

CHARACTERIZATION OF *DROSOPHILA* SCAP:
ANALYSIS OF MUTANTS AND EVIDENCE FOR A RETENTION FACTOR

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To my parents

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by

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The SREBP pathway is one of the major regulators of lipid homeostasis and it is highly conserved among metazoans. SREBP is a transcription factor whose precursor is an endoplasmic reticulum (ER) transmembrane protein. In order to be activated it must travel to the Golgi apparatus via interaction with an escort protein, Scap. Scap, in turn can interact with components of the coatamer protein complex II (COPII) when lipid levels fall. In the Golgi, SREBP is cleaved sequentially by two proteases, S1P and S2P. By contrast to mammalian cells, which cannot survive without S2P or Scap, flies lacking Scap or S2P can activate SREBP. These mutants survive owing to non-canonical mechanisms of SREBP activation.

Scap has a intrinsic tendency to travel to Golgi. In vertebrates, the ER retention factor, Insig, anchors the Scap:SREBP complex to the ER membrane when *de novo* lipid synthesis is not required. In *Drosophila* dSREBP pathway there is no Insig orthologues. However, our data suggest that there should be an analogous component that retains dScap in the ER.

In order to discover the putative retention factor and other modifiers of the dSREBP, I set up a high through-put genome-wide screen. Employing luciferase as reporter, knocking-down each gene in genome through RNA interference will reveal the genes that modulate the activity of dSREBP.

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LIST OF DEFINITIONS

Balancer: a chromosome which has multiple nested inversion mutations and carrying visible marker mutations that permit the identification of flies harboring the balancer by visual inspection. These multiple inversions suppress recombination.

BSA: Bovine serum albumin.

Deficiency: An allele that lacks multiple loci due to large deletion

Null: A mutation that totally abolishes the function of a gene.

CHAPTER ONE

Introduction

All cells are defined by lipid-bilayer membranes. By rendering the cell a closed system, membranes make life thermodynamically possible. These membranes are essentially impermeable to polar molecules and ions. By contrast to prokaryotic cells, eukaryotes possess additional internal membrane systems that further sub-compartmentalize the cell. This additional compartmentalization helps the eukaryotic cell spatially organize its constituents, specialize each compartment for a certain function, and allow incompatible chemical reactions to occur under different conditions within the same cell. For example, extreme pH values (*e.g.* lysosomes), differing ionic concentrations or redox potential (*e.g.* ER).

The membranes are mostly made of phospholipids (Berg et al., 2002). A phospholipid molecule consists of a polar head group and two fatty acid molecules attached to glycerol backbone. In a typical phospholipid bilayer, water-seeking polar head groups are exposed to aqueous surroundings on both faces of the bilayer and the fatty acyl chains are oriented towards the water-fearing inner moiety.

In addition to the phospholipids found in almost all membranes, eukaryotic membranes also contain a distinct class of lipids called sterols. Sterols alter the permeability and fluidity of the bilayer. The main sterol in vertebrate membranes is cholesterol. Cholesterol decreases the void volume between phospholipid molecules, especially at the steroid ring region (Falck et al., 2004). The packing of unsaturated fatty acyl chains is

increased by cholesterol. However, the presence of cholesterol has the opposite effect for saturated fatty acyl chains. Thus cholesterol increases the temperature range over which the bilayer membrane is in a fluid phase. Cholesterol localizes to both the inner and outer leaflets of the membrane, perpendicular to the plane of bilayer. The 3 β -hydroxyl group is exposed to the hydrophilic surface and the iso-octyl side chain merges into the hydrophobic interior of the membrane. Because of its shape, cholesterol interacts tightly with acyl chains of membrane lipids. The composition of phospholipid bilayer determines the fluidity and permeability of the membrane. The ratio of saturated and unsaturated fatty acids in phospholipids and percentage of cholesterol are precisely adjusted based on the needs of the cell.

In addition to its structural role in cell membranes, cholesterol has several other vital functions. It is also a precursor molecule for steroid hormones, vitamin D, and bile acid synthesis. Cholesterol is essential for proper functioning of several G-protein coupled receptors (Burger et al., 2000; Paila and Chattopadhyay, 2010; Pucadyil and Chattopadhyay, 2006)

Cell membranes are dynamic structures. *De novo* synthesis of membranes is vital for survival and proliferation of the cells. Transport of molecules inside the cell or to the extracellular environment requires the formation of vesicles from plasma membrane (Hurley et al., 2010). Membrane-containing organelles such as the ER and the Golgi apparatus are likewise dynamic structure because of this vesicle trafficking. Also, prior to cell division, the cell membrane expands so that it can encompass the components of the daughter cells.

Besides these crucial structural and signaling functions, lipids are important sources of energy. Unlike carbohydrates which cells must store in a hydrated form, lipids are hydrophobic and do not mix with water. Whereas each gram of glucose stored as glycogen requires approximately 2 g of water along with it, lipids store energy essentially without water. Thus they store energy more efficiently than the carbohydrates on a mass/energy basis (Berg et al., 2002).

By definition, lipids readily dissolve in non-polar solvents but only sparingly in water. Within the aqueous environment of a cell, excess intracellular lipids aggregate to form protein-coated lipid droplets. Cholesterol overload of blood increases the risk of arteriosclerosis (Brown and Goldstein, 1983). However, if cells lack sufficient lipid, they are unable to synthesize membrane and may not be able to produce high amounts of energy. To complicate matters a little bit more, lipids also function as signaling molecules. As lipids are not encoded by genome directly, but synthesized *de novo* or supplied by diet, cells must tightly regulate their lipidogenic pathways and lipid intake.

THE SREBP PATHWAY

The sterol regulatory element binding protein (SREBP) pathway is a central regulator of lipid metabolism. This pathway is activated when cells need lipid and is inactive when lipid supplies are sufficient. SREBP is a member of the basic-helix-loop-helix-leucine zipper (bHLH-zip) family of transcription factors (although there are multiple forms of SREBP in mammals, I will use the singular here for clarity). It is synthesized as a precursor that is a transmembrane protein located in the ER and nuclear envelope (Brown

and Goldstein, 1997). In the ER membrane, the precursor adopts a conformation like that of a hairpin. Both the amino and carboxy terminal domains of SREBP are oriented towards the cytoplasm. These two cytoplasmic domains are linked via two membrane-spanning helices that are separated by a short luminal loop. There are critical amino acid sequences within the first transmembrane helix and the luminal loop that are required for activation of SREBP.

The amino terminal portion of SREBP is the transcription factor domain. The carboxy terminus of the precursor is a protein-protein interaction domain. Through this domain, SREBP binds to a polytopic ER membrane protein, SREBP cleavage activating protein, Scap. This interaction is crucial for the regulation of SREBP activity and thus this domain is referred to as the regulatory domain of SREBP.

The SREBP precursor is synthesized in the ER membrane and has no known activity as an intact protein. In order to be activated it must travel to the Golgi apparatus. The SREBP precursor is escorted from ER to Golgi via Scap.

The amino terminal portion of Scap has 8 transmembrane helices. Its carboxy terminus binds to the carboxy terminus of SREBP. Of Scap's eight membrane-spanning helices, helices 2-6 are designated as the Sterol Sensing Domain (SSD) (Hua et al., 1996a; Hua et al., 1996b). An SSD was first identified in the membrane anchor domain of HMG-CoA reductase. In the presence of cholesterol, the full-length form of this enzyme is degraded 10 times faster than other ER proteins. By contrast, a truncated form of the enzyme that

lacks the membrane anchor is much more stable and its degradation rate is independent of sterols (Gil et al., 1985). In addition to Scap and HMG-CoA reductase, Patched, Dispatched, and NPC-1 also have SSDs.

Interaction with Scap stabilizes the SREBP precursor in the ER membrane (Matsuda et al., 2001; Rawson et al., 1999). When the cell has sufficient lipid, the Scap:SREBP complex resides in ER membrane as an inactive complex due Scap's increased affinity towards an ER-resident protein, Insig (Yang et al., 2002)

When the cell requires *de novo* lipid synthesis, the Scap:SREBP complex travels from the ER to the Golgi via COPII-coated vesicles (Nohturfft et al., 1999). These vesicles bud from ER and move to Golgi where two proteases that target SREBP, site-1 protease (S1P) and site-2 protease (S2P), reside (Rawson et al., 1999; Sakai et al., 1998). S1P cleaves the SREBP precursor within the luminal loop that separates the two membrane-spanning helices. After that cleavage, the amino terminal domain becomes a substrate for S2P. S2P cleaves SREBP within the first membrane spanning helix and this cleavage likely occurs within the plane of the lipid bilayer (Rawson et al., 1997). With that cut, the transcription factor domain is released from the Golgi membrane and travels to nucleus. As a homodimer, SREBP binds to sterol regulatory element (SRE) sequences which are present in the promoter regions of the genes encoding the LDL receptor and more than 20 lipogenic genes, such as 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA Reductase, the enzyme that catalyzes the rate limiting step in cholesterol synthesis)

(Brown and Goldstein, 1999; Edwards and Ericsson, 1999; Horton and Shimomura, 1999; Kim and Spiegelman, 1996; Magana et al., 1997; Sul and Wang, 1998).

