ALTERNATIVE PROCESSING OF SREBP IN SITE 2 PROTEASE

AND SCAP MUTANTS DURING LARVAL DEVELOPMENT

IN *DROSOPHILA MELANOGASTER*

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To my parents

For your love and constant support.

ALTERNATIVE PROCESSING OF SREBP IN SITE 2 PROTEASE AND SCAP MUTANTS DURING LARVAL DEVELOPMENT IN *DROSOPHILA MELANOGASTER*

by

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ALTERNATIVE PROCESSING OF SREBP IN SITE 2 PROTEASE AND SCAP MUTANTS DURING LARVAL DEVELOPMENT IN *DROSOPHILA MELANOGASTER*

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Lipid metabolism is regulated by the membrane-bound transcription factor, sterol regulatory element binding protein (SREBP). SREBP requires release of the amino terminus from the membrane to activate transcription of genes involved in cholesterol and fatty acid synthesis. In response to low sterol levels, Scap escorts SREBP from the ER to the Golgi where it is cleaved by Site-1 and Site-2 proteases. The SREBP pathway is conserved in *Drosophila* despite these

organisms being cholesterol auxotrophs. dSREBP is essential for activating genes involved in the uptake and synthesis of fatty acids which are required for rapid growth during larval development. I have demonstrated that processing of SREBP in *Drosophila* does not require the S2P or Scap, in contrast to the mammalian system.

Flies lacking dS2P are viable and still process dSREBP. dS2P homozygotes were subviable, only emerging at 40% of the expected ratio. This phenotype can be rescued completely by supplementation with fatty acids. dSREBP activity was detected in the fat body of dS2P mutant larvae and to a lesser extent in the oenoctyes. Additionally, SREBP target genes were expressed at higher levels in dS2P homozygotes compared to dSREBP mutants, though less than wild type. dS2P mutants were viable due to alternative cleavage of dSREBP within the juxtamembrane region by the effector caspase, Drice. Flies lacking both dS2P and Drice, or the initiator caspase Dronc, exhibited an early larval lethality that could be rescued by lipid supplementation. Caspase cleavage was dependant upon the aspartic acid at residue 386 in dSREBP.

dScap was not essential for larval growth or dSREBP processing in *Drosophila*. dScap mutants were relatively healthy, emerging at 70% of the expected numbers. dSREBP was actively cleaved in midgut and oenocytes, but

significantly reduced in fat body. Levels of dSREBP mRNA and precursor were reduced in larvae lacking dScap, thus demonstrating that *Drosophila* SREBP is subject to feed-forward activation of its own transcription. Addition of soy lipids suppress dSREBP processing in dScap mutants, but whether this regulation is translational or post-translational is unknown. Furthermore, flies lacking both dScap and dS2P are viable, but survive less well than either single mutant alone. Membrane-bound intermediate dSREBP accumulates in double mutants, suggesting that dSREBP is processed normally by dS1P and dS2P in dScap single mutants. Thus, dScap mutants escape the larval lethality seen in dSREBP mutants due to alternative processing of dSREBP, but through different mechanism than that seen in dS2P mutants.

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PUBLICATIONS

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Matthews K, Kunte A, Ebot-Tambe, E and Rawson R. 2008. Alternative processing of SREBP during larval development in *Drosophila melanogaster*. Genetics. In press.

Kunte A, **Matthews K**, and Rawson R. 2006. Fatty acid auxotrophy in *Drosophila* larvae lacking SREBP. Cell Metabolism. 3(6):439-48.

Huang J, Wu S, Barrera J, **Matthews K**, and Pan D. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by Yorkie, the *Drosophila* homolog of YAP. Cell. 122(3):421-34.

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LIST OF DEFINITIONS

Balancer – a chromosome containing multiple inversions and phenotypic markers; prevents recombination of homologous chromosomes in female flies; used to maintain lethal mutant alleles.

Deficiency – a large deletion removing multiple loci.

FRT – Flp recombinase target sequence from the yeast 2µ plasmid; used for site-specific excision

GAL4/UAS reporter system – A binary reporter system used for temporal and tissue specific gene expression; 1) reporter: the yeast transcription factor GAL4 under a specific promoter of choice; 2) responder: the yeast GAL4 promoter 'upstream activating sequence' driving expression your gene of interest.

hsFlp – the Flp recombinase from the yeast 2µ plasmid under the control of an inducible *hsp70* heat shock promoter; gene expression is induced at 37˚.

P element – a *Drosophila* transposon; used to generate imprecise excisions upon remobilization; requires a transposase source.

piggyBac element – a lepidopteron-derived transposable element; inserts randomly within the genome; precise excision upon removal.

CHAPTER ONE

Introduction

Lipids are major components of cell membranes and fundamental building blocks for many molecules. While lipids are essential for cell survival and growth, an excess of lipids leads to cell toxicity and disease. To maintain lipid homeostasis, cells tightly regulate the synthesis and uptake of lipids.

THE SREBP PATHWAY

Sterol Regulatory Element Binding Proteins (SREBPs) are transcription factors of the basic-helix-loop-helix-leucine zipper (bHLH-zip) family that maintain lipid homeostasis by end-product feedback regulation. The mammalian genome contains two SREBP genes encoding three proteins that activate transcription of genes with sterol regulatory elements (SREs) in their promoter regions. SREBP-1 encodes two isoforms, SREBP-1a and SREBP-1c, through the use of alternative transcription start sites (Hua et al., 1995). SREBP-1a activates target genes mediating both cholesterol and fatty acid synthesis, whereas SREBP-1c is predominately responsible for fatty acid synthesis (Horton et al., 2002). SREBP-2 encodes one protein that specifically activates transcription of genes mediating cholesterol synthesis (Horton et al., 2002).

Studies using knockout and transgenic animals have demonstrated the importance of maintaining proper levels of SREBP activation. In mice, loss of SREBP-1 is 50-85% embryonic lethal and loss of SREBP-2 is 100% embryonic lethal (Shimano et al., 1997). Those SREBP-1 homozygotes that do survive are healthy due to compensation by SREBP-2. Over-expression of SREBP-1a in the liver increased the rate of fatty acid synthesis 25-fold over wild type resulting in fatty liver due to the massive accumulation of triglycerides and cholesterol esters (Shimano et al., 1996). Over-expression of SREBP-2 in the liver and adipose tissue results in a 28-fold increase in the rate of cholesterol synthesis (Horton et al., 1998).

Transcriptional activation of SREBP is regulated through proteolytic processing (Figure 1-1) (Brown and Goldstein, 1997). SREBPs are inserted into the endoplasmic reticulum (ER) membrane in a hairpin-like fashion as inactive precursors. The amino terminus which encodes the bHLH-zip DNA binding domain and the carboxyl terminus which encodes a protein-protein interacting domain face the cytosol and are connected through a transmembrane spanning luminal loop. For SREBP to activate transcription of target genes in the nucleus, the amino terminal transcription factor domain must be released from the membrane. The proteins that coordinate SREBP processing are Scap, site-1 protease (S1P) and site-2 protease (S2P). Scap escorts SREBP from the ER to the

Golgi where it is cleaved by S1P within the luminal loop, cutting the protein in half. The amino terminal membrane-bound intermediate is then a substrate for S2P which cleaves SREBP within the first transmembrane region releasing the DNA binding domain to travel to the nucleus.

In mammals, regulation occurs in the transport of SREBP between the ER and Golgi and involves the ER retention protein Insig (Yang et al., 2002). When sterol levels are low, Scap binds SREBP and this complex is incorporated into coat protein complex II (COPII)-coated vesicles through the interaction of Scap and the coat protein Sec24 (Sun et al., 2005). An increase in sterol levels promotes binding of Insig to Scap which blocks the interaction of the Scap:SREBP complex with Sec24 and incorporation into COPII vesicles (Sun et al., 2007). Thus, SREBP is retained in the ER membrane and transcription of target genes is suppressed.

The SREBP processing machinery are absolutely required for survival of mammalian cells. Cell lines carrying defects in the genes encoding Scap, S1P, or S2P fail to process SREBP (Rawson et al., 1998; Rawson et al., 1999; Rawson et al., 1997). These mutant cells fail to synthesize cholesterol de novo and require supplementation of cholesterol and fatty acids to the media for survival. In mice, loss of S1P is embryonic lethal due to the complete absence of processing of each

of the three SREBP proteins (Yang et al., 2001). Targeted knockdown of Scap in the liver results in 71% and 84% reductions in the rate of cholesterol and fatty acid synthesis, respectively (Matsuda et al., 2001), but overall these mice are healthy due to compensation by adipose tissue (Kuriyama et al., 2005). In contrast, cells deficient of Insig fail to regulate SREBP processing in response to sterol levels and SREBP is constitutively cleaved (Lee et al., 2005). Mice lacking Insig exhibit severe facial deformities due the overproduction of cholesterol and the accumulation of cholesterol intermediates (Engelking et al., 2006).

DROSOPHILA AS A MODEL ORGANISM FOR UNDERSTANDING METABOLIC REGULATION

Drosophila has long been utilized as a model organism for the study of development and growth, but only recently has *Drosophila* been seen as a model for metabolism (reviewed in (Baker and Thummel, 2007)). The fruit fly offers many advantages over other model organisms, from the relative ease of genetic manipulation to an established array of genetic tools. From a practical view, *Drosophila* are easily grown on a large scale making up for lack of size and have a well characterized life cycle of 10 days from embryo to adult. Furthermore, there is a considerable degree of conservation in cellular pathways between mammals and *Drosophila* which allows for the knowledge gained in this simpler system to be applied to more complex animal models.

The fly life cycle is divided into four stages: embryogenesis, three larval instars (from 24 to 84 hours after egg laying (AEL)), pupation, and adult. The larval stages are characterized by extensive feeding and rapid growth during which there is a 200 fold increase in mass over a three day period (Church and Robertson, 1966). Maximum growth is reached around 72 hours AEL at which time wild type larvae exit the food in search of a pupation site (Church and Robertson, 1966). Larval tissues are geared towards nutrient accumulation to support growth and ultimately pupation, whereas adult tissues are predominately geared towards flight and reproduction. To this end, *Drosophila* offers two different models in one animal. Thus, the *Drosophila* larva is an excellent animal in which to study lipid homeostasis and metabolism.

THE SREBP PATHWAY IN *DROSOPHILA*

The SREBP pathway is highly conserved in *Drosophila* despite their being cholesterol auxotrophs. Like all arthropods, *Drosophila* lacks several key enzymes required for the *de novo* synthesis of cholesterol from acetyl CoA and must obtain cholesterol or a close derivative of cholesterol from their diet (Clark and Bloch, 1959). The *Drosophila* genome contains one SREBP gene encoding one protein, designated dSREBP, and orthologs of the basic processing machinery, dScap, dS1P, and dS2P (Seegmiller et al., 2002). However, no Insig-

like protein has been identified to date based on sequence homology (Rawson, 2003). Studies in *Drosophila* cell culture and flies have shown that dSREBP more closely resembles SREBP-1c, regulating transcription of genes mediating fatty acid synthesis, including acetyl CoA carboxylase (ACC), acetyl CoA synthase (ACS), and fatty acid synthase (FAS) (Kunte et al., 2006; Seegmiller et al., 2002). *Drosophila* SREBP also plays a role in the uptake of lipids from the diet (Kunte et al., 2006).

Drosophila SREBP is essential for larval development

Drosophila SREBP is essential in the synthesis and uptake of fatty acids during larval development (Kunte et al., 2006). Larvae lacking SREBP exhibit normal growth up until 48 hours AEL, but fail to transition from second to third instar (Figure 1-2A). This lethality is due to reduced transcription of dSREBP target genes (Figure 1-2B). Knockout or knock-down of dSREBP in either larvae or in *Drosophila* S2 cells, respectively, results in significant reductions in ACC, ACS, and FAS transcripts (Kunte et al., 2006; Seegmiller et al., 2002). In consequence, the amount of total fatty acid in first instar dSREBP mutants is reduced by 30% compared to wild type (Kunte et al., 2006). Furthermore, uptake and utilization of lipids from the diet is also affected by decreased transcription of CG6295, a putative lipid lipase expressed only in the midgut (Kunte et al., 2006)(Bill Amarneh, unpublished data). Lethality due to loss of dSREBP can be rescued by

supplying larvae with the end-products of dSREBP target genes: fatty acids, either in the form of soy lipids or specific long-chain unsaturated fatty acids (Kunte et al., 2006).

Drosophila SREBP is actively processed throughout all three larval instars in tissues involved in the uptake, storage, and synthesis of lipids: midgut, fat body, oenocytes, and ring gland (Figure 1-3)(Kunte et al., 2006). dSREBP is involved in the uptake of fatty acids from the larval gut. Expression of active dSREBP is restricted to a specific region of the midgut, which correlates with expression of the lipid lipase, CG6295 (Bill Amarneh, unpublished data). The larval fat body is equivalent to mammalian liver and adipose. The primary function of the larval fat body is the accumulation and storage of lipids in order to support the animal during metamorphosis. Church and Robertson (1966) showed that lipids accounted for 6% of larval body weight in first instars and increased to 15% prior to pupation. This accumulated lipid is almost completely consumed during metamorphosis so that little of the larval fat body is present in new eclosed adults. dSREBP is actively cleaved in the oenocytes which are clusters of 5-7 cells per abdominal hemi-segment of incompletely characterized function. Gutierrez et al. (2007) suggest that these cells function similar to hepatocytes in coordinating lipid mobilization from the fat body during starvation. Furthermore, dSREBP is abundantly processed in the corpus allatum of the ring gland, the

endocrine organ of *Drosophila* larvae, although the significance of dSREBP activity in this tissue is not understood.

Like the mammalian system, dSREBP is subject to end-product feedback regulation. Studies of the suppression of dSREBP cleavage in *Drosophila* cell culture identified the regulatory molecule to be a phosopholipid, phosophtidlyethanolamine (Dobrosotskaya et al., 2002). Furthermore, addition of excess soy lipids to larval food suppresses processing of dSREBP and larvae deficient of dSREBP are rescued to adulthood by supplementation of the diet with myristate and oleate (Kunte et al., 2006). By analogy to cultured Drosophila cells, the regulation of dSREBP processing by fatty acids in larvae is likely to be due to their incorporation into phosopholipids. The mechanism by which phospholipids regulate dSREBP is unknown.

PROJECT GOAL

The goal of my project was to determine the physiological role of the dSREBP processing machinery, specifically dS2P and dScap, in the whole animal. Previous work in cultured *Drosophila* cells and in flies defined the role of dSREBP as a transcription factor essential for activation of genes mediating the synthesis and uptake of fatty acids required for the normal growth and development of *Drosophila* larvae (Dobrosotskaya et al., 2002; Kunte et al., 2006;

Seegmiller et al., 2002). Work in cell culture suggested that dScap was required for cleavage of dSREBP, but the role of dS2P in *Drosophila* remained unknown (Seegmiller et al., 2002). To determine the role of dS2P and dScap in *Drosophila*, I addressed the following questions: 1) Are *dS2P* and *dScap* essential genes in *Drosophila*? 2) Are these genes required for dSREBP activation during larval development? 3) What other proteins cleave dSREBP in *Drosophila*?

To answer these questions, I generated loss-of-function alleles of *dS2P* and *dScap* and assessed their effects on dSREBP activation and *Drosophila* physiology. Since previous work showed that dSREBP does not play an essential role in the adult fly, I focused on the activity of dS2P and dScap during larval development.

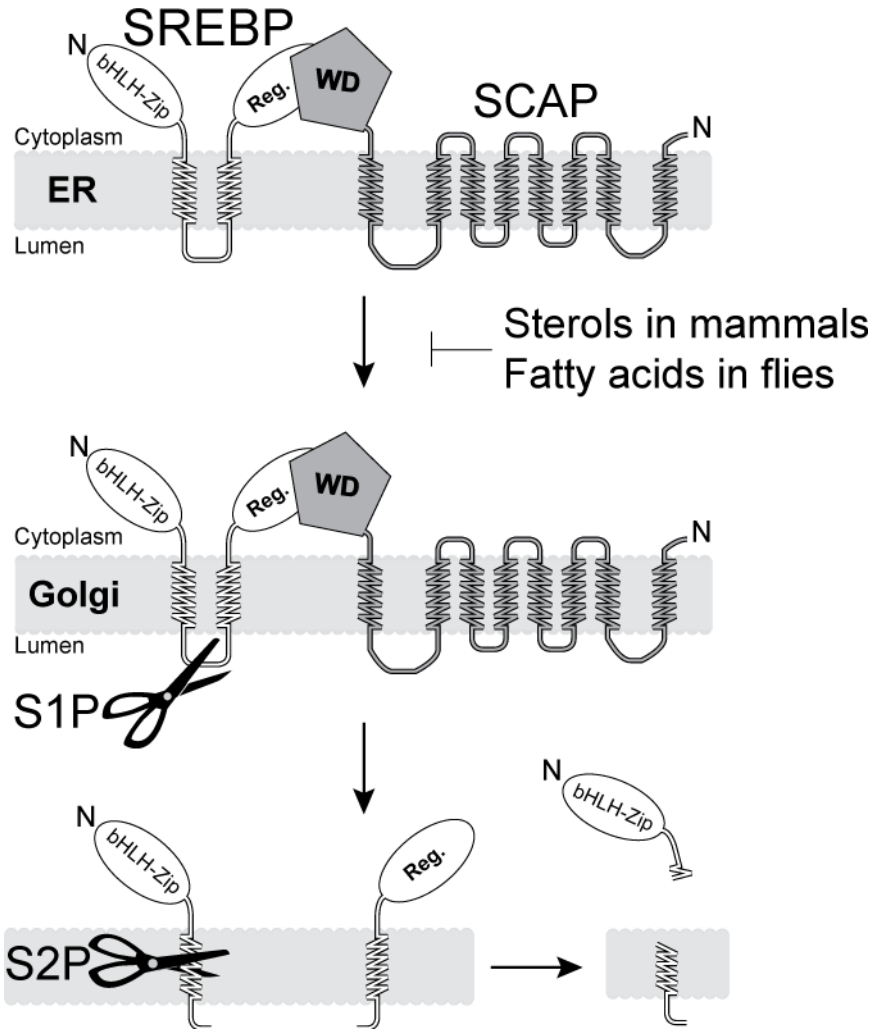


Figure 1-1.

Schematic depiction of the SREBP pathway. Modified after (Rawson, 2003).

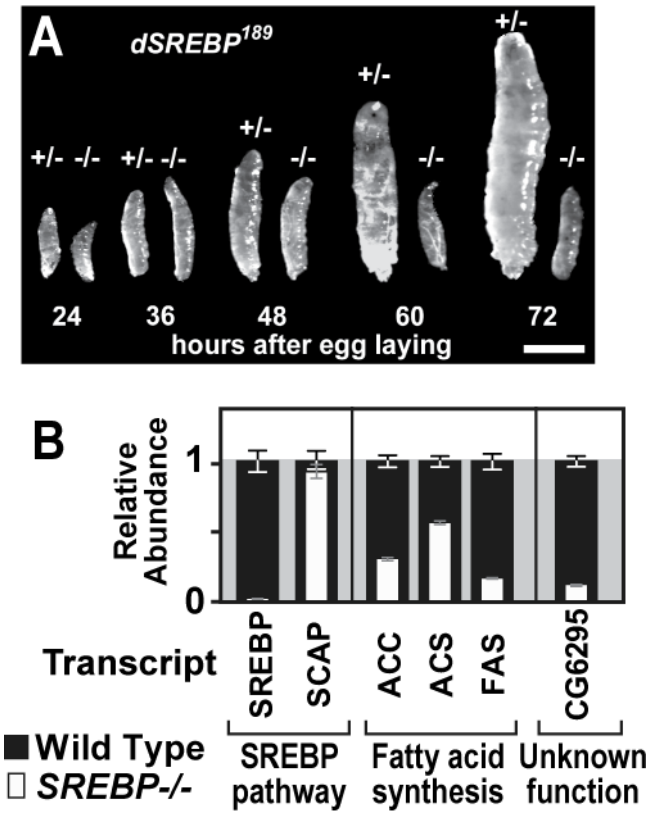


Figure 1-2.

(A) Comparison of larval growth between dSREBP homozygous and heterozygous (+/-) mutants. Larvae from *dSREBP*^{189} */TM3,Actin-GFP, Ser* stocks were collected at each time point and photographed at the indicated time points. Scale bar = 1 mm. **(B)** Quantitative analysis of transcripts from wild type and dSREBP mutant first instar larvae. Adapted from (Kunte et al., 2006).

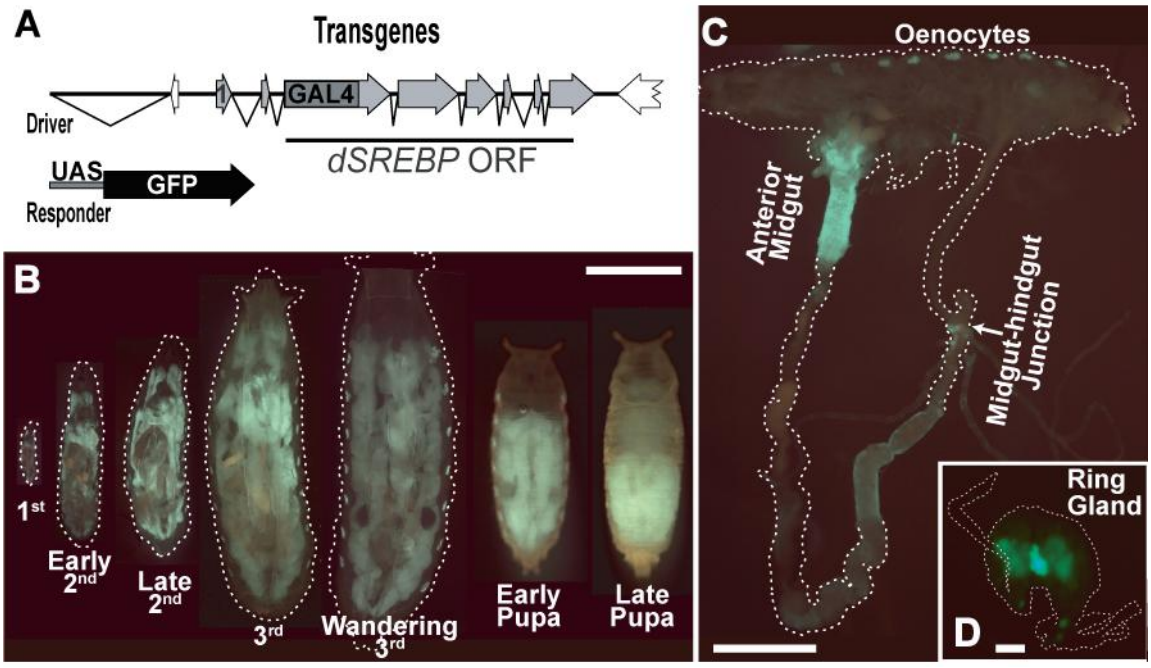


Figure 1-3.

Localization of dSREBP cleavage. (**A**) A binary reporter system for dSREBP activity. The transcription factor domain of pP{*dSREBPg*} was replaced by a GAL4-VP16 transcription factor to generate pP{*GAL4-dSREBPg*}. Animals transgenic for both P{*GAL4-dSREBPg*} and P{UAS-GFP} were examined for spatial localization of GFP fluorescence. **(B)** Dorsal views of larvae and early pupae. At all larval stages, fluorescence is detected in fat body, midgut and oenocytes. The contents of the gut autofluoresce (Figure S4). In late pupae, fluorescence can be detected throughout the animal. (**C)** Dissection of a third instar larva shows two domains of dSREBP activity 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. In this preparation, the fat body has been removed so that the oenocytes are more distinctly visible. (**D)** 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.
Adapted from (Kunte et al., 2006)**.**

CHAPTER TWO

Alternative Processing of SREBP during Larval Development in *Drosophila*

INTRODUCTION

Increased transcription of genes targeted by SREBP requires proteolytic release of the NH_2-terminal transcription factor domain from the membrane-bound precursor. This process, which involves two separate cleavages by two different proteases, is an example of regulated intramembrane proteolysis (Rip; (Brown et al., 2000)). Once SREBP is cleaved in the luminal loop of the precursor at site-1, the second cleavage occurs at site 2, which lies within the first membrane-spanning helix of SREBP (Duncan et al., 1998). This cleavage requires a highly hydrophobic integral membrane protein that contains a metalloprotease active site motif (Rawson et al., 1997). This protein is thus designated site-2 protease (S2P) and its homologues are found throughout all kingdoms of life. Recent structural analysis of an archaebacterial S2P shows that its active site is highly similar to that of thermolysin (Feng et al., 2007). Importantly, all currently-known functions of SREBP require its cleavage by S2P (Bengoechea-Alonso and Ericsson, 2007).

S2P is absolutely required for the survival of mammalian cells under standard culture conditions (Rawson et al., 1997). Cells lacking S2P cannot

process SREBPs and are therefore deficient in the transcription of many genes needed for synthesis and uptake of lipid (e.g. genes of the biosynthetic pathways for cholesterol and unsaturated fatty acids, and the low-density lipoprotein receptor gene). Mutant cells survive when the ultimate products of SREBP activation, cholesterol and unsaturated fatty acids, are added to the medium (Goldstein et al., 2002b; Limanek et al., 1978), demonstrating that the essential role for S2P in cultured mammalian cells is to process SREBPs and thereby enable them to mediate the transcriptional upregulation of the genes of lipid metabolism.

The SREBP pathway is also found in insects (Seegmiller et al., 2002), even though they cannot make cholesterol from acetyl-Coenzyme A and must therefore get sterols from their diet (Clark and Bloch, 1959). Accordingly, cleavage of their single isoform of SREBP (also called *HLH106* (Theopold et al., 1996)) is regulated by phospholipids rather than sterols (Dobrosotskaya et al., 2002). We have shown that dS2P is required for release of dSREBP from the membranes in *Drosophila* S2 cells (Seegmiller et al., 2002). An asparagine-proline (NP) motif found in the first membrane-spanning helix of all SREBP homologues is necessary for cleavage by S2P (Ye et al., 2000a). When $N_{462}P$ is mutated to phenylalanine-leucine (FL), dSREBP is still correctly inserted into the membrane but no longer serves as a substrate for dS2P (Seegmiller et al., 2002).

In *Drosophila* larvae, *dSREBP* itself is an essential gene. Without it, larvae raised on standard cornmeal-molasses-agar culture medium die at second instar (Kunte et al., 2006). Supplementing the culture medium with fatty acids affords substantial rescue of *dSREBP* mutant flies. Mutant flies can also be rescued by expressing a truncated form of dSREBP that ends before the first membrane spanning helix and therefore bypasses proteolytic regulation. Transcription of dSREBP target genes is restored to rescued larvae. The remaining portions of dSREBP neither rescue mutants nor are required for their rescue (Kunte et al., 2006). Once *dSREBP* mutants reach adulthood, dSREBP is dispensable (Cherry et al., 2006). These data indicate that the essential role for dSREBP in larvae requires it to reach the nucleus and mediate the increased transcription of target genes required for fatty acid synthesis and uptake.

There are currently no known animal models lacking S2P and the consequences of its loss in whole animals are therefore not known. To address the role of S2P in the SREBP pathway *in vivo*, we isolated mutant *Drosophila* lacking *dS2P* owing to deletion of the locus. We also obtained mutants harboring a transposon insertion in exon 3. These mutations eliminate dS2P function. Unexpectedly, we find that flies lacking dS2P function are viable.

dS2P mutant larvae show modest transcriptional deficits in some dSREBP target genes but the deficits are less severe than those observed in *dSREBP* mutants. The present data indicate that some protease(s) other than dS2P can release the transcriptionally active NH_2-terminal domain of dSREBP from the membrane, freeing it to go to the nucleus. This alternative cleavage thus supports larval development in the absence of dS2P.

