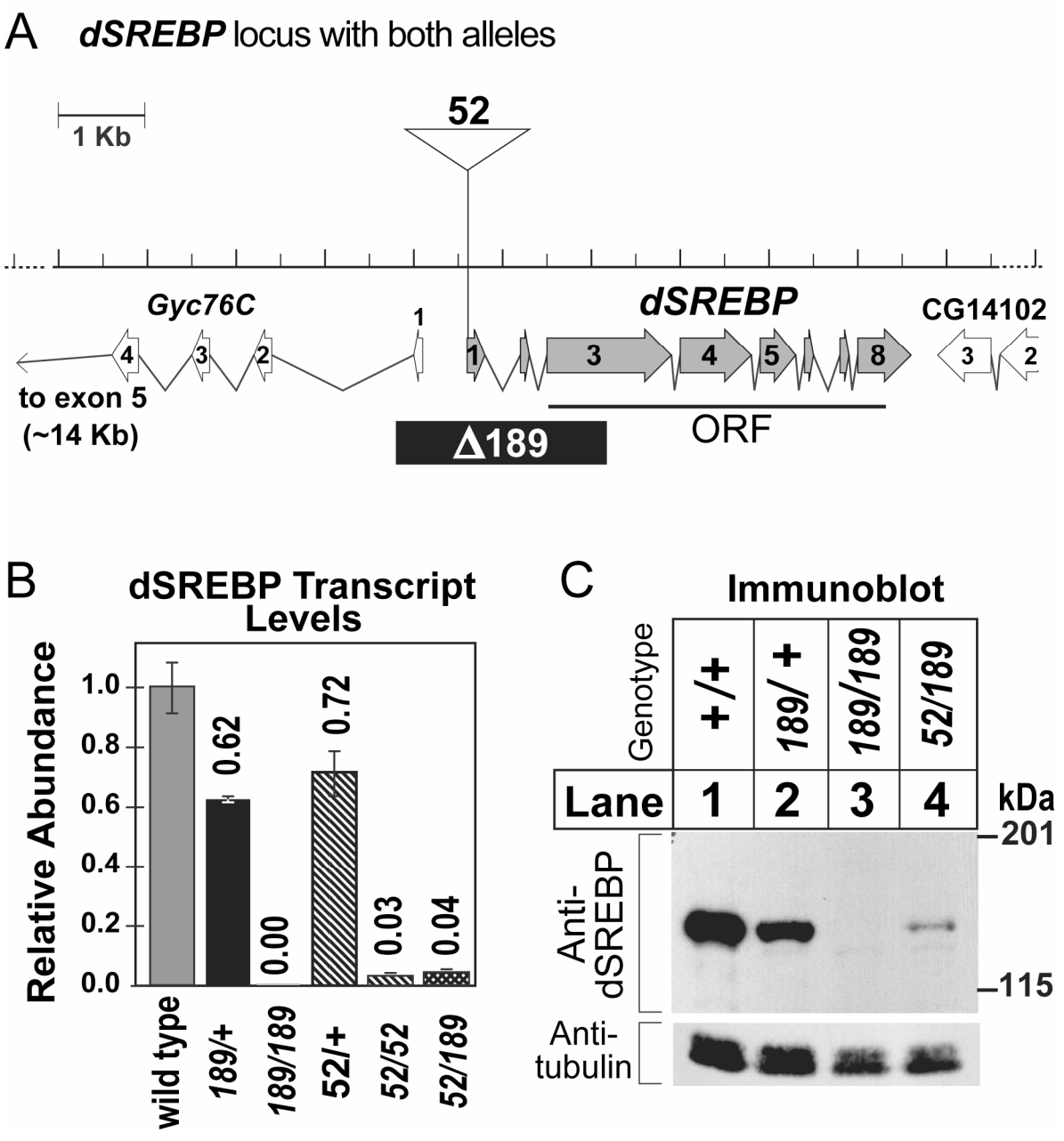

A
dSREBP locus with both alleles
1 Kb
52
Gyc76C
dSREBP
CG14102
to exon 5
(~14 Kb)
Δ189
ORF
B
dSREBP Transcript Levels
Relative Abundance
1.0
0.8
0.6
0.4
0.2
0.0
0.62
0.00
0.72
0.03
0.04
wild type
189/+
189/189
52/+
52/52
52/189
C
Immunoblot
Genotype
+/+
189/+
189/189
52/189
Lane
1
2
3
4
kDa
201
115
Anti-dSREBP
Anti-tubulin

Figure 4-4

(**A**) **Lethal phase of *dSREBP* mutants**: Larvae from *dSREBP*^{189} /*TM3, Actin-GFP, Ser* or *dSREBP*^{52}/*TM3, Actin-GFP*, *Ser* stocks were collected at each time point and the ratio of homozygous to heterozygous larvae was determined. The ratio was used to calculate survival as a percentage of the expected ratio (0.5). A mean of 500 larvae were scored at each time point (range = 367-821).

(**B**) **Lethal phase of transheterozygous mutants**: Larvae from *dSREBP*^{52}*/TM3*, *actin-GFP, Ser* virgin females crossed to *dSREBP*^{189}/*TM3*, *actin-GFP, Ser* males were collected at each time point. The ratio of *dSREBP*^{52}*/dSREBP*^{189} larvae to heterozygotes was calculated. The ratio was used to calculate survival as a percentage of the expected ratio (0.5). A mean of 410 larvae were scored at each time point (range = 396-420).

Figure 4-4

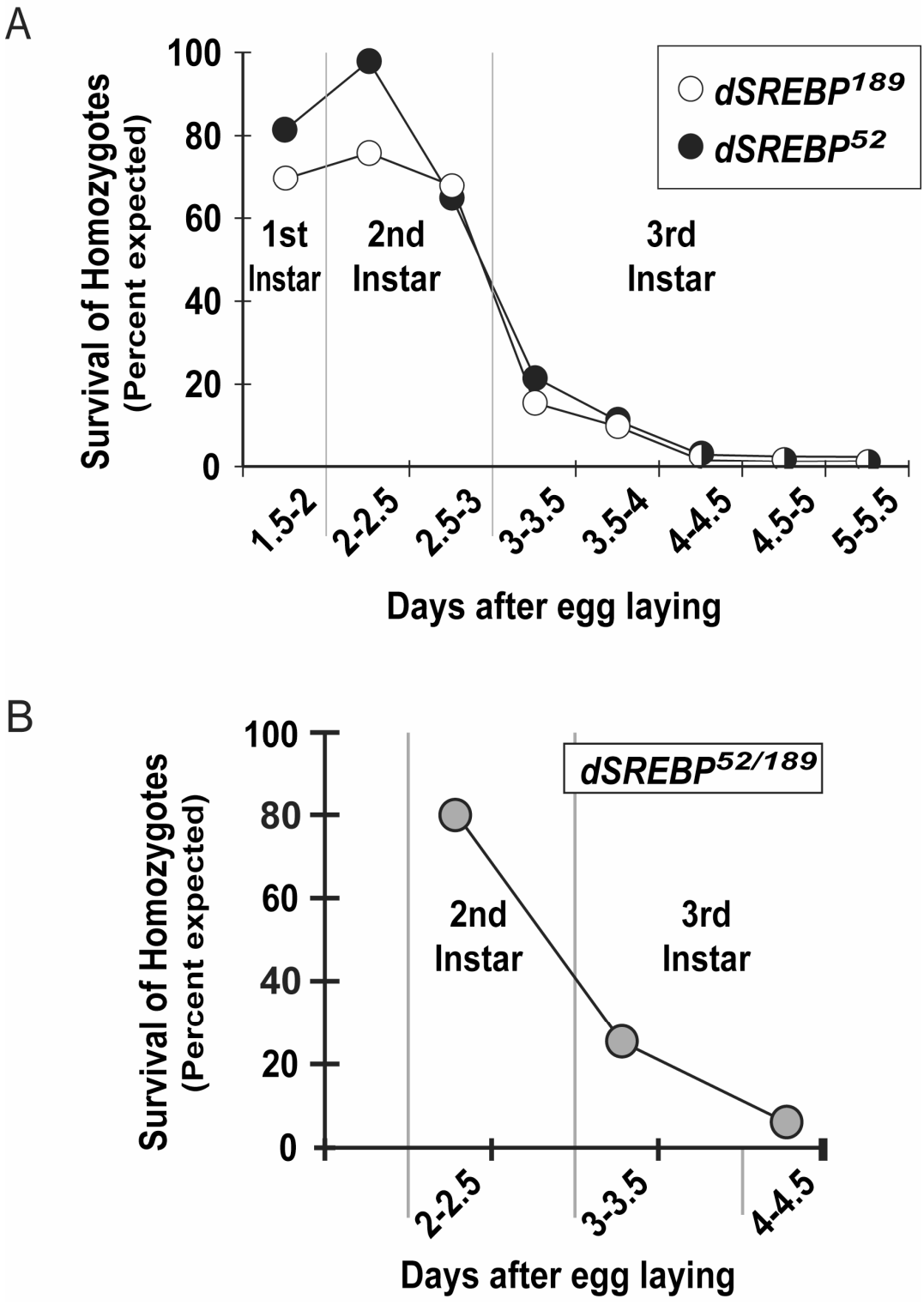

A
Survival of Homozygotes
(Percent expected)
100
80
60
40
20
0
dSREBP189
dSREBP52
1st
Instar
2nd
Instar
3rd
Instar
1.5-2
2-2.5
2.5-3
3-3.5
3.5-4
4-4.5
4.5-5
5-5.5
Days after egg laying
B
Survival of Homozygotes
(Percent expected)
100
80
60
40
20
0
dSREBP52/189
2nd
Instar
3rd
Instar
2-2.5
3-3.5
4-4.5
Days after egg laying

Figure 4-5

***dSREBP* null larvae fail to grow in second instar**: Comparison of size differences between *dSREBP*^{189} heterozygous (+/-) and homozygous (-/-) larvae. Embryos from a *dSREBP*^{189} /*TM3, Actin-GFP, Ser* stock were collected, cultured and larvae photographed at the indicated time points.

Figure 4-5

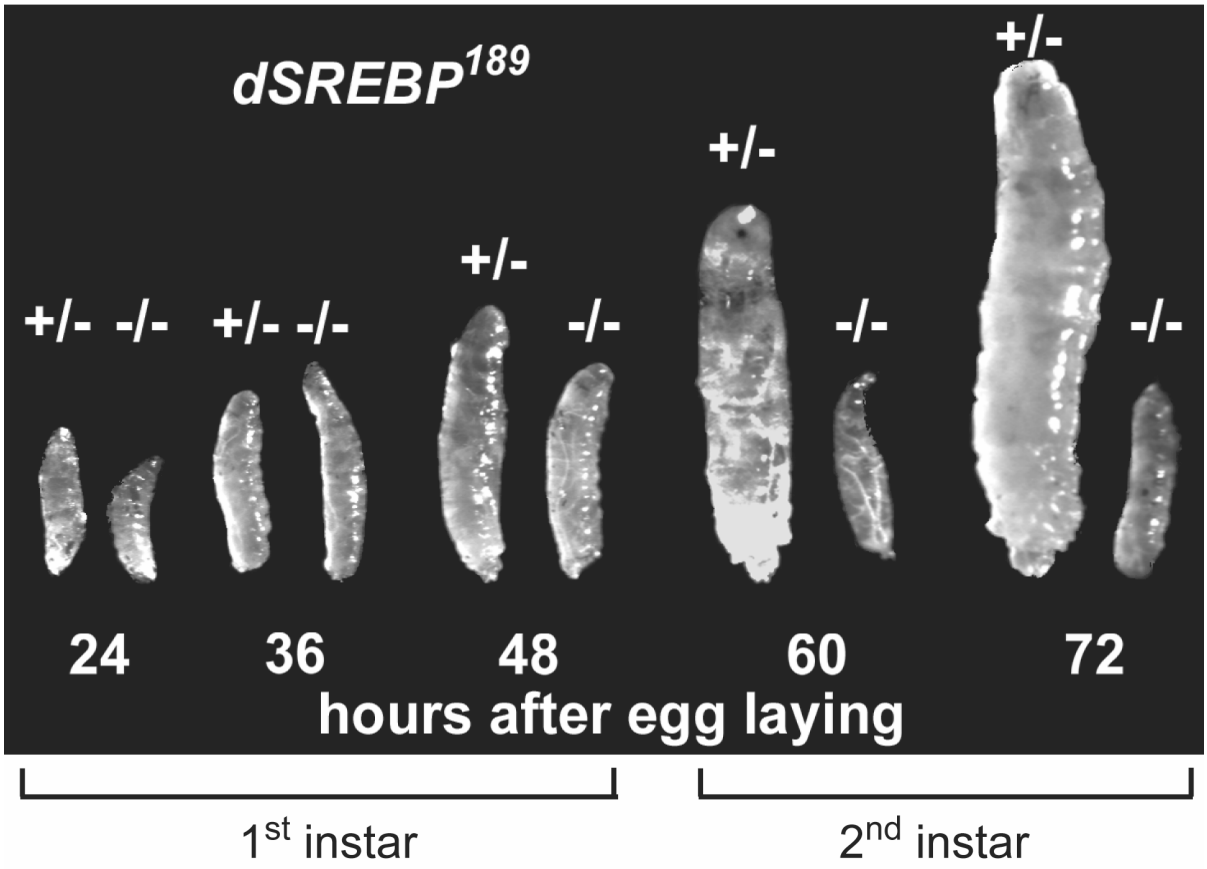

dSREBP189
+/-
-/-
+/-
-/-
+/-
-/-
+/-
-/-
+/-
-/-
24
36
48
60
72
hours after egg laying
1st instar
2nd instar

Figure 4-6

(**A**) **Quantitative RT-PCR analysis of transcripts:** The indicated transcripts were measured in wild type and *dSREBP*^{52}*/dSREBP*^{189} first instar larvae. The larval cultures were harvested at 36 hours after egg laying, before any obvious growth deficit is detected.