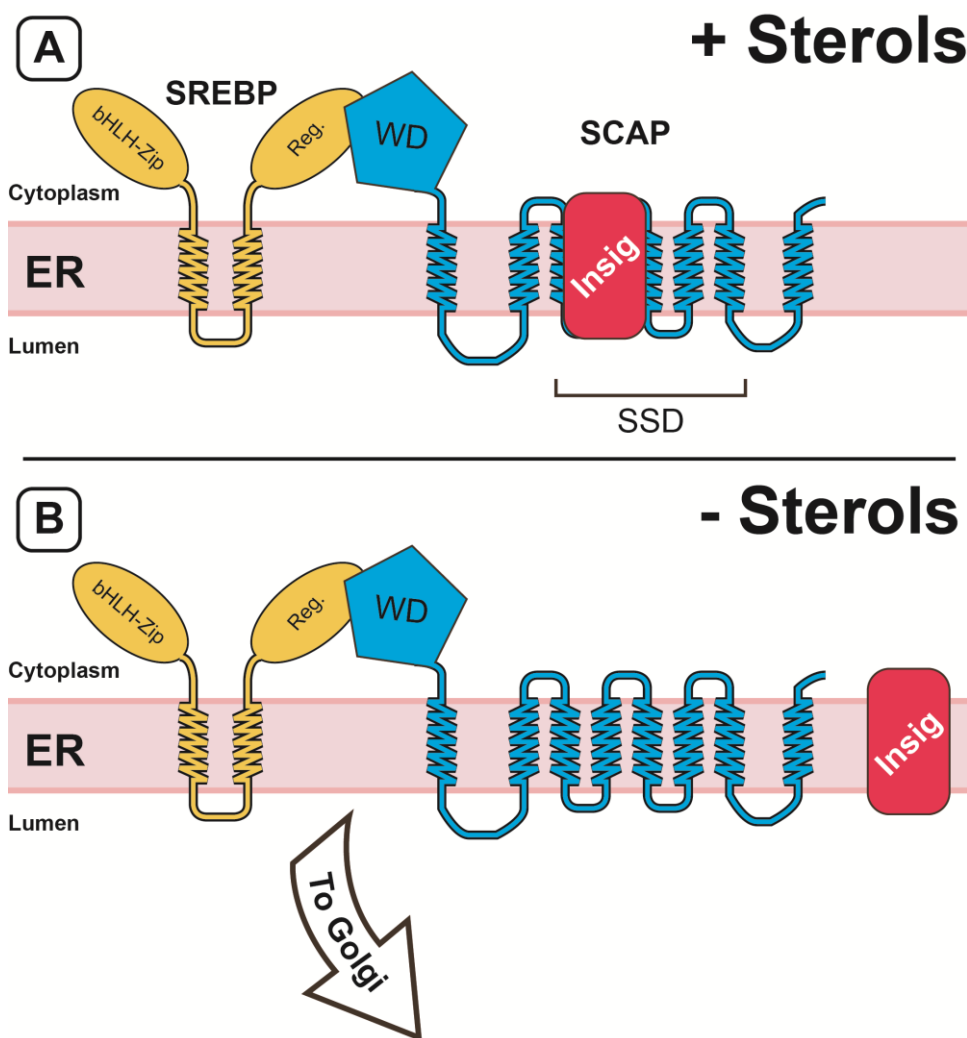


Figure 1. Schematic of SREBP pathway (A) Sterol regulatory element binding protein (SREBP) is an ER membrane protein interacting with SREBP-cleavage-activating protein (Scap). In the presence of sterols, complexes of SREBP and Scap are anchored in the endoplasmic reticulum (ER) membrane via an ER resident protein, Insig.

(B) In the absence of sterols, Scap does not bind to Insig. Scap mediates the interaction with COPII vesicle trafficking system and drags SREBP to the Golgi apparatus. bHLH-zip, basic helix-loop-helix leucine-zipper; Reg, regulatory; SSD, sterol-sensing domain; WD, aspartate-tryptophan motif. (Adapted from (Rawson, 2003))

REGULATION

SREBP pathway is a textbook example for end-product regulation of a metabolic pathway (Berg et al., 2002). The ultimate end products of the SREBP pathway are phospholipids and sterols. The ER membranes contain only about 1% of the total cellular cholesterol (Lange, 1991). Depletion of these lipids is sensed by Scap in the ER membrane. It makes a lot of sense to sense the cholesterol level in ER, for two reasons: first of all, cholesterol is scarce in the ER so that a small increase or decrease in sterol levels can be a many-fold change that may be detected easily; second, the ER membrane is also the host of many cholesterol-synthesizing enzymes.

The critical readout for a cell to switch-on cholesterol synthesis is the molar ratio of ER membrane cholesterol to other ER lipids (Radhakrishnan et al., 2008). When ER membrane cholesterol exceeds **5%** (moles of cholesterol/ moles of total ER lipid), Scap binds to Insig and resides in ER. If the cholesterol level is less than **5%**, Scap no longer binds to Insig. Instead, Scap undergoes a conformational change that exposes a cytoplasmic sequence (MELADL) that interacts with the COPII machinery. The COPII proteins Sec 23/24 bind to Scap and recruit the Scap:SREBP complex into COPII-coated vesicle (Espenshade et al., 2002). Thus, Scap escorts the SREBP precursor to the Golgi (Radhakrishnan et al., 2008).

Analysis of mutant Scap proteins revealed that the hexapeptide MELADL sequence on loop 7 is the recognition site for recruiting Scap into COPII-vesicles (Sun et al., 2005).

This recognition region for COPII vesicle transporting system functions as a switch for the SREBP pathway. The MELADL sequence is exposed to the cytoplasm when Scap is not bound to cholesterol (Espenshade et al., 2002). Cholesterol blocks access of the COPII machinery to the MELADL on loop 7 so that the SREBP: Scap resides in ER. The conformation change that is induced by cholesterol binding has been the subject of intense investigation (Adams et al., 2004; Brown et al., 2002; Radhakrishnan et al., 2008).

The Scap:SREBP complex is retained in ER via Scap's interaction with another ER membrane protein, Insig. Besides cholesterol binding to Scap, oxysterol binding to Insig induces Insig:Scap interaction (Radhakrishnan et al., 2007).

The spatial separation of SREBP and the SREBP cleaving enzymes (S1P and S2P) is maintained during mitosis (Bartz et al., 2008). Active S1P is kept in vesicular compartments that have Golgi matrix proteins. These are distinct from the vesicles that contain SREBP precursors.

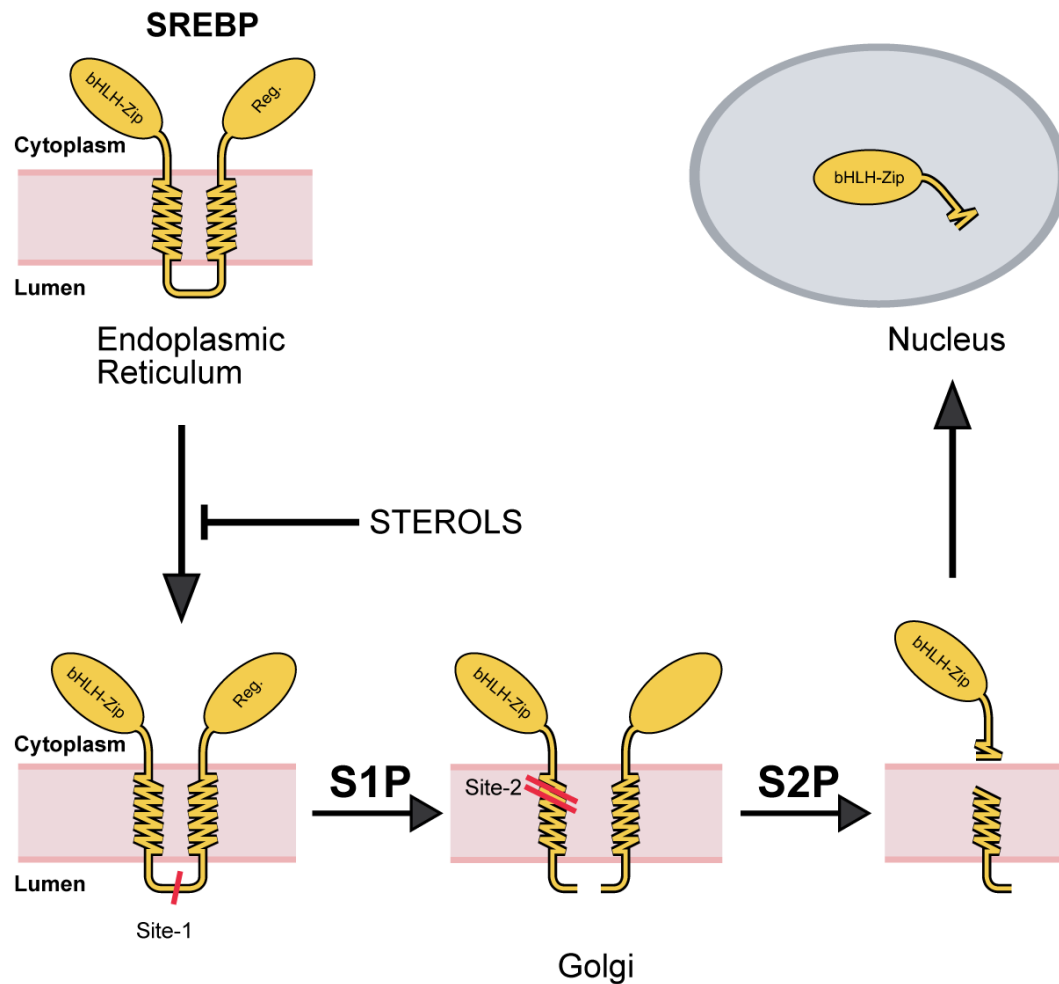


Figure 2: The processing of SREBP

The SREBP precursor is located in the endoplasmic reticulum (ER) membrane. Both the amino-terminal transcription-factor domain (bHLH-zip) and the carboxy-terminal regulatory domain (Reg) are located in the cytoplasmic compartment. When there is not a sufficient amount sterols in the ER membrane, the SREBP precursor protein travels to the Golgi apparatus, where the site-1

protease (S1P) cleaves at site-1 in the luminal loop (red line), producing the membrane-bound intermediate form. The intermediate form is the substrate for the site-2 protease (S2P), which cleaves the intermediate at site-2 (double red line), which is located three amino acids into the membrane-spanning helix. This second cleavage releases the transcription-factor domain from the membrane, freeing it to enter the nucleus and direct the increased transcription of target genes. bHLH-zip, basic helix–loop–helix leucine-zipper.
(Adapted from (Rawson, 2003))

S1P

The Site-1 protease (S1P) is one of the secretory proprotein convertases. The human genome encodes eight other members of this family of proteases. All of them are serine-type peptidases that are synthesized as zymogens that are activated by cleavage of an N-terminal peptide. Many of these proteases have functions in lipid metabolism. For example, PCSK9 (proprotein convertase subtilisin /kexin-like 9), destabilizes the low-density lipoprotein receptor (LDLR). S1P, on the other hand, mediates the transcriptional regulation of lipid metabolism via cleavage of SREBP. S1P cleaves the peptide bond of SREBP-2 between leucine and serine of the sequence R₅₁₉SVLS (Duncan et al., 1997). In addition to SREBP, S1P also cleaves activating transcription factor 6 (ATF6). ATF6 is a transcription factor whose precursor is ER transmembrane protein. In response to the ER stress ATF6 moves to Golgi where it is cleaved by S1P. Its N-terminal transcription factor domain goes to the nucleus and activates genes involved in the unfolded protein response (ER stress)

S2P

Following cleavage of the luminal loop of the SREBP precursor by S1P, S2P targets the newly-generated intermediate form of SREBP. S2P is a member of the S2P family of metalloproteases (M50 in MEROPS protein database (Harris *et al.*, 1991) (Harris *et al.*, 1991) of metallo-proteases. It was first characterized in CHO cells as a part of the cholesterol homeostasis machinery (Rawson *et al.*, 1997). It is an unusual metalloprotease because it cleaves peptide bonds that typically reside within the hydrophobic milieu of the membrane lipid bilayer. As reviewed by Chen and Zhang, many prokaryotes such as *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Caulobacter crescentus* also have S2P orthologs (Chen and Zhang, 2010).

INSIG

When Scap is overexpressed in mammalian cells, it has a tendency to move from ER to Golgi regardless of sterol content of the cell. It drags SREBP precursor along with it. This suggested presence of an anchor protein that retains Scap:SREBP in ER membrane. Further research has shown that Insig (Insulin-induced gene) is the anchor for the Scap:SREBP complex in ER membrane (Yang, *et al.*, 2002). Insig is a polytopic ER transmembrane protein. When cholesterol binds to Scap, Scap binds to Insig and remains in ER membrane (Radhakrishnan *et al.*, 2007). When Scap is overexpressed cholesterol treatment changes the conformation of Scap with the endogenous levels of Insig (Adams *et al.*, 2004). This experiment could not be performed using mutant CHO-derived cells which lack endogenous Insig because these cells are refractory to transfection.