MATERIALS AND METHODS

Plasmids: pUAS-dSREBP is described elsewhere (Kunte et al., 2006). pUAS-dSREBP(NP-FL) was constructed by subjecting pUAS-dSREBP to *in vitro* mutagenesis using the Quickchange-XL kit (Stratagene). The primers used for mutagenesis were 5'-GCCATCCTGGCCGTCTTTCTCTTCAAGACCTTTCTCC -3' and 5'-GGAGAAAGGTCTTGAAGAGAAAGACGGCCAGGATGGC -3'. The mutant dSREBP cDNA fragment was then excised and re-cloned into the original pUAST vector and the open reading frame was completely sequenced. P{*GAL4-dSREBPg*} and P{UAS-GFP} are described in (Kunte et al., 2006).

Genetic strains: All marker mutations and balancer chromosomes are described in and referenced by FlyBase (2003). Crosses were maintained at 25 ºC in vials containing freshly yeasted cornmeal-molasses-agar (Kunte et al., 2006) except where noted. *OreR* flies served as wild type. P element transposon insertion lines EP(2)2245 (~1Kb upstream of *dS2P*), and KG08356 (in exon 3 of *dS2P*) were obtained from the Bloomington *Drosophila* stock center. Transposon alleles were allowed to freely recombine with wild type for three generations prior to being formally isogenized and tested for lethal and sterile phenotypes. Deletion mutants were obtained as described (Kunte et al., 2006). $dSREBP^{189}$ is a deletion extending into the open reading frame of *dSREBP* isolated in a screen for

imprecise excisants of a nearby P element (Kunte et al., 2006). The UAS-dSREBP and UAS-dSREBP(NP→FL) transgenes used are inserted on the 2nd chromosome. These stocks were created as described (Kunte et al., 2006). The 6487 GAL4 driver line is a P{GAWB} enhancer trap insertion (P{*w*[+*mW.hs*]=GawB}OK376) obtained from the Bloomington stock center. The P{*GAL4-dSREBPg*} and P{UAS-GFP} transgenes were recombined onto a single third chromosome.

Characterization of alleles: The following primers were used in PCR analysis and sequencing of mutant alleles (F, forward; R, reverse. Number indicates nucleotide position relative to the predicted start site of transcription): 5'-GGAATTCCATGGATCCCTTCGTGTTCTTCATA-3' (F, 285), 3'-GTGTAAACACCTACTTAAATTTGGC-3'(F, -2381), 5'-CTAGTCTAGATTCTTAAAGCAGGGGTCGCAG-3' (R, 1915), 5'-CTCAGTTAAGGTGAACTTGGTGGTGG-3' (F, -1041), 5'-CATATAAGACTTTTGCCGACTTGC-3' (R, -256), 5'-GTATTTTAAGTCACTTAACACAATGG-3' (F, -202), 5'-GGTGAGGTCTCAAGATGTCATTGG-3' (R, 258), 5'-CGACGACTCAGGGTCAAGAGCGAGG-3' (F, -3977), 5'-GTGCATAGGTTTAACCAGCGTTGGG-3' (R, -3338), 5'-

CCCAACGCTGGTTAAACCTATGCAC-3' (F, -3338, 5'-GTTGGCAATTCTATCAAGAAACCCGG-3' (R, 3441).

cDNA Rescue Experiments: The 6487 GAL4 driver was first crossed into a *dSREBP* ^{189} background to generate w^{-};P[w^{+}, *GAL4*]/ P[w^{+}, *GAL4*]; *dSREBP* ^{189} / *TM6B, Tb Hu e* flies. Similarly, the responder transgenes were crossed into the *dSREBP* ^{189} background in order to generate w^{-};P[w^{+}, *UAS-dSREBP*]/ P[w^{+}; *UAS-dSREBP*]; *dSREBP* ^{189} / *TM6B, Tb Hu e*. This was also done for P[w^{+}, *UAS-dSREBP(NP-FL*) stocks. 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. Genotype of sampled individuals was verified by PCR analysis.

Quantitative analysis of transcripts: Transcript abundance was determined by real-time PCR as described (Kunte et al., 2006). Briefly, total RNA was prepared from approximately 100 larvae for each genotype and time point examined using the RNA-Stat 60 reagent (Tel-Test, Inc). Real-time PCR was performed on an ABI 7900HT instrument, using SYBR Green fluorescent probe and the primers described (Dobrosotskaya et al., 2002; Kunte et al., 2006). The

relative abundance of all mRNAs were calculated using the Comparative CT method as described in User Bulletin #2 (PE Applied Biosystems).

Viability: Standard cornmeal-molasses-agar supplemented with 0.075 % (w/v) Na myristate and 0.15% (w/v) Na oleate was prepared as described (Kunte et al., 2006). Embryos from the indicated crosses were collected overnight at 25 C. 2 mg embryos were added to vials containing 9 ml supplemented medium. Adults were scored as they emerged and scoring was repeated multiple times daily through day 21 after egg laying (AEL) so that no mature adults remained in the cultures to produce F_1 offspring. Percent rescue was calculated by dividing the observed ratio of homozygotes to heterozygotes by the expected ratio (the expected ratio is 1 for crosses of heterozygotes with homozygotes and 0.5 for crosses of heterozygotes with heterozygotes owing to embryonic lethality of balancer chromosome homozygotes). The day of median eclosion is that day by which $\geq$ 50% of adults had emerged from each culture.

Mass of flies: Mass was determined by placing 3-10 flies/tube into 8-10 preweighed tubes for each sex and genotype. These were then re-weighed on a Mettler/Toledo XS105 dual range balance and the initial mass subtracted from the subsequent mass to determine the mass of flies in each tube. This value was divided by the number of flies to determine mass/fly.

RESULTS

Flies Lacking *dS2P* are Viable

We used a P element excision approach (Robertson et al., 1988) to isolate events that removed transposon EP(2)2245 and extended into the *dS2P* locus. The extent of each candidate deletion was determined using Southern blotting, PCR, and sequencing. Excision line #74 harbors a deletion that removes all P element sequences and encompasses the entire *dS2P* locus (Figure 2-1A). We designate this allele $dS2P^{1}$. We also obtained a P element insertion in the *dS2P* locus from the Bloomington Stock Center (KG08356). We designate this allele $dS2P^{2}$.

We determined the site of transposon insertion in $dS2P^{2}$ to be 255 bp into exon 3 (Figure 2-1A) by sequencing multiple PCR products generated from mutant genomic DNA using primers specific for *dS2P* and for P element sequences. This insertion disrupts the open reading frame of the transcript at codon 261 of the 508 amino acid coding sequence (Figure 2-1B). We detected no dS2P transcripts from $dS2P^{1}$ homozygous larvae by northern blot analysis and only a truncated, ~1.4 Kb transcript from $dS2P^{2}$ homozygous larvae (not shown).

Sequencing of PCR-amplified cDNAs from $dS2P^{2}$ mutants revealed an in-frame stop codon arising from P element sequences four codons after the insertion

site and no additional *dS2P*-derived sequence thereafter. A putative protein produced from this transcript could comprise only the first half of dS2P, plus three amino acids encoded by P element sequences. The last 247 amino acids of dS2P include an aspartate residue at position 453 that is the third coordinating ligand for the active site metal atom (Feng et al., 2007; Kinch et al., 2006) and is essential for S2P function. In all S2P homologues tested, alteration of this aspartate renders S2P inactive (Rudner et al., 1999; Zelenski et al., 1999). Thus, any protein product of the $dS2P^2$ allele cannot be proteolytically active.

The $dS2P^1$deletion also removes a recently predicted gene (CG34229, annotation of release 5.2 of the *Drosophila melanogaster* genome). Two independent transposon insertions within CG34229 exhibit no associated phenotypes (FlyBase (2003)). To eliminate possible phenotypic effects due to loss of this putative gene in $dS2P^1$mutants, we performed experiments with mutants transheterozygous for $dS2P^1$and $dS2P^2$.

Figure 1C shows the results of a real-time PCR analysis of dS2P transcript abundance in first instar mutant larvae relative to wild type larvae. The primers used here are specific for exon 1, which is present in the aberrant $dS2P^2$ transcript. No transcript is detectable from $dS2P^1$ mutants, only low levels of the aberrant

transcript in *dS2P*^{2} mutants, and levels intermediate to these are apparent in transheterozygotes.

We also examined cleavage of dSREBP in adult flies. dSREBP is the only confirmed substrate for dS2P in flies (Seegmiller et al., 2002). Both in wild type and in heterozygous adult flies, cleaved dSREBP is clearly detected in nuclear extracts (Figure 2-1D). No nuclear dSREBP is seen in homozygotes (lanes 3 and 5). Instead, the intermediate form, which is the product of cleavage of dSREBP at site-1 (and which is the substrate for dS2P), accumulates in membranes (lanes 3 and 5, upper panel). Thus, in adults, both alleles of *dS2P* are profoundly deficient for cleavage of dSREBP.

Mammalian cells lacking S2P die unless grown in medium supplemented with cholesterol and unsaturated fatty acids (Rawson et al., 1997). This is owing to their inability to cleave SREBPs at site 2 and the consequent loss of transcriptional upregulation of target genes. *dSREBP* is itself essential in flies (Kunte et al., 2006). We expected that loss of *dS2P* would phenocopy loss of *dSREBP* due to inability of d*S2P* mutants to cleave dSREBP. It was therefore surprising that *Drosophila* mutants lacking *dS2P* survive well enough to be easily maintained as homozygous stocks.

We have maintained both homozygous and balanced, heterozygous stocks of d*S2P*^{1} for more than 200 generations (and of *dS2P*^{2} for >100 generations) without intentional selection. Maintenance of homozygous stocks demonstrates that in flies, *dS2P* is not essential for viability. By contrast, maintenance of the lethal-allele-carrying balancer chromosome at high frequency in the heterozygous stocks for so many generations indicates that loss of *dS2P* puts homozygotes at a substantial competitive disadvantage relative to their heterozygous culture mates (see below).

_dS2P_ mutants grow more slowly than heterozygotes

We compared the growth of *dS2P*^{1}/*dS2P*^{2} mutants (from crosses of *dS2P*^{2}/ *dS2P*^{2} virgin females with *dS2P*^{1}*/CyO* males) to wild type larvae raised in parallel cultures. Up to 48 hours after egg laying (AEL), there is no difference in size between *dS2P*^{1}/*dS2P*^{2} mutants and wild type larvae (Figure 2-2A). By 60 hours AEL *dS2P*^{1}/*dS2P*^{2} mutants are distinctly smaller than wild type. Disparity in size persists through 84 hours. By the time mutants reach adulthood, they display a greater variability of body size than do their heterozygous siblings (males shown; Figure 2-2B) and are somewhat less massive, on average (Figure 2-2C). Morphologically, mutants are normal. These data show that the homozygotes grow more slowly than wild type or heterozygotes, taking longer to approach normal size.

Figure 2-3A shows typical emergence data from a heterozygous cross of *dS2P*^{1}*/ dS2P*^{1} virgin females with *dS2P*^{2}*/CyO* males. While the majority of heterozygotes emerge by day 11 AEL, the bulk of their *dS2P*^{1}*/dS2P*^{2}siblings emerge two days later. In multiple experiments, we consistently observe this approximately two-day delay irrespective of the alleles used or the direction of the transheterozygous cross. This delay becomes more pronounced with crowding (Figure 2-3B). We set up cultures with the indicated masses of embryos on standard medium and scored adults as they emerged. The delay is shown as the day AEL of median eclosion for homozygotes minus the day of median eclosion for heterozygotes. At 10 mg embryos per culture, the delay for *dS2P*^{1}*/dS2P*^{1} flies was 2 days. Doubling the mass of embryos in the culture increased the delay to five days. At 40 or 70 mg embryos, the delay extends to about two weeks. Results from flies lacking dSREBP (*dSREBP*$^{18\ 9}$ (Kunte et al., 2006)) are shown for comparison.

Maternally supplied dS2P functions in *dS2P* mutant larvae

We conducted extensive fertility, fecundity, and viability studies on *dS2P* mutant stocks. In the course of these studies, we noted that the frequency of emergence of homozygotes was strongly affected by the maternal genotype. In experimental cultures, the homozygous offspring of heterozygous mothers

emerged at about the expected frequencies (Figure 2-4A, left, white bars). By contrast, the homozygous offspring of homozygous mothers survived markedly less well on unsupplemented medium, emerging at less than half the expected frequency (Figure 2-4A, middle, white bars). To determine if reduced viability resulted from disruption of fatty acid metabolism subsequent to deficient processing of dSREBP, we tested sibling cultures on medium supplemented with fatty acids (Kunte et al., 2006). Supplementation with fatty acids permitted near-expected survival of the homozygous offspring of homozygous mothers (Figure 2-4A, middle, grey bars). *dSREBP*^{189} flies served as a control for rescue by fatty acid supplementation (Figure 2-4A, right).

Differential survival of homozygotes depending on the maternal genotype indicates that maternally supplied *dS2P* ameliorates the effects of lacking *dS2P* in the zygotic genome. We tested the hypothesis that at least some maternal dS2P activity is supplied via mRNA. Figure 4B shows real-time PCR analysis of transcript abundance in 0-2.5 hour AEL embryos and 36 hour AEL larvae. At 0-2.5 hours AEL, before the onset of most zygotic transcription, the offspring of heterozygous mothers show significant levels of dS2P transcript, about 1/3 of wild type levels, while no dS2P transcript is detectable in the offspring of homozygous mothers. By 36 hours AEL, no dS2P transcript is detectable in *dS2P* mutant larvae irrespective of the maternal genotype.

Transcript abundance of CG6295, a highly transcriptionally responsive target of dSREBP (Kunte et al., 2006), is shown as an indicator of dSREBP activity in these larvae. We found reduced transcript abundance in the homozygous offspring of heterozygous mothers and a much more substantial deficit in offspring of homozygous mothers (Figure 2-4C). Interestingly, these later animals show greater abundance of CG6295 transcript than do *dSREBP*^{189} larvae, even in the complete absence of detectable dS2P transcripts (see below).

dSREBP mutated at site-2 rescues dSREBP null flies

The NH_2-terminal transcription factor domain of dSREBP, which is the product of cleavage by dS2P, is needed to rescue dSREBP mutants (Kunte et al., 2006). Cleavage of dSREBP by dS2P requires an asp_{462}pro motif in the first membrane-spanning helix of dSREBP (Ye et al., 2000a). When N_{462}P is mutated to phe-leu, dSREBP cleavage is abolished (Seegmiller et al., 2002). Since flies entirely lacking *dS2P* can survive, cleavage of dSREBP by dS2P is not essential for survival. Therefore, an N_{462}P→FL mutant dSREBP that cannot be cleaved by dS2P should be able to rescue flies otherwise lacking dSREBP.

To test this hypothesis, we prepared transgenic flies expressing either wild type or N_{462}P→FL mutant dSREBP cDNAs under the control of the yeast GAL4

upstream activating sequence. Expression was driven by the 4687 GAL4 enhancer trap line, which we have previously show is able to rescue *dSREBP* null mutant animals to adulthood when driving expression of dSREBP (Kunte et al., 2006). These transgenes were tested in a *dSREBP* null background. Samples of emerging flies were analyzed by sequencing PCR products to confirm the presence of the indicated *dSREBP* transgenes. Table One shows that both wild type and mutant SREBPs can substantially rescue *dSREBP* null flies to adulthood, although the NP→FL mutant does so less efficiently than wild type dSREBP.

Alternative cleavage of dSREBP in flies

Nuclear dSREBP is essential for larval survival but cleavage of dSREBP by dS2P is not. This implies that transcriptionally-active dSREBP must be present in the nuclei of $dS2P^{1}/dS2P^{2}$larvae owing to a mechanism that does not require dS2P. To test this hypothesis, we used the previously described P{*GAL4-dSREBPg*} and P{UAS-GFP} binary reporter system (Kunte et al., 2006) to assess dSREBP processing in *dS2P* mutants (Figure 2-5A). Virgin females homozygous for either $dS2P^{1}$ or $dS2P^{2}$ on the second and homozygous for both the P{*GAL4-dSREBPg*} and P{UAS-GFP} transgenes on the third chromosome were crossed to males of the same genotype heterozygous on the second. Embryos were raised on standard medium until 3rd instar when they were examined by fluorescence microscopy. Fluorescence owing to GFP expression is

readily detectable in *dS2P* mutants (Figure 2-5B, top and middle panels), although at levels lower than seen in heterozygous siblings (Figure 2-5B, lower panel). Thus, release of the amino-terminal transcription factor domain from dSREBP occurs even in the absence of dS2P.

We noted above (*c.f.* Figure 2-4) that the $dS2P^{1}/dS2P^{2}$ homozygous offspring of homozygous mothers showed transcription of CG6295 that was greater than in *dSREBP* mutants. This is consistent with the presence of the dSREBP transcription factor domain in the nuclei of *dS2P* larvae. To determine if this pattern held true for other target genes, we performed real-time PCR analysis. Figure 2-6 shows mRNA abundance at 36, 48, and 60 hours AEL for the indicated dSREBP target genes (Dobrosotskaya et al., 2002; Kunte et al., 2006). At 36 hours, $dS2P^{1}/dS2P^{2}$ and wild type larvae show similar abundance of transcripts for acetyl coenzyme A carboxylase (ACC), synthase (ACS), fatty acid synthase (FAS). These transcripts are less abundant in $dSREBP^{189}$ larvae. This pattern continues through 60 hours. By contrast, transcripts for CG6295 are much less abundant in $dS2P^{1}/dS2P^{2}$ than in wild type, more closely matching their abundance in $dSREBP^{189}$ larvae. We consistently observe the small increase in abundance in $dS2P^{1}/dS2P^{2}$ larvae versus $dSREBP^{189}$ larvae. Thus, $dS2P^{1}/dS2P^{2}$ larvae have less severe transcriptional deficits than do $dSREBP^{189}$ larvae.

DISCUSSION

We isolated mutant *Drosophila melanogaster* harboring a deficiency that removes the entire *dS2P* transcription unit (Figure 2-1A). No dS2P mRNA is detectable in these animals and no dSREBP processing is observed in mutant adults under conditions where it is readily observed in wild type flies. Instead, the substrate for dS2P cleavage, the intermediate form of dSREBP, accumulates in membranes (Figure 2-1D). Therefore, the $dS2P^{1}$ deletion is a null allele of *dS2P*.

Phenotypes of the P element insertion allele, $dS2P^{2}$, are indistinguishable from $dS2P^{1}$ and are no more severe *in trans* to the deletion allele. Transcripts from $dS2P^{2}$ cannot yield catalytically-active dS2P (Figure 2-1B). Thus $dS2P^{2}$ is a null allele by genetic and molecular criteria. Surprisingly, animals harboring either allele are viable and can be readily maintained as homozygous stocks. Reciprocally, $dSREBP^{189}$ flies can be rescued by expressing a dSREBP cDNA harboring an $N_{462}P\rightarrow FL$ mutation that renders dSREBP refractory to cleavage by dS2P (Table 1). Thus, the site-2 protease is not essential for the development and growth of *Drosophila melanogaster*.

The $dS2P^{1}$ allele must also be null for the predicted gene CG34229 (Figure 2-1A) that encodes a putative component of the higher eukaryotic NADH

complex. The predicted sequence of the encoded polypeptide is highly conserved, supporting the case for this gene.

Are there consequences of the loss of CG34229 that influence the phenotypes we report? We cannot absolutely exclude the possibility that some phenotypes could be owing, in part, to haploinsufficiency for CG34229 in *dS2P* transheterozygotes. However, CG34229 cannot be an essential gene; $dS2P^{1}$ homozygotes are viable. We performed most of the experiments presented here with mutants transheterozygous for $dS2P^{1}$ and $dS2P^{2}$. In parallel experiments, we found indistinguishable results with flies homozygous for either $dS2P^{1}$ *or* d$S2P^{2}$ (not shown) which indicates that the phenotypes we observe are not the result of loss of CG34229. Further, the reduced survival of *dS2P* mutants is rescued by feeding fatty acids, a treatment that also rescues lethality in animals lacking *dSREBP*. This indicates that reduced survival is a consequence of reduced dSREBP activity.

The phenotype informative for the most important finding described here is cleavage of dSREBP in the absence of dS2P (Figure 2-5B). Whether or not insufficiency for CG34229 (or any gene yet to be identified in this region) contributes in some way to reduced viability, smaller average size, or delayed

development in *dS2P* homozygotes, dS2P *is* absent and dSREBP *does* reach the nucleus without cleavage by dS2P (Figures 2-5, 2-6).

In mammals, S2P is needed to process other membrane-bound transcription factors, ATF-6-α and -β that play a crucial role in the ER-stress response (also known as the unfolded protein response or UPR (Ye et al., 2000b)). The *Drosophila* genome encodes a protein highly similar to mammalian ATF-6, CG3136. In mammals, ATF6 is required to transcribe XBP1 mRNA and mutant cells lacking S2P are deficient in the induction of the spliced form of XBP1 mRNA (Yoshida et al., 2006). When *dS2P*$^{-}$ larvae are challenged with dithiothreitol or tunicamycin, treatments that elicit the UPR, we see no difference in XBP1 splicing compared to wild type larvae (Supplemental Figure 2-1). If the *Drosophila* UPR is closely similar to the mammalian UPR, these data suggest that ATF6 processing is relatively unimpaired in dS2P minus larvae. It might be that the *Drosophila* homologue of ATF6 is not required for the fly UPR or that its activity does not require cleavage by dS2P. If dS2P *is* required to activate this homolog in flies, the observed developmental delay of *dS2P*$^{-}$ larvae may result from defects in ATF6 activation. Nevertheless, while these putative additional functions of dS2P may be important in some circumstances, the crucial function of dS2P in flies is to process dSREBP.

In striking contrast to *dS2P*$^{-}$ adults, which lack nuclear dSREBP under conditions where it is readily detected in wild type, dSREBP can reach the nucleus and activate transcription of target genes in *dS2P* mutant larvae (Figure 2-5B). Thus, *Drosophila* larvae lacking *dS2P* have an alternative means of releasing the nuclear, transcription factor domain of dSREBP from the membrane-bound precursor. This explains the greater abundance of dSREBP target transcripts in *dS2P*^{1}/*dS2P*^{2} mutants compared with *dSREBP*^{189} mutants (Figures 2-4, 2-6).

What is the role of this alternative mechanism for producing nuclear dSREBP? The current data show only that it occurs in the absence of dS2P. We do not yet know if it is a normal, physiologically-relevant mechanism or whether it happens fortuitously, only in the absence of normal dSREBP processing. It is, however, sufficient to afford the survival, over many generations, of flies completely lacking dS2P.

How is the transcription factor domain of dSREBP produced in *dS2P* mutants? A possible mechanism is production of alternative transcripts that encode only the dSREBP transcription factor domain without the membrane-spanning helices. These might arise from different promoter usage or from differential splicing. Arguing against these possibilities is the fact that only a

single transcript is detected for dSREBP in flies, from embryogenesis through adulthood and in various tissues examined (Theopold et al., 1996). We likewise observe a single band on northern blots for dSREBP (not shown). Any putative alternative transcripts or splice forms would have to be present at levels too low to be detected in these assays, while the activity of nuclear dSREBP in *dS2P*^{1}/*dS2P*^{2} larvae is readily detected (Figures 2-5, 2-6). Moreover, a cDNA construct harboring the $N_{462}P\rightarrow FL$ mutation and under control of a single, heterologous promoter rescues *dSREBP* mutants (Table 1). This construct has no exons; it is not subject to alternative splicing nor is it cleaved by dS2P (Seegmiller et al., 2002).

We favor the hypothesis that in larvae lacking dS2P, dSREBP is released from the membrane by some other protease(s). This posited protease is unlikely to cleave within the first membrane-spanning helix of dSREBP: flies have no other S2P homologues and other intramembrane-cleaving proteases display different substrate preferences (c. f. Hooper and Lendeckel (2007)). The signal peptide peptidase (SPP) is an intramembrane protease of the ER (Weihofen et al., 2002). SPPs are unlikely candidates for cleavage of SREBPs, however. Like S2P, the SPPs require prior cleavage of the substrate by a separate protease. Chinese hamster ovary (CHO) cells express active SPP (Dev et al., 2006) but multiple, independently-isolated lines of CHO cells lacking S2P show no

processing of SREBPs (Sakai et al., 1996). If SPPs could cleave SREBPs, one would expect some evidence of SREBP processing in S2P$^-$ cells. Cleavage of dSREBP *following* its first membrane-spanning helix cannot release the NH_2-terminal domain. Therefore, it is most probable that the alternative cleavage occurs in the cytoplasm, between the transcription factor domain and the first membrane-spanning helix of dSREBP. We term this portion of dSREBP the 'stalk'.

Cleavage of SREBPs within the stalk has been reported previously. Wang and colleagues showed that caspases 3 and 7 could each cleave mammalian SREBPs (Pai et al., 1996; Wang et al., 1995) and that this cleavage was detectable during apoptosis (Wang et al., 1996). The physiological significance of this cleavage is presently unclear. The caspase cleavage sites identified by Wang and colleagues are highly conserved among vertebrate SREBP isoforms, however, and all metazoan SREBPs (save those from nematoda) contain potential caspase cleavage sites within their stalk regions (RBR, unpublished observations). Using reporter constructs, Ioannou and colleagues showed that SREBP cleaved during apoptosis by caspases can be transcriptionally active (Higgins and Ioannou, 2001b). There is precedent, therefore for caspase cleavage of SREBPs releasing the functional transcription factor.

Current data do not suggest that the production of nuclear dSREBP in dS2P mutants has any involvement with apoptosis. However, non-apoptotic roles of caspases have been found in *Drosophila* (Huh et al., 2004) and other systems (reviewed in (Algeciras-Schimnich et al., 2002)). Cleavage of dSREBP in the absence of dS2P may be an example of a non-apoptotic caspase function. We are currently testing the hypothesis that dSREBP is cleaved by caspases to produce transcriptionally active dSREBP in *dS2P*$^{-}$ larvae.

Transgene Construct Lines		*Either* Transgene (GAL4 *or* UAS-dSREBP)		*Both* Transgenes (GAL4 *and* UAS-dSREBP)		
		***dSREBP*^{189}**	***dSREBP*^{189}**	***dSREBP*^{189}**	***dSREBP*^{189}**	
GAL4 Driver	**UAS-dSREBP**	**+**	**-**	**+**	**-**	**percent rescue**
6487	**NP→FL (A)**	140	0	658	176	63.3%
	NP→FL (B)	325	0	658	114	44.3%
	NP→FL (C)	419	0	574	155	63.8%
	Totals	**884**	**0**	**1890**	**445**	
6487	**wt (A)**	282	2	473	219	94.9%
	wt (B)	348	1	601	189	71.8%
	wt (C)	28	0	739	288	84.1%
	wt (D)	291	2	502	213	89.4%
	Totals	**949**	**5**	**2315**	**909**	

Table 2-1.

Rescue of *dSREBP* lethality by wild type and mutant *dSREBP* cDNA. We prepared P element based germline transformation constructs that encode either wild type (wt) *dSREBP* cDNA or cDNA carrying a mutation $N_{462}P\rightarrow FL$, which abolishes cleavage by dS2P. Independent second chromosome insertions of each transgene were isolated (designated A, B, and C, etc) and used to generate stocks of the genotypes w^{-};P{ w^{+}, *UAS-dSREBP*}/ P{ w^{+},*UAS-dSREBP*}*; dSREBP* ^{189} / *TM6B, Tb Hu e* (for homozygous viable transgene insertions) and w^{-}*; UAS-dSREBP/CyO; dSREBP* ^{189} / *TM6B, Tb Hu e* (for homozygous lethal transgene insertions). These were crossed to flies carrying the 6487 GAL4 driver (expressed predominantly in anterior gut, fat body and oenocytes), of the genotype w^{-};P{ w^{+}, *GAL4*}/ P{ w^{+}, *GAL4*}*; dSREBP* ^{189} / *TM6B, Tb Hu e*. Adult progeny were scored for homozygosity at the *dSREBP* locus and for the presence of the responder transgene. Wild type and mutant *dSREBP* cDNA transgenes rescue *dSREBP* mutants (range 44.3-63.8% for NP->FL and 71.8-94.9% for wild type).