(**B**) **Total fatty acid content of first instar larvae (μg fatty acid/mg protein)**. Triplicate samples were measured for each genotype and the mean plotted. Error bars represent the standard error of the mean. Samples were prepared and analyzed as described in *Experimental Procedures*. The *dSREBP* homozygous and transheterozygous samples differ significantly from the control samples (wild type and heterozygotes) at $p<0.001$ by Student's two-tailed t-test.

(**C**) **Fatty acid composition of wild type and *dSREBP*^{52}*/dSREBP*^{189} first instar larvae**: Plotted as % of total for all species detected at >0.1 % of total. Table 4-2 lists, in tabular form, this data and that for the other mutant genotypes. The fatty acid composition does not differ significantly among any of the genotypes tested as determined by a χ^2 test of independence ($p > 0.8$ for each genotype).

Figure 4-6

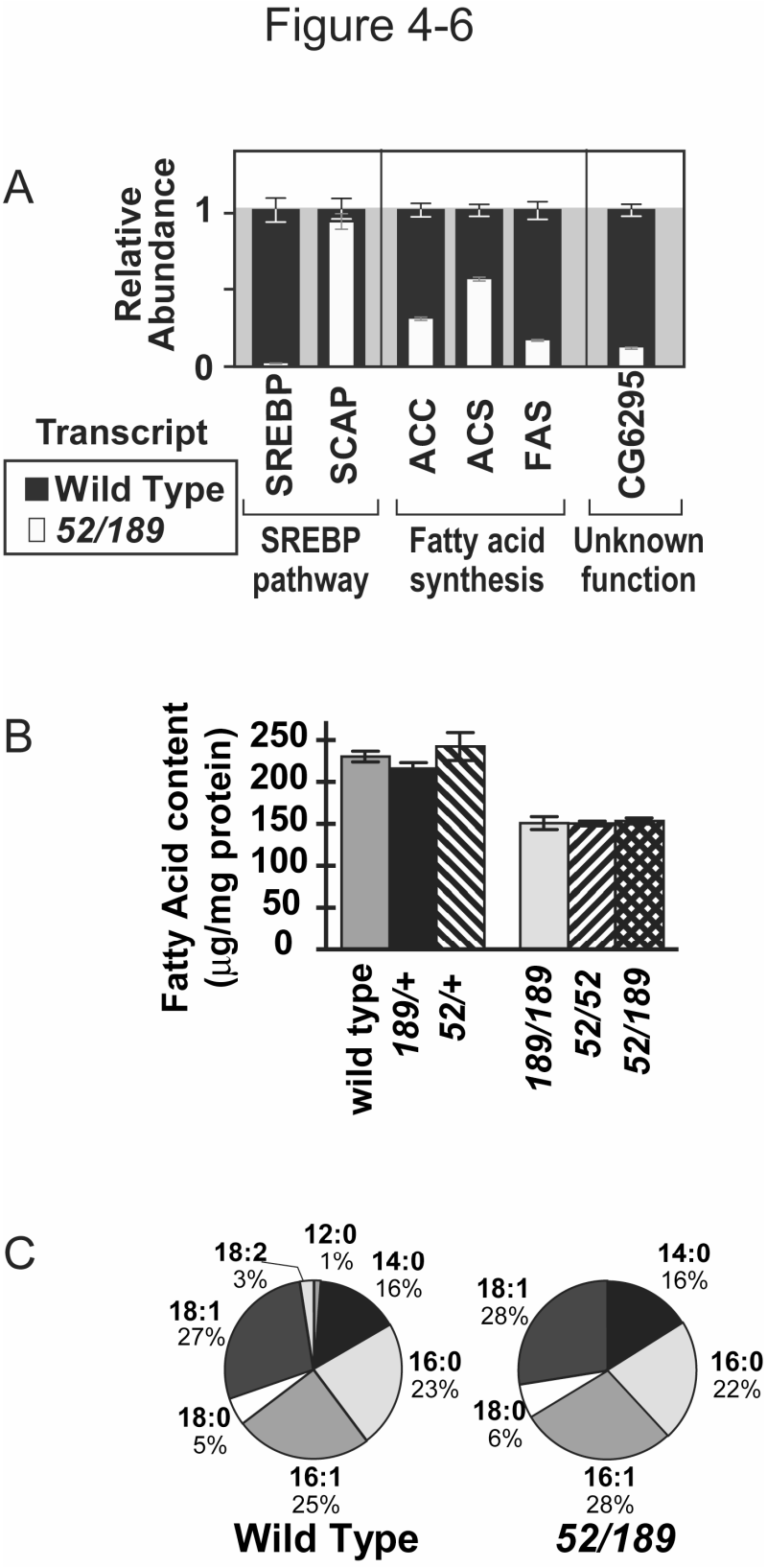

A
Relative Abundance
1
0
Transcript
Wild Type
52/189
SREBP
SCAP
ACC
ACS
FAS
CG6295
SREBP pathway
Fatty acid synthesis
Unknown function
B
Fatty Acid content (μg/mg protein)
250
200
150
100
50
0
wild type
189/+
52/+
189/189
52/52
52/189
C
12:0 1%
14:0 16%
16:0 23%
16:1 25%
18:0 5%
18:1 27%
18:2 3%
Wild Type
14:0 16%
16:0 22%
16:1 28%
18:0 6%
18:1 28%
52/189

Figure 4-7

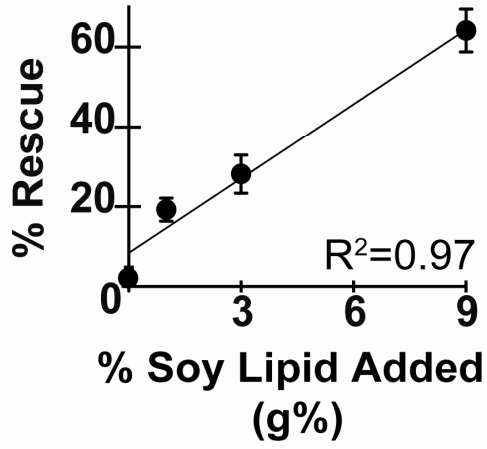
(**A**) **Soy lipid extract rescues *dSREBP^{189}* mutant animals to adulthood**: Embryos from a *dSREBP^{189}/TM3 Actin-GFP, Ser* stock were seeded (at 1 mg/vial) into vials containing either regular cornmeal-molasses-agar medium or medium supplemented with increasing concentrations of soy lipid extract. Emerging adults were scored for their *dSREBP* genotype and the ratio of homozygotes/heterozygotes was used to calculate the survival of homozygotes as a percentage of the expected ratio (0.5 = 100%). At concentrations greater than 9 %, the soy lipids rendered the medium unable to support even wild type flies due to its altered consistency.

(**B**) **Rescued *dSREBP* null adults have normal mass**: Mass of *dSREBP^{189}* heterozygous (+/-) and homozygous (-/-) adults reared on medium supplemented with 9 % soy lipid as compared to wild type (+/+) adults reared on medium with or without supplementation. Mean mass was calculated for 3 replicates of ten adults for each condition. Error bars indicate standard error of the mean.

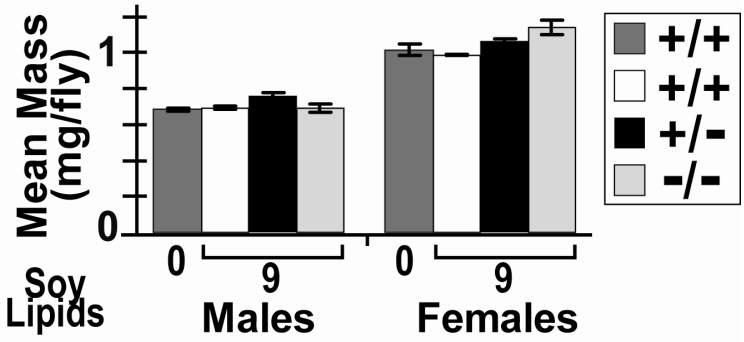
(**C**) **Rescued *dSREBP* null adults have normal morphology**: *dSREBP^{189}* heterozygous (+/-) or homozygous (-/-) adults were raised on medium supplemented with 9 % soy lipid extract.

Figure 4-7

A



B



C

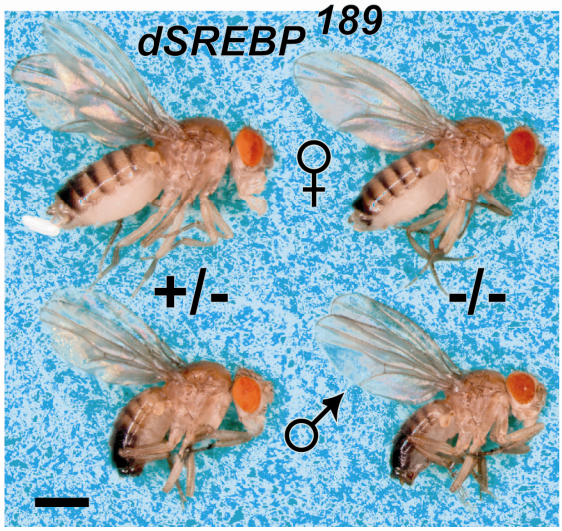


Figure 4-8

(**A**) **A reporter system for dSREBP activity**: The transcription factor domain of the *dSREBP* genomic rescue construct (pP{*dSREBPg*}) was replaced by a GAL4-VP16 transcription factor to generate pP{*GAL4-dSREBPg*}. Flies transgenic for this construct can be crossed with appropriate reporter strains (eg. UAS-GFP transgenic flies).

(**B**) **GAL4-dSREBP is subject to the same physiological regulation as endogenous dSREBP**: On day 0, S2 cells were transfected with either an empty vector or two concentrations of pP{GAL4-*dSREBPg*}. The transfection procedure and subsequent treatments were as described (Dobrosotskaya et al., 2002). Briefly, on day 3, cells were treated with 100 μM Na palmitate and 100 μM ethanolamine as indicated. After 5 hours, the cells were harvested and fractionated into membranes and nuclear extracts as described (Seegmiller et al., 2002). For endogenous dSREBP, 25 μg of membranes and 3 μg of nuclear extracts were analyzed by immunoblotting with anti-dSREBP. For detection of GAL4-dSREBP, 25 μg of membranes and 6 μg of nuclear extracts were analyzed by immunoblotting with anti-GAL4 (Covance). Like endogenous dSREBP, GAL4-dSREBPg is synthesized as a membrane-bound precursor. Likewise, the GAL4-VP16 transcription factor domain accumulates in the nucleus only when dSREBP is cleaved (in the absence of palmitate + ethanolamine). **P**; membrane-bound dSREBP precursor. **N**; nuclear form. The asterisk denotes a cross-reactive band that is also present in untransfected cells.