Insig also binds to HMG-CoA reductase, which catalyzes the rate limiting step in mevalonate pathway. As for Scap, this interaction is via the sterol-sensing domain. Insig recruits the Endoplasmic Reticulum Associated Degradation (ERAD) components to the complex which results in degradation of HMG-CoA reductase and thus *de novo* cholesterol synthesis is inhibited.

Although the SREBP pathway exists in all the metazoans studied so far, Insig is known only from organisms that can synthesize cholesterol *de novo* (Rawson, unpublished observations). A possible exception is flatworms, though the only genome sequence available is for a parasitic form that may lack genes present in free-living flatworms. As I discussed above, lipid synthesis is essential for all animals and has to be strictly regulated. However invertebrates such as nematodes and arthropods (including *Drosophila*) do not have any protein similar in sequence to Insig.

THE MAMMALIAN SREBP PATHWAY

Historically, the consensus sites (SRE sequences) in the promoter regions of the LDL receptor and several lipogenic genes led to the search for a transcription factor that binds to them. From mammalian tissue culture, SREBPs were discovered (Briggs et al., 1993) (Tontonoz et al., 1993; Wang et al., 1993)

In mammalian genomes, there are two SREBP genes, namely *SREBP-1* and *SREBP-2*. Due to alternative splicing of exon1, the *SREBP-1* encodes two isoforms, SREBP-1a and SREBP-1c (Hua et al., 1995). SREBP-1a is a more potent transcriptional activator than -1c, which activates transcription of the genes related to *de novo* synthesis of fatty acid (phospholipids and triacylglycerols) such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Horton et al., 2002). SREBP-2 preferentially regulates the transcription of genes involved in cholesterol biosynthesis and cellular uptake, such as HMG-CoA synthase and HMG-CoA reductase (Horton et al., 1998; Pai et al., 1998) (Horton et al., 2002).

SREBP-2 is an essential gene. Homozygous *SREBP-2* knock-out mice are embryonic lethal. By contrast, *SREBP-1* nulls exhibit 30% of the expected survival due to a compensatory upregulation of SREBP-2 (Horton and Shimomura, 1999). Similarly mouse *SIP* knockouts are lethal at the very beginning of embryonic development (Mitchell et al., 2001; Yang et al., 2001)

Mice harboring a liver-specific knockout of SREBP-2 are viable. In the livers of these mice, SREBP-2 mRNA is decreased 80-90% and SREBP-2 protein is significantly decreased. The transcripts of SREBP2-regulated genes are also diminished as expected. As a result, hepatic cholesterol synthesis is cut down by 6-fold. Surprisingly, SREBP-1 mRNA and protein also decrease in addition to SREBP-2 mRNA and protein. Liver fatty acid synthesis decreases 6-fold (Jay Horton lab, unpublished data)

As in mammalian model organisms, Scap, S1P and S2P are essential components in cultured mammalian cells. Mutant CHO cells lacking any of those proteins cannot activate SREBP and cannot express the genes required for lipid synthesis and cellular uptake. Thus, these mutant cells can only survive in media supplemented with free cholesterol and unsaturated fatty acids (Goldstein et al., 2002).

This role in fatty acid and phospholipid synthesis is evolutionary conserved in all animals even in animals that cannot synthesize *de novo* sterols (Osborne and Espenshade, 2009)

In mammalian systems there are two Insig isoforms, Insig-1 and Insig-2. They are 59% identical in sequence and both have 6 membrane-spanning helices. Sterol treatment increases the half-life of insig-1 from less than 30 min to more than 2 hrs (Gong et al., 2006). Increased stability of Insig causes longer retention of the Scap:SREBP complex in the ER membrane. Thus, *de novo* sterol synthesis is inhibited when a cell has enough sterols. When cells do not have enough sterols, gp78, an E3 ubiquitin ligase that binds to Insig-1 and ubiquitinates it. This marks the protein for proteasome-mediated degradation and thus shortens the half life of Insig (Lee et al., 2006)

UBXD8

Unsaturated fatty acids have been shown to suppress SREBP pathway in mammalian cells (Hannah et al., 2001). Unsaturated fatty acids prevent polyubiquitinated Insig from degradation via keeping it in the ER membrane. Unsaturated fatty acid binds to Ubx8 so that it cannot mediate the interaction between p97 and polyubiquitinated Insig (Lee et al.,

2008). Ubx_{d8} functions as an intracellular long-chain fatty acid sensor and helps in inhibiting the SREBP pathway when the cell does not need *de novo* fatty acid synthesis.

DROSOPHILA AS A MODEL ORGANISM

Genetics offers the opportunity for researchers to get a mutation of interest and observe its effects in the context of a complex organism. The fruit fly, *Drosophila melanogaster*, provides a cost-efficient invertebrate model organism which has many strengths for genetic screens. Flies may be cultured in small volumes of vials in the lab. The *Drosophila* life cycle is only ten to fifteen days. The next generation is obtained in a comparatively short amount of time. This is a great advantage for genetic experiments. It is relatively easy to manipulate the fly genome with classical genetics and with recombinant DNA. Moreover, there are thousands of fly stocks available harboring mutations in over 70% of *Drosophila* genes (2003)

Compared to most vertebrates, *Drosophila* has less redundancy in its genome. In other words, for genes for which a mammalian system has redundant paralogs, *Drosophila* typically has only one ortholog. These characteristics make *Drosophila* a simpler but more effective system for certain experiments.

Balancer chromosomes are one of the most useful genetic tools in *Drosophila*. They are chromosomes that have several nested inversions. These mutations disrupt the synapsis

regions so that meiotic recombination is suppressed. Balancers are typically marked with a visible mutation and a lethal mutation, which may be the same mutation. This means that flies that are homozygous for the same balancer are not viable (Greenspan). This enables maintenance of lethal (or sterile) alleles of genes of interest, such as dSREBP (Kunte, *et al.*, 2006). Because neither the balancer nor the chromosome carrying the (lethal) gene of interest will survive as homozygotes, only heterozygotes are present in the population. Thus, the mutation of interest is balanced, in a Hardy-Weinberg sense. Together with the suppression of recombination, which prevents the emergence of a chromosome carrying wild-type copies of the two lethal mutations, this means that a lethal allele may be maintained in a fly population perpetually.

Although *Drosophila* research has resulted in the award of 4 Nobel Prizes (Morgan, 1933; Nusslein-Volhard, Wieschaus, and Lewis, 1995), it is just beginning to be appreciated as a model system for studying energy metabolism research (Baker and Thummel, 2007). Essentially the core components of energy metabolism are highly conserved from humans to worms and flies (Canavoso *et al.*, 2001; Gutierrez *et al.*, 2007; McKay *et al.*, 2003). As in mammals, sugar concentration in circulating body fluids is regulated tightly and energy is stored in glycogen and lipids (Palanker *et al.*, 2009). A marvelous study in 2007 has provided a new perspective on the relationship between human liver and insect oenocytes. In that study Gutierrez *et al.*, discovered that insect oenocytes (which are first known as a 6-cell cluster that synthesize and secrete hydrocarbon (Demerec, 1950)) have striking similarities to hepatocytes (Gutierrez *et al.*, 2007). Orthologs of more than 22 hepatocyte-specific enzymes are expressed in insect

oenocytes. Even the differentiation of hepatocytes and oenocytes are induced by the same transcription factor: nuclear receptor, namely HNF-4 α .

Like most invertebrates (*e.g.* insects and nematodes), fruit flies are sterol auxotrophs. Hence the dSREBP pathway only regulates *de novo* synthesis of fatty acid and not sterols. Yet sterols are important components of fly cell membranes. Cholesterol is the precursor for insect molting hormone (ecdysone). That makes dietary sterol uptake and transport more crucial than it is for mammalian system.

Cholesterol and fatty acids are carried as lipophorin and lipid transport particles (Chino et al., 1981). When this lipid load is internalized through cell membrane receptors, cholesterol is handled by Niemann-Pick C (NPC) proteins in lysosomes (Wang et al., 2011a). With the advantage of available fly genetics tools, *Drosophila* is a favorable model organism for studying the cholesterol transport from lysosome to the ER.

Drosophila has one SREBP gene encoding one single transcript, dSREBP or HLH106 (Theopold et al., 1996). In mammalian systems, different SREBPs can partially compensate for the loss of one another but dSREBP is indispensable for flies unless their diet is supplemented with fatty acids. The *Drosophila* Scap ortholog, dScap, has 24% amino acid sequence identity with human Scap (Seegmiller et al., 2002). Its sterol sensing domain is more conserved with 47% identity between human Scap and *Drosophila* Scap, as is the WD domain with 40% identity (Seegmiller et al., 2002).

Knocking down dScap in *Drosophila* S2 cells for prolonged periods causes cell death (Irina Dobrosotskaya and Rob Rawson, unpublished data).

Cholesterol or oxysterol treatments have no effect on dSREBP pathway in S2 cells (Seegmiller, *et al.*, 2002). Instead, palmitate and ethanolamine treated S2 cells no longer activate dSREBP cleavage (Dobrosotskaya *et al.*, 2002). As palmitate and ethanolamine are incorporated into phosphatidylethanolamine (PE) it is suggested PE is the end product regulator of dSREBP pathway. This is confirmed by dsRNAs and inhibitors for each enzyme responsible for the PE synthesis from palmitate and ethanolamine.

The *Drosophila* genome does not encode any protein similar in sequence to Insig (Rawson, 2003). The presence of Insig strongly correlates with the capacity of *de novo* sterol ring synthesis. The dSREBP pathway does not regulate cholesterologenic genes such as hydroxymethylglutaryl CoA reductase and hydroxymethylglutaryl CoA synthase (Dobrosotskaya *et al.*, 2003) (Seegmiller *et al.*, 2002). Interestingly, if mammalian Insig-1 or Insig-2 co-overexpressed with the membrane domain of hamster 3-hydroxy-3-methylglutaryl-coenzyme A reductase, in *Drosophila* S2 cells, sterol-accelerated degradation of HMG-reductase is mimicked (Nguyen *et al.*, 2009). As insects are sterol auxotrophs (Clark and Block, 1959) and they lack any Insig ortholog, this data suggested that Insig is the minimal requirement to link the SREBP pathway with the feedback-regulation of cholesterol synthesis.

Among all sterol auxotrophic animals whose sequence is known, the insects classified in the order diptera, have relatively different loop1, and the rest of the sterol auxotroph animals have a loop 1 that is similar to that of mammalian Scap (Rob Rawson, unpublished observations).