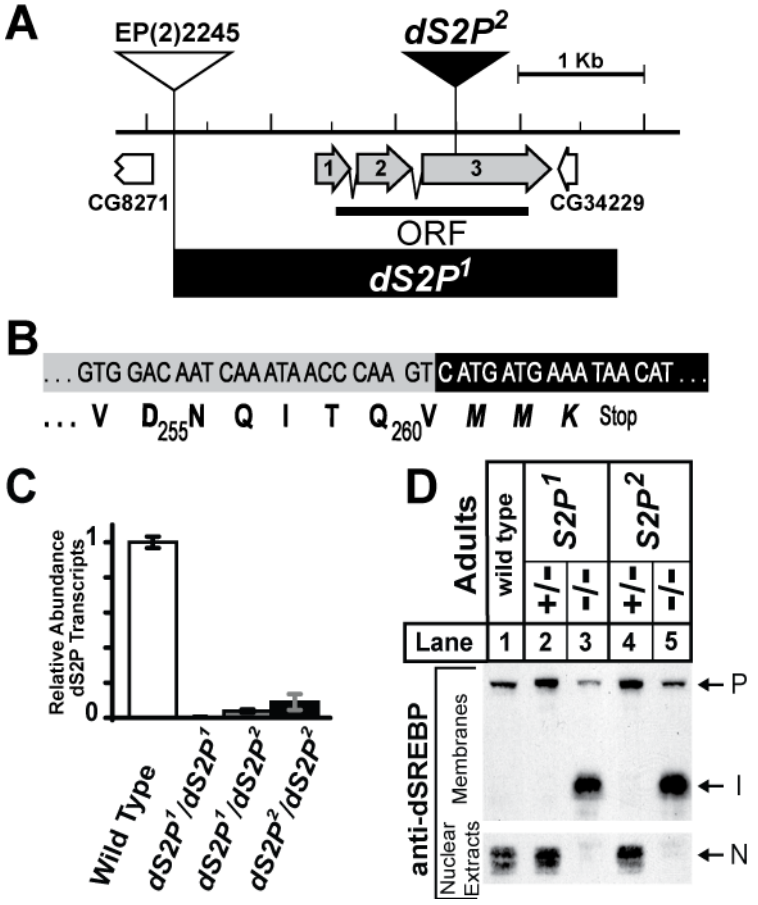


Figure 2-1.

dS2P^{1} and *dS2P*^{2} alleles. **(A)** Map of the dS2P locus. Grey arrows represent the position of exons. The extent of the open reading frame (ORF) is shown by the heavy black line. The location of the P element that was mobilized to yield the *dS2P*^{1} is indicated by the white triangle. The extent of the deficiency is shown by the black box. It includes the entire dS2P gene. The black triangle represents the location of the *dS2P*^{2} P element insertion (KG08356) at bp 255 of exon 3. CG34229 is a predicted gene encoding a putative component of the higher eukaryotic NADH complex. **(B)** Sequence of the dS2P transcript from *dS2P*^{2} flies at the junction with P element sequences. Sequences from the dS2P gene are indicated by the grey box. Sequences from P element KG08356 are indicated by the black box. The encoded protein sequence is shown below, numbered as the wild type sequence. **(C)** *dS2P*^{1} and *dS2P*^{2} are null alleles. Quantitative real-time PCR measurements of dS2P transcripts in wild type (white), *dS2P*^{1} (grey), *dS2P*^{1}/*dS2P*^{2} mutants (dark grey) and *dS2P*^{2} (black) homozygous 1st instar larvae, using probe against exon 1, which is present in both wild type and truncated, chimeric dS2P transcripts.

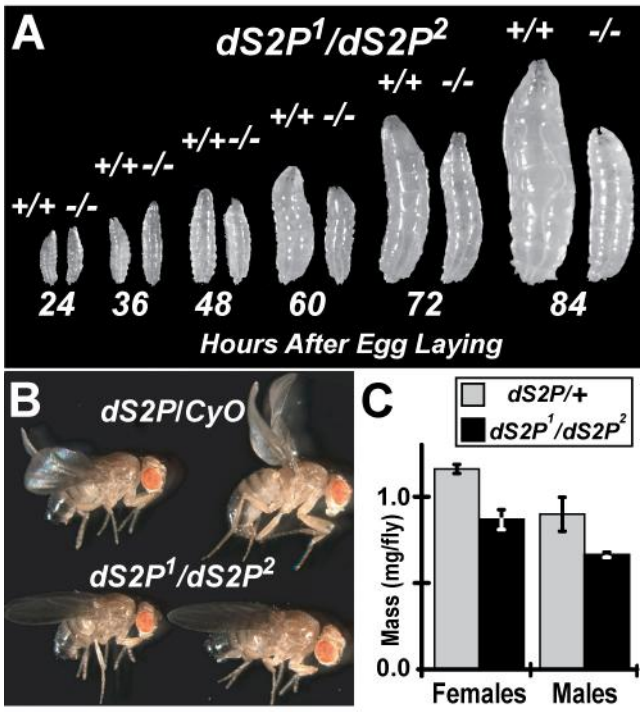


Figure 2-2.

dS2P^{1}/*dS2P*^{2} mutants grow more slowly than wild type. **(A)** Representative larvae of each genotype were photographed at the indicated time after egg laying (24-84 hrs). By 84 hours wild type have reached the 3rd instar based on mouth hook and anterior spiracle morphology. **(B)** Male offspring of mothers homozygous for dS2P. Heterozygotes above, transheterozygotes below. **(C)** Mass of offspring of mothers homozygous for dS2P. Grey bars indicate heterozygotes, black represent transheterozygotes. Male and female transheterozygotes show a similar reduction in average mass compared with heterozygotes. Error bars represent the SEM.

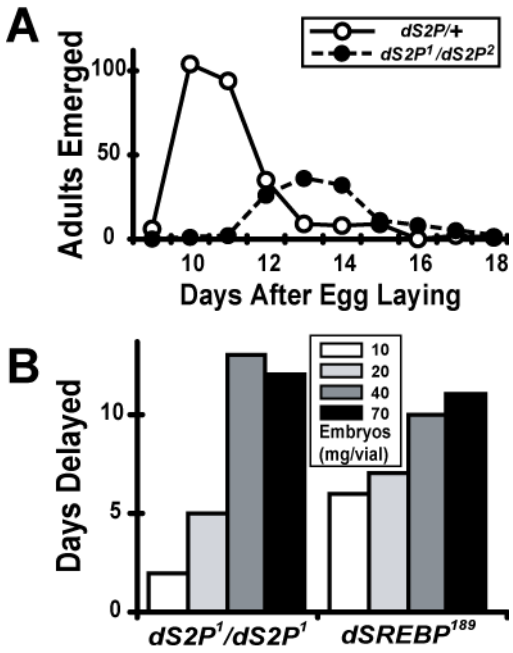


Figure 2-3.

dS2P mutants develop more slowly than wild type. **(A)** Plot of the number of adults emerging versus days after egg laying. On day 0, three mg of embryos was introduced into vials of standard cornmeal-molasses-agar medium. Beginning on day 9, and each day thereafter, adults were cleared from the culture and counted. **(B)** Crowding substantially exacerbates the developmental delay. The indicated mass of embryos was introduced into flasks of standard cornmeal-molasses-agar medium (~80 ml/flask) on day 0. Beginning on day 9, and each day thereafter, adults were cleared from the culture, scored and counted. 'Days delayed' is calculated as the day of median eclosion for homozygotes minus that of heterozygotes.

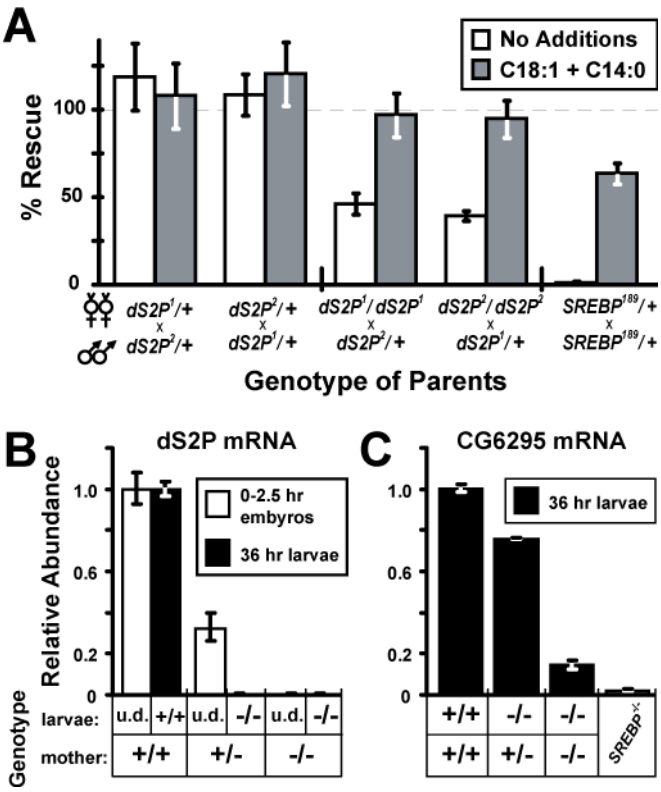


Figure 2-4.

Effect of maternal *dS2P* genotype. **(A)** Survival of homozygous offspring. Virgin females and males of the indicated genotypes were crossed. Embryos from these crosses were collected and cultures were set up on standard cornmeal-molasses-agar medium or on medium supplemented with fatty acids as described in *Materials and Methods*. On day 10 AEL, each vial was cleared and the newly-emerged adults were scored for *dS2P* genotype. Standard culture medium - white bars; culture medium supplemented with fatty acids - grey bars. Error bars represent SEM. **(B, C)** Virgin females homozygous for $dS2P^2$ were crossed to males heterozygous for $dS2P^1$. A minus ("-") indicates *dS2P* allele. Embryos from these crosses were collected and divided into two groups that were allowed to develop for the indicated times after which total RNA was isolated and subjected to real-time PCR analysis with primers for the indicated transcripts. Larvae were genotyped based on expression of a GFP transgene on the balancer chromosome. The 0-2.5 hour embryos were not genotyped owing to the lack of zygotic transcription at this early time in development (u.d., undetermined). Transcript abundance is plotted relative to wild type controls. Error bars represent the SEM.

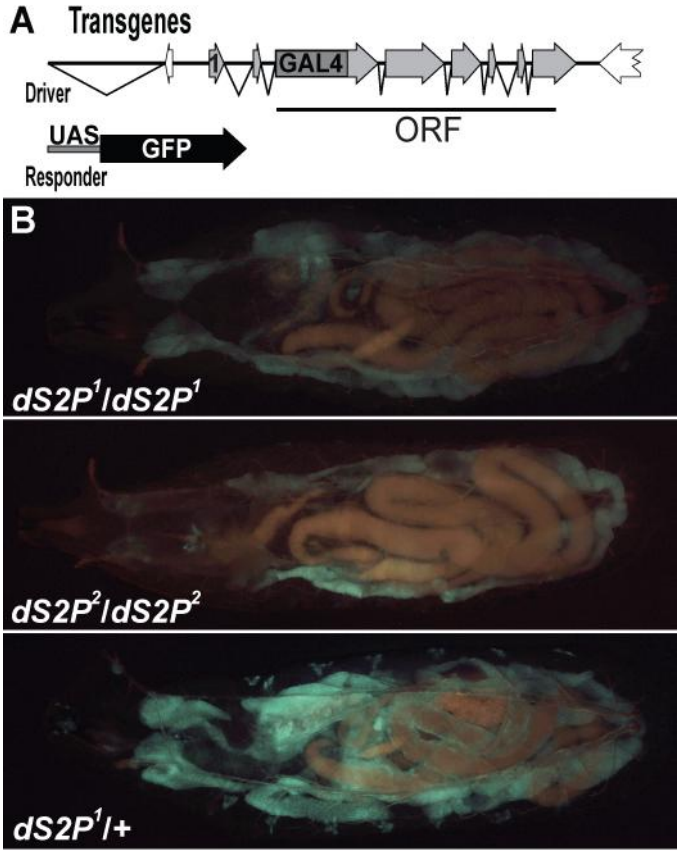


Figure 2-5.

Larvae lacking *dS2P* nevertheless process dSREBP. **(A)** A binary reporter system for dSREBP activity (Kunte et al., 2006). The transcription factor domain of pP{*dSREBPg*} was replaced by a GAL4-VP16 transcription factor to generate pP{*GAL4-dSREBPg*}. **(B)** Animals homozygous for both P{*GAL4-dSREBPg*} and P{UAS-GFP} transgenes in the indicated *dS2P* background were examined for spatial localization of GFP fluorescence. In larvae homozygous for either *dS2P* allele, fluorescence is detectable in fat body but levels are decreased relative to heterozygous siblings. No fluorescence is detectable in the mid gut of *dS2P* homozygotes, in contrast to heterozygotes. Although not clearly visible in photographs, we detect a faint fluorescence in the oenocytes of many *dS2P* homozygotes. All larvae are the offspring of mothers homozygous for the indicated dS2P allele. Images are 1 second exposures taken 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.

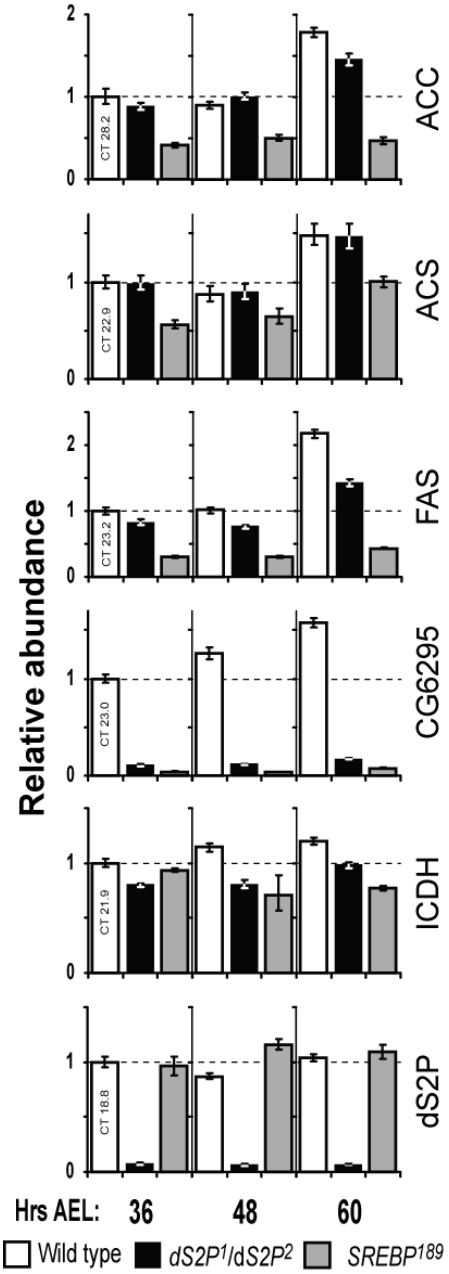


Figure 2-6.

Larvae null for *dS2P* show a less severe transcriptional deficit in genes of fatty acid synthesis than *dSREBP* null larvae. Total RNA was extracted from larvae at the indicated times and transcript abundance was determined by real-time PCR as described in *Materials and Methods*. Relative abundance was normalized to wild type at 36 hrs AEL.

CHAPTER THREE

Cleavage of SREBP by the caspase Drice during *Drosophila* development

INTRODUCTION

Genetic systems offer powerful tools for understanding the role of proteolysis in a physiological context. Studies of mutants lacking one or more proteases can reveal physiologically relevant details not accessible from biochemical approaches alone. One proteolytic signaling pathway that has yielded to the combination of genetics and biochemistry is the sterol regulatory element binding protein (SREBP) pathway that plays a central role in the regulation of lipid metabolism (Goldstein et al., 2002a). SREBPs are membrane-bound transcription factors found in all animals, from cnidarians (Putnam et al., 2007) to mammals (Wang et al., 1993). The ~120 kDa precursor form is anchored to the membranes of the endoplasmic reticulum (ER) by two membrane-spanning helices. The large amino- and carboxy-terminal domains reside in the cytoplasm while a short loop projects into the ER lumen. In the ER membrane, SREBP forms a complex with Scap, a polytopic membrane protein harboring a sterol sensing domain.

When cellular demand for lipid rises, SREBP:Scap complexes exit the ER via COPII coated vesicles and travel to the Golgi apparatus. Once there, SREBP is cleaved at two sites to release the soluble, active, amino-terminal transcription

factor domain. The site-1 protease (S1P) cuts within the luminal loop that separates the two membrane-spanning helices. The membrane-bound amino-terminal fragment, which harbors the transcription factor domain, is the substrate for the site-2 protease (S2P). This intermediate form of SREBP only accumulates in the absence of cleavage by S2P. S2P cleaves SREBP within the first membrane-spanning helix, between Leu and Cys residues that normally lie within the plane of the membrane. (Espenshade and Hughes, 2007).

Drosophila melanogaster has a single SREBP gene, *dSREBP* (also called *HLH-106* (Theopold et al., 1996)), as well as orthologues of S1P, S2P, and Scap (Seegmiller et al., 2002). In mammalian cells and in flies, transcriptional upregulation of the genes of lipid metabolism by SREBP is essential for survival. Mammalian cells lacking S1P, S2P or Scap cannot activate SREBP and do not survive unless their culture medium is supplemented with free cholesterol and unsaturated fatty acids (Goldstein et al., 2002a). Similarly, *Drosophila* lacking dSREBP die at the end of second instar but can be rescued by supplementing their diet with the ultimate end products of dSREBP activation, fatty acids (Kunte et al., 2006). Unlike vertebrates, insects cannot synthesize cholesterol from acetyl coenzyme A and therefore always have a requirement for sterols in their diet (Clark and Bloch, 1959; Sang, 1956).

We have recently shown that, in striking contrast to mammalian cells, for which loss of S2P is lethal, flies lacking dS2P survive rather well. In *dS2P* mutants, dSREBP continues to be activated. Thus they exhibit a less severe deficit in the transcription of genes involved in lipid metabolism than larvae lacking dSREBP (Kunte et al., 2006; Matthews et al., 2008). This explains why they survive while larvae lacking dSREBP do not. In the present work, we identify the mechanism by which *dS2P* mutants activate dSREBP.

In mammalian cells, SREBP can also be cleaved during apoptosis by caspases-3 and -7 (Pai et al., 1996; Wang et al., 1996). These cysteine proteases nearly always cleave following an Asp residue. Caspase cleavage of SREBP occurs at a cytoplasmic site that lies in the juxtamembrane domain between the DNA binding domain and the first membrane spanning helix (Wang et al., 1995). The transcription factor domain released by caspase cleavage can activate a reporter gene under control of a synthetic SREBP target promoter (Higgins and Ioannou, 2001a) but the physiological significance of cleavage of SREBPs during apoptosis is unknown.

The genome of *Drosophila melanogaster* encodes seven caspases: *damm* (48C5), *dcp1* (59E3), *decay* (89B18), *dream* (or s*trica*, 42A8), *dredd* (1B12-13), *drice* (99C1), and *dronc* (or *Nc*, 67D2). Dronc, Dredd, and Dream are thought to

be initiator (or apical) caspases that activate other caspases by cleaving them, while Damm, Dcp1, Decay, and Drice, are effector (or executioner) caspases that cleave various target proteins. *Drosophila* offers an attractive system in which to combine genetic and biochemical techniques to investigate the role of caspases in the activation of SREBP.

We find that two *Drosophila* effector caspases, Drice and Dcp1, can cleave dSREBP *in vitro* and in cultured *Drosophila* S2 cells undergoing apoptosis. The initiator caspase Dronc is not able to cleave dSREBP *in vitro*. Furthermore, flies lacking *dS2P* and either *drice* or *dronc* (but not *dcp1*) phenocopy loss of *dSREBP* itself, dying during the second larval instar. Importantly, this synthetic lethality is rescued by supplementing the culture medium with fatty acids, just as seen for *dSREBP* mutants. Thus Drice can cleave dSREBP and is necessary for its cleavage in the absence of dS2P, enabling the survival of *dS2P* mutants.

MATERIALS AND METHODS

Expression plasmids: The plasmid MTAL-Grim, and plasmids harboring cDNAs of Dcp-1, Drice, Dronc, Damm, Decay, Dream, and Dredd, were gifts from John Abrams (UT Southwestern) and were used as a template to synthesize DNA fragments for RNAi synthesis. pDS47_HSV-dSREBP was described earlier (Seegmiller et al., 2002).

Due to enhanced expression of genomic constructs encoding dSREBP under apoptotic conditions and to assess transfection efficiencies of various dSREBP mutant constructs in S2 cells, a genomic construct of dSREBP tagged at its amino terminus with EYFP (CLONTECH) and also double tagged with the HSV epitope was engineered such that tags are inserted in frame at the unique AscI restriction site described earlier (Kunte et al., 2006). This is designated YFP-dSREBP.

Site directed mutagenesis using PCR and/or the QuickChange Site-Directed Mutagenesis Kit (Stratagene) was employed to construct desired mutations, and the mutagenesis was confirmed by multiple sequencing runs for each strand.

Germline transformation of *Drosophila* was performed by BESTGENE (Chino Hills CA)

Cell Culture: Transfection of S2 cells was performed as described earlier (Seegmiller et al., 2002). For RNAi studies, S2 cells were transfected with MTAL-Grim using Celfectin for 4 hrs.

Apoptotic induction: Cells were treated with 0.7 mM CuSO4 in SF900 serum free media for 4 hours prior to harvest. SDS-Page and immunoblot analysis was performed as described (Seegmiller et al., 2002).

Caspase *in vitro* assays: Recombinant Dcp1 and Drice were generous gift from Dr. Xiaodong Wang (UT Southwestern). Membrane fractions of HSV and EYFP tagged dSREBP were purified from transfected S2 cells as described (Seegmiller et al., 2002). The assays were done in 0.15 ml of 25 mM Tris, 25 mM NaCl, 2mM DTT (pH 7.4) containing 100 microgram of membrane fractions and 200 nM of caspase. The reaction was incubated up to 60 minutes at 25 C and was stopped by addition of sample loading buffer and boiling in preparation for SDS-PAGE.

Genetic strains: All marker mutations and balancer chromosomes are described in and referenced by FlyBase (2003). Crosses were maintained at 25 ºC in vials containing freshly yeasted cornmeal-molasses-agar (Kunte et al., 2006)

except where noted. *OreR* flies served as wild type. *dS2P*^{1} is a deletion encompassing the *dS2P* locus and *dS2P*^{2} is a transposon insertion within the dS2P open reading frame. They are described elsewhere (Matthews et al., 2008). *dSREBP*^{189} is a deletion extending into the open reading frame of *dSREBP* isolated in a screen for imprecise excisants of a nearby P element (Kunte et al., 2006). Caspase mutants *dcp1*^{Prev1}(Laundrie et al., 2003), *drice*$^{\Delta 1}$ (Muro et al., 2006), and *dronc*^{51} (Chew et al., 2004), were from Kimberly McCall, Bruce Hay, and John Abrams, via the Abrams lab. The P{dSREBPg} and P{dSREBPg(D386A)} transgenes used are inserted on the 2nd chromosome.

dS2P, caspase **double mutant assays:** *Drosophila* S2P and caspase double mutant lines were maintained as heterozygous stocks: *dS2P*/ CyO, twist-GFP; *drice*$^{\Delta 1}$*/ TM3, Ser, actin-GFP* and *dS2P/ CyO, twist-GFP; dronc*^{51}*/ TM3, Ser, actin-GFP*. All crosses were set up using virgin females homozygous for *dS2P* and heterozygous for *drice* or *dronc*. These animals survive poorly on standard medium (see below). Thus, in order to collect sufficient numbers of females, embryos from the heterozygous stocks were plated onto dishes containing semi-defined media supplemented with fatty acids. Third instar larvae homozygous for *dS2P* and heterozygous for *drice* or *dronc* were scored using the twist- and actin-GFP markers and transferred to vials containing regular-cornmeal-molasses agar medium. Adult females were collected as they emerged.

Virgin *dS2P*^{2}/*dS2P*^{2}; *drice*$^{\Delta 1}$ (or *dronc*^{51})/ *TM3, Ser, actin-GFP* females were crossed to *dS2P*^{1}/*CyO, twist-GFP; drice*$^{\Delta 1}$ (or *dronc*^{51})/ *TM3, Ser, actin-GFP* males. On Day 0, embryos from an overnight collection were plated at 10 mg embryos/dish onto duplicate 60mm dishes containing semi-defined media with no additions ("No Addition") and 60mm dishes containing semi-defined media supplemented with 0.075% myristate and 0.15% oleate ("14:0 + 18:1"). Two days later, larvae from one "No Addition" and one "14:0 + 18:1" plate were separated from the food by floatation on a salt cushion and scored using the twist- and actin-GFP markers. On Day 4, this procedure was repeated for the remaining two dishes.

In order to calculate the percent expected, the observed ratio (calculated by dividing the number of larvae for each genotype by the expected total) was divided by the predicted Mendelian ratio. The total number of larvae expected was calculated based on the emergence of the doubly heterozygous mutants, which are expected to comprise 1/3 of the larvae owing to embryonic lethality of animals homozygous for the third chromosome balancer. Thus, the expected total is (# doubly heterozygous larvae/ 0.33).

Rescue of *dSREBP^{189}* lethality by genomic dSREBP transgenes:

Independent insertions of P{dSREBPg(D_{386}A)} on the second chromosome were recombined onto both *dS2P^{1}* and *dS2P^{2}* alleles and then crossed into a *dSREBP^{189}* background. Recombinants were genotyped by PCR analysis and sequencing of the P{dSREBPg(D_{386}A)} transgene. Lines homozygous or heterozygous for the second chromosome were generated: *dS2P, P{dSREBPg(D_{386}A)}/ dS2P, P{dSREBPg(D_{386}A)}; dSREBP^{189}/ TM6B, Tb Hu e* and *dS2P, P{dSREBPg(D_{386}A)}/ CyO, twist-GFP; dSREBP^{189}/ TM6B, Tb Hu e*. Virgin *dS2P^{2}, P{dSREBPg(D_{386}A)}/ dS2P^{2}, P{dSREBPg(D_{386}A)}; dSREBP^{189}/ TM6B, Tb Hu e* females were crossed to *dS2P^{1}, P{dSREBPg(D_{386}A)}/ CyO, twist-GFP; dSREBP^{189}/ TM6B, Tb Hu e* males. On Day 0, embryos from an overnight collection were seeded into 10 vials (at 1 mg embryos/vial) containing regular-cornmeal-molasses agar ("No Addition") and 10 vials containing regular-cornmeal-molasses agar supplemented with 0.075% myristate and 0.15% oleate ("14:0 +18:1"). Emerging adults were scored using *twist-GFP* and *Hu* markers until no further adults emerged (approximately day 19 after plating). The data is presented as percent of the expected calculated as described above.

RESULTS

dSREBP is cleaved during apoptosis in *Drosophila* S2 cells

Mammalian SREBPs were among the first experimentally-verified substrates for cleavage by caspases (Wang et al., 1995). Therefore, to identify the protease(s) other than dS1P and dS2P that activate dSREBP in *dS2P*$^{-}$ larvae, we first tested the hypothesis that dSREBP can be cleaved by *Drosophila* caspases.