Figure 4-8

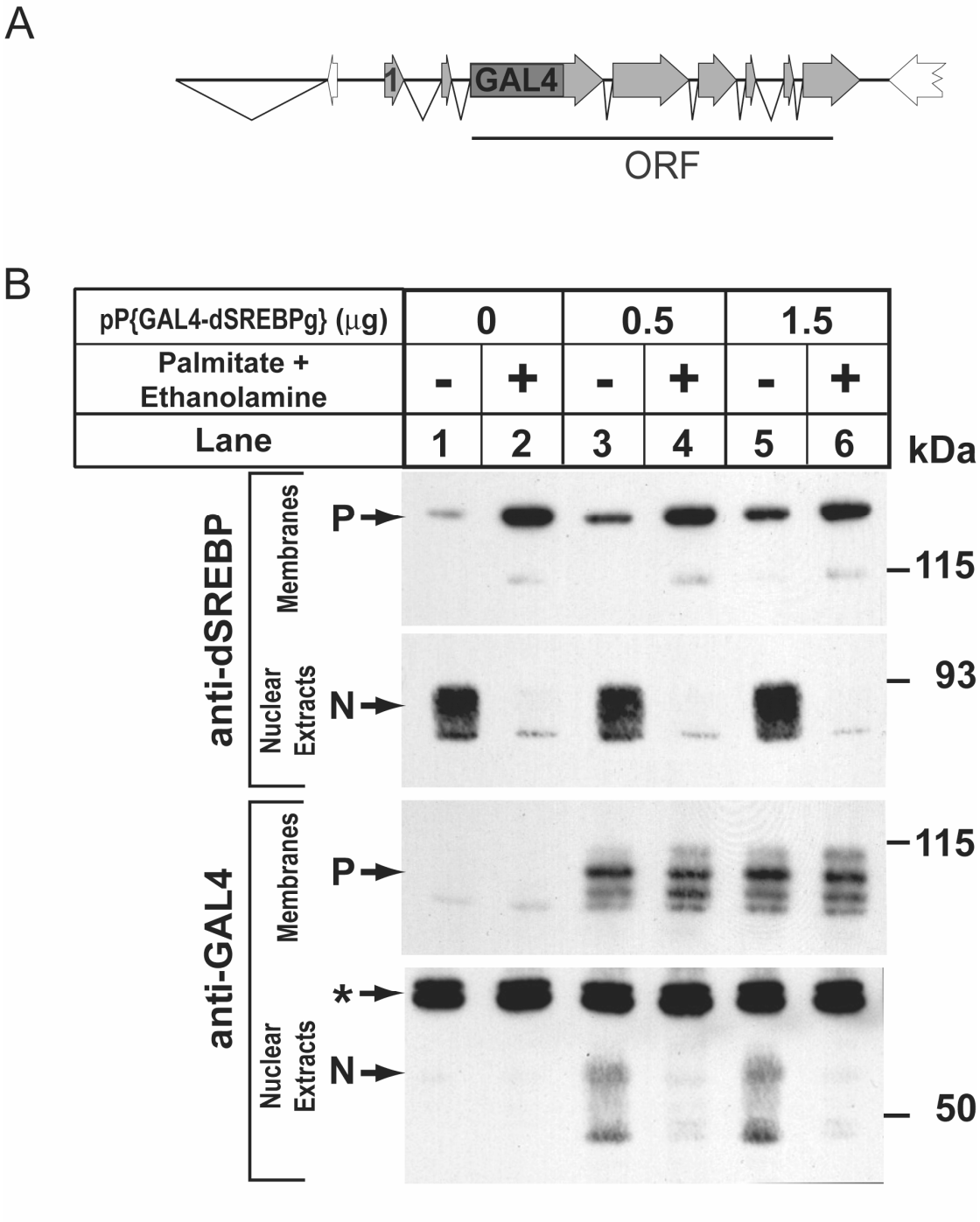

A
1
GAL4
ORF
B
pP{GAL4-dSREBPg} (μg)
0
0.5
1.5
Palmitate + Ethanolamine
- + - + - +
Lane
1 2 3 4 5 6
kDa
anti-dSREBP
Membranes
P
115
Nuclear Extracts
N
93
anti-GAL4
Membranes
P
115
Nuclear Extracts
*
N
50

Figure 4-9

(**A**) **Dorsal views of larvae and early pupae**: Transgenic flies carrying GAL4-dSREBP were crossed to flies carrying a UAS-GFP transgene. The progeny were photographed. At all larval stages, fluorescence is detected in fat body, midgut and oenocytes. The contents of the gut autofluoresce with a brownish color (Appendix- Figure S5). In late pupae, fluorescence can be detected throughout the animal. Scale bar = 1mm. Dashed lines denote the extent of larval tissues.

(**B, C, D) Dissection of a third instar larva**: **B**) Two domains of dSREBP activity are detectable in the midgut: 1) a strong signal in the anterior midgut and 2) weaker signal in a region encompassing the posterior portion of the midgut. The oenocytes are attached to the cuticle. Panel (**C**) shows GAL4-dSREBP activity in the ring gland of a wandering third instar larva. The corpus allatum shows intense fluorescence. Fluorescence is also detectable in the lateral portions of the gland. Panel (**D)** shows a dissected piece of the fat body.

Figure 4-9

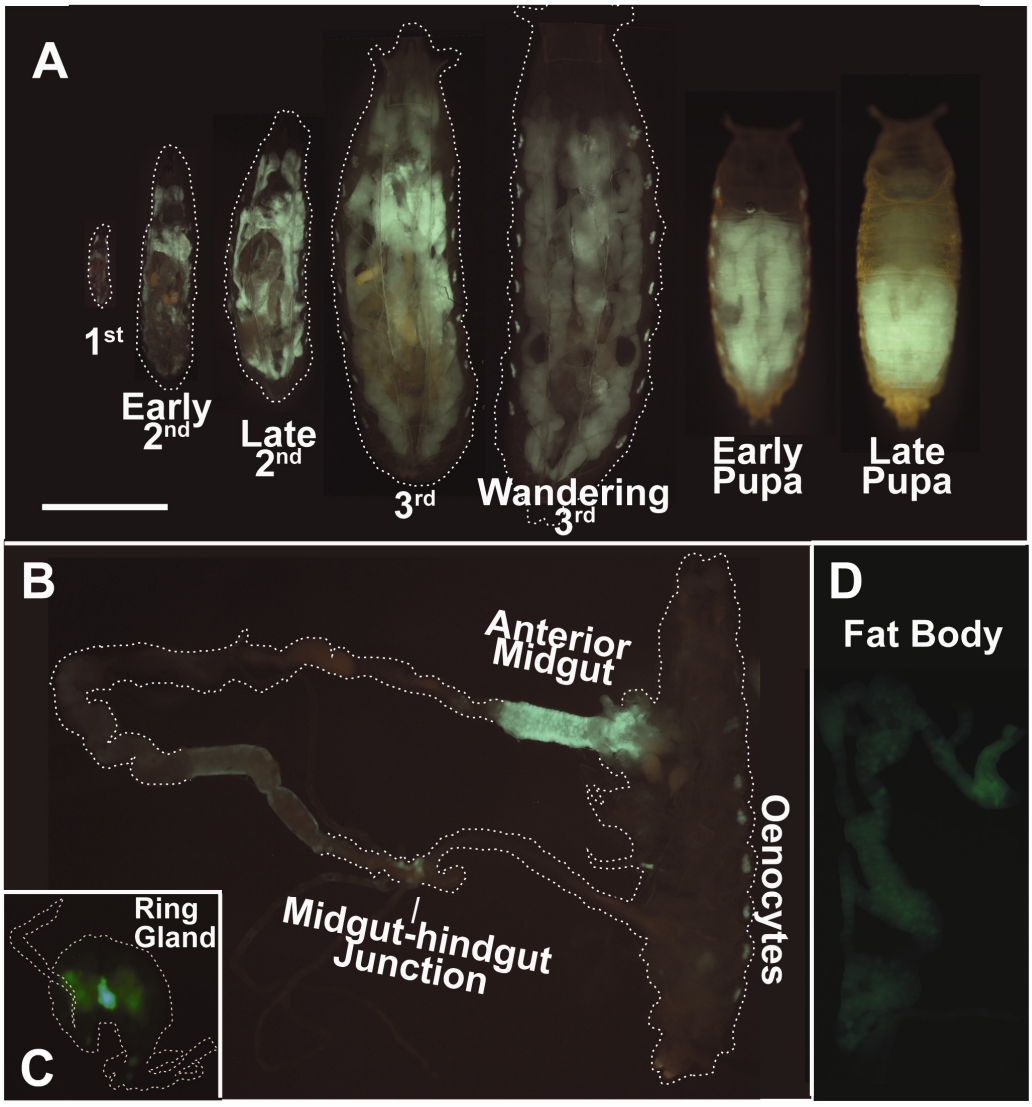

A
1st
Early 2nd
Late 2nd
3rd
Wandering 3rd
Early Pupa
Late Pupa
B
Anterior Midgut
Oenocytes
Midgut-hindgut Junction
C
Ring Gland
D
Fat Body

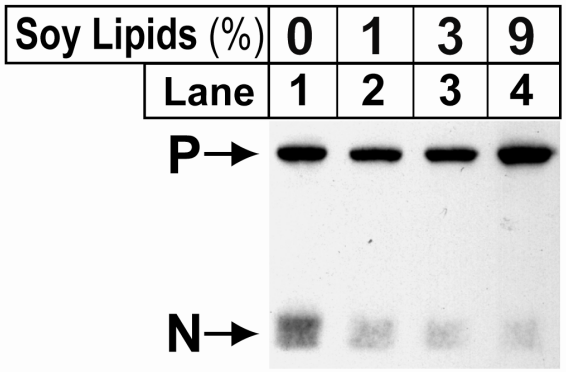
Figure 4-10

Supplementation with soy lipid extract suppresses dSREBP cleavage in wild type larvae: (**A**) Third instar larvae raised on semi-defined medium supplemented with increasing concentrations of soy lipid extract were harvested and whole-larva lysates were prepared. Samples (60 μg) were analyzed by immunoblotting with anti-dSREBP. P; membrane-bound dSREBP precursor. N; nuclear form.

(**B**) Wild type larvae carrying UAS-GFP and either P{*GAL4-dSREBPg*} (left panels) or, as a control, the $dSREBP^{52}$ insertion (right panels) are shown. Larvae were cultured on either unsupplemented cornmeal-molasses-agar medium (0%) or the same medium supplemented with 9 % soy lipid extract (9%). In larvae carrying P{GAL4- *dSREBPg*}, supplementation causes a disappearance of GFP signal. The brownish color of the guts is due to autofluorescence of gut contents (Appendix – Figure S5). Thus, soy lipid supplementation suppresses dSREBP activity. Suppression is not seen in larvae expressing soluble GAL4 under control of the *dSREBP* promoter. The $dSREBP^{52}$ piggyBac insertion also encodes an enhanced yellow fluorescent protein (EYFP) marker that is expressed in brain and hindgut (Figure 10B, right panels; also see Appendix- Figure S2). Dashed lines denote the extent of larval bodies.

Figure 4-10

A



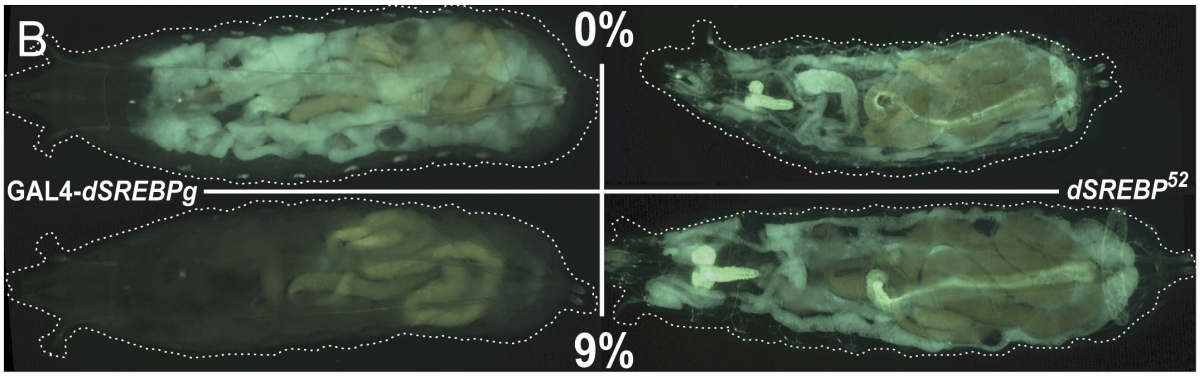


Figure 4-11

dSREBP target genes are suppressed by lecithin feeding: Quantitative analysis of transcripts of known or putative dSREBP target genes in wild type first instar larvae raised on semi-defined medium (-) or medium supplemented with 9 % soy lipid extract (+).