A note on nomenclature: in this dissertation thesis, the prefix 'd' in gene names denotes the *Drosophila* orthologues of mammalian genes. Gene names are written in lower-case *italics* while their protein products are in roman face and capitalized. As we and other groups have shown (Rosenfeld and Osborne, 1998; Seegmiller et al., 2002), dSREBP activity is not regulated by sterols. Hence the dSREBP DNA-binding sites are not 'Sterol Regulatory'. In that regard 'dSREBP' is a misnomer. However, because of the homology between the *Drosophila* and mammalian SREBPs I will stick to this conventional name. I will likewise refer to the dSREBP binding sites as SREs.

PROJECT GOAL

Scap escorts the precursor of SREBP from the ER to the Golgi in all model systems studied, from fission yeast to humans (Espenshade and Hughes, 2007). My first aim is to characterize *dScap* mutant flies and observe the function of dScap in the complexity of a model organism and determine whether the absence of Scap can be tolerated via compensatory mechanisms.

Different membrane bound transcription factors have different mechanisms for spatial regulation. For example, Activating transcription factor 6 (ATF6) resides in ER via

binding to BiP through its luminal tail. However CREB-H has its own ER retention domain and controls its own travel to the Golgi apparatus (Llarena et al., 2010).

In vertebrates the SREBP precursor is retained in the ER membrane via an ER-resident protein, Insig. There is no Insig ortholog in the *Drosophila* genome. However dScap functions similarly to mammalian Scap. My aim is to discover whether there is a retention factor for dScap analogous in function to the vertebrate Insig or whether dScap intrinsically and self-sufficiently stays in ER when there is enough lipids.

My second aim is to establish conditions for a luciferase reporter-based screening system that can be exploited to conduct a genome-wide screen for modifiers of dSREBP pathway.

CHAPTER TWO

Results

CHARACTERIZATION OF MUTANTS

GENERATION OF dSCAP MUTANTS

In order to analyze the functions of dSREBP machinery *in vivo*, our lab generated null alleles of *dsrebp* (Kunte et al., 2006), and *ds2p* (Amarneh et al., 2009). Krista Matthews, then a graduate student in our lab, generated *dscap* null fly lines via flipase (FLP) mediated recombination (SC4) and via transposase-mediated P element excision method (the deficiency *dscap*⁹¹⁰ line).

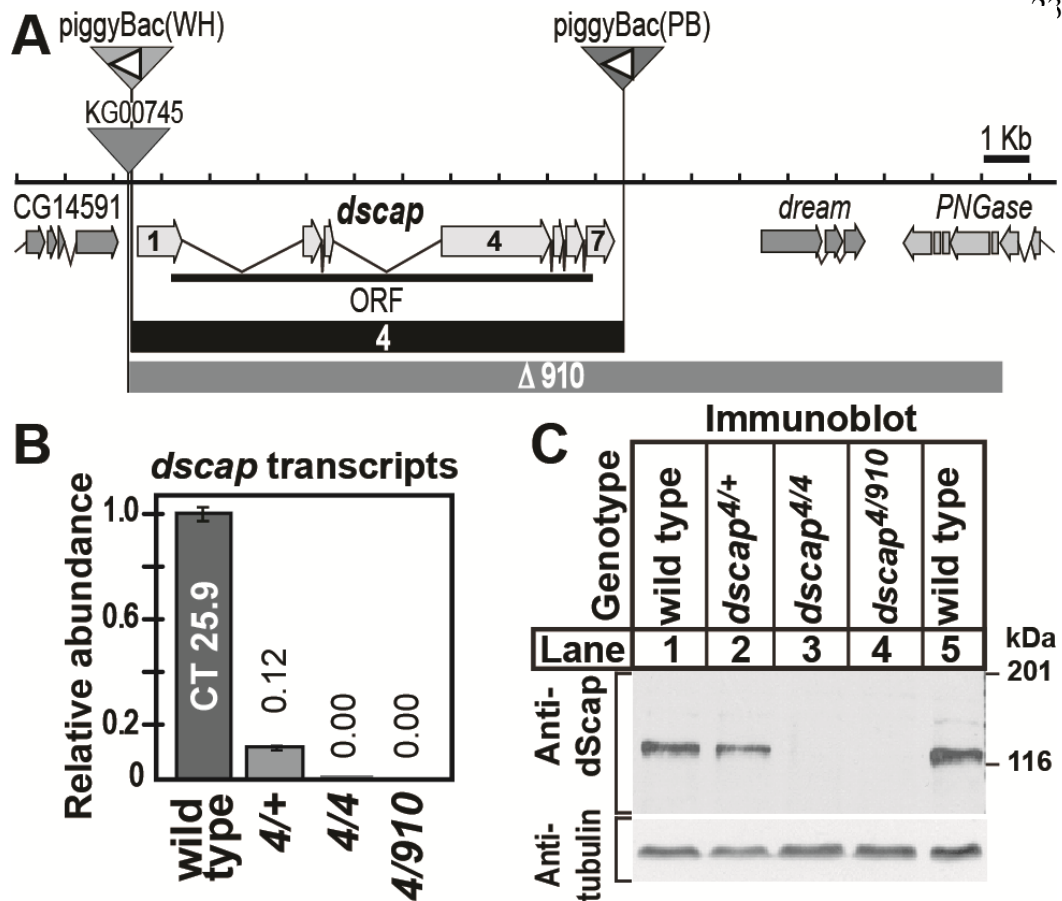


Figure 3. Generation of *dscap* null mutants

(A) Map of *dscap* locus. The *dscap* gene comprises seven exons (block arrows with light shading) encoding one protein (ORF, thick solid 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. PiggyBac (PB)c00785 is inserted 88 bp after *dscap* exon 7. The orientation of the FRT sites within both piggyBac elements are indicated by the open triangles. The extent of *dscap*⁴ and *dscap* ^{$\Delta 910$} deletions are indicated by solid boxes and boxes with dark shading, respectively. (B) Quantitative analysis of dScap mRNA from *dscap*⁴ homozygous and *dscap* ^{$\Delta 910$} /*dscap*⁴ transheterozygous larvae 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 μ g 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 semidefined media at 10 mg/dish. Larvae were isolated from the food by salt flotation and homozygous larvae were scored for absence of act-GFP fluorescence. The membrane

was probed with monoclonal antibody IgG7A8 against dScap TM1-8 (1-min-30-sec exposure), stripped, and reprobed with anti-acetylated tubulin (2-sec exposure). From (Matthews, *et al.*, 2010).

As shown in Figure 3, *dscap⁴* excises the whole *dscap* open reading frame and does not delete neighboring genes. RT-PCR data revealed that homozygous *dscap⁴* flies do not have any dScap mRNA, and western blot showed that homozygous *dscap⁴* flies do not have any dScap protein. These data prove that *dscap⁴* is a null allele by molecular criteria.

In the 910 deficiency allele, the *dscap* ORF is completely removed but this deficiency also lacks *dream* and *PNGase*. This deletion is homozygous-lethal and is designated as a deficiency allele (a deletion covering more than one locus). The fly line *dscap⁴/dscap^{Δ910}* transheterozygotes do not have dScap mRNA or protein. The phenotype of *dscap⁴/dscap^{Δ910}* transheterozygotes is no more severe than that of *dscap⁴/dscap⁴* homozygotes. Thus *dscap⁴* is a null allele by genetic criteria as well.

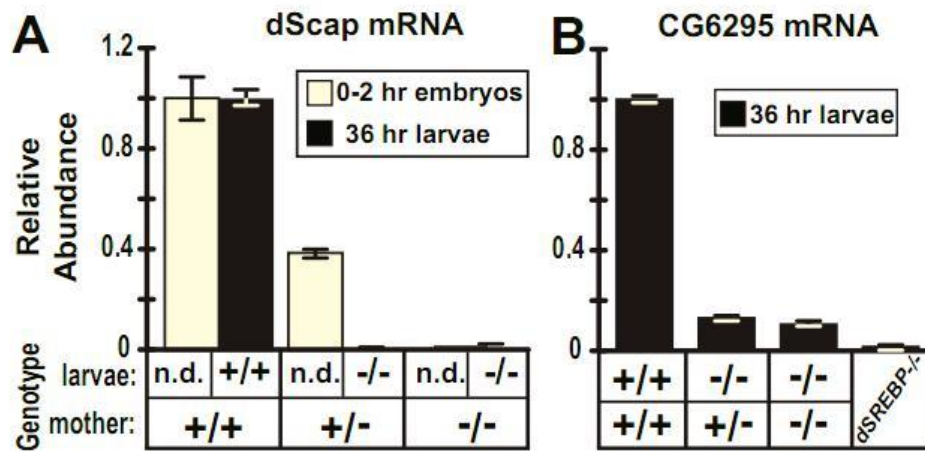


Figure 4. Maternal contribution in *dscap* mutants

Maternal contribution of dScap mRNA. (A) Quantitative RT-PCR analysis of dScap mRNA in 0-2 hour embryos (white bars) and first instar larvae (black bars). Embryos from wild-type or virgin *dscap4/dscap4* females crossed to *dscap4/Cyo*, act-GFP males were collected for 2 hours. Embryos were either collected for immediate RNA isolation or seeded (10mg/ 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. *dsrebp189* nulls are included for comparison. Quantitative RT-PCR was performed as described in *Materials and Methods*. The relative abundance of embryonic and larval transcripts was calculated relative to wild-type 0-2 hour embryos and 36 hour larvae RNA, respectively. *Methods*. The relative abundance of embryonic and larval transcripts was calculated relative to wild-type 0-2 hour embryos and 36 hour larvae RNA respectively.

(Adapted from Matthews et. al.,2010)

One factor that can complicate our analysis of *dscap* null flies is a potential maternal contribution of *dscap* mRNA to the oocyte. As shown in Figure 4A, heterozygous mothers contribute dScap mRNA to the embryo. Thus the following experiments are all performed using larvae from homozygous-null mothers. The transcriptional activation of dSREBP is measured by the expression level of dSREBP target gene, CG6295 as shown in Figure 4B.

dSCAP IS DISPENSABLE

Interestingly, *dscap* null flies can be kept as homozygous stocks. In crosses of homozygous *dscap* mothers with heterozygous *dscap* fathers, homozygous larvae survive at about 70% of the rate of their heterozygous siblings (= the expected rate). The 30% lethality is totally abolished when the larvae are fed with fatty acid-supplemented food. This rescue by fatty acid supplementation suggests that lethality is caused by deficiency in fatty acid or phospholipid metabolism.