To increase caspase activity in *Drosophila* S2 cells, we induced apoptosis by expression of the apoptotic activator, grim (Chen et al., 1996) under control of the metallothionine promoter. Following addition of copper sulfate ($CuSO_4$), caspase activity in grim-transfected cells increased dramatically as monitored by cleavage of the fluorescent substrate Ac-DEVD-AMC (not shown).

Figure 3-1 shows cells co-transfected with tagged versions of dSREBP (YFP-dSREBP), either wild type or harboring specific mutations that block cleavage by dS1P and dS2P (Seegmiller et al., 2002). The YFP-dSREBP constructs show similar levels of the precursor form ("P") in the membrane fraction (Figure 3-1, upper panel, lanes 1-8). For dSREBP with wild-type sequences at site-1 and site-2 (YFP-dSREBP WT), the nuclear form of dSREBP ("N") is detected in both the absence and presence of $CuSO_4$ (Figure 3-1, lower panel, lanes 1 and 2). A novel, more rapidly-migrating, band (hereafter

designated “fragment C”) also appears in the nuclear extract of the $CuSO_4$-treated sample (lane 2) but not the untreated one (lane 1). Mutating the crucial Arg at position 486 in site-1 to Ala blocks cleavage by dS1P (Seegmiller et al., 2002) and no nuclear dSREBP is observed in the absence of $CuSO_4$ (lower panel, lane 3). The smaller, novel fragment C appears when apoptosis is induced (lower panel, lane 4). Mutating an Asp-Pro motif within the first membrane spanning helix of dSREBP blocks cleavage by the site-2 protease but leaves cleavage at site-1 unaffected (Seegmiller et al., 2002). The intermediate form (“I”) of dSREBP is the product of dS1P cleavage and remains attached to the membrane because it retains a single membrane spanning helix. The intermediate form appears in both the presence and absence of $CuSO_4$ (upper panel, lanes 5 and 6). The normal nuclear form (“N”) is absent from the nuclear extract (lower panel, lanes 5 and 6) but fragment C appears during apoptosis (lower panel, lane 6). The same pattern holds true when both sites -1 and -2 are mutated (lower panel, lanes 7 and 8). We observed the same results using versions of dSREBP tagged with the HSV epitope alone (not shown). Thus, dSREBP can be cleaved when caspase activity is upregulated in S2 cells and this cleavage does not require prior cleavage by S1P or S2P.

dSREBP is cleaved by Drice and Dcp1

To further investigate the role of caspases in the production of fragment C, we performed RNAi experiments in *Drosophila* S2 cells targeting *dronc*, *dredd*, *decay*, *drice*, *dream*, *damm*, and *dcp1* and determined whether fragment C still was produced upon induction of apoptosis (Supplemental Figure 3-1). When apoptosis was induced by expression of grim, RNAi against *drice* blocked the appearance of fragment C (lane 7). RNAi against *dcp1* partially reduced the accumulation of fragment C (lane 10). The evident effect of RNAi treatment against Drice and Dcp1 indicates that these enzymes can mediate cleavage of dSREBP during grim-induced apoptosis in S2 cells. The lack of effect for RNAi against other caspases is inconclusive.

To test whether Drice and Dcp1 can cleave dSREBP directly, we prepared membrane fractions from S2 cells transfected with YFP-dSREBP and incubated these membranes *in vitro* with purified, recombinant Drice or Dcp1 (Figure 3-2). At 0 minutes, no cleavage product is observed with either protease (lanes 1 and 5). Increasing accumulation of fragment C is observed over time (lanes 2-4 and 6-8). No product is observed at 60 minutes in the absence of added protease (lane 10). These data demonstrate that Drice and Dcp1 can cleave membrane-bound dSREBP *in vitro*.

We performed parallel experiments with purified, recombinant Dronc, shown in Supplemental Figure 3-2. No cleavage is observed when Dronc is incubated with wild type dSREBP, although this substrate is cleaved by either Drice or Dcp1 (lanes 11, 12).

Cleavage of dSREBP by caspases requires an Asp residue at position 386

As their name indicates (Alnemri et al., 1996), caspases have a decided preference for an Asp residue in the P_1 position. Drice and Dcp1 have cleavage specificities similar to mammalian caspase 3, with the sequence DEVD being optimal for cleavage by Drice *in vitro* (Fraser and Evan, 1997). Figure 3-3A shows sequence alignment of the juxtamembrane stalk region (between the transcription factor domain and the first membrane-spanning helix) of SREBPs from each of the 12 SREBP sequences available from the genus *Drosophila*. There are 4 Asp residues within this region of dSREBP (black boxes) and these four are conserved among all species, save *D. willistoni*. We note that many attributes of this species are exceptional among the twelve species sequenced (Clark et al., 2007; Vicario et al., 2007).

We individually mutated each of these Asp residues to Ala in the context of YFP-dSREBP and assessed the ability of each construct to be cleaved during apoptosis (Figure 3-3B). The apoptosis-dependent fragment C appears in wild

type dSREBP (lanes 2 and 12) and in Asp to Ala mutants at positions 395, 398, and 407 (lanes 6, 8, and 10). No such fragment is observed when the Asp at 386 is mutated to Ala (lane 4). This indicates that cleavage during apoptosis occurs at the sequence $F_{383}TTD\downarrow A_{387}$ in dSREBP.

This was somewhat surprising as Drice and Dcp1 are thought to have a preference for the sequence DXXD, as is present at 395. In transfected S2 cells, fragment C co-migrates with a truncated version of dSREBP that has a stop codon at position 387 (not shown). We further tested the requirement of Asp_{386} for cleavage by *drice* by incubating membrane fractions from S2 cells transfected with YFP-dSREBP, either wild type or harboring the $D_{386}A$ mutation (Figure 3-4A). Accumulation of fragment C proceedes in a time-dependant fashion with the wild type substrate (lanes 3-6), but no fragment C is produced from the $D_{386}A$ mutant (lanes 7-10). Cleavage during apoptosis is also abolished when Asp_{386} is substituted by Glu or Asn (Figure 3-4B, lanes 6 and 8).

Both *dronc* and *drice* are required for the survival of *dS2P* mutant larvae

In S2 cells and using purified enzyme in vitro, Drice and Dcp1 can cleave dSREBP after the Asp at 386. We hypothesized that this cleavage of dSREBP by caspases is responsible for the survival of flies lacking dS2P. To test this, we constructed stocks harboring null mutations both in *dS2P* as well as in one of

three caspases using the alleles *dcp1*^{Prev1}, *drice*$^{\Delta 1}$, and *dronc*^{51}(Chew et al., 2004; Laundrie et al., 2003; Muro et al., 2006). If cleavage of dSREBP by one of these caspases were responsible for the survival of *dS2P* mutants, then flies lacking both that caspase and *dS2P* would be unable to activate dSREBP. The doubly-mutant animals should evince the phenotype observed in flies completely lacking dSREBP and die at the end of second larval instar owing to deficient transcription of dSREBP target genes. Importantly, if this synthetic lethality were indeed owing to deficient dSREBP activation, then supplementing the larval diet with free fatty acids should afford substantial rescue of these double mutants, just as seen for *dSREBP* mutants (Kunte et al., 2006).

Flies homozygous for *dcp1*^{Prev1} are viable (Laundrie et al., 2003). We find that the *dS2P, dcp1*^{Prev1} doubly mutant animals survive about as well as *dS2P* mutants alone (not shown). Therefore, cleavage of dSREBP by Dcp1 is not required by flies lacking dS2P.

Flies homozygous for *dSREBP* die at the end of second instar whereas flies homozygous either for *drice*$^{\Delta 1}$ or for *dronc*^{51} mostly die during pupation (Chew et al., 2004; Muro et al., 2006). This leaves a window of 2-3 days (between the middle of second instar, when larvae die owing to insufficient dSREBP activity, and the onset of pupariation, when larvae die if they lack Drice

or Dronc) in which to assess synthetic lethality in the *dS2P, caspase* double mutants. Therefore, we scored the survival of the doubly mutant larvae at day two AEL, before dSREBP-dependent lethality, and again at day 4 AEL, after dSREBP-dependent lethality but before pupariation.

Synthetic lethality occurs in both the *dS2P; drice* and *dS2P; dronc* double mutants (Figure 3-5). At day two after egg laying (AEL), on unsupplemented medium, the doubly mutant larvae are present in the population at about 2/3 of their expected frequency (open bars, upper panel, left, center). They survive somewhat better on medium supplemented with fatty acids (solid bars). Their survival is comparable to $dSREBP^{189}$ larvae (right). By day four AEL, on unsupplemented medium, $dS2P^{1}/dS2P^{2}$; $drice^{\Delta 1}$/+ and $dS2P^{1}/dS2P^{2}$; $drice^{\Delta 1}$/ $drice^{\Delta 1}$ larvae are almost undetectable in the population, while $dS2P^{1}$/+; $drice^{\Delta 1}$/ $drice^{\Delta 1}$ larvae survive about as well as $dS2P^{1}$/+; $drice^{\Delta 1}$/+ larvae (lower panel, left). On medium supplemented with fatty acids, many more $dS2P^{1}/dS2P^{2}$; $drice^{\Delta 1}$/+ and $dS2P^{1}/dS2P^{2}$; $drice^{\Delta 1}$/ $drice^{\Delta 1}$ larvae survive, comparable to the survival of the $dSREBP^{189}$ homozygotes on this medium (lower panel, right). A similar pattern is observed for $dS2P^{1}/dS2P^{2}$; $dronc^{51}/dronc^{51}$ larvae (center). Thus, flies lacking dS2P and partly or completely lacking either Drice or Dronc die at the end of second instar owing to deficient activation of dSREBP.

To confirm that the lethality observed in animals lacking Drice or Dronc and dS2P is owing to failure to cleave dSREBP at Asp386, we prepared animals transgenic for P{dSREBPg ($D_{386}A$)}. This transposon carries a genomic DNA fragment encompassing the *dSREBP* locus and its upstream sequences (Kunte et al., 2006) and harbors the $D_{386}A$ mutation that blocks cleavage of dSREBP by caspases. This was recombined onto second chromosomes harboring $dS2P^{1}$ or $dS2P^{2}$ alleles. The recombinant chromosomes were then moved into a $dSREBP^{189}$ /TM6B background. This permitted us to test the hypothesis that, in flies lacking dS2P, cleavage of dSREBP at Asp_{386} is essential for survival.

Figure 3-6 shows that animals completely lacking *dSREBP* but harboring P{dSREBPg($D_{386}A$)} and heterozygous for *dS2P*, survive at 60% the rate of their doubly heterozygous siblings. Thus, one copy of wild type dS2P and one copy of dSREBP harboring the D386A mutation are sufficient to afford substantial survival. On standard medium, in the complete absence of *dS2P* and in the presence of two copies of P{dSREBPg(D386A)} and one wild type copy of *dSREBP*, the animals survive at less than a quarter of the expected rate. This may reflect haploinsufficiency for caspase-cleavable dSREBP in these animals. That is, if you cannot cleave dSREBP at site-2 owing to lack of dS2P, then a single caspase-cleavable copy of dSREBP (the wild type copy) is insufficient to support fully normal rates of survival. This is in some sense the converse of the case seen

in animals lacking *dS2P* and having only one copy of *drice* or *dronc* (Figure 3-5). In the complete absence of *dS2P* and *dSREBP*, animals carrying two copies of P{dSREBPg(D386A)} fare even more poorly, surviving at 10% of the expected rate. In each of these latter cases, survival is substantially restored by supplementing the culture medium with free fatty acids (Figure 3-6, solid bars). This demonstrates that the greatly reduced survival of $dS2P^{1}$, dSREBPg($D_{386}A$)/ $dS2P^{2}$, dSREBPg($D_{386}A$); $dSREBP^{189}/dSREBP^{189}$ larvae results from reduced dSREBP activity.

DISCUSSION

Cleavage of SREBP by caspases was first reported in 1995 (Pai et al., 1996; Wang et al., 1995; Wang et al., 1996) but the significance of those observations has remained unknown. The present data demonstrate that *dS2P* mutants survive because Drice cleaves dSREBP during development. Both in transfected S2 cells and *in vitro*, with purified enzyme, Drice cleaves dSREBP at residue 386 (Figures 3-3, 3-4), releasing the amino terminus. In *dS2P* mutant larvae, this enables it to travel to the nucleus and increase transcription of genes involved in lipid metabolism; *dS2P* mutant larvae have a much less severe deficit in the transcription of dSREBP target genes than do larvae lacking dSREBP itself (Matthews et al., 2008).

When cleavage of dSREBP by Drice is blocked, either because of substitution of Asp_{386} by Ala at the cleavage site, or because of null mutations in *drice*, $dS2P^{1}/dS2P^{2}$ larvae die at the end of second instar (Figures 3-5 & 3-6). The doubly-mutant larvae are substantially rescued by dietary supplementation with fatty acids. The extent of rescue is similar to the rescue of $dSREBP^{189}$ homozygotes on the same media, confirming that lethality results from deficient activation of dSREBP.

Experiments with *dronc*^{51} demonstrate that *dS2P* mutants also require this caspase. The results with the *dS2P*^{1}/*dS2P*^{2}; *dronc*^{51}/ *dronc*^{51} double-mutants are very similar to the results of experiments with *drice*$^{\Delta 1}$ (Figure 3-5) even though purified Dronc cannot cleave dSREBP directly (Supplemental Figure 3-2). This likely reflects a requirement for Dronc cleavage of Drice, such that in the absence of Dronc, Drice is not processed and cannot cleave dSREBP. This interpretation is consistent with the observation that cleavage of Drice by Dronc is required for its activation during apoptosis (Hawkins et al., 2000; Meier et al., 2000; Muro et al., 2006).

Little apoptosis is observed in *Drosophila* between embryogenesis and pupariation. Cleavage of dSREBP by Drice during larval growth does not, therefore, appear to be related to apoptosis. Even in the absence of substantial apoptosis, however, mRNAs for Drice and Dronc are detected at low levels in larvae (Dorstyn et al., 1999; Fraser and Evan, 1997). Our genetic data indicate that at least some of the message is translated to yield active enzyme.

Interestingly, Dcp1, which is similar in sequence to Drice and which can cleave dSREBP *in vitro*, is not required by *dS2P* mutants. *dS2P*^{1} *dcp1*^{Prev1}/*dS2P*^{2} *dcp1*^{Prev1} double mutants are no less viable than *dS2P*^{1}/*dS2P*^{2} mutants. Despite their similarity to one another, these two caspases must have significantly

different roles in *Drosophila* larvae. Differing roles for these similar enzymes are observed elsewhere in development. For example, flies lacking d*rice* die during the pupal stage while *dcp1* mutants survive quite well (Muro et al., 2006) and some cell types require only Drice for apoptosis while others require both Drice and Dcp1 (Xu et al., 2006). Drice is important for spermatid individualization, a nonapoptotic process, but its role is apparently distinct from that of Dcp1 (Muro et al., 2006). Cleavage of dSREBP during larval life is another process in which the functions of Drice and Dcp1 do not overlap significantly.

Does caspase cleavage of dSREBP play a role in normal larval physiology or is this phenomenon seen only when dS2P is absent? The present data do not allow firm conclusions but an inference may be drawn from sequence data. The caspase site Asp in dSREBP is well conserved among *Drosophila* SREBPs (Figure 3-3). Similarly, the site of caspase cleavage in SREBP-1 is conserved among all its vertebrate homologues. The site of caspase cleavage in SREBP-2 is not homologous to the caspase site in SREBP-1 but it too is conserved among all vertebrate SREBP-2s. Examination of 91 SREBP homologues from cnidaria, nematotoda, arthropoda, mollusca, echinodermata, chordata, and vertebrata show that all of these (save *Caenorhabditis elegans*) harbor a potential caspase site within the juxtamembrane stalk region, as predicted using the algorithm of Wee, *et al.* (Wee et al., 2006). In the case of mammalian SREBP-1 and -2, and now for

dSREBP, these putative sites have been validated experimentally. Conservation of these sites suggests that cleavage by caspases is both ancient and significant for some SREBP function in *Drosophila* and in mammals. What might this significance be?

Cleavage of dSREBP is normally tightly controlled by end-product feedback regulation. The membrane-bound precursor form of SREBP resides in membranes of the ER where it forms a complex with an escort factor, Scap, that is also involved in sensing lipid levels. When lipid levels are low, the SREBP:Scap complex is released from the ER and travels to the Golgi apparatus where SREBP is cleaved in two sequential steps by S1P and S2P to release the transcriptionally active amino-terminal domain of SREBP. As lipid levels rise, the SREBP:Scap complex is retained in the endoplasmic reticulum, away from S1P and S2P. Thus the normal cleavage does not happen, fresh SREBP does not enter the nucleus, and levels of SREBP in the nucleus decline rapidly owing to its proteasome-mediated degradation. Consequently, the transcription of target genes, such as fatty acid synthase, declines as well. If SREBP in the ER membrane is the substrate for caspase cleavage, this would bypass feedback regulation, which relies of the regulation of ER-to-Golgi transport of SREBP.

Under what circumstances might a cell or organism need to bypass end-product-mediated feedback suppression of the transcription of the genes of lipid synthesis? Rapid deposition of large stores of lipid may be such a case. The mass of the *Drosophila* larvae increases about 200 fold between the time it emerges from the egg and the onset of pupariation about 5 days later. The majority of this increase in mass results from the storage of lipid in the fat body, which is needed to fuel metamorphosis. End-product mediated suppression of the transcription of the genes of lipid synthesis may be incompatible with the need for continued high levels of lipid synthesis in the presence of high levels of lipids already stored.

In *dS2P* mutant larvae, activation of dSREBP is readily detected in the fat body (Matthews et al., 2008) owing to (as shown here) the action of Drice. This permits the survival of the mutant animals. However, in the complete absence of dS2P, the mutant offspring of mutant mothers survive only half as well as their heterozygous siblings (Matthews et al., 2008). Their reduced survival results from a deficit in lipid metabolism; they survive at nearly the expected rate on medium supplemented with fatty acids. Therefore, cleavage of dSREBP by Drice is not fully redundant with the normal processing mechanism. Instead, it may normally serve to augment dSREBP activation to support the rapid deposition of lipid stores during larval life.

It is possible, conversely, that SREBP must be cleaved to perform some unknown role during apoptosis and that the cleavage of dSREBP by Drice in the larval fat body is a fortuitous consequence of the presence of a caspase site in the juxtamembrane stalk and the expression of Drice in the larval fat body.

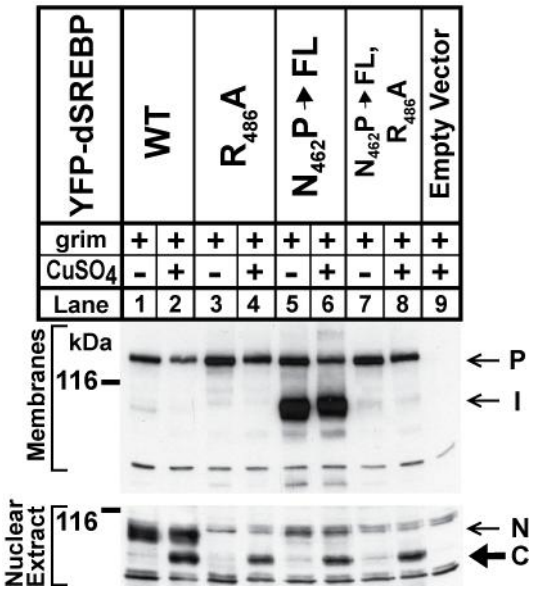


Figure 3-1.

Cleavage of dSREBP by during apoptosis in S2 cells. Cells were co-transfected with the indicated YFP-dSREBP constructs and with Mtal-Grim as described in Materials and Methods. We added 0.7 mM $CuSO_4$ to induce expression of grim. Cells were fractionated and subjected to immunoblot analysis using an anti-HSV antibody as described in Materials and Methods. Some membrane-bound intermediate form is apparent in the nuclear fractions in lanes 5 and 6. P, precursor; I, intermediate form; N, normal nuclear form; C, caspase-dependent band.

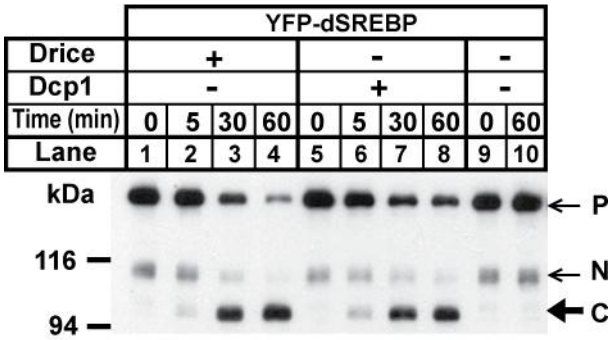


Figure 3-2.

Membrane fractions were purified from S2 cells transfected with YFP-dSREBP as described. The membranes were incubated along with the indicated purified, recombinant caspase in caspase reaction buffer (see Materials and Methods). The reaction was stopped at the indicated times by the addition loading dye. The membranes were then subject to immunoblot analysis using an anti-HSV antibody as described in Materials and Methods. P, precursor; N, normal nuclear form; C, caspase-dependent band.

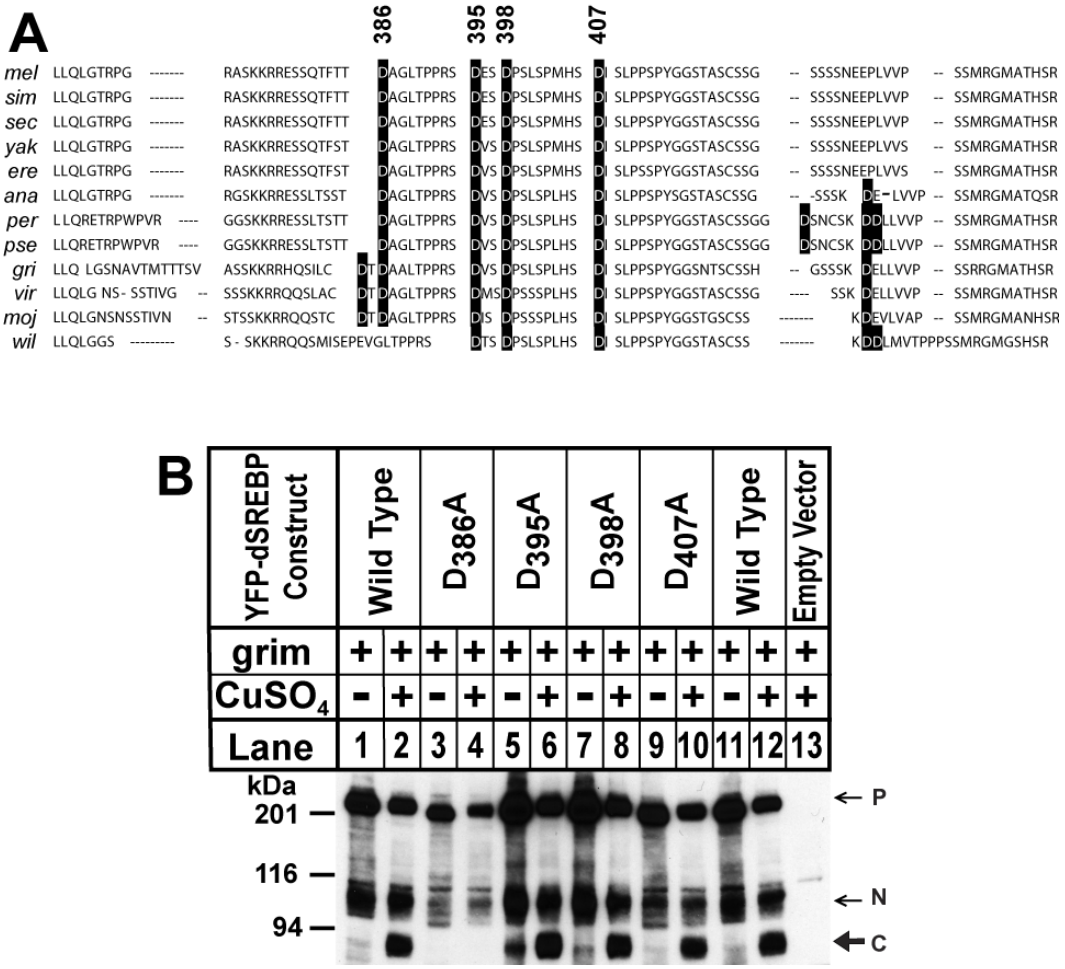


Figure 3-3.

Caspases cleave dSREBP between residues Asp_{386} and Ala_{387}. **(A)** Sequence alignment of SREBP homologues from 12 Drosophila species. In the abbreviated names, "mel" indicates melanogaster (CG8522-PA); sim, simulans (GD14825-PA); sec, sechellia (GM19644-PA), yak, yakuba (GE19622-PA), ere, erecta (GG16056-PA); ana, anassae (GF23590-PA); per, persimilis (GL15732-PA); pse, pseudoobscura (GA21134-PA); gri, grimshawii (GH14653-PA), wil, willistoni (GK17496-PA); vir, virilis (GJ11320-PA); moj, mojavensis (GI11638-PA). **(B)** Drosophila S2 cells were transfected with constructs encoding wild type YFP-dSREBP or YFP-dSREBP harboring an Ala in place of Asp residues at position 386, 395, 398, or 407 in the juxtamembrane region. Expression of grim was induced with $CuSO_4$ and whole cell lysates were subjected to immunoblot analysis using anti HSV antibody as described in Materials and Methods. P, precursor; N, normal nuclear form; C, caspase-dependent band.

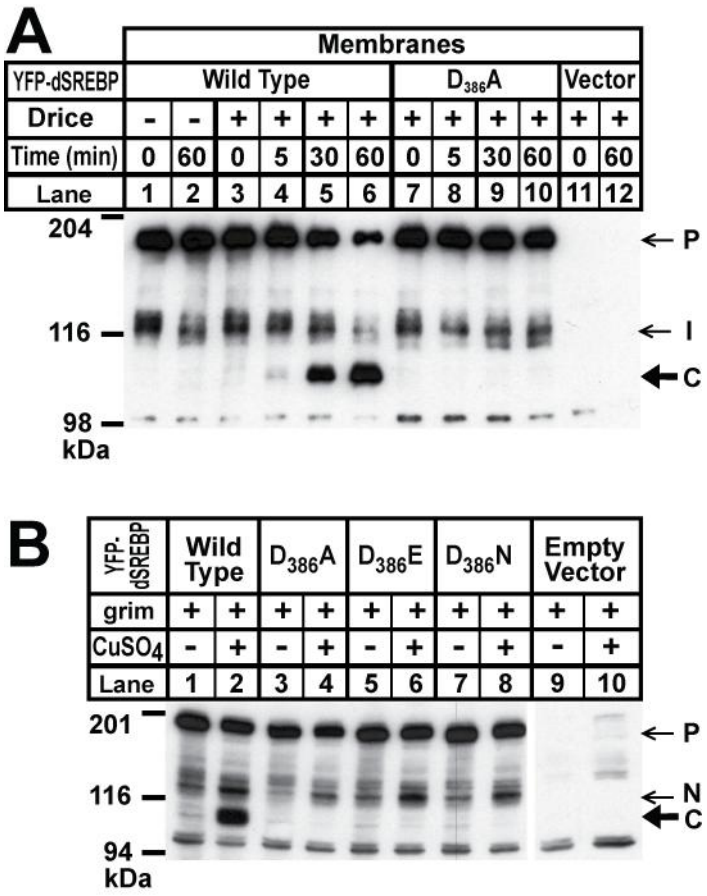


Figure 3-4.