Figure 4-11

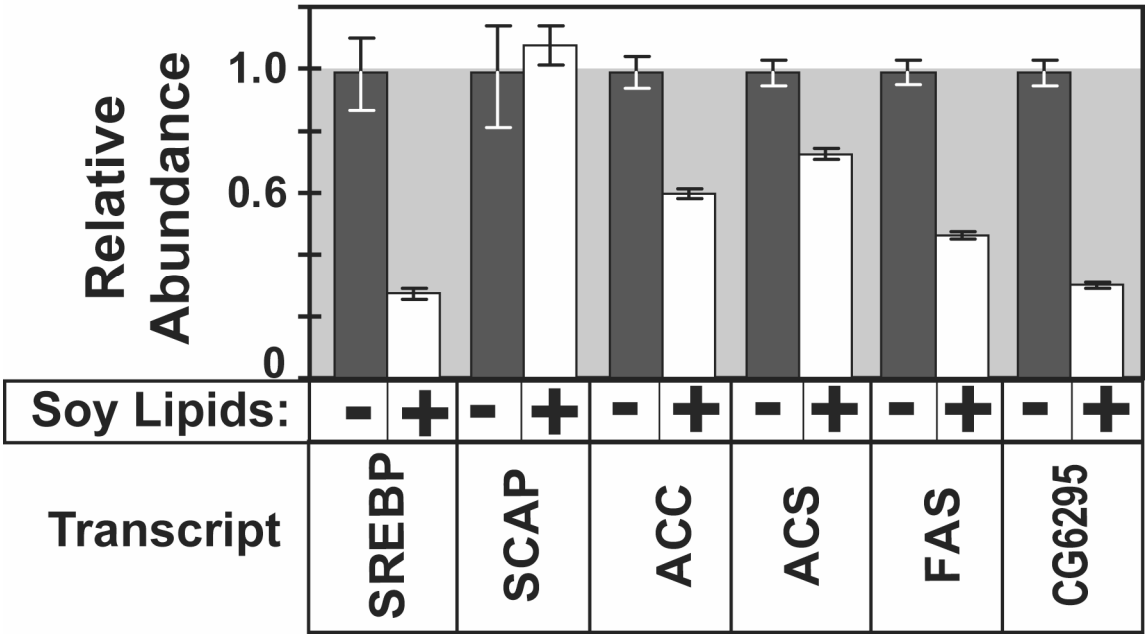

Relative Abundance
1.0
0.6
0
Soy Lipids:
- + - + - + - + - + - +
Transcript
SREBP
SCAP
ACC
ACS
FAS
CG6295

Figure 4-12

(**A**) **Characterization of flies lacking dSREBP in adulthood**: *dSREBP^{189}/ dSREBP^{189}* flies were rescued through larval development by using the P{Switch} system to express a dSREBP cDNA during the larval period. *dSREBP^{189}* homozygous adults thus obtained have no detectable dSREBP protein (lane 5) when cultured in the absence of RU-486. Homozygous adults cultured in the presence of RU486 express dSREBP strongly (lane 6). Adult males were collected within 24 h of eclosion and cultured for an additional 4 days in the presence or absence of 200 μM RU486 mixed into wet yeast paste. 50 μg aliquots (upper panels) or 80 μg (lower panels) of whole fly lysate from each sample was analyzed by immunoblotting with anti-dSREBP. **P;** membrane-bound dSREBP precursor. **N**; nuclear form. Membranes were stripped and re-probed with anti-tubulin antibody as a loading control.

(**B**) **Longevity of flies lacking dSREBP in adulthood**: Adults lacking dSREBP were generated as above and collected within 24 hours of eclosion. Flies were then cultured in the absence of RU-486 in yeasted vials containing cornmeal-molasses-agar. Males and females were separated and flies were kept at 15 flies/vial. Heterozygous flies collected from the same bottles, and wild type flies collected on the same day served as controls. A total of 345 flies/genotype (165 males, 180 females) were present at the beginning of the assay. Vials were scored for dead flies everyday and the number was used to calculate the percent alive. Flies were transferred to fresh vials every three days.

Figure 4-12

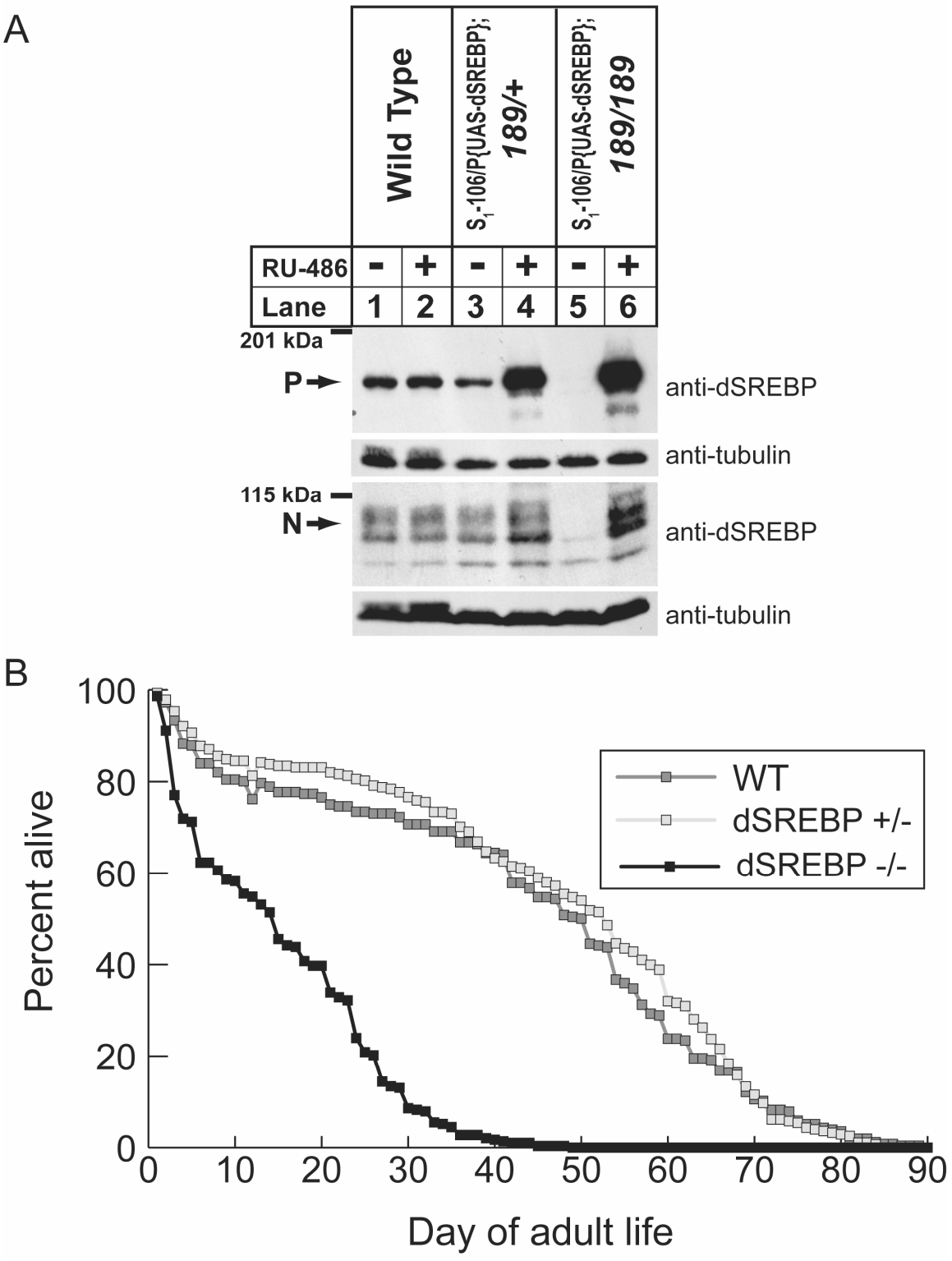

A
Wild Type
S1-106/P{UAS-dSREBP}; 189/+
S1-106/P{UAS-dSREBP}; 189/189
RU-486 - + - + - +
Lane 1 2 3 4 5 6
201 kDa
P
anti-dSREBP
anti-tubulin
115 kDa
N
anti-dSREBP
anti-tubulin
B
Percent alive
Day of adult life
WT
dSREBP +/-
dSREBP -/-
0 20 40 60 80 100
0 10 20 30 40 50 60 70 80 90

Figure 4-13

GAL4-SREBP activity in adult flies:

(**A**) In adults of either sex, signal can be detected in oenocytes (female shown).

(**B**) Dorsal view of a female abdomen showing GFP fluorescence in bands of oenocytes.

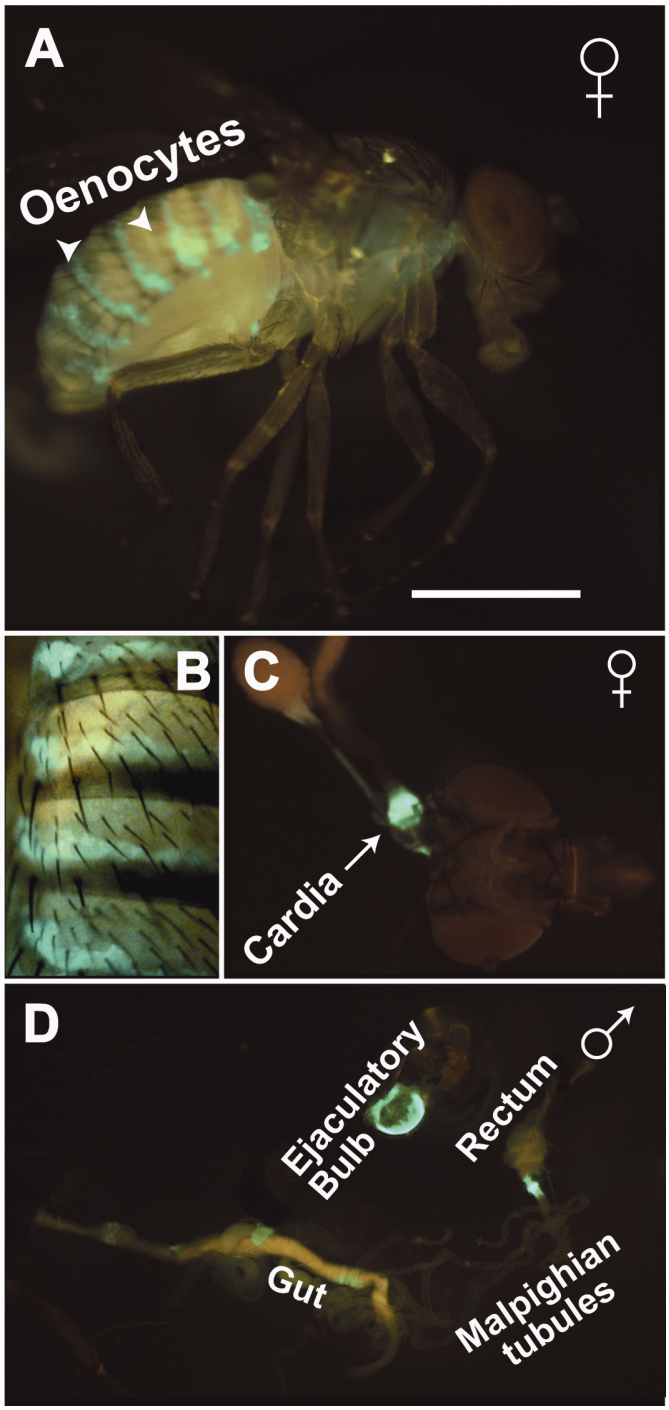
(**C**) The cardia shows intense fluorescence in both sexes (female shown).

(**D**) In males, strong GFP fluorescence can be detected in the ejaculatory bulb.

In either sex, a region anterior to the rectum along with isolated regions of the posterior midgut also show activity (male shown).