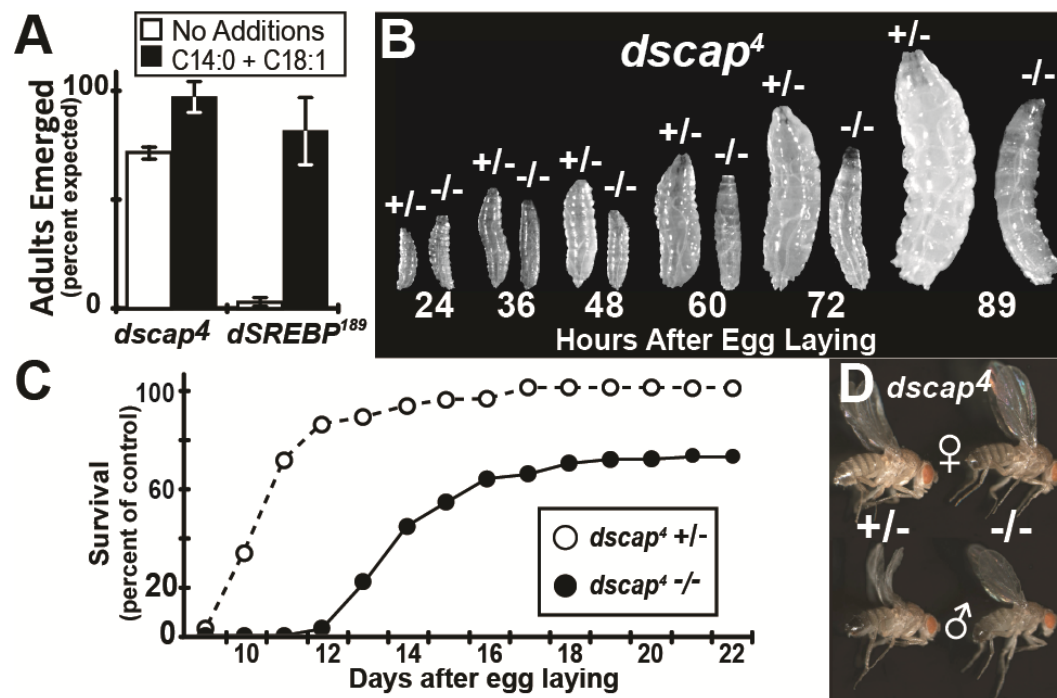


Figure 5. Viability of *dscap* null flies

(A) Embryos from virgin *dscap*⁴/*dscap*⁴ females crossed to *dscap*⁴/CyO, act-GFP males were collected and set up in vials ($n = 10$ for each condition) containing standard medium or supplemented medium (C14:0 + C18:1) as described

in MATERIALS AND METHODS. Starting on day 10 AEL, adults were collected from the vials twice daily and scored for genotype until day 20 AEL, after which no further adults emerged. Shown is the mean of three independent determinations. Error bars represent the SEM. (B) Comparison of 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 semidefined media. Larvae were scored for genotype on the basis of GFP fluorescence and photographed at the indicated time points. (C) Emergence of adult flies homozygous (solid circles) or heterozygous (open circles) for *dscap*⁴. Embryos from virgin *dscap*⁴/*dscap*⁴ females crossed to *dscap*⁴/CyO, act-GFP males were collected and set up on standard medium and emerging adults scored as described in MATERIALS AND METHODS. (D) Comparison of size between *dscap*⁴ homozygous ($-/-$) and heterozygous ($+/-$) adults reared on standard medium.

From (Matthews, *et al.*, 2010).

Figure 5B shows the growth rate of heterozygous and homozygous *dscap* null larvae at successive time points starting 24 hours After Egg Laying (AEL) and ending at 89 hrs AEL. At 24hrs AEL, the heterozygous larvae are indistinguishable from heterozygous larvae. With increasing time, the developmental delay in homozygotes is more evident, becoming very drastic at 89 hrs AEL. This growth defect translates into delay in pupariation and ~2-day-later emergence of adults from the pupal case. Despite of this retardation, adult homozygotes are morphologically indistinguishable from heterozygotes.

dscap null flies can be kept as homozygous stock with regular fly food. Hence dScap is not essential under laboratory culture conditions. This is in contrast to other model organisms and cell culture studies, which demonstrated a need for Scap to escort SREBP from the ER to the Golgi (Espenshade and Hughes, 2007). dSREBP transcriptional activity is essential for the transition from the second to third larval instar, as we learn from the dSREBP null flies. When dScap is knocked down via dsRNA in *Drosophila* S2

cells, cells do not survive (Irina Dobrosotskaya and Rob Rawson, unpublished data).

Surprisingly, Scap is dispensable for the fly.

dSREBP IS CLEAVED IN *dscap* NULL FLIES

In all model systems studied, SREBP is carried from ER to Golgi via Scap-mediated vesicular transport (Espenshade and Hughes, 2007). In the absence of Scap, transport does not occur and no proteolytic activation of SREBP is observed in these model systems (*e.g.* mammalian cultured cells). Since Scap is dispensable but transcriptional activation of target genes by dSREBP is not, animals lacking dScap must have an alternative means of producing transcriptionally-active dSREBP. Previously, our lab has shown that the caspase Drice, can cut the juxtamembrane stalk of dSREBP in *ds2p* null flies (Amarneh et al., 2009). This cleavage is sufficient to activate dSREBP and support the survival of flies lacking dS2P.

Figure 6 is a western blot of whole larval extracts from wild-type, *dscap* null, and *dsrebp* null flies. The membrane is blotted with the 3B2 antibody which recognizes the transcription factor domain of dSREBP. Despite loading double the amount of total protein in the second lane, the nuclear form of dSREBP is highly reduced. That is still intriguing that in the absence of dScap dSREBP continues to be processed, albeit at low levels.

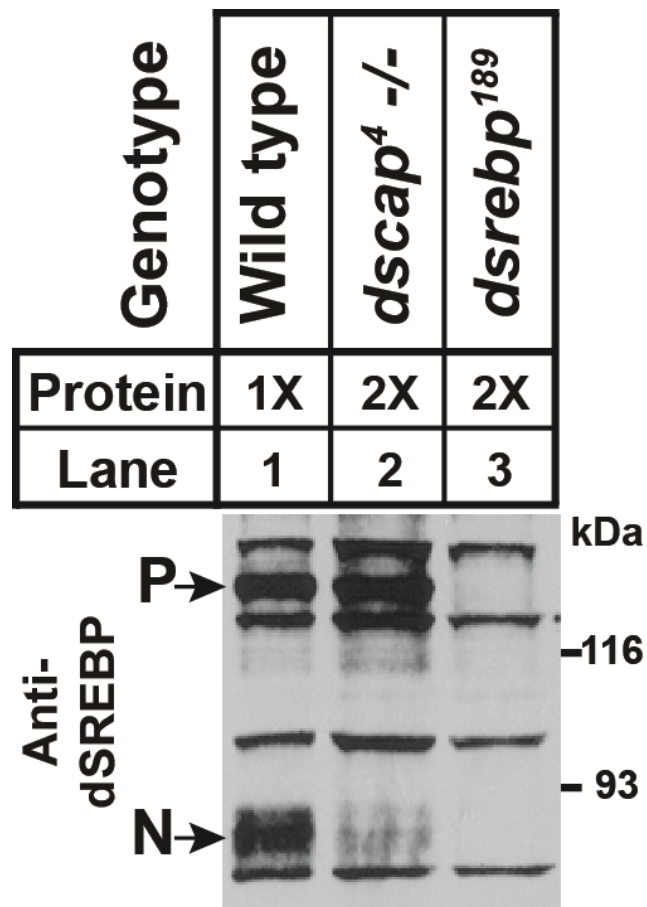


Figure 6. Immunoblot analysis of *dscap* null larvae lysates

Immunoblot analysis of whole larval lysates from first instar larvae probed with monoclonal antibody 3B2 against the amino terminus of dSREBP. Wild-type lysates were loaded at 30 $\mu\text{g/ml}$; *dscap*⁴ and *dsrebp* lysates were loaded at 60 $\mu\text{g/ml}$. Membranes were exposed to film for 2 min. P, precursor; N, nuclear form. From (Matthews, *et al.*, 2010).

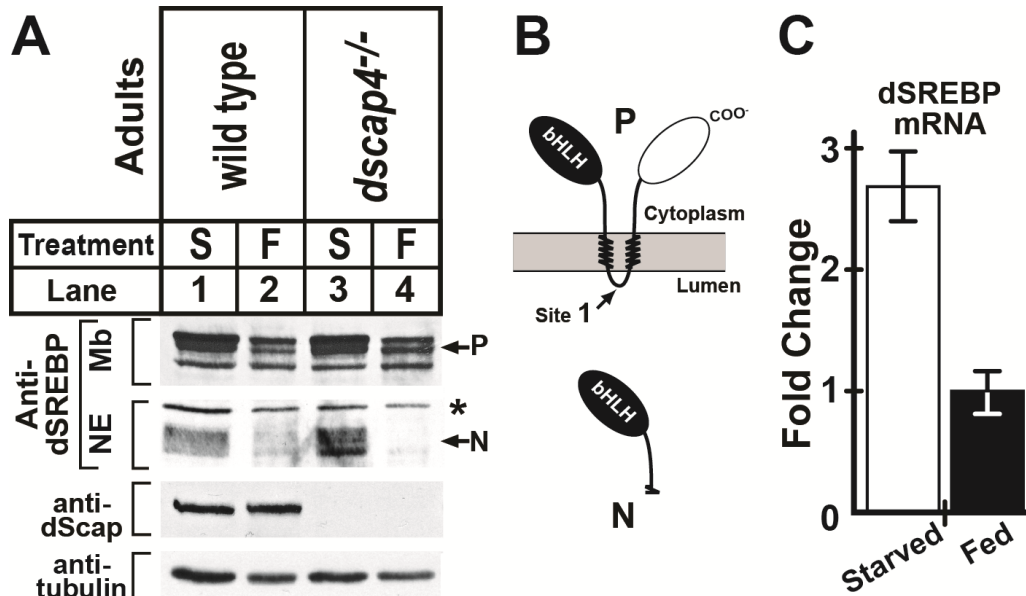


Figure 7. Immunoblot analysis of extract from *dscap* null flies

(A) Immunoblot analysis of membrane and nuclear fractions of wild type and *dscap* null mutant adult flies. Adults were either starved or starved and refed (indicated by S and F respectively) as described in Materials and Methods. The membrane fractions (75 μ g) and nuclear extracts (25 μ g) were subjected to immunoblot analysis as described in Materials and Methods. A parallel membrane blot was probed with IgG-7A8 against dScap. A membrane fraction blot was stripped and re-probed with anti-tubulin as a loading control. P, precursor; N, nuclear form. The asterisk indicates a cross-reacting band.

(B) Schematic showing the orientation and topology of dSREBP and its cleavage fragments. bHLH, the transcription factor domain; COO-, carboxy terminal regulatory domain; Site 1, site of cleavage by S1P. The cytoplasm and lumen are indicated.

(C) Quantitative RT-PCR analysis of dSREBP transcripts in starved (white bars) and refed (black bars) wild-type flies. RNA was prepared and transcripts quantified as described in Materials and Methods. Error bars represent the SEM.

From (Matthews, *et al.*, 2010).

After fractionation, dSREBP is detected in the nuclear fraction of both wild type and *dscap* null starved flies. Nuclear accumulation of dSREBP is greatly reduced in refed

flies. We do not know why *dscap* null flies are more responsive to feeding and starvation.