Cleavage by Drice requires an Asp at position 386. **(A)** Membrane fractions were purified from S2 cells transfected with YFP-dSREBP or YFP-dSREBP($D_{386}A$). Membranes were incubated for the indicated times in caspase reaction buffer in the presence or absence of purified, recombinant Drice (see Materials and Methods). The reaction was stopped at the indicated times by addition of loading dye. The membranes were then subject to immunoblot analysis using an anti-HSV antibody as described in Materials and Methods. **(B)** S2 cells were co-transfected with Mtal-grim and the indicated YFP-dSREBP construct and the experiment was conducted as described in the legend to Figure 1. Note that intervening lanes between lanes 8 and 9 have been omitted for clarity. P, precursor; N, normal nuclear form; C, caspase-dependent band.

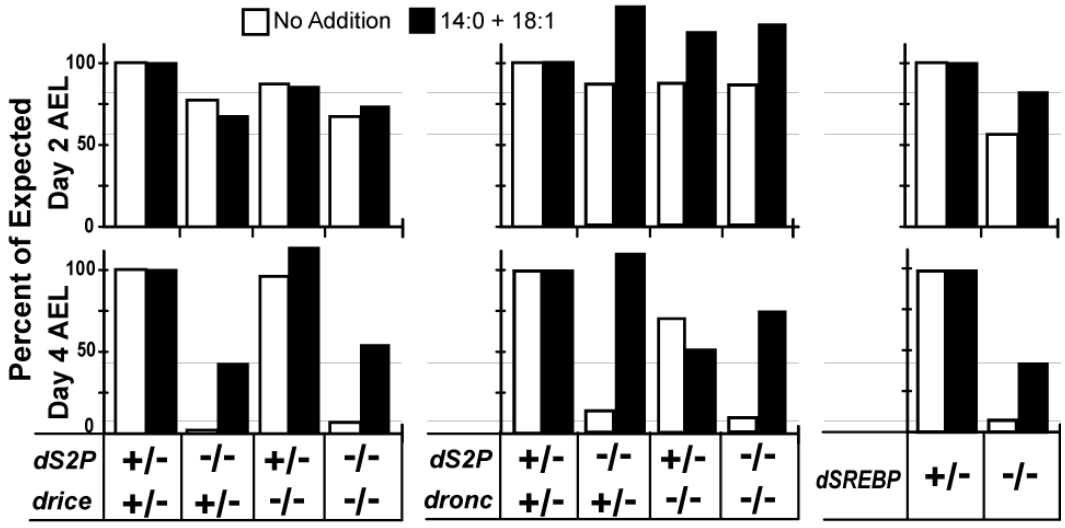


Figure 3-5.

Caspase cleavage of dSREBP is essential for the survival of larvae lacking dS2P. Strains harboring mutations in both *dS2P*^{1} or *dS2P*^{2} and in *drice*$^{\Delta 1}$ or *dronc*^{51} were constructed as described in Materials and Methods. Virgin females homozygous for *dS2P*^{2} and heterozygous for *dronc* or *drice* were crossed to males heterozygous for *dS2P*^{1} and *dronc* or *drice*. Note that the dS2P homozygous, caspase heterozygous females were raised on medium supplemented with fatty acids to enable efficient recovery of these flies. Parallel cultures were inoculated with 1 mg/vial of embryos on regular or supplemented medium. Larvae were scored on day 2 and day 4 AEL. *dSREBP*^{189} null mutant larvae served as a control for the efficacy of rescue in this experiment. Light lines corresponding to the number of *dSREBP* ^{189} larvae under each condition are shown to facilitate comparison.

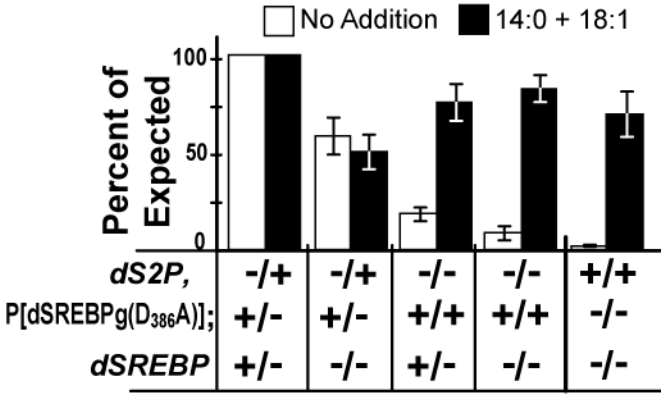


Figure 3-6.

Virgin dS2P^{2}, P{dSREBPg(D_{386}A)}/ dS2P^{2}, P{dSREBPg(D_{386}A)}; dSREBP^{189}/ TM6B, Tb Hu e females were crossed to dS2P^{1}, P{dSREBPg(D_{386}A)}/ CyO, twist-GFP; dSREBP^{189}/ TM6B, Tb Hu e males. On Day 0, embryos from an overnight collection were seeded into 10 vials (at 1 mg embryos/vial) containing regular-cornmeal-molasses agar (white bars, "No Addition") and 10 vials containing regular-cornmeal-molasses agar supplemented with 0.075% myristate and 0.15% oleate (black bars, "14:0 +18:1"). Emerging adults were scored using twist-GFP and Hu markers until no further adults emerged (approximately day 19 after plating). The data is presented as percent of the expected.

CHAPTER FOUR

Scap independent processing of SREBP in *Drosophila* larvae

INTRODUCTION

Activation of membrane bound SREBP requires transport from the ER to the Golgi for sequential cleavage by S1P and S2P and release the soluble N' terminal transcription factor domain (Rawson et al., 1999; Sakai et al., 1998). Thus, SREBP transcriptional activity is regulated by the transport or retention of SREBP in the ER or its transport to the Golgi apparatus. Work in mammalian cell culture identified Scap as the "SREBP cleavage activating protein"(Hua et al., 1996). Scap serves two roles in the processing of SREBP: 1) escort of SREBP to the Golgi and 2) sensor of membrane sterols. Accordingly, Scap protein can be divided into two functional domains: 1) the C terminus which contains a WD repeat domain that facilitates binding to SREBP, and 2) the N terminus which contains eight transmembrane spanning helices of which two through six contain a sterol sensing domain (SSD) (Hua et al., 1996).

Upon synthesis and insertion of SREBP into the ER membrane, Scap binds and stabilizes the SREBP precursor (Matsuda et al., 2001; Rawson et al., 1999). In the absence of sterols, Scap binds to COPII vesicle proteins and the Scap:SREBP complex is transported to the Golgi (Brown et al., 2002; Sun et al.,

2005). In mammalian cells, this transport of SREBP to the Golgi and subsequent proteolysis by S1P requires Scap (Sakai et al., 1998). Cells deficient of Scap fail to process SREBP and are thus cholesterol auxotrophs (Rawson et al., 1999). An excess sterols induces a conformational change in Scap that blocks the Scap:SREBP complex from entering COPII vesicles (Espenshade et al., 2002; Sun et al., 2005). This conformational change is accomplished through either the direct interaction of cholesterol with Scap's SSD or indirectly through the interaction of oxysterols with the ER retention factor Insig (Brown et al., 2002; Radhakrishnan et al., 2007; Radhakrishnan et al., 2004; Yang et al., 2002). Both result in the binding of Scap to Insig and the suppression of SREBP activity (Adams et al., 2004). Mutations in Scap that block the interaction with Insig results in constitutive SREBP activity independent of sterol levels (Yabe et al., 2002).

The study of Scap in whole organisms has been limited to conditional knockdown models as germ-line knockout mice were predicted to exhibit embryonic lethality similar to SREBP-2 null mice (Matsuda et al., 2001). Knockdown of Scap in mouse liver results in a significant decline in transcript levels of SREBP target genes and a 71% and 84% decrease in cholesterol and fatty acid synthesis, respectively (Matsuda et al., 2001). Also, SREBP-1 and -2 mRNA and protein levels are reduced due to decreased feed forward activation of

SREBPs by SREs present in both genes promoter regions. Furthermore, SREBP-2 fails to up-regulate target genes involved in sterol synthesis in response to cholesterol deprivation. Interestingly, mice deficient of Scap in the liver are relatively healthy (Matsuda et al., 2001). These mice display normal levels of whole-body fatty acid synthesis due to a compensatory increase in fatty acid synthesis in adipose tissue (Kuriyama et al., 2005).

The *Drosophila* genome encodes a Scap homolog (designated dScap) that shares 24% identity in amino acid sequence with human Scap (Seegmiller et al., 2002). *Drosophila* Scap also contains a SSD and WD repeat domain which share 47% and 40% identity with human Scap, respectively. Similar to the mammalian system, Scap is required for processing of dSREBP in *Drosophila* S2 cells (Seegmiller et al., 2002). In addition to the complete disappearance of nuclear SREBP, there is also a marked reduction in dSREBP precursor levels. However, unlike mammals, *Drosophila* are cholesterol auxotrophs lacking key enzymes in the cholesterol pathway and solely rely on sterols from the diet (Clark and Bloch, 1959). In fact, in vitro binding studies have shown that dScap binds cholesterol with much lower affinity as compared to hamster Scap (A. Radhakrishnan, personal communication). Furthermore, the *Drosophila* genome does not encode an Insig-like protein based on sequence homology (Rawson, 2003). What lipid(s) regulates SREBP processing in *Drosophila* and does dScap act as a lipid sensor in

flies as it does in mammals? Dobrosotskaya et al. (2002) demonstrated in S2 cell culture that dSREBP processing was blocked in the presence of palmitate and ethanolamine. Their data suggest that these substrates are incorporated into phosophtidylethanolamine and that dSREBP processing in *Drosophila* is regulated by membrane phosopholipid levels. Furthermore, Kunte et al. (2006) showed that feeding larvae fatty acids significantly reduced levels of nuclear dSREBP. However, whether dScap plays a direct or indirect role in this regulation has yet to be determined.

To determine the role of dScap in *Drosophila*, I generated mutations in the *dScap* gene. Surprisingly, flies lacking *dScap* are viable (with homozygotes emerging at 70% of the expected ratio) and can be maintained as a homozygous stock. Furthermore, this lethality can be completely rescued by supplementation of the diet with fatty acids. We find that in larvae lacking dScap, dSREBP is still transported to the Golgi and actively processed by dS1P and dS2P. These results show that *dScap*, unlike *dSREBP*, is not essential in *Drosophila* and suggest an alternative mechanism for the transport and regulation of dSREBP during larval development.

METHODOLOGY

Genetic strains: All marker mutations and balancer chromosomes are described and referenced by the FlyBase Consortium (2003). Crosses were carried out at 25˚ in vials containing freshly yeasted cornmeal-molasses-agar except were noted. *OreR* flies served as wild type. P element transposon lines KG00745, P{w^+, *UAS-GFP}* (inserted on the third chromosome) and P{*ry*, *hsFlp}*; *Adv*/*CyO* were obtained from the Bloomington stock center. PiggyBac transposon insertion lines PB(WH)f04534 and PB(PB)c00785 were obtained from the Exelixis collection at the Harvard stock center (Thibault et al., 2004). *dSREBP*^{189} and *dS2P*^{1} are deletion alleles previously described in (Kunte et al., 2006) and (Matthews et al., 2008), respectively. The P{w^+, *GAL4-dSREBPg}* transgene is inserted on the third chromosome (Kunte et al., 2006).

FLP-FRT recombination: Transposons PB(WH)f04534 and PB(PB)c00785 contain the piggyBac vectors (WH) and (PB), respectively (Thibault et al., 2004). Both vectors contain a mini-white marker gene and yeast FLP recombination target (FRT) sequences. In addition, the (WH) vector contains Su(*Hw*) insulator sequences and a terminal UAS site. Orientation of the transposon insertion was determined by PCR using one primer within the piggyBac transposon and one primer within the genome.

To induce FLP-FRT mediated recombination, I used a P{*ry, hsFLP}* insertion on the X chromosome (Golic and Lindquist, 1989) which was crossed into a PB(WH)f04534 background. Virgin w^{118}/ w^{118}; PB(PB)c00785/ PB(PB)c00785 females were crossed to P{*ry, hsFLP}*/Y; PB(WH)f04534 /*CyO* males in bottles. After two days, larvae and adults were incubated at 37 degrees for one hour to induce recombination as described in (Golic et al., 1997). Adults were removed at the end of the day. Larvae were incubated at 37 degrees for one hour each day for an additional four days. Single virgin P{*ry, hsFLP}*/ +; PB(WH)f04534/ PB(PB)c00785 females displaying mosaic eye color were then crossed to balanced w^{118}/ w^{118}; *Sp/ CyO* males. Single F_3 males with solid red eyes were crossed to balanced females and lines were established. Recombinants were verified by Southern blot using restriction enzymes Stu I and Sph I, and PCR using primer sets within the *dScap* locus.

Buffers: Buffer F is 125 mM Tris-HCl (pH 6.8), 8 M Urea, and 5% SDS.

Monoclonal antibodies: IgG-3B2 against *Drosophila* SREBP is described in (Seegmiller et al., 2002). IgG-7A8 was generated in mice against *Drosophila* Scap transmembrane domains 1-8. IgG-611B-1 against acetylated tubulin was obtained from Sigma (St. Louis).

Whole-larval lysis: For Figure 4- 1C, six third instar larvae of the indicated genotype were homogenized in buffer F. For Figure 4-9, 5 mg of embryos were seeded onto filter paper wet with IPL-41. First instar larvae were collected from the filter paper and homogenized in buffer F. Homogenates were filtered through a 100 µM Nytex mesh at 1000g for 1 min. Total protein concentrations were determined using BCA protein assays (Promega).

Analysis of larval growth: Embryos were collected for 2 hours and plated at 10 mg/plate onto semi-defined media (Backhaus, 1984). Representative larvae were selected at each time point and photographed next to a ruler. Images were aligned using Adobe Photoshop.

Adult emergence assays: Embryos were collected overnight and seeded into vials at 1 mg embryos/ vial containing cornmeal-molasses-agar with either no additions or supplemented with 0.075% Na-myristate and 0.15% Na-oleate (Sigma). Larvae were allowed to develop and emerging adults were scored based on phenotypic markers on a daily basis for 10-19 days AEL.

Quantitative analysis of transcripts: Embryos were collected for 2 hours and plated at 10 mg/plate onto semi-defined media (Backhaus, 1984). Larvae were collected at the desired time points and total RNA and cDNA were

generated as previously described (Kunte et al., 2006). Real-time quantitative PCR analysis was performed as previously described (Dobrosotskaya et al., 2002; Kunte et al., 2006).

Larval Imaging: Larvae were photographed 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 enhanced over background using the auto-curve function with 50% fading in Photoshop CS2.

RESULTS

Generation of *dScap* deletion alleles

To generate deletions in the *dScap* open reading frame (ORF), I used transposase-mediated P element excision (Robertson et al., 1988) of transposon KG00745 located 442 base pairs upstream of the *dScap* ORF (Figure 4-1A). We screened 1,200 independent excision lines by PCR and Southern blotting analysis (Figure 4-2) and identified 3 lines that extended into the *dScap* ORF. Two of these lines removed only the first exon and start of the ORF. However, further analysis of these deletions showed that a truncated *dScap* transcript was still produced. One deletion, designated $dScap^{\Delta 910}$, extended 22 kilobases, removing the entire *dScap* gene and two adjacent genes, *Dream* and *PNGase* (Figure 4-2). By definition, this line is designated a deficiency allele. The $dScap^{\Delta 910}$ line was homozygous lethal, whereas the other two deletion lines were homozygous viable, suggesting that these smaller deletions maybe hypomorphic alleles of *dScap*.

To generate a deletion of *dScap* that removes the entire ORF without disrupting neighboring genes, I induced FLP-mediated recombination between two piggyBac transposon lines from the Exelixis collection containing FLP recognition target (FRT) sequences (Golic et al., 1997; Sadowski, 1995; Thibault et al., 2004) (Figure 4-1A). Line f04534 contained a piggyBac(PB) element

inserted 814 base pairs upstream of the *dScap* ORF and line c00785 contains a piggyBac(WH) element located 88 base pairs after *dScap* exon 7.

The orientation of the FRT sequences with respect to each other dictates whether the gene located between the two transposons will be deleted or duplicated upon recombination (Thibault et al., 2004). Recombination of FRT sequences in the same orientation result in gene deletion, whereas FRT sequences in opposite orientations result in gene duplication upon recombination. The FRT sequences within piggyBac lines f04534 and c00785 are oriented in the same direction with respect to the *dScap* gene (Figure 1A, red arrows) and therefore, recombination between the two FRT sites should delete the *dScap* gene (Figure 3A). However, the piggyBac (PB) vector in line c00785 contains two FRT sequences flanking a mini-white marker gene generating two possible recombination outcomes: 1) A recombined allele containing two mini-white marker genes or 2) a recombined allele containing one mini-white marker gene (Figure 4-3A).

Males heterozygous for piggyBac(WH) f04534 and expressing a heat-shock inducible FLPase on the X chromosome where crossed to virgin females homozygous for piggyBac(PB) c00785 (Figure 4-3B). FLP-mediated recombination was induced by incubating the larval progeny at 37 degrees. From

these bottles, 500 F_2 virgin females displaying mosaic eyes were individually crossed to balanced males. F_3 males with red eyes indicating the presence of two mini-white marker genes and recombination in the germ line, were selected. Each line was then screened by Southern blot analysis to verify recombination of chromosomes and deletion of *dScap* (Figure 4-4). Seventy independent lines successfully recombined between the two FRT containing piggyBac elements resulting in the deletion of the entire *dScap* gene. One line, designated $dScap^4$, was selected for further study.

dScap is not essential in _Drosophila_

Surprisingly, in contrast to flies lacking *dSREBP*, flies deficient of d*Scap* are viable. Analysis of dScap transcript and protein verify that both $dScap^4$ and $dScap^{\Delta 910}$ are null alleles (Figure 4-1B & C). It is surprising that $dScap^4$ heterozygous larvae show such a severe reduction in dScap transcripts although this decrease does seem to translate to protein levels (which appear about half of wild type levels).

Larvae lacking dScap develop more slowly than heterozgyotes. Beginning around 48 hours after egg laying (AEL), $dScap^4$ homozygous larvae are notably smaller than their heterozygous siblings (Figure 4-5A). This difference in size becomes more pronounced with time, such that by 89 hours AEL the majority of

heterozygotes have transitioned to third instar, whereas most homozygous larvae are still second instars based on spirical morphology. As a result, the majority of homozygotes emerge from their pupal cases about two days after their heterozygous siblings (Figure 4-5B).

Homozygous adults appear morphologically similar to their heterozygous siblings (Figure 4-6A). However, I noted that *dScap*^{4} homozygotes emerge at about 70% of expected numbers (Figure 4-6B). Supplementing food with the end products of dSREBP targets, fatty acids, rescues this lethality. Thus, the lethality seen in 30% of *dScap* homozygotes is a consequence of reduced transcription by dSREBP.

As indicated in Figure 4-7, dScap mRNA is maternally loaded into embryos. Before the start of zygotic transcription, dScap transcript is present in embryos collected 0-2 hours AEL from mothers either wild type or heterozygous for *dScap*^{4}, but not in embryos deposited from *dScap*^{4} homozygous mothers (Figure 4-7A). By 36 hours AEL, no dScap mRNA is detected in larvae from either heterozygous or homozygous mothers. We used the levels of CG6295, a dSREBP target gene whose transcription strongly correlates to dSREBP activation, to determine the effect of maternal loading from heterozygous mothers (Figure 4-7B). In 36 hour AEL larvae, CG6295 transcript levels are significantly

reduced in *dScap*^{4} homozygotes despite the genotype of the mother. Therefore, maternal loading of dScap transcript into embryos does not replace zygotic transcription in larvae.

dSREBP is processed in *dScap* mutants

Since *dScap*^{4} homozygotes are viable, in contrast to *dSREBP* mutants, I wanted to determine if this viability was due to the processing dSREBP in the absence of dScap. To visualize dSREBP activation in larvae, I utilized the GFP reporter system described in (Kunte et al., 2006)(Figure 4-8A). In wild type larvae, GAL4-SREBP is actively processed in the fat body, midgut and oenocytes as determined by GFP fluorescence (Figure 4-8A, upper larva). In the absence of dScap, GFP fluorescence is greatly diminished in the fat body, and to a lesser in the midgut (Figure 4-8A, lower larva). However, the fluorescence is similar in the oenocytes of homozygous and heterozygous larvae (Figure 4-8B).

I probed whole larval lysates from either wild type or *dScap*^{4} homozygous larvae with an antibody against *Drosophila* SREBP (Figure 4-9). Lysates from wild type larvae show two bands corresponding to the full length precursor form (P) and a faster migrating band corresponding to the full processed amino terminus (N)(lane 1). Immunoblot analysis of *dScap*^{4} homozygous lysates (lane 2) shows a reduction in the SREBP precursor level (note that twice as much total

protein as wild type is loaded in lane 2). Furthermore, there is a band that comigrates with nuclear dSREBP. The intensity of this band is significantly reduced compared to wild type, in agreement with the reduced fluorescence mentioned above. Thus, dSREBP is still processed in the absence of dScap, albeit at significantly reduced levels.

I next evaluated the transcript levels of dSREBP target genes at different time points during development in larvae transheterozgyous for $dScap^{4}$ and $dScap^{\Delta 910}$ (Figure 4-10). As previously described (Kunte et al., 2006; Seegmiller et al., 2002), *Drosophila* SREBP activates genes involved the uptake and synthesis of fatty acids including acetyl coenzyme A carboxylase (ACC), acetyl coenzyme A synthase (ACS), fatty acid synthase (FAS), and the putative lipase, CG6295. The transcript levels of dSREBP target genes are significantly reduced in dScap transheterozygotes compared to wild type larvae at 48 and 60 hours AEL. However, by 72 hours AEL the transcript levels of ACC and FAS, but not ACS, in $dScap^{4}$ mutants are intermediate to wild type and *dSREBP* null larvae. Furthermore, CG6295 transcripts show a slight increase in *dScap* homozygotes as compared to *dSREBP* mutants (see also Figure 4-7), whereas transcript levels for dS2P, which is not a target of dSREB, are unchanged.

To determine if dSREBP processing in the absence of dScap is regulated, I grew dScap^4 larvae on semi-defined medium or on medium supplemented with soy lipids. In the presence of added soy lipids, no GFP fluorescence was detected in either the midgut or oenocytes (Figure 4-11A, lower larva) as compared to larvae grown on regular medium (upper larve). Furthermore, I tested dSREBP transcript levels in dScap larvae in response to excess soy lipids. Figure 4-11B show quantitative real time PCR results for 60 hour AEL larvae grown in the presence of 0% or 9% soy lipids. dSREBP and CG6295 mRNA levels decrease in response to soy lipids in wild type larvae and to a lesser extent in dScap^4 homozygotes.

SREBP is normally processed by S1P and S2P in the Golgi. Previously, I generated a null alleles of dS2P (designated dS2P^1) and determined that larvae lacking dS2P are viable due to an alternative cleavage of the Drosophila caspase, Drice (Matthews et al., 2008). Since Drice is not as efficient at cutting dSREBP as is dS2P, the dSREBP membrane-bound intermediate form, which is the product of dS1P cleavage, accumulates. To determine if dSREBP is cleaved by dS1P and dS2P in the absence of dScap, I recombined the dScap^4 allele onto the dS2P^1 chromosome. Surprisingly, flies lacking both dScap and dS2P are viable though not as healthy as either single mutation alone. We performed immunoblot analysis on lysates from third instar larvae wild type or mutant for either dScap,

dS2P, or both (Figure 4-12). In wild type larvae (lane 1), both precursor (P) and nuclear (N) dSREBP are present. In $dScap^4$ single mutants (lane 2) only precursor dSREBP is visible (nuclear dSREBP is not visible at this exposure), whereas in $dS2P^1$ single mutants I see the presence of the intermediate form (I). In larvae double mutant for dScap and dS2P (lanes 4-6), the intermediate form of dSREBP is detected at reduced levels. Lysate from larvae lacking dSREBP is loaded in lane 7 as a negative control. Thus, in larvae lacking dScap, dSREBP is still processed by dS1P and dS2P.

DISCUSSION

I report that *dScap*, unlike *dSREBP*, is dispensable during larval development in *Drosophila melanogaster*. Seventy percent of *dScap* homozygotes emerge as adults, whereas 100% of the expected emerge when grown in the presence of fatty acids (Figure 4-6B). While larvae lacking dScap exhibit a delay in development (Figure 4-5A), adults are morphologically similar to their heterozygous siblings (Figure 4-6A).

Mammalian cells lacking Scap die unless supplemented with cholesterol and fatty acids (Rawson et al., 1999). In contrast, my data indicate that dSREBP is still processed in larvae deficient for dScap. First, GAL-SREBP is actively cleaved in the oenocytes and to a lesser extent the midgut, whereas GFP signal is significantly reduced in the fat body (Figure 4-8). Second, I detect a band corresponding to nuclear dSREBP in whole larval lysates from *dScap* mutants (Figure 4-9). Third, dSREBP target genes, FAS and ACC, are expressed in *dScap* homozygous larvae at levels greater than in *dSREBP* nulls (Figure 4-10). Together these data demostrate that *Drosophila* have an alternative mechanism for processing dSREBP in the absence of dScap during larval development.

How is SREBP processed in the absence of Scap? I have demonstrated that the dSREBP amino terminus is released from the membrane (Figures 4-8 and

4-9) and up regulates transcription of target genes without the escort protein dScap (Figure 4-10). Thus, in dScap mutants dSREBP must be cleaved after the basic helix-loop-helix DNA binding domain, but before or within the first transmembrane domain. This cleavage is normally conducted by the S2P following proteolysis by S1P. It is therefore possible that dSREBP is cleaved normally by dS1P and dS2P in dScap mutants. I can not rule out the possibility that another protease cleaves dSREBP within this juxtamembrane region as I have previously shown in the dS2P single mutants. To address this question, I generated flies doubly mutant for dScap and dS2P. In flies lacking only dS2P, the intermediate form of dSREBP, product of dS1P, accumulates in membranes. Therefore, if dSREBP is processed by dS1P in dScap single mutants, then the dSREBP intermediate form should be present in larvae lacking *both* dScap *and* dS2P. However, if dSREBP is processed by another protease within the juxtamembrane region, then there should be little or no membrane-bound intermediate form present. Using immunoblot analysis of larval lysates from double mutants, I detect the membrane-bound intermediate form of dSREBP (Figure 4-12). This indicates that dSREBP is processed normally by dS1P and dS2P in larvae lacking *only* dScap.

Where does proteolysis of dSREBP occur in dScap mutants? In mammalian cell culture, Scap is required for transport of SREBP between the ER

and Golgi and its subsequent proteolysis by S1P (Sakai et al., 1998). I postulate that dSREBP is processed normally by dS1P and dS2P in the absence of dScap. Therefore, dSREBP is either 1) conveyed to the Golgi without dScap, where it is then cleaved by dS1P and dS2P, or 2) cleaved by dS1P and dS2P within the ER.

Examples of bulk flow between the ER and Golgi have been reported for secretory proteins (Lee et al., 2004). The yeast mating pheromone, gpαf, is normally targeted to COPII vesicles by the transmembrane cargo-receptor, Erv29p. However, in the absence of Erv29p, gpαf is still transported from the ER to the Golgi by non-specific incorporation into COPII vesicles (Belden and Barlowe, 2001). However, this method is extremely inefficient as <1% of gpαf protein is present in vesicles (Malkus et al., 2002). It seems unlikely that transport by random sampling would supply growing larvae with sufficient dSREBP to meet lipid demands during development. To this point, previous work by Kunte et al. (2006) has shown that the small amount of dSREBP protein in the hypomorphic allele $dSREBP^{52}$ was nevertheless insufficient to permit larval survival. dSREBP might also be cleaved by dS1P and dS2P within the ER membranes if these proteases were active in the ER or through some fusion of ER and Golgi membranes. While these seem unlikely, I cannot distinguish among these possibilities based on our current data.