Scale bar = 1 mm

Figure 4-13


A
♀
Oenocytes
B
C
♀
Cardia
D
Ejaculatory Bulb
Rectum
♂
Gut
Malpighian tubules

CHAPTER FIVE
Conclusions and Recommendations

dSREBP and the maintenance of fatty acid prototrophy

The main goal of these studies was to elucidate the role of the SREBP pathway in *Drosophila* physiology. The results demonstrate that dSREBP is essential for larval development. Importantly, the results permit the conclusion that this essential developmental role of SREBP resides in its lipogenic activity. The single most convincing piece of evidence in favor of this conclusion is the dramatic reversal of lethality observed when the *dSREBP* null animals are fed either soybean lipids or pure fatty acids (Figure 4-7A, Table 4-5). Inasmuch as the emerging adults are indistinguishable from their heterozygous siblings in size and morphology (Figure 4-7 B, C), this result suggests that these treatments are able to largely substitute for the presence of active SREBP. Corroborating evidence includes the following: 1) dSREBP is cleaved primarily in tissues with a known role in either lipid synthesis (fat body and oenocytes) or acquisition (midgut) (Figure 4-9). These are also the tissues where dSREBP activity is required for survival (Table 4-4). 2) known lipogenic target mRNAs (FAS, ACC and ACS) are down-regulated in the absence of SREBP (Figure 4-6A). Importantly, this downregulation cannot be reversed by lipid supplementation, suggesting that these are direct effects and not secondary consequences of end-product depletion. 3) Larvae lacking dSREBP have lower fatty acid content (Figure 4-6B). 4) In a classic end-product mediated feedback inhibition loop, dSREBP cleavage in growing larvae is strongly suppressed by dietary lipid supplementation (Figure 4-10A,B).

That lipid synthesis is the critical function of the SREBP pathway was implied from previous studies, but had not been rigorously demonstrated in a whole animal system.

Lipid acquisition is a prominent feature in larval development. The larval lipid/protein ratio increases more than two-fold during the second and third instar (Church and Robertson, 1966). It is interesting, then, that wild type flies can develop on defined medium lacking all lipids save cholesterol (Sang, 1956) and are thus fully prototrophic for fatty acids. *dSREBP* mutant larvae, however, are unable to grow even on regular cornmeal-molasses-agar unless supplemented with fatty acids. Therefore, flies lacking *dSREBP* are fatty acid auxotrophs (Davis and Mingioli, 1950) and an important role of *dSREBP* in *Drosophila* physiology is the maintenance of fatty acid prototrophy.

While *Drosophila* larvae can satisfy their entire fatty acid requirement by endogenous synthesis, it is also clear that they can utilize dietary lipids for the same purpose (Keith, 1967b). In this situation, a system to control *de novo* synthesis in response to dietary supply would be beneficial to the organism. The data demonstrate that dSREBP cleavage responds to dietary lipids (Figures 4-10, 4-11 and S7) and is thus likely responsible for this function. The existence of such a system should allow the growing larva to allocate available metabolic resources efficiently between the synthesis of various macromolecules and thus achieve the most rapid growth possible. SREBPs have been shown to similarly regulate cholesterol synthesis in the liver in mice and hamsters (Brown and Goldstein, 1997). The benefits of balancing endogenous synthesis with dietary input and lipid demand likely provide the selective pressure for conservation of the SREBP pathway in evolution.

In these studies, I identified multiple genes involved in *de novo* fatty acid synthesis as dSREBP targets (Figure 4-6A, 4-11). I did not detect changes in transcript abundance for genes involved in the elongation or desaturation of fatty acids (not shown). This differs from mouse liver, where manipulation of the SREBP pathway causes transcriptional changes leading to altered fatty acid composition (Shimomura et al., 1998). In *dSREBP* mutants, we observed only a global deficit in the fatty acid content but no change in the relative abundance of the various species (Figure 4-6B, C, Table 4-2). In addition, dietary supplementation with any of the major fatty acids of flies served to compensate for lack of dSREBP, albeit with varying efficiency (Table 4-5). These data indicate that the mechanisms necessary for interconversion among various fatty acid species continue to function in the absence of dSREBP.

Activation of the SREBP pathway in mammals results in the preferential production of oleate (C18:1). This may reflect the need for a substrate for the esterification and storage of the other major product of the SREBP pathway, cholesterol (Repa et al., 2000; Shimomura et al., 1998). In *Drosophila,* the SREBP pathway is not involved in cholesterol synthesis (Seegmiller et al., 2002) and this distinction may underlie the observed difference in SREBP mediated fatty acid production between mammals and *Drosophila*.

The reduced fatty acid content of dSREBP mutant larvae is unlikely to result from a selective deficit in a particular class of lipids (neutral lipids vs membrane lipids). An inference may be drawn by comparing the abundance of myristate (C14:0) and oleate (C18:1). Myristate is relatively enriched in di- and tri-glycerides, while oleate is enriched in phospholipids (de Renobales and Blomquist, 1984). The lack of change in the relative

abundance of C14:0 and C18:1 suggests a co-ordinate decrease in the production of both classes of lipid (Table 4-2).

The role of dSREBP in organismal growth

Rapid growth is the predominant characteristic of larval development in insects. *Drosophila* larvae increase >200 fold in mass in a period of approximately 48 hours, corresponding to the second and early third instars ((Church and Robertson, 1966), Figure 4-5). Rapid growth undoubtedly places a great demand on lipid metabolism. Growing larvae must make additional cell membrane to accommodate increasing cell size. Larvae lacking dSREBP fail to initiate this growth spurt, remaining the size of first instar larvae (Figure 4-5). The simplest hypothesis to explain growth arrest is that larvae lacking dSREBP are unable to generate the membrane lipids required to accommodate growth. The fact that dSREBP null larvae have a markedly reduced lipid/protein ratio and a deficit in all fatty acid classes lends credence to this hypothesis. The small amount of growth that does occur in the mutants is likely the result of maternally loaded lipids.

It is not currently clear how the growth arrest in *dSREBP* null animals translates into lethality. Nutritionally deprived animals may be expected to undergo a prolonged growth arrest (like the dauer state in *C. elegans*) and not lethality. Indeed, when wild type larvae are starved for nutrients that must be acquired exogenously, such as certain amino acids, choline and cholesterol, pyrimidines, or vitamins, growth is arrested but the larvae can survive for an extended period. Transfer to complete medium within 6-8 days permits these starved animals to finish development (Britton and Edgar, 1998). The basis for this phenomenon is still under

investigation, but a number of recent studies implicate suppression of insulin signaling as the effector of growth arrest (Britton et al., 2002; Colombani et al., 2005; Colombani et al., 2003; Ikeya et al., 2002; Mirth, 2005; Rulifson et al., 2002). While flies have mechanisms such as arrested growth and delayed development for coping with deficits in essential nutrients, these mechanisms may not respond to a deficit in nutrients that can be fully supplied endogenously. In the absence of a coordinated suppression of all metabolic processes, accumulation of toxic intermediates may cause lethality. Analysis of the activity of the insulin signaling pathway in *dSREBP* null larvae should help answer this question.

Conversely, it is also interesting to speculate whether, in addition to the cell-autonomous response to lipid levels, the SREBP pathway also responds to global growth regulatory pathways. Interaction of insulin signaling with the SREBP pathway has already been demonstrated in mice (Shimomura et al., 1999; Yabe et al., 2003b). It is possible that insulin induction of the SREBP pathway is conserved in evolution. The availability of numerous insulin pathway alleles and the creation of an *in situ* reporter of dSREBP activity should allow this question to be answered.

dSREBP in the midgut

Experiments with the GAL4-dSREBP reporter system revealed that dSREBP is active in the midgut throughout larval development. Furthermore, the results presented in Table 4-4 demonstrate that this activity is essential for optimal survival. Since this activity can be substituted by dietary fatty acid supplementation, it must involve the accumulation of fatty acids, either by *de novo* synthesis or absorption from the gut. It is interesting, then, that I

identified a gene with similarity to mammalian pancreatic lipases (CG6295) as a *bona fide* dSREBP target gene. As shown in Figure 5-1, an alignment with porcine and human pancreatic lipases shows that CG6295 contains a conserved catalytic triad and also has high identity in residues immediately adjacent to those involved in catalysis. Finally, Table 4-5 reveals that lecithin, which contains only esterified fatty acids, rescued *dSREBP* null larvae at much higher concentrations (optimal at 9g%) than free fatty acids such as oleate and myristate (optimal at 0.075 and 0.15 g%, respectively). Lack of a dSREBP dependent lipase would be one explanation for this observed difference. Experiments are currently underway to test whether transgenic expression of CG6295 in the midgut will allow low concentrations of complex lipids (triglycerides or phospholipids) to rescue *dSREBP* null animals.

It is also possible that in addition to (or instead of) a direct role in fatty acid absorption, dSREBP activity in the midgut is required for *de novo* fatty acid synthesis. Fatty acid synthesis in the midgut may be required for the proper assembly of lipophorin particles that are essential to transport numerous hydrophobic metabolites between tissues. The lethality and arrest in larval development induced by lowered lipophorin levels attest to the importance of proper lipophorin function (Panakova et al., 2005).

In hamster intestine, the SREBP isoform most closely associated with fatty acid biosynthesis (Srebp-1c) is most highly expressed in the jejunum. Srebp-1c expression is concentrated towards the tips of the jejunal villi, the regions most responsible for nutrient absorption (Field et al., 2001). This expression pattern is similar to the localization of dSREBP in the more proximal regions of the gut. It is thus likely that any lessons learned

from the *Drosophila* system may illuminate hitherto unexplored functions of the SREBP pathway in the mammalian gut.

dSREBP during adult life

Unlike the larval phase, adult *Drosophila* do not exhibit any growth. In this situation, membrane biosynthesis is expected to occur at maintenance levels except in actively replicating tissues, such as the germline. It is not surprising, then, that a loss of dSREBP does not have the same catastrophic consequences during adult life as it does in larvae. Most dSREBP null adults survive for days to weeks after eclosion and are able to mate during this time. As might be expected from failure of a system involved in somatic maintenance, these animals have a reduced median lifespan and senesce earlier than their heterozygous siblings (Figure 4-12, Table 4-7).

In agreement with a reduced need for dSREBP, GAL4-dSREBP activity in adults is absent in the anterior midgut and is weak in the fat body. These are tissues in which dSREBP activation is the most prominent and essential during larval life. There are, however, other tissues which demonstrate strong activation of GAL4-dSREBP in adulthood (Figure 4-13)- the oenocytes and cardia in both sexes, and the ejaculatory bulb in the male. It is not yet clear what role dSREBP plays in these tissues, but the ability to generate dSREBP null adults in two ways (the p{Switch}system and nutritional rescue) should render these problems amenable to further study. The best known function of oenocytes is the synthesis of cuticular hydrocarbons, which are generated by elongation and decarboxylation of medium chain fatty acids (Jallon et al., 1997). Hydrocarbons serve two functions: a) they confer dessication

resistance due to their hydrophobic nature and b) they function as sex pheromones during courtship (Fan et al., 2003; Ferveur et al., 1997; Romer, 1980). Direct analysis of hydrocarbon profiles is possible and should allow a role for dSREBP to be tested (Jallon et al., 1997; Ueyama et al., 2005). The ejaculatory bulb is the site of maximal concentration of cis-vaccenyl acetate (cVA or 11-cis-octadecenyl acetate), a male specific aggregation pheromone present in the seminal fluid (Bartelt et al., 1985; Chertemps et al., 2005). Experiments are ongoing to determine whether cVA levels are altered in *dSREBP* null animals. The cardia is a funnel shaped structure present at the foregut-midgut junction. It is the site of production of the peritrophic membrane- a proteinaceous coat that a) protects the single layered intestinal wall from gut luminal contents, and b) contains digestive enzymes embedded in it (Miller, 1994). It is unclear what role, if any, dSREBP could have in the function of the cardia.

Future prospects- forward genetics

As stated in the introduction, a major goal of this project was to facilitate the application of forward genetic techniques to the SREBP pathway and its interactions. Knowledge of the physiological role of dSREBP should serve as a fundamental building block on which forward genetic screens can be designed. An example of the use of the knowledge and reagents obtained in this study is presented in Figure 5-2. This is an F1 screen aimed at uncovering dominant mutations that would prevent lecithin suppression of dSREBP cleavage. This design would allow thousands of mutagenized chromosomes to be screened

daily. Since the screen will be performed in live animals, the mutagenized chromosomes can be recovered by simply growing the larvae of interest up to adulthood.