GFP REPORTER SYSTEM SHOWS ACTIVE TISSUES

Although dSREBP is a transcription factor, its precursor is a transmembrane protein that is activated via subcellular transport and regulated intramembrane proteolysis (Brown et al., 2000). Thus, simply determining where *dsrebp* is transcribed or where the protein is present will not necessarily reveal where dSREBP is transcriptionally active. In order to visualize the activation of dSREBP our lab has come up with a neat reporter system. The transcription factor domain of dSREBP is replaced with a chimeric protein of yeast GAL4 DNA-binding domain and the VP16 viral transactivation domain. This is done in a transgene construct harboring several kilobases of DNA up- and down-stream from the start site of transcription of *dsrebp*. This ensures that this construct fully replicates wild-type patterns of *dsrebp* transcription and its proteolytic activation is responsive to the same physiological regulatory signals as is the endogenous, wild-type dSREBP (Kunte et al., 2006). We designate this construct P{GAL4-dSREBPg}. The GAL4-VP16 chimera is highly transcriptionally active and specific for the yeast GAL4 upstream activating sequence (UAS). This target sequence is not present in the *Drosophila* genome and flies harbor no factors that target this sequence.

We used P-element-mediated germline transformation to produce transgenic flies expressing GAL4-dSREBP. We crossed these flies with flies transgenic for a UAS-driven jellyfish green fluorescent protein (UAS-GFP) construct. When endogenous

dSREBP is activated, the GAL4-dSREBP chimeric construct is also activated. The GAL4-dSREBP protein travels to the Golgi apparatus where it is released from the membrane-bound portion of the dSREBP precursor. It is then free to travel to the nucleus.

In nuclei, GAL4-Vp16 binds to the UAS promoter sequence that drives the expression of GFP mRNA (Kunte et al., 2006). In wild-type, third instar larvae, the reporter system shows activation of dSREBP most intensely in fat body, midgut, and oenocytes. In *dscap* null larvae the GFP signal is greatly reduced in fat body and midgut. However dSREBP continues to be highly activated in the oenocytes. This binary reporter system revealed some variability in dSREBP processing in mutant larvae. The GFP signal was significantly reduced in all *dscap* larvae compared to wild type ($n > 50$), however some larvae had scattered patches of GFP signal in the anterior midgut. (Figure 8D). In all *dscap* null larvae, a cluster of cells immediately posterior to the proventriculus exhibit strong GFP signal (Figure 8 D and F). The early pupae of mutants have very weak signal compared to wild type (Figure 8E), late pupae have almost no signal at all (data not shown).

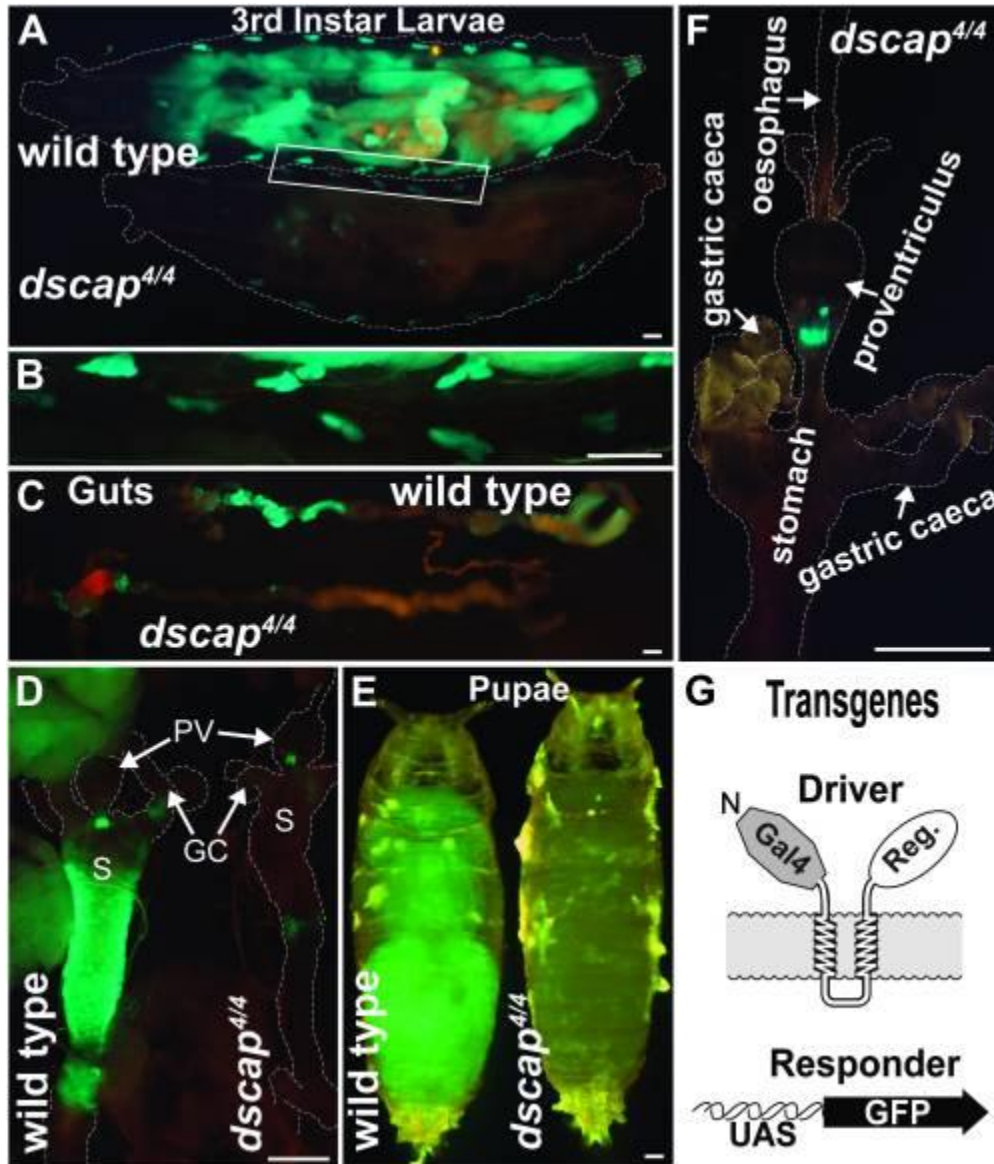


Figure 8. Analysis of dSREBP activation in *dscap* vs. WT larvae

(A) Ventral views of dSREBP activity in wild-type (upper) and *dscap*⁴ (lower) third instar larvae visualized using the GAL4-dSREBP/UAS-GFP reporter system. Fluorescence is readily detected in *dscap*⁴ oenocytes, and to a lesser extent in regions of the anterior midgut. 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. Dashed lines denote extent of larval bodies. Exposure = 10 sec. (B) Higher

magnification view of the region indicated by the white box in A showing wild-type and *dscap*⁴ oenocytes. Exposure = 4 sec. (C) Midguts showing reduced fluorescence in *dscap*⁴ larvae compared to wild type. Exposure = 8 sec. (D) Anterior midguts from wild-type and *dscap*⁴ larvae showing greatly reduced dSREBP activity in *dscap*⁴ larvae. GC, gastric caeca; PV, proventriculus; S, stomach; exposure = 4 sec. (E) Wild-type and *dscap*⁴ pupae (stage P5)(Bainbridge and Bownes, 1981). Exposure = 5 sec. (F) Anterior midgut from a *dscap*⁴ larva exhibiting fluorescence only at the posterior end of the proventriculus. Exposure = 3 sec. (G) Schematic of the GAL4-dSREBP; UAS-GFP reporter system. Scale bars, 0.2 mm. The variability of dSREBP activity in the midgut of *dscap*⁴ larvae is evident among the different individuals shown in C, D, and F From (Matthews, *et al.*, 2010).

As shown by our reporter system, in the absence of *dscap*, dSREBP continues to be processed in a subset of tissues where dSREBP normally is activated. If that is true for the endogenous, wild-type dSREBP, the transcriptional deficiency in *dscap* null larvae should not as dramatic as that seen in *dsrebp* null flies(Kunte et al., 2006)

dSREBP TARGET GENE TRANSCRIPTS ARE DIMINISHED IN *dscap* NULL FLIES

As previously reported (Kunte et al., 2006; Seegmiller et al., 2002) dSREBP modulates the transcription of the enzymes that are responsible for *de novo* fatty acid synthesis, namely acetyl coenzyme A synthase (ACS), acetyl coenzyme A carboxylase (ACC), and fatty acid synthase (FAS) etc.,. These genes are not solely regulated by dSREBP but their mRNA levels are affected by defects in dSREBP pathway. CG6295 expresses a putative lipase whose transcription is most strongly (and presumably solely) regulated by dSREBP (Kunte et al., 2006; Matthews et al., 2009). *dscap* null larvae have much less CG6295 mRNA but slightly more than *dsrebp* null mutant larvae.(Matthews et al., 2010)

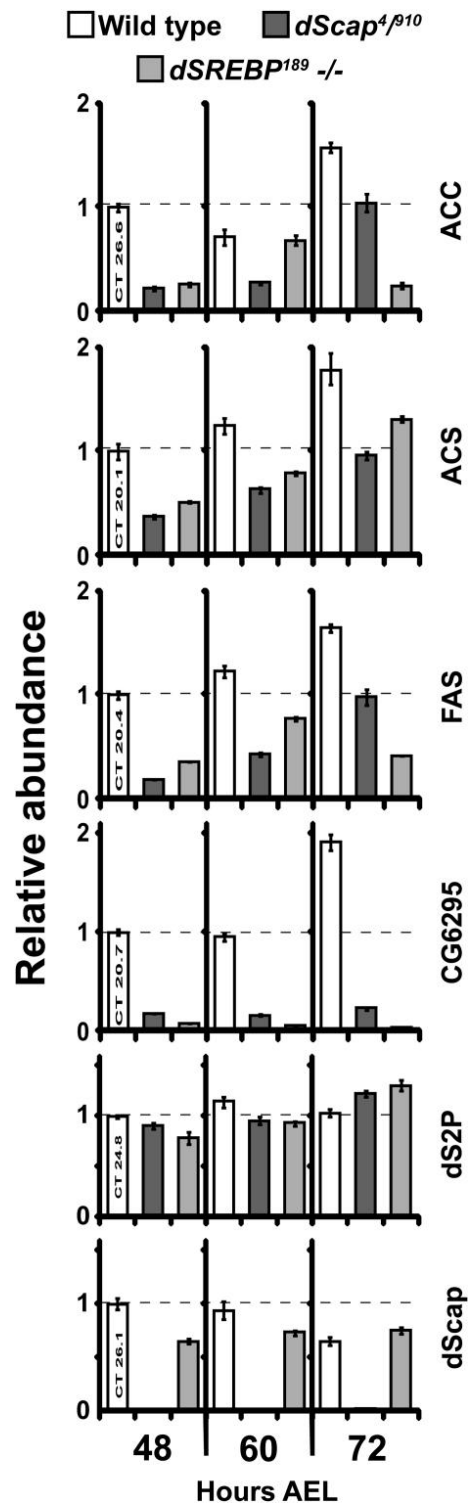


Figure 9. Quantitative real-time PCR analysis of mRNAs of *dSREBP* target genes from *dscap*^{4/910} transheterozygous mutant larvae compared to wild-type and *dsrebp* mutants. These results are representative of three independent determinations. mRNA was isolated from 48, 60, and 72 hr AEL larvae as described in METHODS AND MATERIALS. Fold change was calculated relative to 48-hr wild-type RNA levels. Error bars represent the SEM. From (Matthews, *et al.*, 2010).