Is dSREBP cleaved by Drice in the absence of dScap as it is in dS2P single mutants? This seems unlikely based on several observations. First, flies lacking dScap are relatively healthy (emerging at 70% of the expected numbers) as compared to dS2P transheterozygous mutants which emerge at 40% of the expected numbers (Matthews, 2008). Second, in the two mutants, dSREBP is active in different tissues as visualized by the GAL-dSREBP reporter system. In dS2P homozyotes, fluorescence is detected predominately in the fat body and to a lesser extent the oenocytes, but is notably absent from the midgut. In contrast, fluorescence is detected in the midgut and oenocytes of dScap homozygous larvae, but severely reduced in the fat body. However, I am currently testing the potential roles for caspase cleavage by generating flies doubly mutant for dScap and Drice.

How is SREBP regulated in *Drosophila*? Regulation of mammalian SREBP cleavage is owing to its ER-to-Golgi transport by Scap (Adams et al., 2004; Brown et al., 2002; Sun et al., 2005). However, I show in Figure 4-11A that SREBP activity is still suppressed in dScap homozygous larvae grown in the presence of soy lipids. It is likely that this reduction in SREBP activation is due to the coordinate decrease in dSREBP transcript levels (Figure 4-11B). dSREBP precursor levels are reduced when dScap is lacking either in dScap mutant larvae or S2 cells treated with RNAi against dScap (Seegmiller et al., 2002). This

treatment results in reductions in dSREBP transcript levels by 45% and 66%, respectively. Additionally, dSREBP mRNA is decreased in larvae grown in the presence of added soy lipid, which suppress dSREBP processing (see Figure 4-11B)(Kunte et al., 2006). Mammalian SREBPs are subject to transcriptional regulation through a feed-forward mechanism due to the presence of SREs within the promoter/enhancer regions of both genes (Amemiya-Kudo et al., 2000; Sato et al., 1996). We have identified a putative SRE sequence in the region upstream of dSREBP (Bill Amarneh, unpublished data). However, Kunte et al. (2006) showed that feeding larvae increasing concentrations of soy lipids results in increased suppression of nuclear SREBP without significant reductions in precursor levels. This suggests that there are transcriptional and post-transcriptional mechanism that regulate SREBP activity in *Drosophila*.

Is dScap involved in the post-transcriptional regulation of dSREBP? Extensive work in mammalian cell culture has demonstrated that Scap physically binds cholesterol resulting in a conformational change in the Scap:SREBP complex, initiating binding to the ER retention factor Insig. This model explains how the cell senses and responds to membrane sterols. How does the cell sense and regulate other lipids? Is SREBP-1c, whose targets predominately include the genes involved in fatty acid synthesis, solely regulated by membrane sterol levels or is there a second level of regulation involving fatty acids? *Drosophila* is an

excellent organism in which to address these questions as its single SREBP protein is not involved in sterol synthesis; *Drosophila* are cholesterol auxotrophs. Previous work is S2 cells suggests that dSREBP activation may be regulated by phosophlipids. Does dScap play a role in sensing phospholipid levels? And if so, how?

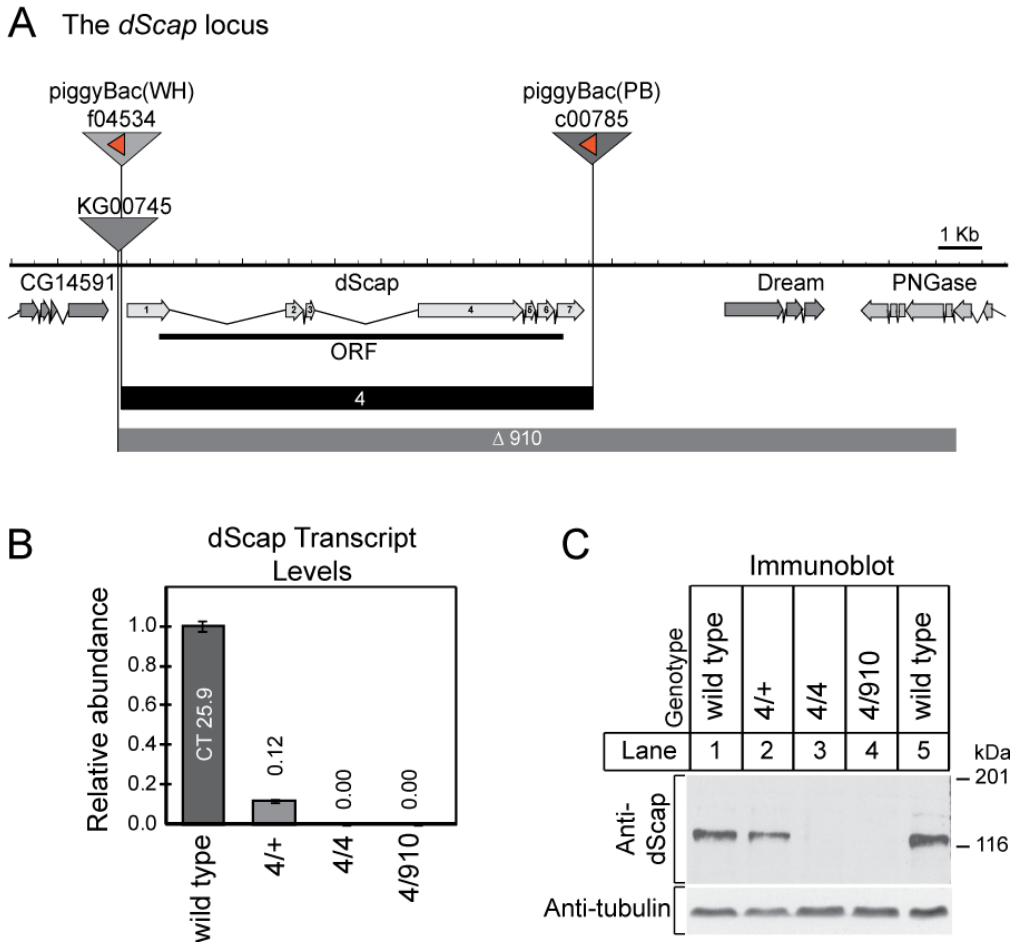


Figure 4-1

(A) Map of *dScap* locus. The *dScap* gene contains seven exons (light grey block arrows) encoding one protein (ORF is indicated by a thick black line). Sites of transposon insertion are indicated by inverted triangles. P element transposon KG00745 and piggyBac(WH)f04534 are inserted prior to the start of *dScap* of exon 1 and after CG14591's ORF. PiggyBac(PB)c00785 is inserted 88 base pairs after *dScap* exon 7. The orientation of the FRT sites with both piggyBac elements are indicated by the red triangles. The extent of $dScap^{4}$ and $dScap^{\Delta 910}$ deletions are indicated by black and dark grey boxes, respectively. **(B)** Quantitative analysis of dScap mRNA from $dScap^{4}$ and $dScap^{\Delta 910}$ homozygous or transheterozygous larvae as compared to wild type (wt = 1). Numbers above bars indicate the relative abundance of transcript. Error bars represent the standard deviation. **(C)** Immunoblot analysis of whole larval lysates from third instar larvae of the indicated genotype (60 µg total protein/ lane). Virgin $dScap^{4}$/ $dScap^{4}$ females were crossed to either $dScap^{4}$/ *CyO*, act-GFP or $dScap^{\Delta 910}$/ *CyO*, act-GFP males. Embryos were seeded onto dishes containing semi-defined media at 10 mg/ dish. Larvae were isolated from the food by salt floatation and homozygous larvae were scored based on GFP fluorescence. The membrane was probed with an antibody against dScap TM1-8 (1 min 30 sec exposure), then stripped and re-probed with anti-acetylated tubulin (2 sec exposure).

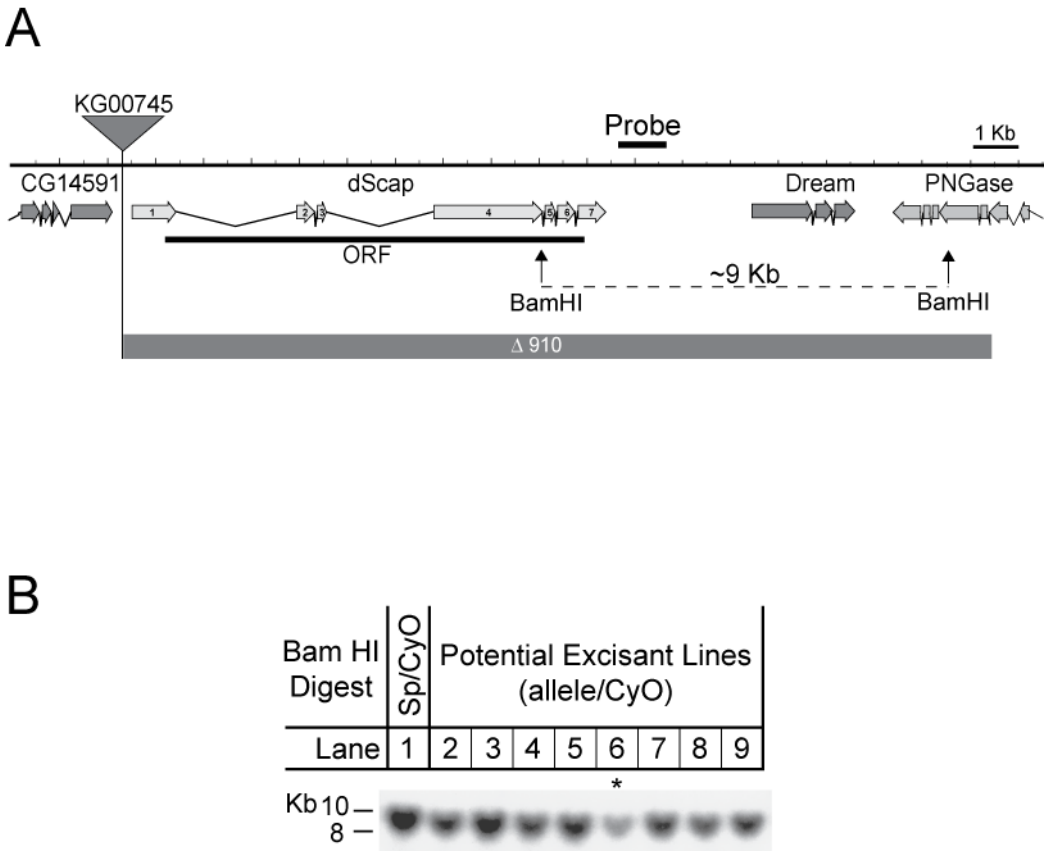
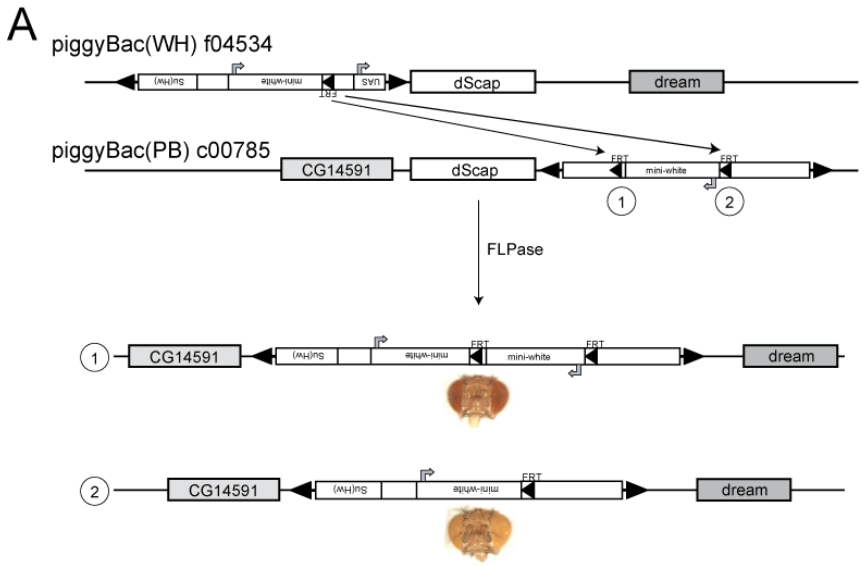


Figure 4-2

(A) Map of *dScap* locus indicating site of P element KG00745 insertion and extent of *dScap*$^{\Delta 910}$ deletion. The predicted fragment (~9 Kb) of BamHI restriction digest (arrows) is indicated by a dashed line. The probe used to analyze fragments is located down stream of *dScap* exon 7. **(B)** Genomic DNA from individual P element excisant lines was digested with BamHI and analyzed by Southern blot. A representative blot showing a line harboring a deletion that removes up to *dScap* exon 7 (indicated by astrick) as indicated by a 0.5 reduction in signal intensity.


A
piggyBac(WH) f04534
Su(Hw)
mini-white
UAS
FRT
dScap
dream
piggyBac(PB) c00785
CG14591
dScap
FRT
mini-white
FRT
1
2
FLPase
1
CG14591
Su(Hw)
mini-white
FRT
mini-white
FRT
dream
2
CG14591
Su(Hw)
mini-white
FRT
dream

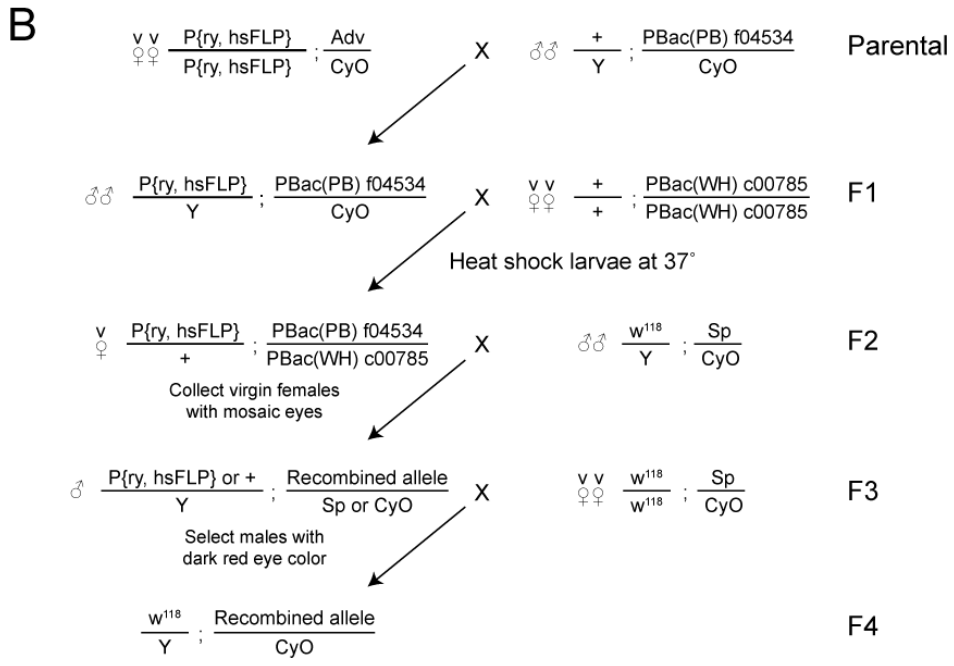
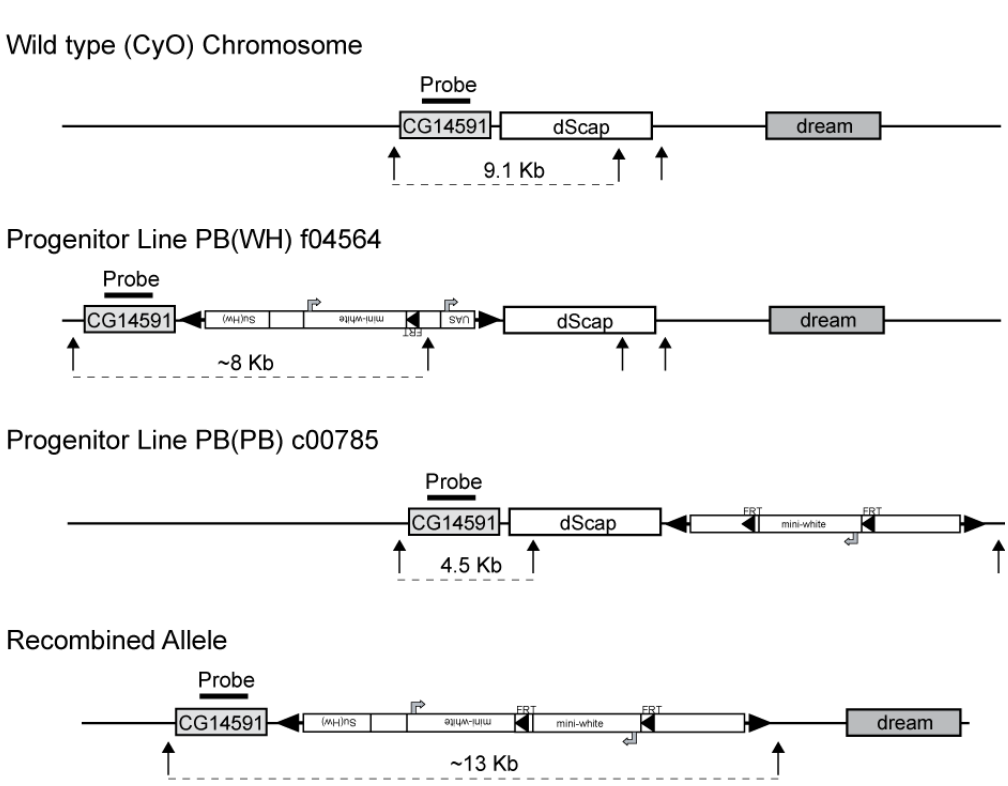

B
v v ♀♀ P{ry, hsFLP} / P{ry, hsFLP} ; Adv / CyO
X
♂♂ + / Y ; PBac(PB) f04534 / CyO
Parental
♂♂ P{ry, hsFLP} / Y ; PBac(PB) f04534 / CyO
X
v v ♀♀ + / + ; PBac(WH) c00785 / PBac(WH) c00785
F1
Heat shock larvae at 37°
v ♀ P{ry, hsFLP} / + ; PBac(PB) f04534 / PBac(WH) c00785
X
♂♂ w118 / Y ; Sp / CyO
F2
Collect virgin females with mosaic eyes
♂ P{ry, hsFLP} or + / Y ; Recombined allele / Sp or CyO
X
v v ♀♀ w118 / w118 ; Sp / CyO
F3
Select males with dark red eye color
w118 / Y ; Recombined allele / CyO
F4

Figure 4-3

(A) Targeting scheme of Flp-mediated recombination between FRT sites located within piggyBac element transposons flanking the *dScap* gene. Since piggyBac(PB)c00785 contains two FRT sequences there are two outcomes upon recombination with the single FRT sequence in piggyBac(WH)f04534. 1) Recombination at the first FRT site results in the replacement of the *dScap* locus with two mini-white marker genes resulting in flies with a dark red eye color. 2) Recombination at the second FRT site replaces the *dScap* locus with a single mini-white marker gene resulting in flies an eye color indistinguishable from the progenitor lines. **(B)** Crossing scheme to generate *dScap* deletion alleles by Flp-mediated recombination.

A


Wild type (CyO) Chromosome
Probe
CG14591
dScap
dream
9.1 Kb
Progenitor Line PB(WH) f04564
Probe
CG14591
Su(Hw)
mini-White
FRT
UAS
dScap
dream
~8 Kb
Progenitor Line PB(PB) c00785
Probe
CG14591
dScap
FRT
mini-white
FRT
4.5 Kb
Recombined Allele
Probe
CG14591
Su(Hw)
mini-White
FRT
mini-white
FRT
dream
~13 Kb

B

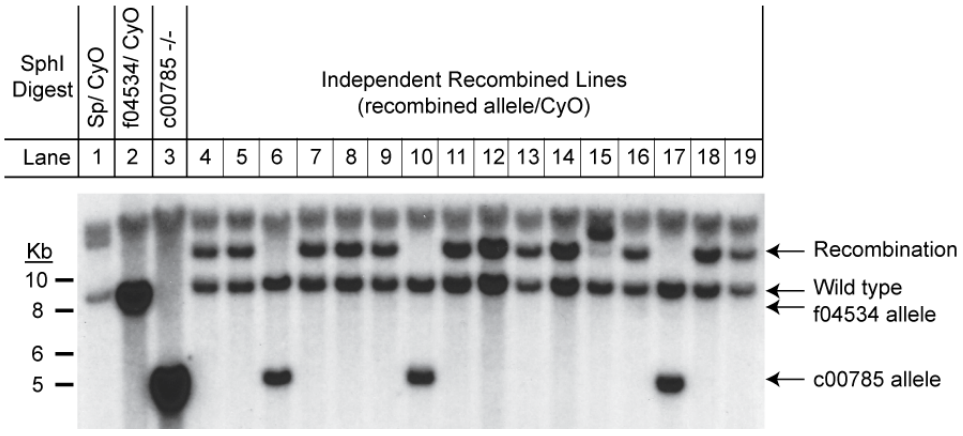

SphI Digest
Sp/ CyO
f04534/ CyO
c00785 -/-
Independent Recombined Lines (recombined allele/CyO)
Lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Kb
10
8
6
5
Recombination
Wild type
f04534 allele
c00785 allele

Figure 4-4

(A) The structures of wild type, progenitor, and recombined chromosomes with respect to the *dScap* locus. Arrows indict *Sph*I restriction sites. Dashed lines indicated the extent of digested fragments. A probe in the upstream gene CG14591 is indicated by a solid line. **(B)** Molecular verification of recombination lines. Genomic DNAs were digested with *Sph*I and analyzed by Southern blotting.

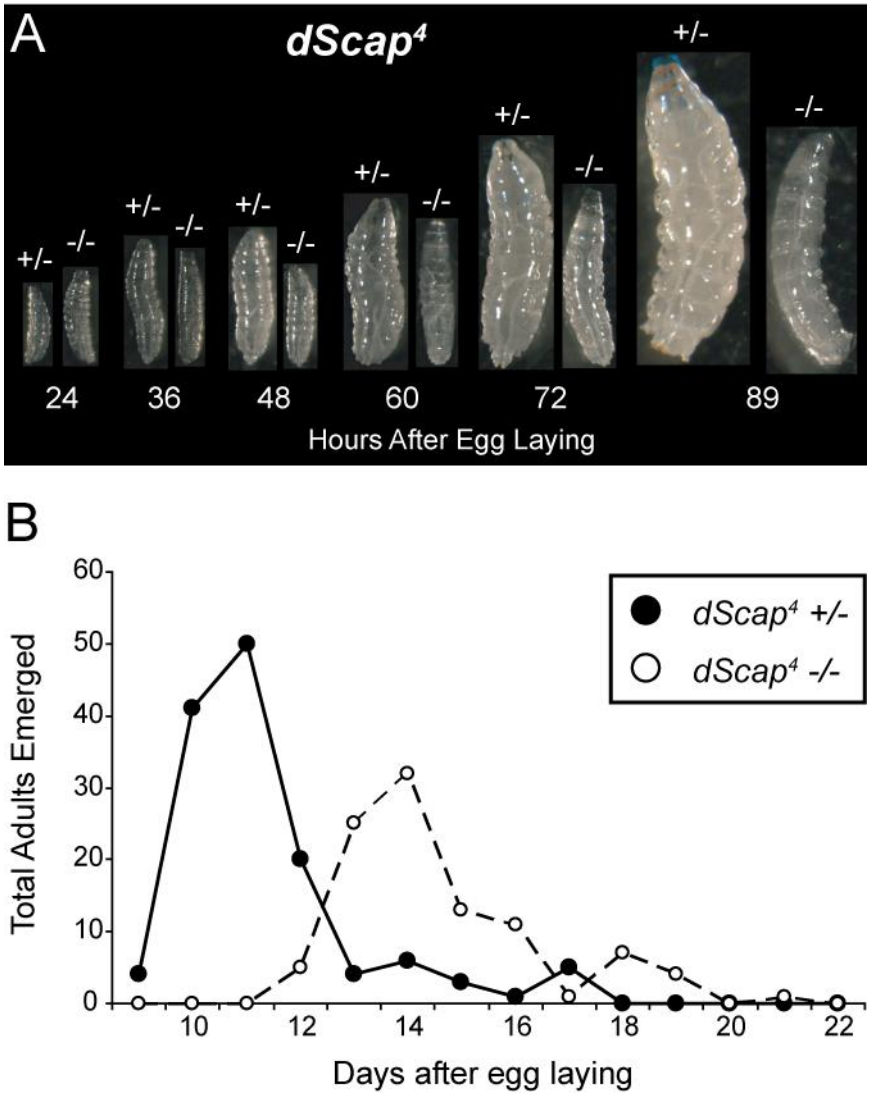


Figure 4-5

(A) Comparison of size between larvae homozygous (-/-) or heterozygous (+/-) for *dScap*^{4}. Virgin *dScap*^{4}/ *dScap*^{4} females were crossed to *dScap*^{4}/ *Cyo*, act-GFP males. Embryos were seeded at 10 mg/ dish onto dishes containing semi-defined media. Larva genotype was scored based on GFP fluorescence and photographed at the indicated time points. **(B)** Emergence of adult flies either homozygous (closed circles) or heterozygous (open circles) for the *dScap*^{4} allele. Virgin *dScap*^{4}/ *dScap*^{4} females were crossed to *dScap*^{4}/ *CyO*, act-GFP males. Embryos were seeded into 10 vials containing cornmeal-molasses-agar medium at 1 mg/ vial. Larvae were allowed to develop at 25˚. Adults were removed from the vial and scored daily using the *Cy* and act-GFP phenotypic markers.

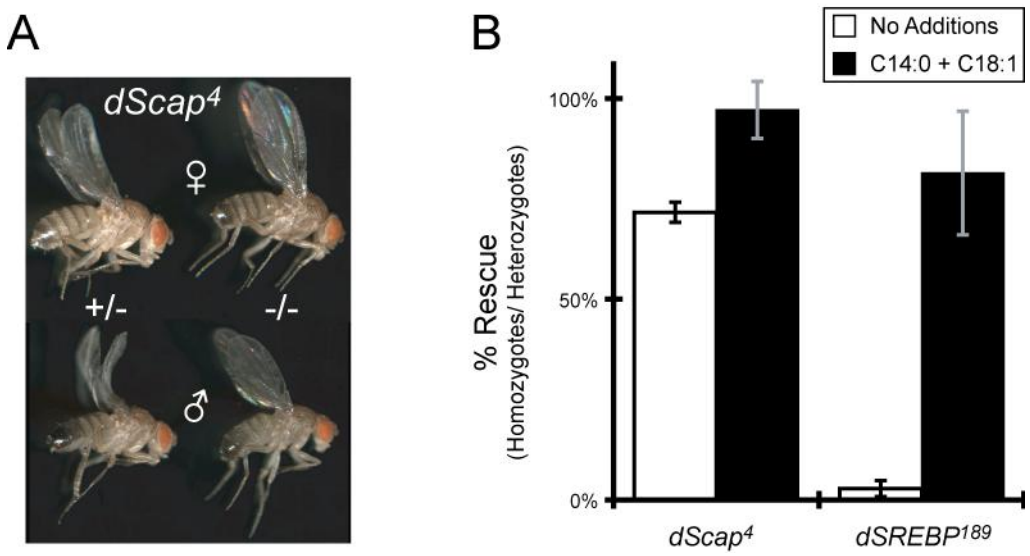


Figure 4-6

(A) Embryos from the *dScap*^{4} cross described above or from *dSREBP*^{189}/ *TM3*, *Ser*, act-GFP were seeded into 10 vials containing cornmeal-molasses-agar medium or medium supplemented with 0.075% Na-myristate and 0.15% Na-oleate at 1 mg/ vial. Percent rescue is calculated as a percent of the expected for the ratio of total homozygotes to heterozygotes (0.5 = 100% for the dScap cross and 0.33 = 100% for the dSREBP cross). **(B)** Comparison of size between *dScap*^{4} homozygotes (-/-) and heterozygous (+/-) adults.