A modification of this design, using the FLP-FRT system, can also be used to generate mosaic F1 larvae that would allow the detection of recessive mutations that confer the same phenotype. While endoreplicative larval tissues are not traditionally used for mosaic analysis, a protocol for doing so has been devised and used successfully (Manfruelli et al., 1999).

Both versions of this screen are likely to produce informative results. Dominant mutations in SCAP led to its initial identification and were instrumental in dissecting the mechanism of mammalian SREBP regulation. Given that there are some fundamental differences in the regulation of mammalian and *Drosophila* SCAP (PE as the regulating moiety and the absence of Insigs), isolation of such mutations in SCAP would be similarly useful in understanding the *Drosophila* pathway. It is, of course, also possible that this approach will uncover gain-of-function mutations in an interacting pathway that is capable of initiating SREBP cleavage. The loss-of-function screen has the potential to uncover components required for the retention of dSCAP in the endoplasmic reticulum, including the *Drosophila* Insig equivalent, if it exists. Other conserved components of the SCAP-SREBP complex may also be found by the mosaic approach, even if they have pleiotropic functions.

In addition to loss of lecithin suppression, this approach can also be used to screen for a failure to cleave SREBP.

It may be argued that an *in vivo* system is less attractive given the availability of whole genome RNAi methods in *Drosophila* and the proven power of mammalian somatic

cell genetics. It is worth reiterating, however, that numerous differences exist between SREBP regulation in immortalized cell lines and tissues *in situ*. 1) In mice, the relative amounts of Srebp-1a and Srebp-1c are different in tissues vs cell lines (Shimomura et al., 1997b), 2) Cleavage of SREBP-1c and SREBP-2 is regulated differentially in the refed liver. 3) The effect of insulin on Srebp-1c is not detectable in the immortalized cell lines used for somatic cell genetics, 4) There exists a liver specific isoform of Insig-2 which responds to insulin signaling (Yabe et al., 2003a), 5) Rat spermatogenic cells produce an alternative SREBP transcript that encodes only the transcription factor domain (Wang et al., 2002). It is likely, then, that there are other tissue specific proteins or pathway interactions that modulate SREBP activity. A genetically tractable whole-animal model system is therefore likely to contribute to the understanding of the SREBP pathway- both in *Drosophila* as well as mammals.

Figure 5-1

Multiple sequence alignments between pig and human pancreatic lipases and CG6295 were generated using the ClustalW algorithm. Conserved residues are highlighted. The sequence ruler is based on pig pancreatic lipase. The catalytic triad of pig pancreatic lipases includes the following residues- S152, D176 and H263 (arrows). The GxSxG motif at the catalytic site, characteristic of lipases and esterases, is also conserved. The output file was formatted using the ESPript formatting tool available on the Expasy website (www.expasy.ch).

FIGURE 5-1

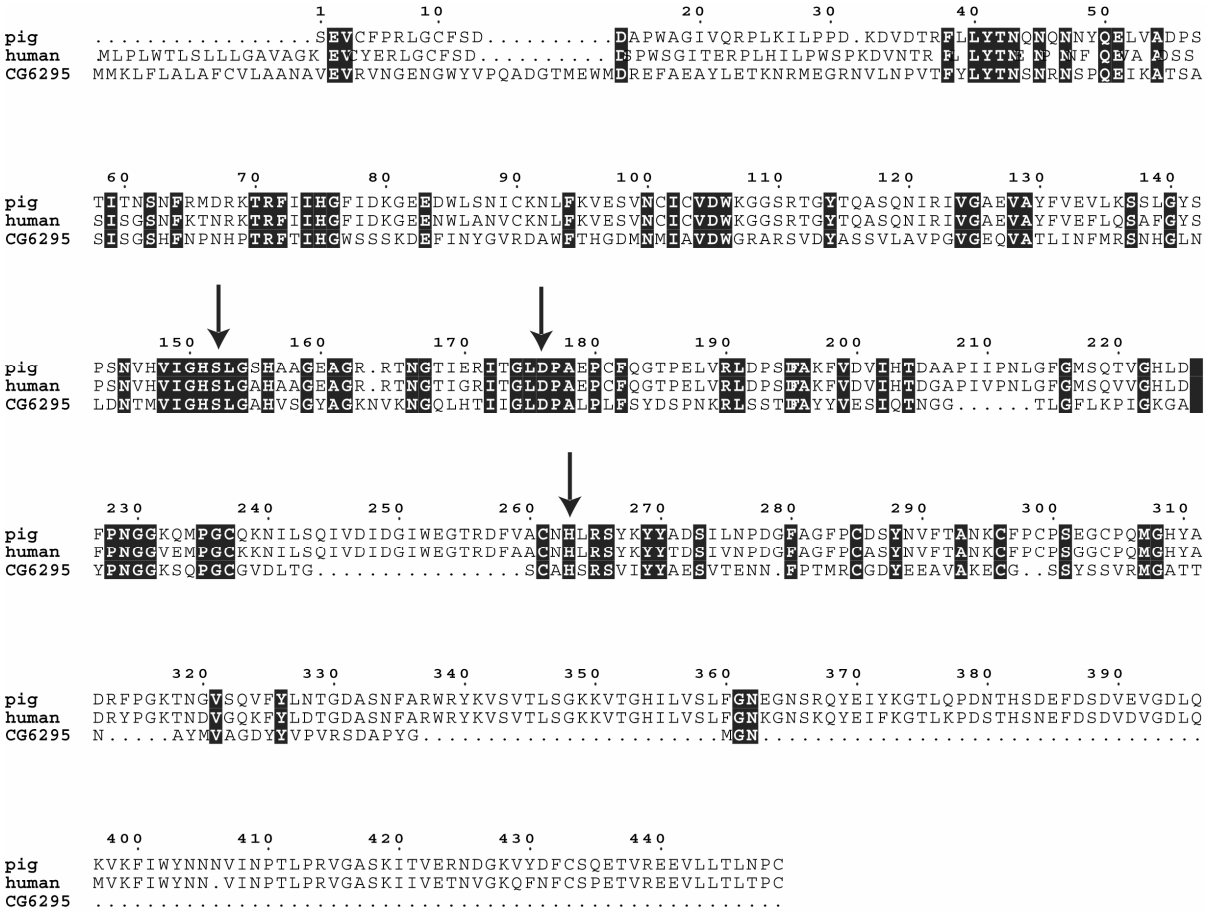
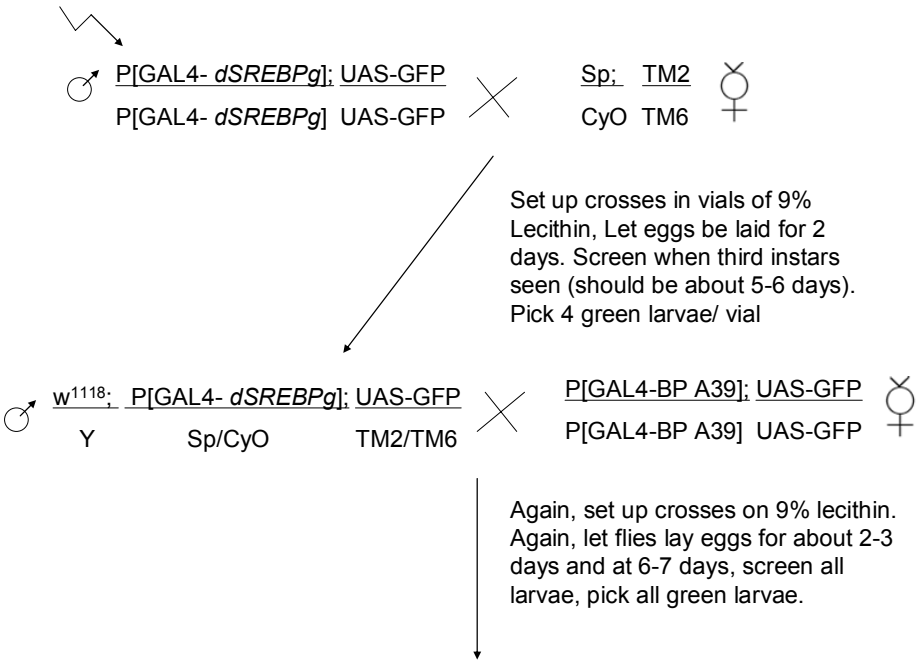


Figure 5-2

Schematic depicting the protocol for an F-1 screen for dominant mutations that prevent suppression of SREBP cleavage.

FIGURE 5-2



- If the mutation is on the X and the parent carrying it is male, all the selected larvae will be female.
- If the mutation is on the 2nd, none of the selected larvae will be Sp or CyO. You will, however, see TM2 or TM6 flies from the green larvae
- If on the 3rd, none of the selected larvae will be TM2 or TM6. You will, however, see Sp or CyO flies from the green larvae
- If the mutation is on the X and the parent carrying it is female, you will see both male and female, Sp/CyO and TM2/TM6 flies among the green progeny. Repeat the cross with a green male to confirm the result

Once the chromosome is known, balance the stock on that chromosome by crossing with Sp/CyO;TM2/TM6 or appropriate X chromosome balancer

APPENDIX

Supplemental Figures

Table S1. Rescue of *dSREBP*^{52}*/dSREBP*^{189} Transheterozygotes by Fatty Acids

Embryos/vial	1 mg		2 mg		3 mg	
	% Rescue	(SEM)	% Rescue	(SEM)	% Rescue	(SEM)
No additions	18.6	(2.4)	16.1	(3.1)	16.3	(2.0)
C12:0	64.4	(11.3)	63.0	(8.1)	49.1	(7.7)
9% Soy Lipids	89.6	(16.8)	77.5	(5.6)	87.9	(13.9)
C14:0	103.8	(19.5)	102.3	(10.6)	88.5	(3.9)
C18:1	106.8	(17.7)	108.4	(16.8)	98.2	(4.5)

Embryos were collected from crosses of *dSREBP*^{52}*/TM3, Actin-GFP, Ser* virgins and *dSREBP*^{189}*/TM3, Actin-GFP* males. Embryos were plated in vials containing cornmeal-molasses-agar supplemented with the indicated lipid (C 12:0- 0.075g%, Soy Lipids- 9 g%, C14:0- 0.075%, C 18:1- 0.15 g%). Emerging progeny were scored to obtain the percent of the expected survival as described in *Experimental Procedures*. Similar to rescue of *dSREBP*^{189} homozygotes, maximum rescue was observed with C14:0 and C18:1. Under these optimized conditions, increased survival of the transheterozygotes compared to the *dSREBP*^{189} null mutants (Table 4-5) may be owing to the very low levels of dSREBP present in these animals (Fig. 4-3C). The numbers in parentheses indicate the standard error of the mean.

Figure S1

Soy lipid extract rescues transheterozygotes to adulthood: Embryos from *dSREBP*^{52}/TM3, *actin-GFP, Ser* virgin females crossed to *dSREBP*^{189}/TM3, *actin-GFP, Ser* males were seeded (at 1 mg/vial) into vials of cornmeal-molasses-agar supplemented with increasing concentrations of soy lipid extract. Emerging adults were scored for their *dSREBP* genotype and the ratio of homozygotes/heterozygotes was used to calculate the survival of homozygotes as a percentage of the expected ratio (0.5 = 100%). At concentrations greater than 9 %, the soy lipids rendered the medium unable to support even wild type flies due to its altered consistency. Similarly to the experiments with *dSREBP*^{189} (Fig. 4-7A), maximum rescue (86.0%) occurred at 9 g% soy lipid extract. Under these optimized conditions, increased survival of the transheterozygotes compared to the *dSREBP*^{189} (Figure 4-7A) null mutants may be owing to the very low levels of dSREBP present in these animals (Figure 4-3C).