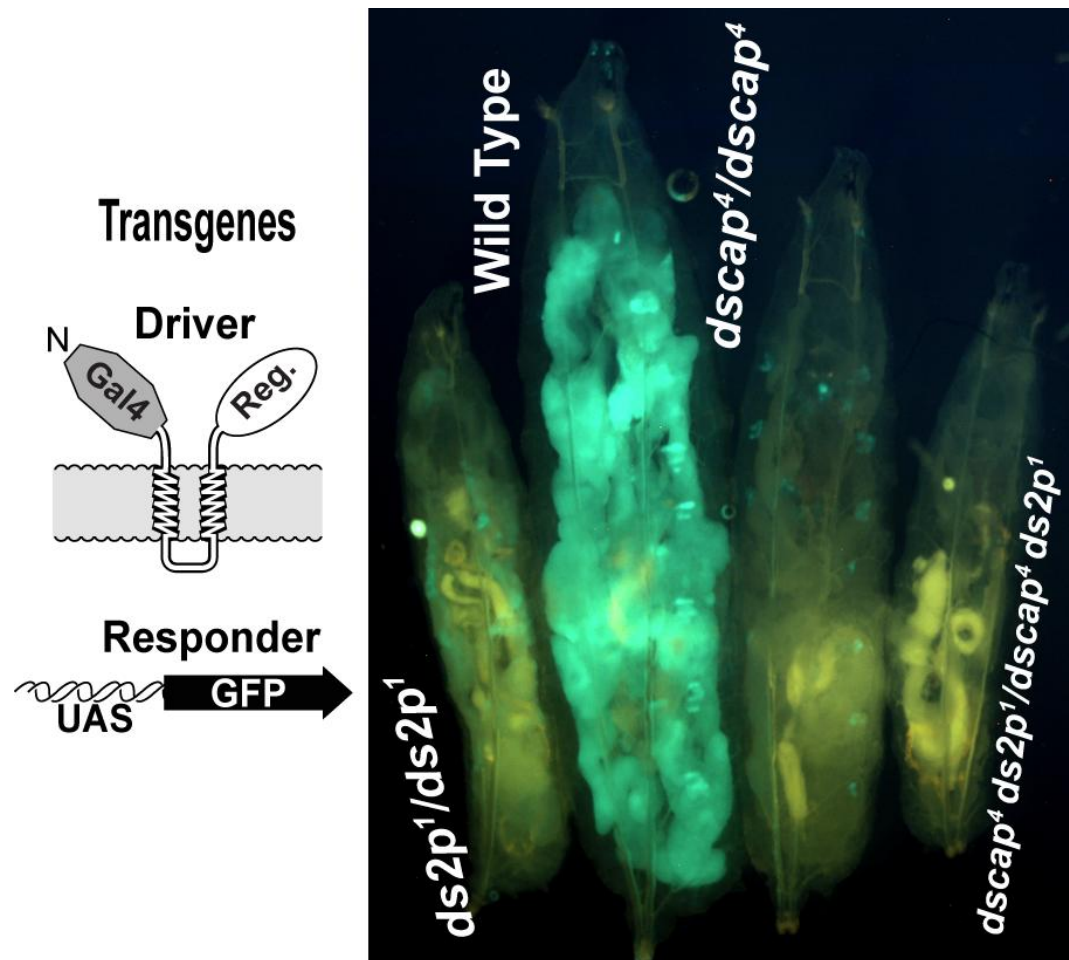


Figure 10. Comparison of dSREBP activation in mutants

Third instar larvae expressing the binary reporter system of wild type, *dscap* null, *ds2p* null, and *dscap;ds2p* double mutants are immobilized in halocarbon oil:chloroform (199:1). The image is taken in fluorescent microscope.

In order to compare the activation pattern of dSREBP in mutants, I examined third instar larvae of wild type, *dscap* null, *ds2p* null, and *dscap;ds2p* double mutants side by side. This ensures that the images are acquired under identical conditions (for example, the

same time of exposure and identical illumination). As seen in Figures 8 and 10, the GAL4-dSREBP; UAS-GFP binary reporter system shows expression of GFP in fat body, oenocytes, and in the anterior midgut of wild-type third instar larvae. It shows that these tissues have accumulated substantial amounts of GFP in these tissues due to the activation of dSREBP. In *ds2p* null larvae, some fat body cells glow green but it is not strong or extensive as in wild type. The precise degree of fluorescence observed in the fat body of these mutants varies (see (Matthews et al., 2010)), but it is always distinctly reduced compared to wild-type larvae. As in the Figures 8 and 10, *dscap* null fly larvae have GFP signal most clearly in oenocytes. Some patches in the anterior midgut are visible, as well as in corpus allatum (the intense spot in the center of *dscap* mutant, about 1/3 the way down the body).

Larvae doubly-mutant for *ds2p* and *dscap* do not show any detectable SREBP activation at this exposure. However *dscap* null larvae show obvious activation (i.e. fluorescence) in oenocytes while the *ds2p* null larvae have activation in the fat body.

Although, 30% of *ds2p dscap* double mutants survive on standard *Drosophila* culture medium, there is no detectable GFP signal. The *srebp* null flies do not survive at all under these conditions, and as the only known function of dSREBP is through its transcriptional activity, there must be some undetected dSREBP activation in the double mutants (perhaps at a time different from that investigated here); otherwise these mutants would phenocopy the *srebp* null flies.

TRANSCRIPTION OF dSREBP TARGET GENES IS RESPONSIVE TO DIET IN *dscap* MUTANTS

As shown in Figure 10, the *dscap* null flies can activate dSREBP in oenocytes and midgut under standard culture conditions. Interestingly, when *dscap* mutants are fed a diet supplemented with 9% soy lipids, no GFP signal is detectable. Thus, proteolytic processing of dSREBP is suppressed.

As shown in Figure 11B, lipid supplementation causes 50% decrease in the mRNA levels of dSREBP and its target gene CG6295, both in wild type and *dscap* mutants. The GAL4-dSREBP construct has the same exact promoter region as endogenous *dsrebp*. Therefore a decrease in GAL4-dSREBP mRNA may partially account for the decrease in GFP signal.

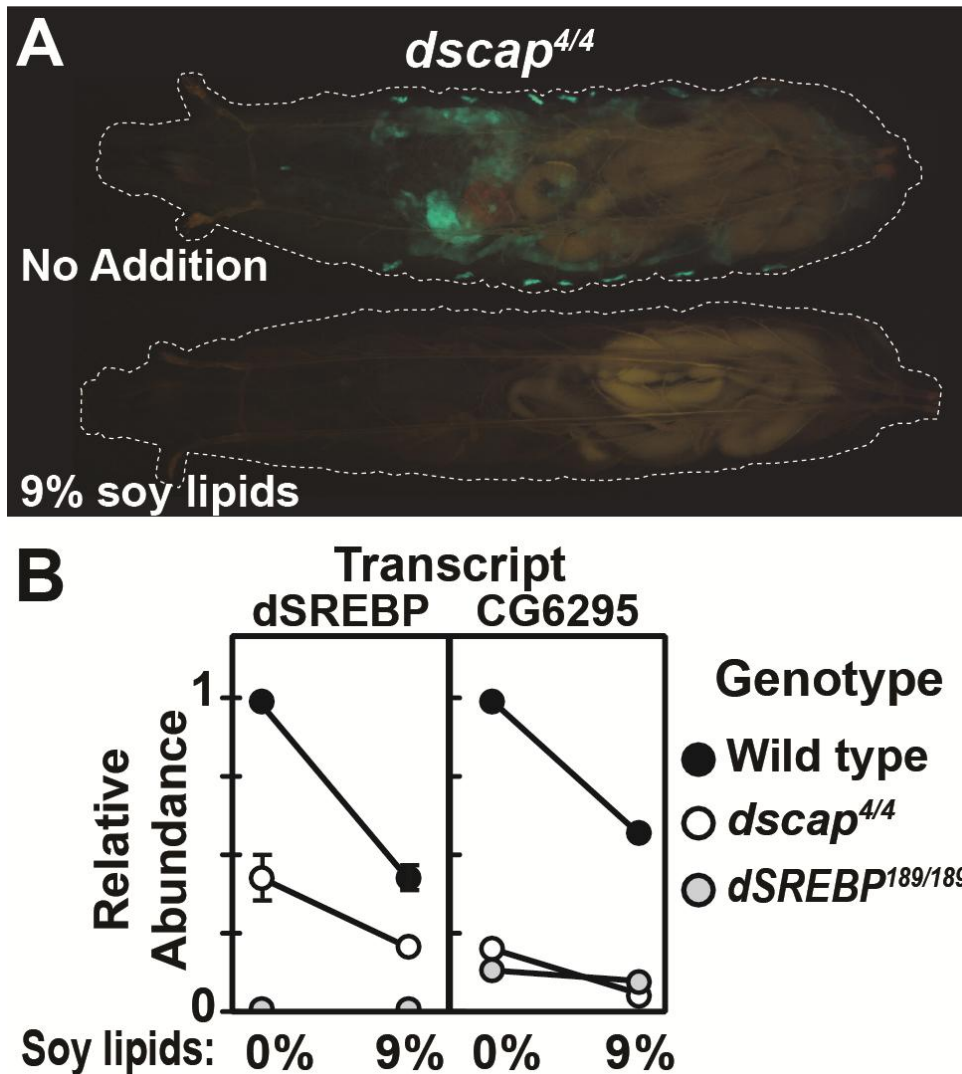


Figure 11. *dscap* mutant larvae are responsive to diet

(A) Comparison of GFP fluorescence in *dscap*⁴ homozygotes grown on semidefined medium (upper) or the same medium supplemented with 9% soy lipids (lower). 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. Exposure = 2.5 sec. (B) Quantitative RT-PCR analysis of dSREBP and CG6295 transcripts of wild-type (black circles), *dscap*⁴ (white circles), and *dsrebp*¹⁸⁹ (gray circles) larvae grown on semidefined medium (0%) or the same medium supplemented with 9% soy lipids (9%). Transcript abundance is relative to wild-type larvae on 0%. Error bars represent the SEM. From (Matthews, *et al.*, 2010).