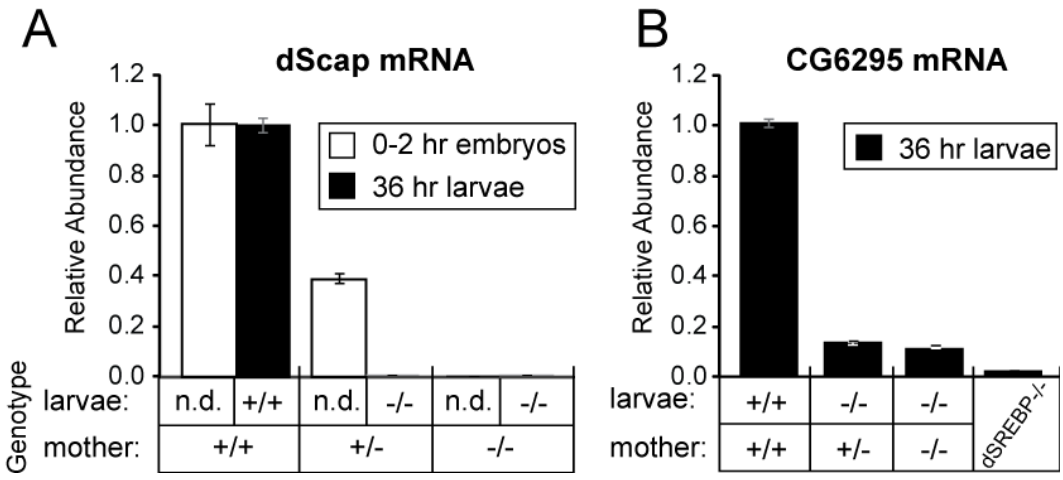


Figure 4-7

(A) Maternal contribution of dScap mRNA in 0-2 hour embryos (white bars) and first instar larvae (black bars). Embryos from wild type or virgin *dScap*^{4}/ *dScap*^{4} females were crossed to *dScap*^{4}/ *Cyo*, act-GFP males were collected for 2 hours. Embryos were either collected for RNA isolation or seeded (10 mg/ dish) onto a dish containing semi-defined media. After 36 hours, larvae were genotyped based on GFP fluorescence and total RNA was isolated. **(B)** Activation of dSREBP was determined in first instar larvae from above by analysis of CG6295 transcript levels. dSREBP^{189} nulls were included for comparison.

Figure 4-8

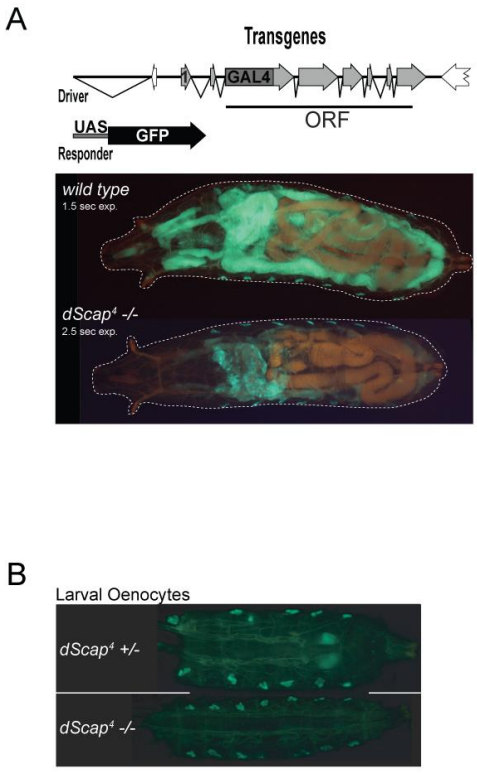


Figure 4-8

(A) Ventral views of dSREBP activity in wild type (upper panel) and dScap^4 (lower panel) third instar larvae visualized using a GAL4-dSREBP/ UAS-GFP reporter system. Fluorescence is detected in dScap^4 -/- in midgut and oenocytes, and to a lesser extent in regions of fat body. Larvae are wild type or homozygous for *dScap^4* on the second chromosome and homozygous for P[GAL4-dSREBPg], P[UAS-GFP] on the third chromosome. Wild type and dScap^4 larva were photographed for GFP fluorescence at a 1.5 and 2.5 sec exposure, respectively. Dashed lines denote extent of larval bodies. **(B)** Comparison of GFP fluorescence in larval oenocytes. Cuticles from *dScap^4* homozygous (-/-) or heterozygous (+/-) larvae were prepared by gently pulling the mouth hooks to remove the intestines and removing internal organs by gently squeezing the cuticle through a pair of forceps. Note that some fat body is still present in the *dScap^4* heterozygous prep.

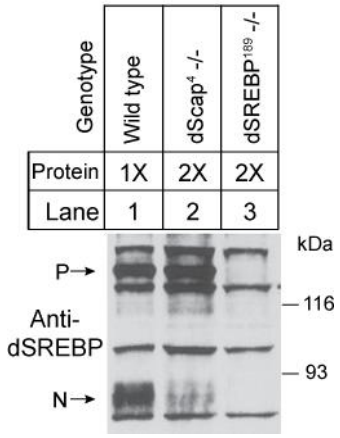


Figure 4-9

Immunoblot analysis of whole larval lysates from first instar larvae probed with a monoclonal antibody against the amino terminus of dSREBP. Wild type lysates were loaded at 30 µg/ml, whereas dScap^4 and dSREBP lysates were loaded at 60 µg/ml. Membranes were incubated with primary antibody overnight at 4˚ and secondary anti-mouse antibody for 45 minutes at room temperature. Membranes were exposed to film for 2 minutes. P, precursor; N, nuclear form.

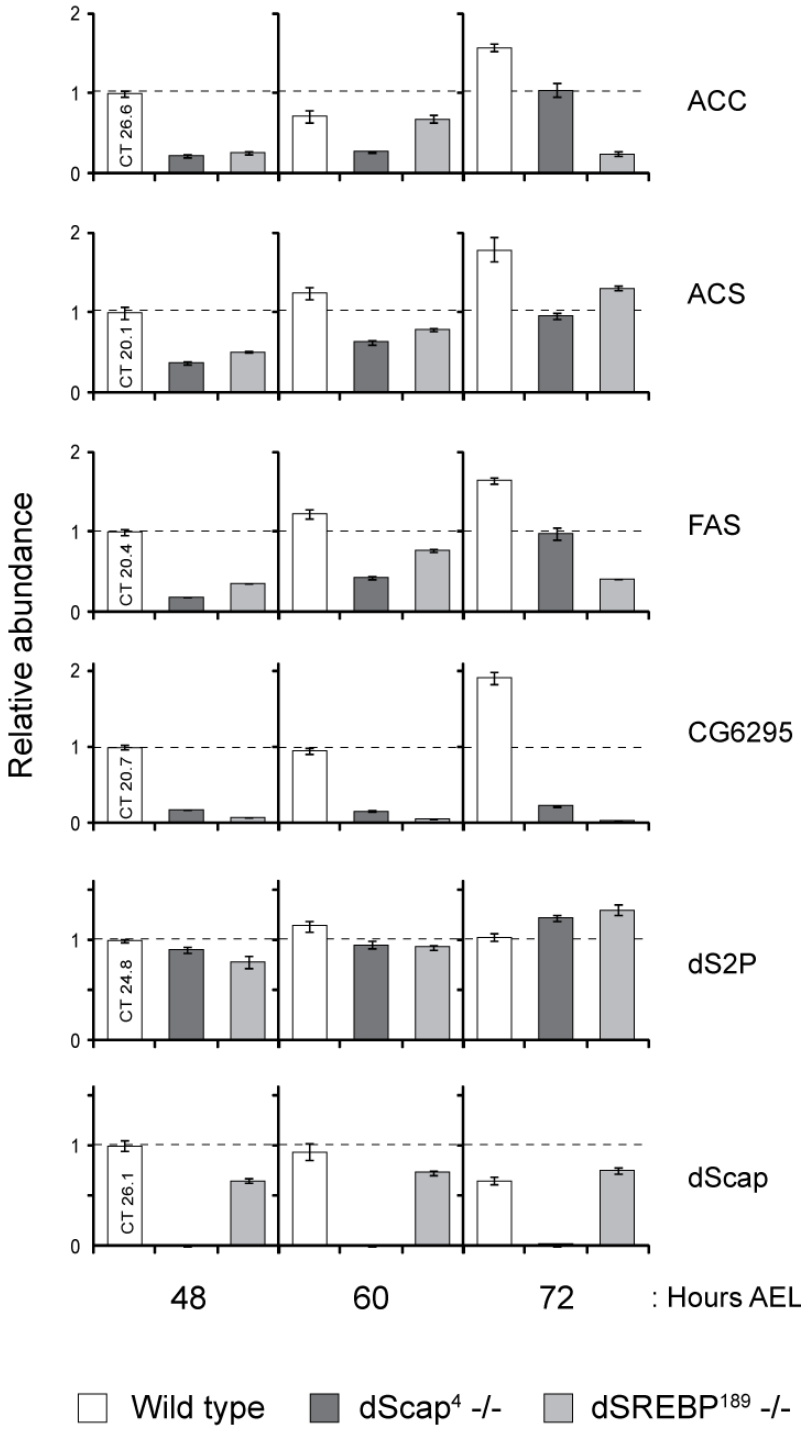

Relative abundance
ACC
ACS
FAS
CG6295
dS2P
dScap
CT 26.6
CT 20.1
CT 20.4
CT 20.7
CT 24.8
CT 26.1
0
1
2
48
60
72
: Hours AEL
Wild type
dScap4 -/-
dSREBP189 -/-

Figure 4-10

Quantitative real-time PCR analysis of mRNA from dScap mutant larvae. Embryos from either wild type, *dScap*^{4}/ *dScap*^{4}, or *dSREBP*^{189}/ *TM3*, *Ser*, act-GFP stocks were seeded (at 10 mg/dish) onto dishes containing semi-defined medium. Larvae were collected from one dish per time point and isolated from food by salt floatation. *dSREBP*^{189} homozgyotes were scored based on GFP fluorescence. Primers were previously described in (Kunte et al., 2006; Seegmiller et al., 2002). Abundance of transcripts was calculated relative to 48 hour wild type RNA levels.

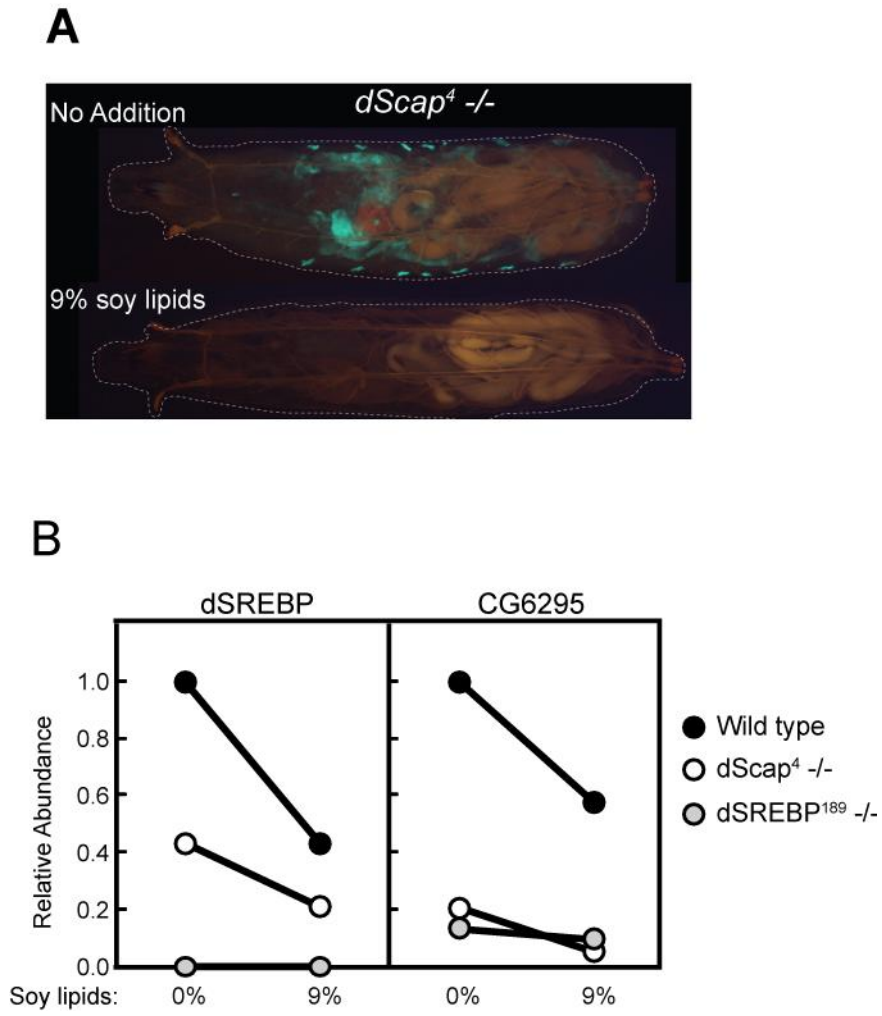


Figure 4-11

(A) Comparison of GFP fluorescence in *dScap*^{4} homozgyotes grown on semi-defined medium (upper panel) or medium supplemented with 9% soy lipids (Avanti Polar Lipids) (lower panel). Larvae are *dScap*^{4} / *dScap*^{4}; *P{GAL4-dSREBPg}, P{UAS-GFP}/ P{GAL4-dSREBPg}, P{UAS-GFP}*. Dashed lines denote extent of larval bodies. Both larva were photographed for GFP fluorescence at a 2.5 sec exposure. **(B)** Quantitative real time PCR analysis of dSREBP and CG6295 transcripts of wild type (black circles), dScap^{4}-/- (grey circles), and dSREBP^{189}-/- (white circles) larvae grown on semi-defined medium (0%) or medium supplemented with 9% soy lipids (9%). Transcript abundance was calculated relative to wild type larvae on 0% RNA levels.

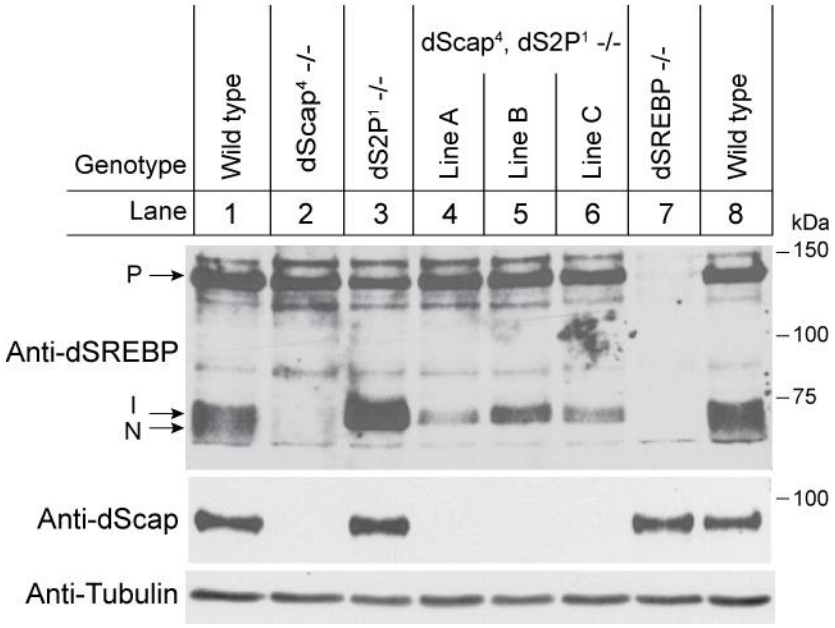


Figure 4-12

Immunoblot analysis of whole larval lysates from first instar larvae doubly mutant for dScap and dS2P. Sixty micrograms of total protein was run on a 7% SDS-PAGE gel. Membranes were probed overnight with anti-dSREBP or anti-dScap at 4˚. The dSREBP membrane was stripped and re-probed with anti-acetylated tubulin. P, precursor; I, intermediate form; N, nuclear form.

CHAPTER FIVE

Conclusions and Recommendations

THE ROLE OF S2P AND SCAP IN *DROSOPHILA*

Loss of dS2P and dScap

The first objective of my project was to determine whether *dS2P* and *dScap* are essential genes in *Drosophila*. To address this question, I generated null alleles of both *dS2P* (Figure 2-1A) and *dScap* (Figure 4-1A) in the whole fly. Surprisingly, flies deficient of either dS2P *or* dScap, or both dS2P *and* dScap are viable, in contrast to loss of dSREBP itself.

Effects on dSREBP processing

A second goal of my research was to determine the role of S2P and Scap in processing *Drosophila* SREBP. Contrary to the mammalian cells, dSREBP is still processed and transcriptionally active in the absence of dS2P and dScap. This was demonstrated using a GAL4-dSREBP reporter system to visualize active dSREBP in whole larvae. In $dS2P^{-}$ larvae (Figure 2-5), dSREBP activity was predominately detected in the fat body and oenocytes, whereas in $dScap^{-}$ larvae (Figure 4-8), dSREBP was mainly processed in the midgut and oenocytes. Cleavage of dSREBP in *dS2P* and *dScap* mutants is significantly reduced compared to wild type, but enough nuclear SREBP is generated to meet lipid

demands of the growing larva. These results were further supported by analysis of dSREBP target genes in both mutants. Transcripts of fatty acid synthetic genes in *dS2P* (Figure 2-6) and *dScap* (Figure 4-10) mutants are more abundant than in *dSREBP* mutants, but less so as compared to wild type. Together, these data show that the known SREBP processing machinery in dispensable in *Drosophila*.

In the *dS2P* mutants, we identified alternative cleavage of dSREBP by the effector caspase, Drice, during larval development. While dSREBP can be cleaved by Drice or Dcp1 *in vitro* (Figure 3-2), I demonstrate that *only* Drice is responsible for cleaving dSREBP in larvae. Larvae lacking both dS2P and Drice can not process SREBP and thus, exhibit early larval lethality similar to SREBP null larvae (Figure 3-5). Importantly, this lethality can be rescued by supplementation with fatty acids demonstrating that this lethality is due to lack of SREBP processing. Furthermore, I show that this Drice-dependent cleavage of dSREBP relies on the initiator caspase Dronc, as larvae lacking both *dS2P* and *Dronc* also exhibit an early larval lethality that can be rescued by fatty acid supplementation (Figure 3-5).

Effects on whole animal physiology

Larvae lacking dS2P or dScap exhibit a delay in development that results in homozygotes emerging approximately two days after their heterozygous siblings

(Figures 2-3A & 4-5B). Beginning around 48 hours AEL, homozygous mutant larvae begin to lag in their growth compared to wild type (Figures 2-2A & 4-5A). This is the same time period when dSREBP mutants arrest growth and die suggesting that this delay seen in *dS2P* and *dScap* mutants is likely to do inefficient processing of dSREBP. Surprisingly, lipid supplementation fails to rescue this delay. It is possible that 1) fatty acids do not substitute for all functions of dSREBP, 2) fatty acids are not the end product of dSREBP target genes and must be incorporated into other molecules, or 3) the absorption of fatty acids is not as efficient as synthesis by dSREBP target genes.

RECOMMENDATIONS AND FUTURE DIRECTIONS

Role of caspase cleavage of SREBP

The current data do address whether the Drice-dependent cleavage of SREBP seen in *dS2P* mutants is physiologically relevant in wild type larvae. The role of Drice in cell death during embryogenesis and pupation has been well characterized, but there is little data on non-apoptotic functions of Drice (and Dronc) in larval tissues. Kanuka et al. (2005) identified a non-apoptotic role of Drice in modulating Wingless signaling through substrate cleavage during neural precursor development in third instar larvae. In imaginal discs, Drice cleaves the kinase Shaggy at a DEVD motif located upstream of the kinase domain. Cleaved Shaggy actively phosphorylates Armadillo leading to its degradation and

preventing localization of the transcription factor Wingless from entering the nucleus.

What would be the role of caspase-dependent cleavage of dSREBP during larval development? We hypothesize that Drice cleavage of dSREBP bypasses the normal feedback regulatory mechanism thereby allowing the larva to accumulate and synthesize excess lipids needed to fuel metamorphosis. In accordance with our hypothesis, larvae expressing a version of dSREBP in which the caspase site has been mutated (P{*dSREBPg(D386A)*}/ P{*dSREBPg(D386A)*}; $dSREBP^{189}$/ $dSREBP^{189}$) should take longer to develop than larvae expressing wild type dSREBP (P{*dSREBPg*}/ P{*dSREBPg*}; $dSREBP^{189}$/ $dSREBP^{189}$).

The conservation of caspase cleavage sequences within the juxtamembrane region of *Drosophila* and mammalian SREBPs suggests some functionally important role for Drice in SREBP processing (Figure 5-1). If such a cleavage occurred in mammals then one would predict caspase cleavage of SREBP during embryogenesis and fetal development. Cholesterol and fatty acids are essential for fetal development, a period of rapid growth requiring lipids for membrane synthesis (reviewed in (Woollett, 2001)). Cholesterol is also required for activation of sonic hedgehog, a signaling molecule important in forebrain patterning (Woollett, 2001). The rates of lipid synthesis in the fetus are increased

compared to adults (Woollett, 2001). For example, cholesterol synthesis in the fetus is 3-4 fold higher than in adults to support new tissue growth (Belknap and Dietschy, 1988; Dietschy et al., 1993). Reduced lipid synthesis in the growing fetus is deleterious resulting in abnormal development and congenital defects (Woollett, 2001).

SREBPs are required for *de novo* lipid synthesis in the embryo and fetus (Woollett, 2001); loss of SREBP-1 or SREBP-2 in mice results in embryonic lethality (Shimano et al., 1997). In the human fetus, high levels of SREBP-1 and its target genes, FAS and ACC, are expressed in proliferative tissues including intestines, kidney, and skin (Wilentz et al., 2000). Similar to *Drosophila* larvae, lipid homeostasis in the fetus differs from the adult; the fetus requiring an excess of lipids compared to adults (Woollett, 2008). Accordingly, Yao et al. (2007) compared sterol synthesis and SREBP-2 activation in hamster adult and fetal tissues in response to dietary cholesterol. Dietary cholesterol completely suppressed sterol synthesis in adult tissues and partially suppressed rates in fetal tissues. However, SREBP-2 processing in fetal tissues was independent of dietary cholesterol levels in contrast to adult tissues. This constitutive activation of SREBP-2 results in part from the increased ratio of Scap to Insig protein in the fetus. It will be interesting to see if caspases also contribute to this constitutive cleavage of SREBPs in the fetus.

Role of dScap in regulating dSREBP cleavage

In mammals, Scap is required for the transport of SREBP from the ER to the Golgi. Furthermore, Insig exerts its regulation on SREBP through interactions with Scap. However, I have demonstrated that dScap is not required for SREBP processing in *Drosophila* larvae and no Insig-like protein has been identified to date. What then is the role of dScap in *Drosophila*?

Does dScap act as a lipid sensor? It is possible that dScap normally acts to regulate dSREBP transport and that the processing of dSREBP seen in the absence of dScap is a fortuitous survival mechanism. dScap transmembrane helices 2 through 6 contain a sterol sensing domain similar in sequence (47% identity) to mammalian Scap suggesting that dScap may play a role in sensing membrane lipids (Seegmiller et al., 2002). Dobrosotskaya et al. (2002) suggest that dScap senses the levels of phosotidylethanolamine, either directly or through variations is membrane curvature. Binding assays similar to those reported in Radhakrishnan et al. could provide useful information as to what lipids (specifically phosopholipids and fatty acids) interact with dScap and whether mammalian Scap, which directly binds cholesterol, can also interact with these other types of lipids.

The binding of cholesterol or Insig to mammalian Scap induces a conformational change in Scap which renders the COPII binding motif inaccessible (Adams et al., 2004; Brown et al., 2002; Sun et al., 2007). Do phospholipids, especially phosotidylethanolamine, induce a similar conformational change in dScap? Now that we have a useful antibody against dScap we can address this question using the trypsin-cleavage assay performed in (Brown et al., 2002). These data should provide clues as to if and how dScap senses membrane lipids. Furthermore, dScap does not contain a sequence similar to mammalian Scap's COPII 'MELADL' binding motif (Sun et al., 2005). Does dScap bind Sec24 or is there another protein that facilitates the entrance of dSREBP into COPII vesicles?

Lastly, are there other proteins that regulate SREBP processing in *Drosophila*? Though the *Drosophila* genome does not encode an Insig-like protein based on sequence homology, it is possible that other proteins interact with dScap to regulate the dScap:dSREBP complex. One could potentially identify proteins that interact with dScap by immunoprecipitation of dScap from S2 cells either treated under dSREBP-inducing or -suppressing conditions. Perhaps, more meaningful data could be gained by immunoprecipitation of dScap from larval lysates grown in the absence or presence of fatty acids. It might also be informative to attempt immunoprecipitation of GAL4-dSREBP with the

polyclonal VP16 antibody to identify proteins that interact with dSREBP's regulatory carboxyl terminus.

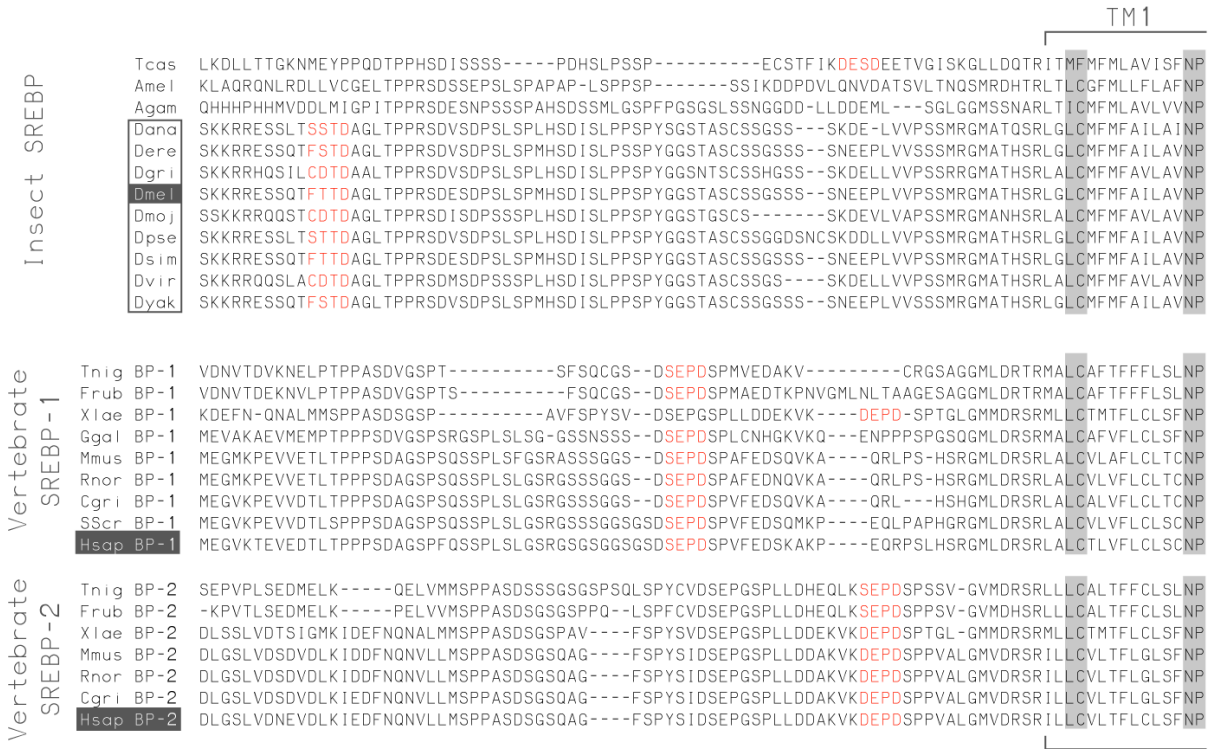


Figure 5-1.