Figure S1

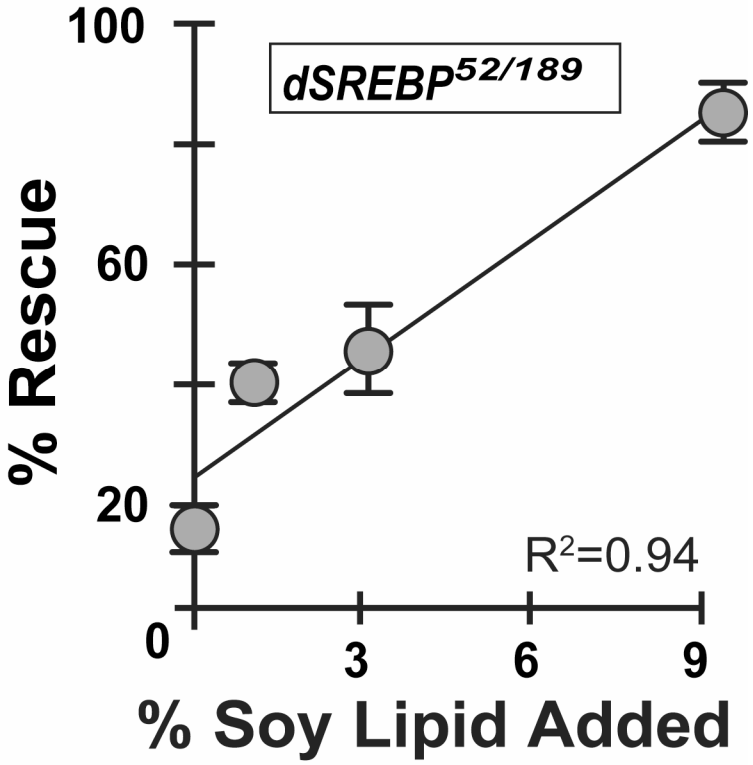


Figure S2

Expression pattern of the *dSREBP^{52}* enhancer trap: The piggyBac transposon used to generate the *dSREBP^{52}* insertion carries a GAL4 cDNA under control of a minimal promoter. Thus, it serves as a *dSREBP* enhancer trap. The transposon also carries a enhanced yellow fluorescent protein (EYFP) cDNA that serves as a marker for presence of the transposon. The YFP is expressed in the brain and hindgut as seen in the '*dSREBP^{52}* alone' panels. When flies carrying *dSREBP^{52}* are crossed to UAS-GFP flies, progeny larvae demonstrate GFP fluorescence in the fat body, midgut, and oenocytes.

Figure S2

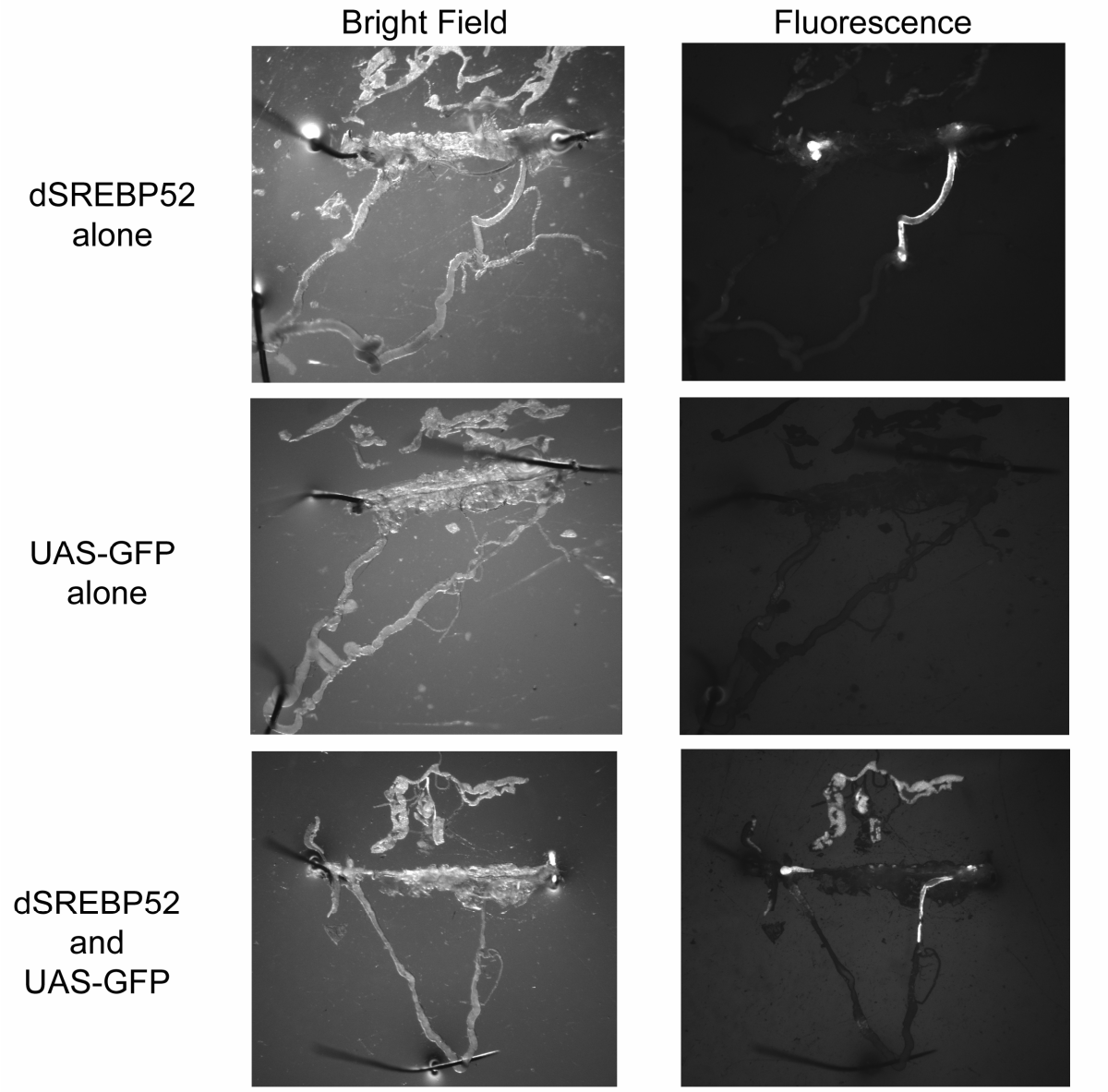

Bright Field
Fluorescence
dSREBP52 alone
UAS-GFP alone
dSREBP52 and UAS-GFP

Figure S3

Visible and fluorescence light micrographs of third instar larvae of the following genotypes: GAL4-dSREBP only (left panels), UAS-GFP only (center panels), and GAL4-dSREBP and UAS-GFP together (right panels). Both the driver and reporter transgenes are required in order for green fluorescence to be detected. In the absence of GFP signal (left and center panels), the contents of the gut are visible under fluorescent light due to autofluorescence. In the presence of GFP signal (right panel), the pale yellow of the gut is highly distinguishable from the bright green visible in the fat body and oenocytes. Larvae are oriented with the anterior up. The views are of the dorsal side.

Figure S3

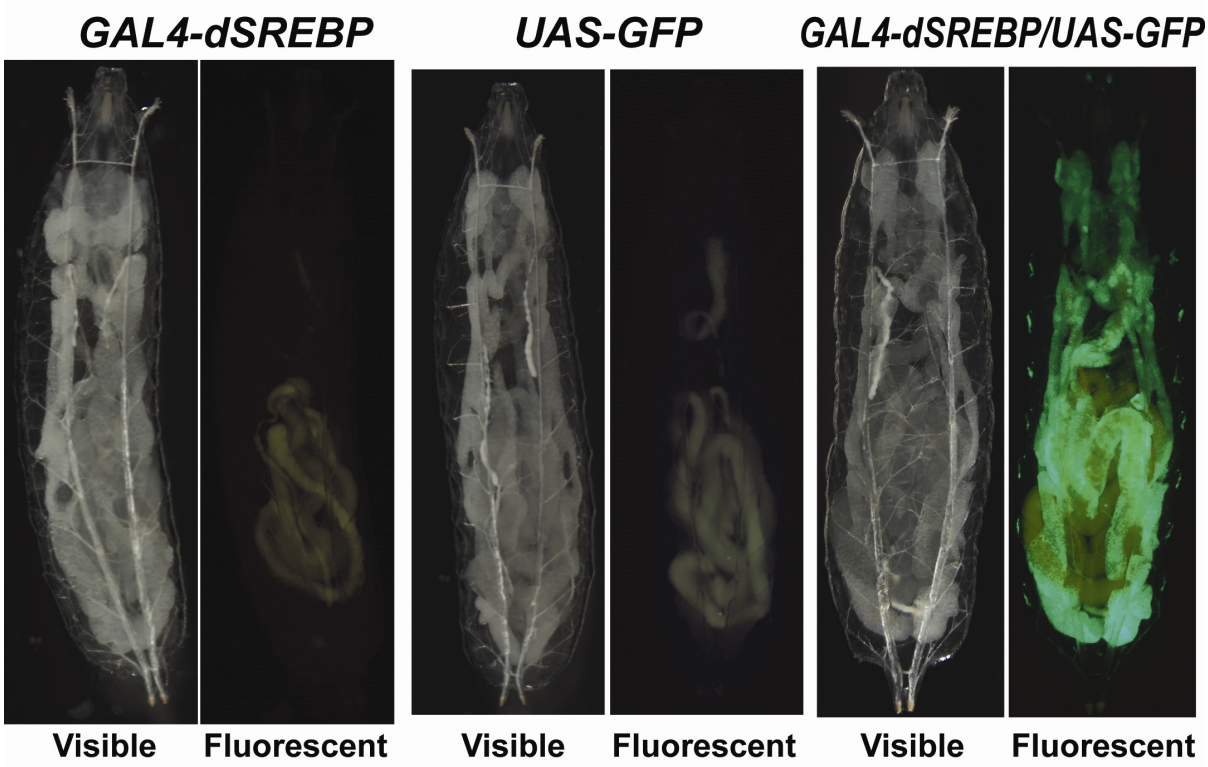

GAL4-dSREBP
UAS-GFP
GAL4-dSREBP/UAS-GFP
Visible
Fluorescent
Visible
Fluorescent
Visible
Fluorescent

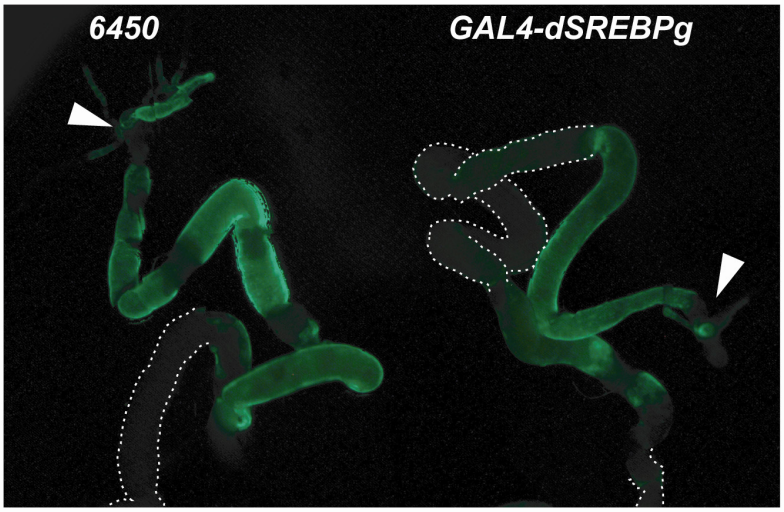
Figure S4

(**A**) **Strength of 6450 versus GAL4-dSREBPg in the midgut**: Comparison of GFP fluorescence intensity between the GAL4 enhancer trap line 6450 and GAL4-dSREBPg in the midgut of dissected third instar larvae. Larvae harbor one copy of either transgene. White arrowheads indicate the mid-hindgut junction.

(**B**) **Strength of DcG-GAL4 versus GAL4-dSREBPg in the fat body**: Comparison of GFP fluorescence intensity in the fat body throughout larval development for larvae harboring single copies of either driver transgene and the same UAS-GFP reporter transgene. GFP fluorescence in dissected fat body (lower left panel) from DcG-GAL4 larvae (left) compared to GAL4-dSREBPg (right). Comparisons shown for each genotype were photographed together in a single image. Dashed lines indicate the extent of larval tissues.