Alignment of insect SREBP and vertebrate SREBP-1 and -2 sequences between the bHLH-zip and first transmembrane (TM1) domains. Sites of caspase cleavage are highlighted in red. 'LC' is the site of S2P cleavage within the first transmembrane. 'NP' are residues critical for proteolysis by S2P. Image modified from R. Rawson.

LITERATURE CITED

Adams, C.M., Reitz, J., De Brabander, J.K., Feramisco, J.D., Li, L., Brown, M.S., and Goldstein, J.L. (2004). Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. J Biol Chem *279*, 52772-52780.

Algeciras-Schimnich, A., Barnhart, B.C., and Peter, M.E. (2002). Apoptosis-independent functions of killer caspases. Curr Opin Cell Biol *14*, 721-726.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. Cell *87*, 171.

Amemiya-Kudo, M., Shimano, H., Yoshikawa, T., Yahagi, N., Hasty, A.H., Okazaki, H., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., *et al.* (2000). Promoter analysis of the mouse sterol regulatory element-binding protein-1c gene. J Biol Chem *275*, 31078-31085.

Backhaus, B., Sulkowski, E., Scholte, F.W. (1984). A semi-synthetic, general-purpose medium for Drosophila melanogaster. Drosphila Information Service *60*, 210-212.

Baker, K.D., and Thummel, C.S. (2007). Diabetic larvae and obese flies-emerging studies of metabolism in Drosophila. Cell Metab *6*, 257-266.

Belden, W.J., and Barlowe, C. (2001). Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. Science *294*, 1528-1531.

Belknap, W.M., and Dietschy, J.M. (1988). Sterol synthesis and low density lipoprotein clearance in vivo in the pregnant rat, placenta, and fetus. Sources for tissue cholesterol during fetal development. J Clin Invest *82*, 2077-2085.

Bengoechea-Alonso, M.T., and Ericsson, J. (2007). SREBP in signal transduction: cholesterol metabolism and beyond. Curr Opin Cell Biol *19*, 215-222.

Brown, A.J., Sun, L., Feramisco, J.D., Brown, M.S., and Goldstein, J.L. (2002). Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. Mol Cell *10*, 237-245.

Brown, M.S., and Goldstein, J.L. (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell *89*, 331-340.

Brown, M.S., Ye, J., Rawson, R.B., and Goldstein, J.L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell *100*, 391-398.

Chen, P., Nordstrom, W., Gish, B., and Abrams, J.M. (1996). grim, a novel cell death gene in Drosophila. Genes Dev *10*, 1773-1782.

Cherry, S., Kunte, A., Wang, H., Coyne, C., Rawson, R.B., and Perrimon, N. (2006). COPI Activity Coupled with Fatty Acid Biosynthesis Is Required for Viral Replication. PLoS Pathog *2*.

Chew, S.K., Akdemir, F., Chen, P., Lu, W.J., Mills, K., Daish, T., Kumar, S., Rodriguez, A., and Abrams, J.M. (2004). The apical caspase dronc governs programmed and unprogrammed cell death in Drosophila. Dev Cell *7*, 897-907.

Church, R.B., and Robertson, F.W. (1966). Biochemical analysis of genetic differences in the growth of Drosophila. Genet Res *7*, 383-407.

Clark, A.G., Eisen, M.B., Smith, D.R., Bergman, C.M., Oliver, B., Markow, T.A., Kaufman, T.C., Kellis, M., Gelbart, W., Iyer, V.N., *et al.* (2007). Evolution of genes and genomes on the Drosophila phylogeny. Nature *450*, 203-218.

Clark, A.J., and Bloch, K. (1959). Absence of Sterol Biosynthesis in Insects. J Biol Chem *234*, 2578-2588.

Consortium (2003). The FlyBase database of the Drosophila genome projects and community literature. Nucleic Acids Res *31*, 172-175.

Dev, K.K., Chatterjee, S., Osinde, M., Stauffer, D., Morgan, H., Kobialko, M., Dengler, U., Rueeger, H., Martoglio, B., and Rovelli, G. (2006). Signal peptide peptidase dependent cleavage of type II transmembrane substrates releases intracellular and extracellular signals. Eur J Pharmacol *540*, 10-17.

Dietschy, J.M., Turley, S.D., and Spady, D.K. (1993). Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. J Lipid Res *34*, 1637-1659.

Dobrosotskaya, I.Y., Seegmiller, A.C., Brown, M.S., Goldstein, J.L., and Rawson, R.B. (2002). Regulation of SREBP processing and membrane lipid production by phospholipids in Drosophila. Science *296*, 879-883.

Dorstyn, L., Colussi, P.A., Quinn, L.M., Richardson, H., and Kumar, S. (1999). DRONC, an ecdysone-inducible Drosophila caspase. Proc Natl Acad Sci U S A *96*, 4307-4312.

Duncan, E.A., Dave, U.P., Sakai, J., Goldstein, J.L., and Brown, M.S. (1998). Second-site cleavage in sterol regulatory element-binding protein occurs at transmembrane junction as determined by cysteine panning. J Biol Chem *273*, 17801-17809.

Engelking, L.J., Evers, B.M., Richardson, J.A., Goldstein, J.L., Brown, M.S., and Liang, G. (2006). Severe facial clefting in Insig-deficient mouse embryos caused by sterol accumulation and reversed by lovastatin. J Clin Invest *116*, 2356-2365.

Espenshade, P.J., and Hughes, A.L. (2007). Regulation of sterol synthesis in eukaryotes. Annu Rev Genet *41*, 401-427.

Espenshade, P.J., Li, W.P., and Yabe, D. (2002). Sterols block binding of COPII proteins to SCAP, thereby controlling SCAP sorting in ER. Proc Natl Acad Sci U S A *99*, 11694-11699.

Feng, L., Yan, H., Wu, Z., Yan, N., Wang, Z., Jeffrey, P.D., and Shi, Y. (2007). Structure of a site-2 protease family intramembrane metalloprotease. Science *318*, 1608-1612.

Fraser, A.G., and Evan, G.I. (1997). Identification of a Drosophila melanogaster ICE/CED-3-related protease, drICE. EMBO J *16*, 2805-2813.

Goldstein, J.L., Rawson, R.B., and Brown, M.S. (2002a). Mutant mammalian cells as tools to delineate the sterol regulatory element-binding protein pathway for feedback regulation of lipid synthesis. Arch Biochem Biophys *397*, 139-148.

Goldstein, J.L., Rawson, R.B., and Brown, M.S. (2002b). Mutant mammalian cells as tools to delineate the sterol regulatory element-binding protein pathway for feedback regulation of lipid synthesis. Arch Biochem Biophys *397*, 139-148.

Golic, K.G., and Lindquist, S. (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. Cell *59*, 499-509.

Golic, M.M., Rong, Y.S., Petersen, R.B., Lindquist, S.L., and Golic, K.G. (1997). FLP-mediated DNA mobilization to specific target sites in Drosophila chromosomes. Nucleic Acids Res *25*, 3665-3671.

Gutierrez, E., Wiggins, D., Fielding, B., and Gould, A.P. (2007). Specialized hepatocyte-like cells regulate Drosophila lipid metabolism. Nature *445*, 275-280.

Hawkins, C.J., Yoo, S.J., Peterson, E.P., Wang, S.L., Vernooy, S.Y., and Hay, B.A. (2000). The Drosophila caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. J Biol Chem *275*, 27084-27093.

Higgins, M.E., and Ioannou, Y.A. (2001a). Apoptosis-induced release of mature sterol regulatory element-binding proteins activates sterol-responsive genes. J Lipid Res *42*, 1939-1946.

Higgins, M.E., and Ioannou, Y.A. (2001b). Apoptosis-induced release of mature sterol regulatory element-binding proteins activates sterol-responsive genes. J Lipid Res *42*, 1939-1946.

Hooper, N.M., and Lendeckel, U. (2007). Intramembrane-Cleaving Proteases (I-Clips). Springer, Dordrecht.

Horton, J.D., Goldstein, J.L., and Brown, M.S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest *109*, 1125-1131.

Horton, J.D., Shimomura, I., Brown, M.S., Hammer, R.E., Goldstein, J.L., and Shimano, H. (1998). Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. J Clin Invest *101*, 2331-2339.

Hua, X., Nohturfft, A., Goldstein, J.L., and Brown, M.S. (1996). Sterol resistance in CHO cells traced to point mutation in SREBP cleavage-activating protein. Cell *87*, 415-426.

Huh, J.R., Vernooy, S.Y., Yu, H., Yan, N., Shi, Y., Guo, M., and Hay, B.A. (2004). Multiple apoptotic caspase cascades are required in nonapoptotic roles for Drosophila spermatid individualization. PLoS Biol *2*, E15.

Kanuka, H., Kuranaga, E., Takemoto, K., Hiratou, T., Okano, H., and Miura, M. (2005). Drosophila caspase transduces Shaggy/GSK-3beta kinase activity in neural precursor development. EMBO J *24*, 3793-3806.

Kinch, L.N., Ginalski, K., and Grishin, N.V. (2006). Site-2 protease regulated intramembrane proteolysis: sequence homologs suggest an ancient signaling cascade. Protein Sci *15*, 84-93.

Kunte, A.S., Matthews, K.A., and Rawson, R.B. (2006). Fatty acid auxotrophy in Drosophila larvae lacking SREBP. Cell Metab *3*, 439-448.

Kuriyama, H., Liang, G., Engelking, L.J., Horton, J.D., Goldstein, J.L., and Brown, M.S. (2005). Compensatory increase in fatty acid synthesis in adipose tissue of mice with conditional deficiency of SCAP in liver. Cell Metab *1*, 41-51.

Laundrie, B., Peterson, J.S., Baum, J.S., Chang, J.C., Fileppo, D., Thompson, S.R., and McCall, K. (2003). Germline cell death is inhibited by P-element insertions disrupting the dcp-1/pita nested gene pair in Drosophila. Genetics *165*, 1881-1888.

Lee, M.C., Miller, E.A., Goldberg, J., Orci, L., and Schekman, R. (2004). Bi-directional protein transport between the ER and Golgi. Annu Rev Cell Dev Biol *20*, 87-123.

Lee, P.C., Sever, N., and Debose-Boyd, R.A. (2005). Isolation of sterol-resistant Chinese hamster ovary cells with genetic deficiencies in both Insig-1 and Insig-2. J Biol Chem *280*, 25242-25249.

Limanek, J.S., Chin, J., and Chang, T.Y. (1978). Mammalian cell mutant requiring cholesterol and unsaturated fatty acid for growth. Proc Natl Acad Sci U S A *75*, 5452-5456.

Malkus, P., Jiang, F., and Schekman, R. (2002). Concentrative sorting of secretory cargo proteins into COPII-coated vesicles. J Cell Biol *159*, 915-921.

Matsuda, M., Korn, B.S., Hammer, R.E., Moon, Y.A., Komuro, R., Horton, J.D., Goldstein, J.L., Brown, M.S., and Shimomura, I. (2001). SREBP cleavage-activating protein (SCAP) is required for increased lipid synthesis in liver induced by cholesterol deprivation and insulin elevation. Genes Dev *15*, 1206-1216.

Matthews, K.A., Kunte, A., Ebot-Tambe, E., and Rawson, R.B. (2008). Alternative processing of SREBP during larval development.

Matthews, K.A., Kunte, A., Ebot-Tambe, E., and Rawson, R.B. (2008). Alternative processing of SREBP during larval development.

Meier, P., Silke, J., Leevers, S.J., and Evan, G.I. (2000). The Drosophila caspase DRONC is regulated by DIAP1. EMBO J *19*, 598-611.

Muro, I., Berry, D.L., Huh, J.R., Chen, C.H., Huang, H., Yoo, S.J., Guo, M., Baehrecke, E.H., and Hay, B.A. (2006). The Drosophila caspase Ice is important for many apoptotic cell deaths and for spermatid individualization, a nonapoptotic process. Development *133*, 3305-3315.

Pai, J.T., Brown, M.S., and Goldstein, J.L. (1996). Purification and cDNA cloning of a second apoptosis-related cysteine protease that cleaves and activates sterol regulatory element binding proteins. Proc Natl Acad Sci U S A *93*, 5437-5442.

Putnam, N.H., Srivastava, M., Hellsten, U., Dirks, B., Chapman, J., Salamov, A., Terry, A., Shapiro, H., Lindquist, E., Kapitonov, V.V., *et al.* (2007). Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. Science *317*, 86-94.

Radhakrishnan, A., Ikeda, Y., Kwon, H.J., Brown, M.S., and Goldstein, J.L. (2007). Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. Proc Natl Acad Sci U S A *104*, 6511-6518.

Radhakrishnan, A., Sun, L.P., Kwon, H.J., Brown, M.S., and Goldstein, J.L. (2004). Direct binding of cholesterol to the purified membrane region of SCAP: mechanism for a sterol-sensing domain. Mol Cell *15*, 259-268.

Rawson, R.B. (2003). The SREBP pathway--insights from Insigs and insects. Nat Rev Mol Cell Biol *4*, 631-640.

Rawson, R.B., Cheng, D., Brown, M.S., and Goldstein, J.L. (1998). Isolation of cholesterol-requiring mutant Chinese hamster ovary cells with defects in cleavage of sterol regulatory element-binding proteins at site 1. J Biol Chem *273*, 28261-28269.

Rawson, R.B., DeBose-Boyd, R., Goldstein, J.L., and Brown, M.S. (1999). Failure to cleave sterol regulatory element-binding proteins (SREBPs) causes cholesterol auxotrophy in Chinese hamster ovary cells with genetic absence of SREBP cleavage-activating protein. J Biol Chem *274*, 28549-28556.

Rawson, R.B., Zelenski, N.G., Nijhawan, D., Ye, J., Sakai, J., Hasan, M.T., Chang, T.Y., Brown, M.S., and Goldstein, J.L. (1997). Complementation cloning of S2P, a gene encoding a putative metalloprotease required for intramembrane cleavage of SREBPs. Mol Cell *1*, 47-57.

Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K., and Engels, W.R. (1988). A stable genomic source of P element transposase in Drosophila melanogaster. Genetics *118*, 461-470.

Rudner, D.Z., Fawcett, P., and Losick, R. (1999). A family of membrane-embedded metalloproteases involved in regulated proteolysis of membrane-associated transcription factors. Proc Natl Acad Sci U S A *96*, 14765-14770.

Sadowski, P.D. (1995). The Flp recombinase of the 2-microns plasmid of Saccharomyces cerevisiae. Prog Nucleic Acid Res Mol Biol *51*, 53-91.

Sakai, J., Duncan, E.A., Rawson, R.B., Hua, X., Brown, M.S., and Goldstein, J.L. (1996). Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment. Cell *85*, 1037-1046.

Sakai, J., Nohturfft, A., Goldstein, J.L., and Brown, M.S. (1998). Cleavage of sterol regulatory element-binding proteins (SREBPs) at site-1 requires interaction with SREBP cleavage-activating protein. Evidence from in vivo competition studies. J Biol Chem *273*, 5785-5793.

Sang, J.H. (1956). The quantitative nutritional requirements of Drosophila melanogaser. J Expt Biol *33*, 45-72.

Sato, R., Inoue, J., Kawabe, Y., Kodama, T., Takano, T., and Maeda, M. (1996). Sterol-dependent transcriptional regulation of sterol regulatory element-binding protein-2. J Biol Chem *271*, 26461-26464.

Seegmiller, A.C., Dobrosotskaya, I., Goldstein, J.L., Ho, Y.K., Brown, M.S., and Rawson, R.B. (2002). The SREBP pathway in Drosophila: regulation by palmitate, not sterols. Dev Cell *2*, 229-238.

Shimano, H., Horton, J.D., Hammer, R.E., Shimomura, I., Brown, M.S., and Goldstein, J.L. (1996). Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. J Clin Invest *98*, 1575-1584.

Shimano, H., Shimomura, I., Hammer, R.E., Herz, J., Goldstein, J.L., Brown, M.S., and Horton, J.D. (1997). Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a targeted disruption of the SREBP-1 gene. J Clin Invest *100*, 2115-2124.

Snipas, S.J., Drag, M., Stennicke, H.R., and Salvesen, G.S. (2008). Activation mechanism and substrate specificity of the Drosophila initiator caspase DRONC. Cell Death Differ *15*, 938-945.

Sun, L.P., Li, L., Goldstein, J.L., and Brown, M.S. (2005). Insig required for sterol-mediated inhibition of Scap/SREBP binding to COPII proteins in vitro. J Biol Chem *280*, 26483-26490.

Sun, L.P., Seemann, J., Goldstein, J.L., and Brown, M.S. (2007). Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Insig renders sorting signal in Scap inaccessible to COPII proteins. Proc Natl Acad Sci U S A *104*, 6519-6526.

Theopold, U., Ekengren, S., and Hultmark, D. (1996). HLH106, a Drosophila transcription factor with similarity to the vertebrate sterol responsive element binding protein. Proc Natl Acad Sci U S A *93*, 1195-1199.

Thibault, S.T., Singer, M.A., Miyazaki, W.Y., Milash, B., Dompe, N.A., Singh, C.M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H.L., *et al.* (2004). A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat Genet *36*, 283-287.

Vicario, S., Moriyama, E.N., and Powell, J.R. (2007). Codon usage in twelve species of Drosophila. BMC Evol Biol *7*, 226.

Wang, X., Briggs, M.R., Hua, X., Yokoyama, C., Goldstein, J.L., and Brown, M.S. (1993). Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. II. Purification and characterization. J Biol Chem *268*, 14497-14504.

Wang, X., Pai, J.T., Wiedenfeld, E.A., Medina, J.C., Slaughter, C.A., Goldstein, J.L., and Brown, M.S. (1995). Purification of an interleukin-1 beta converting enzyme-related cysteine protease that cleaves sterol regulatory element-binding proteins between the leucine zipper and transmembrane domains. J Biol Chem *270*, 18044-18050.

Wang, X., Zelenski, N.G., Yang, J., Sakai, J., Brown, M.S., and Goldstein, J.L. (1996). Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis. EMBO J *15*, 1012-1020.

Wee, L.J., Tan, T.W., and Ranganathan, S. (2006). SVM-based prediction of caspase substrate cleavage sites. BMC Bioinformatics *7 Suppl 5*, S14.

Weihofen, A., Binns, K., Lemberg, M.K., Ashman, K., and Martoglio, B. (2002). Identification of signal peptide peptidase, a presenilin-type aspartic protease. Science *296*, 2215-2218.

Wilentz, R.E., Witters, L.A., and Pizer, E.S. (2000). Lipogenic enzymes fatty acid synthase and acetyl-coenzyme A carboxylase are coexpressed with sterol regulatory element binding protein and Ki-67 in fetal tissues. Pediatr Dev Pathol *3*, 525-531.

Woollett, L.A. (2001). The origins and roles of cholesterol and fatty acids in the fetus. Curr Opin Lipidol *12*, 305-312.

Woollett, L.A. (2008). Where does fetal and embryonic cholesterol originate and what does it do? Annu Rev Nutr *28*, 97-114.

Xu, D., Wang, Y., Willecke, R., Chen, Z., Ding, T., and Bergmann, A. (2006). The effector caspases drICE and dcp-1 have partially overlapping functions in the apoptotic pathway in Drosophila. Cell Death Differ *13*, 1697-1706.

Yabe, D., Xia, Z.P., Adams, C.M., and Rawson, R.B. (2002). Three mutations in sterol-sensing domain of SCAP block interaction with insig and render SREBP cleavage insensitive to sterols. Proc Natl Acad Sci U S A *99*, 16672-16677.

Yang, J., Goldstein, J.L., Hammer, R.E., Moon, Y.A., Brown, M.S., and Horton, J.D. (2001). Decreased lipid synthesis in livers of mice with disrupted Site-1 protease gene. Proc Natl Acad Sci U S A *98*, 13607-13612.

Yang, T., Espenshade, P.J., Wright, M.E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J.L., and Brown, M.S. (2002). Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell *110*, 489-500.

Yao, L., Jenkins, K., Horn, P.S., Lichtenberg, M.H., and Woollett, L.A. (2007). Inability to fully suppress sterol synthesis rates with exogenous sterol in embryonic and extraembyronic fetal tissues. Biochim Biophys Acta *1771*, 1372-1379.

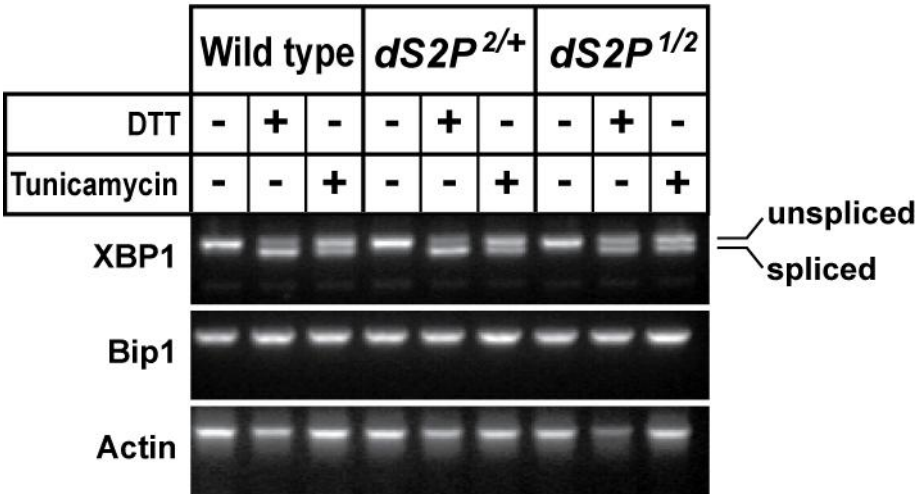
Ye, J., Dave, U.P., Grishin, N.V., Goldstein, J.L., and Brown, M.S. (2000a). Asparagine-proline sequence within membrane-spanning segment of SREBP triggers intramembrane cleavage by site-2 protease. Proc Natl Acad Sci U S A *97*, 5123-5128.

Ye, J., Rawson, R.B., Komuro, R., Chen, X., Dave, U.P., Prywes, R., Brown, M.S., and Goldstein, J.L. (2000b). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell *6*, 1355-1364.

Yoshida, H., Nadanaka, S., Sato, R., and Mori, K. (2006). XBP1 is critical to protect cells from endoplasmic reticulum stress: evidence from Site-2 protease-deficient Chinese hamster ovary cells. Cell Struct Funct *31*, 117-125.

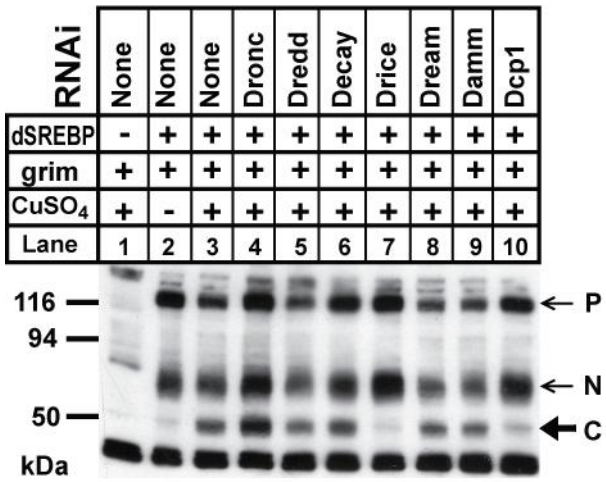
Zelenski, N.G., Rawson, R.B., Brown, M.S., and Goldstein, J.L. (1999). Membrane topology of S2P, a protein required for intramembranous cleavage of sterol regulatory element-binding proteins. J Biol Chem *274*, 21973-21980.

APPENDIX



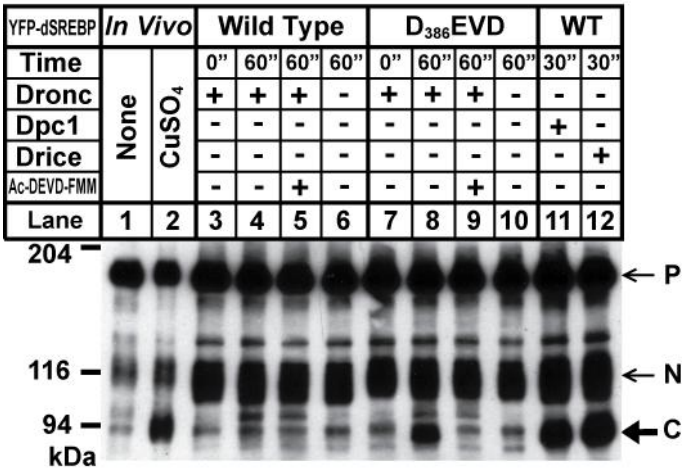
Supplemental Figure 2-1

Activation of the UPR in S2P homozygotes is similar to wild type and heterozygous siblings. Treatment with DTT or tunicamycin induces splicing of XBP1 by Ire1. Third instar larvae were partially dissected to expose tissues in Schneider's media with 10% serum and 1% pen/strep. Larval tissues were then transferred to eppendorf tubes and incubated in Schneider's medium (10% serum and 1% pen/strep) with either 0.5 mM DTT or 10 ug/ml tunicamycin. After six hours, tissues were rinsed with 1x PBS. Total RNA was insolated using RNA-STAT 60 (Tel-Test, Inc.) according to manufacturer's procedures and cDNA was prepared using Superscript First-Strand Synthesis kit (Invitrogen). XBP-1 and actin were amplified using primers described in Plongthongkum et al. 2007.



Supplemental Figure 3-1

Drosophila S2 cells transfected with MTAL-grim were treated with double stranded RNAs targeting the indicated caspase. Apoptosis was induced by addition of $CuSO_4$ to the cultures. Whole cell lysates were prepared and subjected to Immunoblot analysis using IgG-3B2 , against the amino terminus of dSREBP (Seegmiller et al., 2002). P, precursor; N, normal nuclear form; C, caspase-dependent band.



Supplemental Figure 3-2.

Cleavage of dSREBP($D_{386}A$) in vitro. Membrane fractions were prepared from S2 cells transfected with the indicated plasmids and incubated with the indicated enzyme in caspase buffer for the times indicated. Samples shown in lanes 5 and 9 were treated with the caspase inhibitor Ac-DEVD-FMM at the time of enzyme addition. Whole cell lysates from S2 cells transfected with YFP-dSREBP and MTAL-grim (lanes 1 and 2) are shown for comparison of cleaved fragments. Samples were subjected to Immunoblot analysis using anti-HSV antibody as described in Materials and Methods. P, precursor; N, normal nuclear form; C, caspase-dependent band. Incubation of membranes containing wild type dSREBP with either Drice or Dcp1 resulted in substantial accumulation of fragment C (lanes 11 and 12). No accumulation of fragment C was observed with purified Dronc (lane 4). When we introduced a typical caspase cleavage site motif into the juxtamembrane region (F_{383}TTDAGLT was mutated to F_{383}TTDEVD), incubation with Dronc resulted in accumulation of fragment C (lane 8), confirming that the enzyme had activity in this assay. Dronc can cleave Ac-DEVD-AFC (Dorstyn et al., 1999; Hawkins et al., 2000), although it cleaves Ac-TQTE-AFC and Ac-LALD-AFC with greater efficiency (Hawkins et al., 2000; Snipas et al., 2008). With the mutant, Dronc-cleavable, substrate, accumulation of fragment C was blocked by the addition of the caspase inhibitor Ac-DEVD-FMK (lane 9). Recombinant Dronc can cleave a version of dSREBP containing a motif that Dronc has been shown to cleave.