Figure S4

A


6450
GAL4-dSREBPg

B

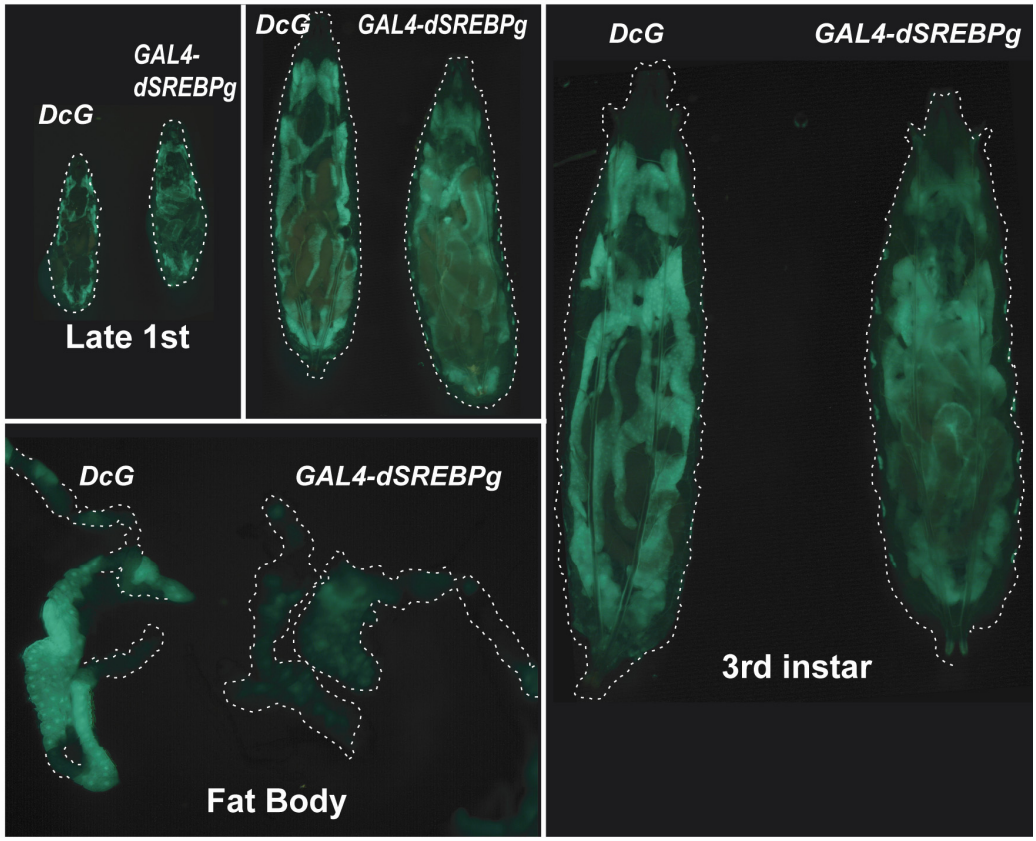

DcG
GAL4-dSREBPg
Late 1st
DcG
GAL4-dSREBPg
DcG
GAL4-dSREBPg
Fat Body
DcG
GAL4-dSREBPg
3rd instar

Figure S5

Demonstration of gut autofluorescence: Fluorescence micrograph of a wild type third instar larva carrying no transgenes demonstrates brownish autofluorescence from the gut contents when fed on cornmeal-molasses-agar medium

Figure S5

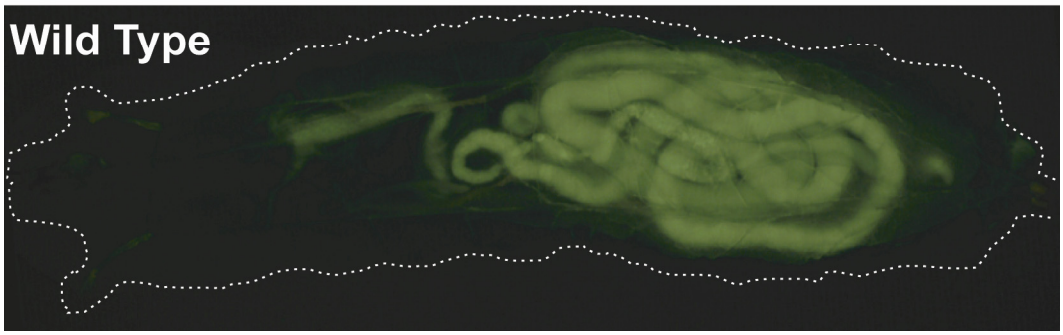
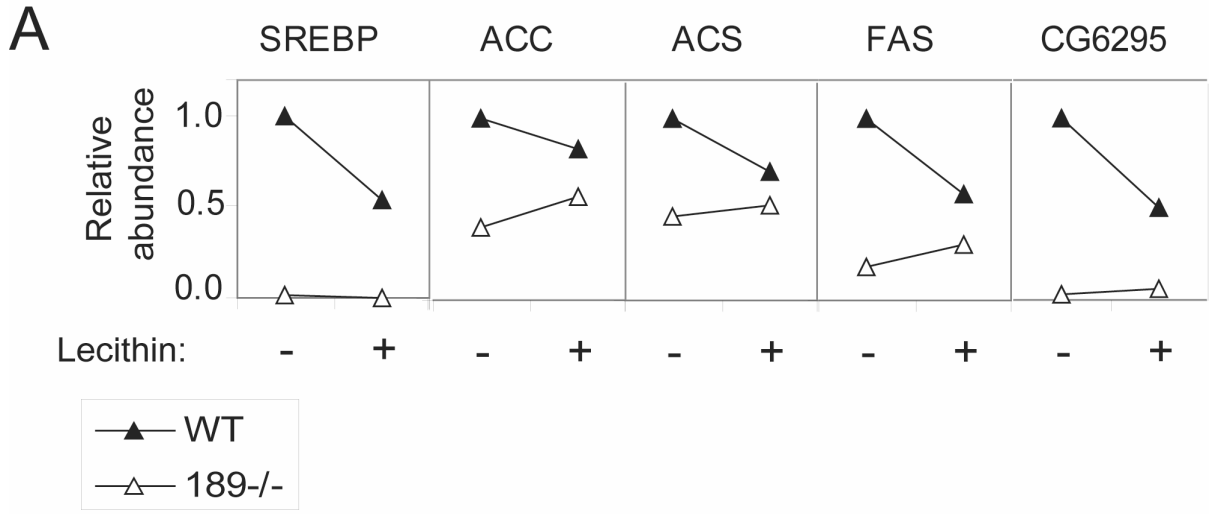


Figure S6

(**A**) **dSREBP target gene abundance is not responsive to lecithin supplementation in *dSREBP* null larvae**: Quantitative analysis of transcripts of known or putative dSREBP target genes in wild type and $dSREBP^{189}/dSREBP^{189}$ first instar larvae raised on semi-defined medium (-) or medium supplemented with 9 % soy lipid extract (+).

(**B**) **dSREBP target gene abundance is not responsive to lecithin supplementation in transheterozygous mutant larvae**: Quantitative analysis of transcripts of known or putative dSREBP target genes in wild type and $dSREBP^{52}/dSREBP^{189}$ first instar larvae raised on semi-defined medium (-) or medium supplemented with 9 % soy lipid extract (+).

Figure S6


A
SREBP
ACC
ACS
FAS
CG6295
Relative abundance
1.0
0.5
0.0
Lecithin:
- + - + - + - + - +
WT
189-/-

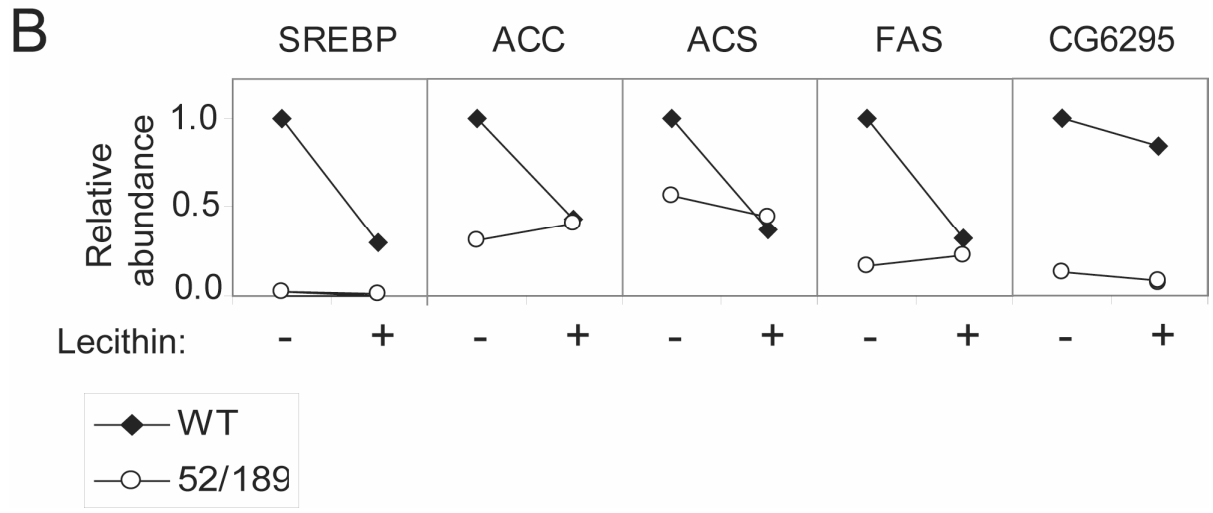
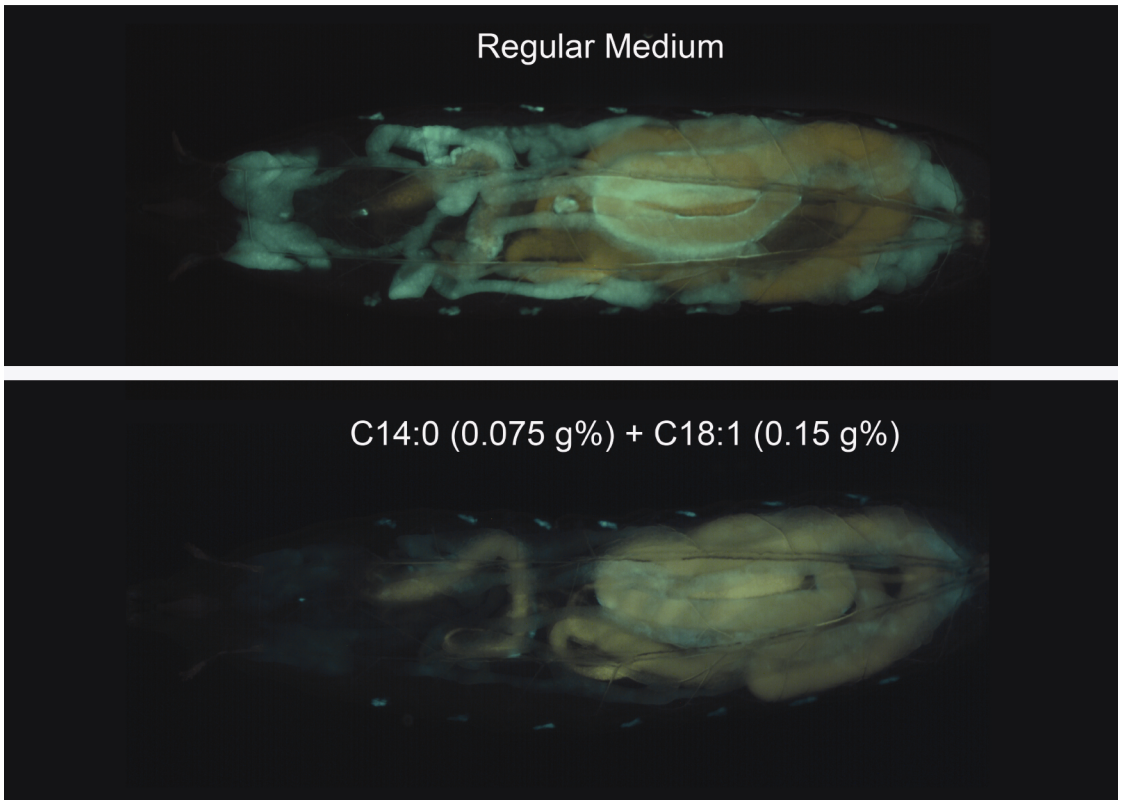

B
SREBP
ACC
ACS
FAS
CG6295
Relative abundance
1.0
0.5
0.0
Lecithin:
- + - + - + - + - +
WT
52/189

Figure S7

Wild type larvae carrying UAS-GFP and P{*GAL4-dSREBPg*} are shown. Larvae were cultured on either unsupplemented cornmeal-molasses-agar medium (0%) or the same medium supplemented with 0.075 g% myristate (C14:0) and 0.15 g% oleate (C18:1). Supplementation causes a disappearance of GFP signal in the fat body and midgut. The brownish color of the gut is due to autofluorescence of gut contents (see Figure S5)

Figure S7



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