MUTANTS ARE DEFECTIVE IN TRIGLYCERIDE ACCUMULATION

Previously, we demonstrated that flies lacking *dsrebp* accumulated less lipid than wild type flies but that its relative composition was unchanged (Kunte et al., 2006). Similarly, we showed that *dscap*, *ds2p*, and *dscap ds2p* adult flies also accumulated less total lipid than wild-type flies (Matthews et al., 2010). We reared these mutants on standard medium and assayed larvae for triglyceride (TG) content. TG abundance is reduced in the mutant larvae (Figure 12), comparable to the reduction in total lipids in the adult flies. While loss of dSREBP (or the machinery needed to process it) has a pronounced effect on the amount of lipid an animal accumulates, little effect is apparent on the classes of lipid accumulated.

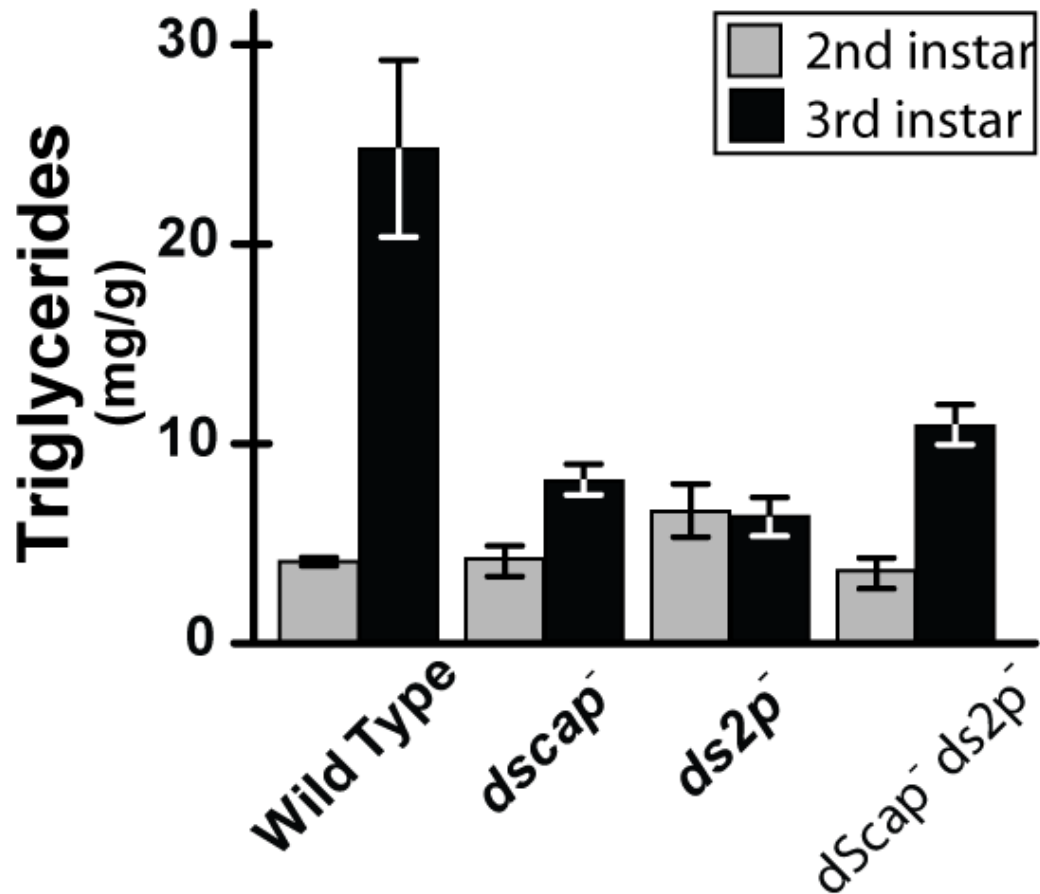


Figure 12. Triglyceride content of larvae.

Embryos were set up on day 0 on rich medium (Kunte et al., 2006). Homozygous larvae (200 per sample) were collected at 48 and 72 hours after egg laying. Samples were extracted and triglycerides assayed as described (Yokode et al., 1990). Adapted from Ozdemir and Rawson 2011 (Ozdemir and Rawson, 2011)

MUTANTS EXHIBIT DELAYED DEVELOPMENT

Figure 13 shows emergence curves for adult flies homozygous for mutations in *dscap*, *ds2p*, or *dsrebp*. The *dscap* mutants (Figure 13B) emerge about five days later than wild-type flies and this delay is observed for the *dscap;ds2p* double mutants as well (Figure 13D). The *dScap* and *ds2p* mutants do not survive as well as wild-type flies but they do survive much better than *dsrebp* mutants (Figure 13E). For the single mutants and for the *dscap ds2p* double mutants, supplementation of the larval diet with exogenous lipid substantially restores normal survival to these animals. This indicates that reduced survival of the mutants under standard culture conditions is largely owing to lipid deficiency arising from a deficit in dSREBP activation.

Even on medium supplemented with additional lipid, the mutants continue to emerge later than wild type flies. Surprisingly this phenotype is consistently more pronounced for *dscap* mutants (Figure 13B) than for *ds2p* mutants (Figure 13C), even though loss of *ds2p* has a more severe effect on overall survival. Thus, loss of *dscap*, *ds2p*, or *dsrebp* confers some phenotypes that are not rescued simply by feeding exogenous lipid. This may reflect a need for endogenous lipid synthesis in some tissue (s) that cannot be compensated by dietary sources. Alternatively, the residual phenotypes may point to lipid-independent roles for components of the SREBP pathway, acting together or individually (Ye et al., 2000).

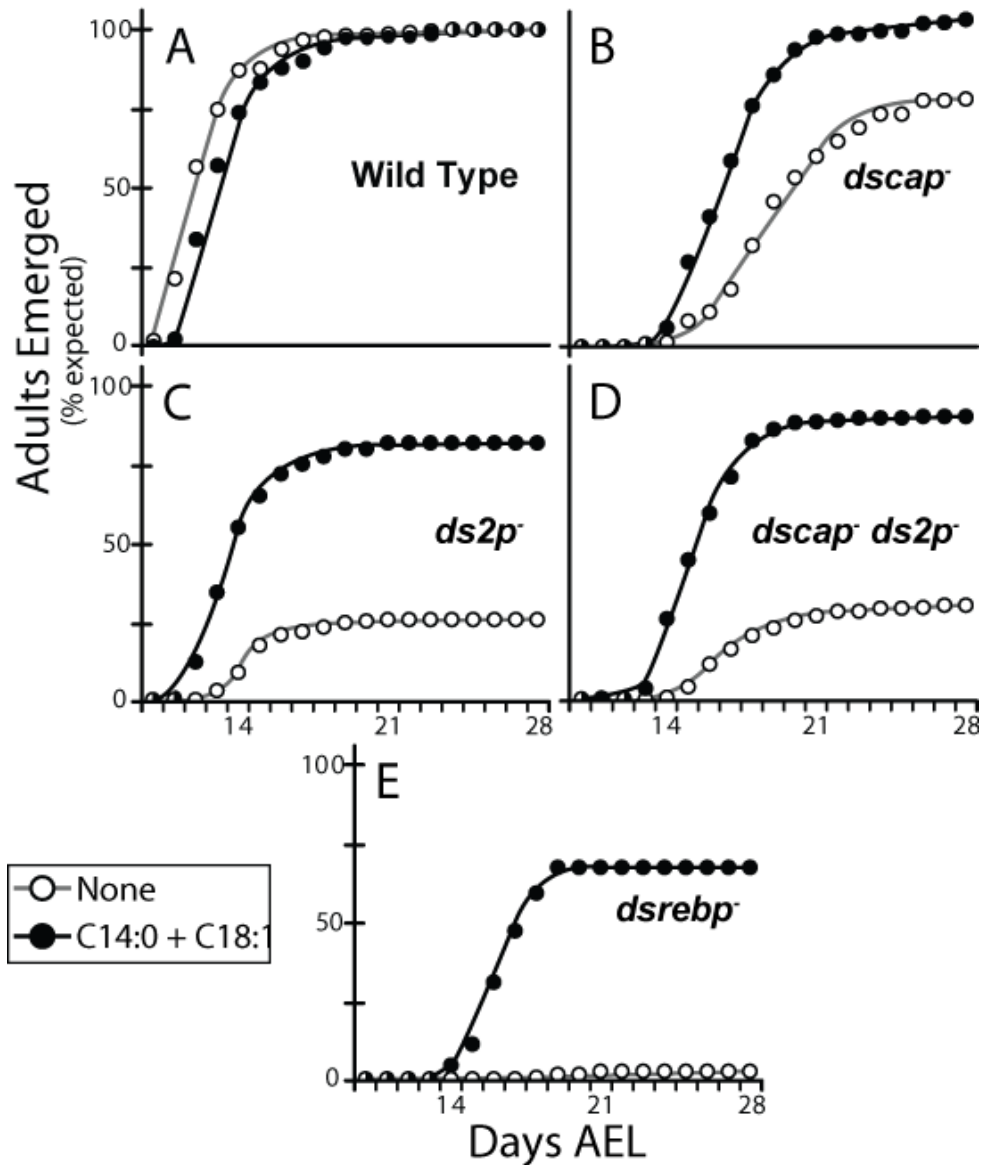


Figure 13. Emergence of homozygotes. Plots show the total number of adults emerged vs. days after egg laying (AEL). On day 0, embryos (3 mg) were introduced into vials of standard cornmeal–molasses–agar medium (open circles) or the same medium supplemented with 0.075% (w/v) myristate and 0.15% oleate (C14:0 + C18:1). Beginning on day 9, and each day thereafter, adults were cleared twice daily from the culture and counted. (A) Wild type. (B) Homozygotes from a cross of *dscap*⁴/*CyO*

virgins x *dscap*⁴/*dscap*⁴ males(Matthews et al., 2010). (C) Homozygotes from a cross of *ds2p*²/*CyO* virgins x *s2p*¹/*s2p*¹ males(Matthews et al., 2009). (D) Homozygotes from a cross of *dscap*⁴ *ds2p*²/*CyO* virgins x *dscap*⁴ *ds2p*¹/*dscap*⁴ *ds2p*¹ males(Matthews et al., 2010). (E) Homozygotes from a cross of *dsrebp*¹⁸⁹ heterozygotes(Kunte et al., 2006) included as a control for rescue efficiency. The expected ratio of homozygotes to heterozygotes emerging is 100% for crosses of heterozygotes with homozygotes and 50% for crosses of heterozygotes with heterozygotes owing to embryonic lethality of balancer chromosome homozygotes. We scored a mean of 508 animals for each cross and condition (range 346 to 829). The data shown are representative of three independent replications. Adapted from(Ozdemir and Rawson, 2011)

LARVAE DOUBLY-MUTANT FOR *ds2p* AND *drice* PHENOCOPY *dsrebp*

MUTANTS

Previously our group has shown that *s2p* null flies survived through alternative cleavage of dSREBP by a caspase, Drice(Amarneh et al., 2009). Like *dsrebp* null larvae, doubly-mutant *ds2p*; *drice* larvae could not proceed to the third instar larval stage. To determine if this similarity was reflected at the level of transcription of dSREBP target genes, we assessed the transcription profile of dSREBP target genes in those flies by real-time, reverse-transcriptase PCR (RT-PCR):

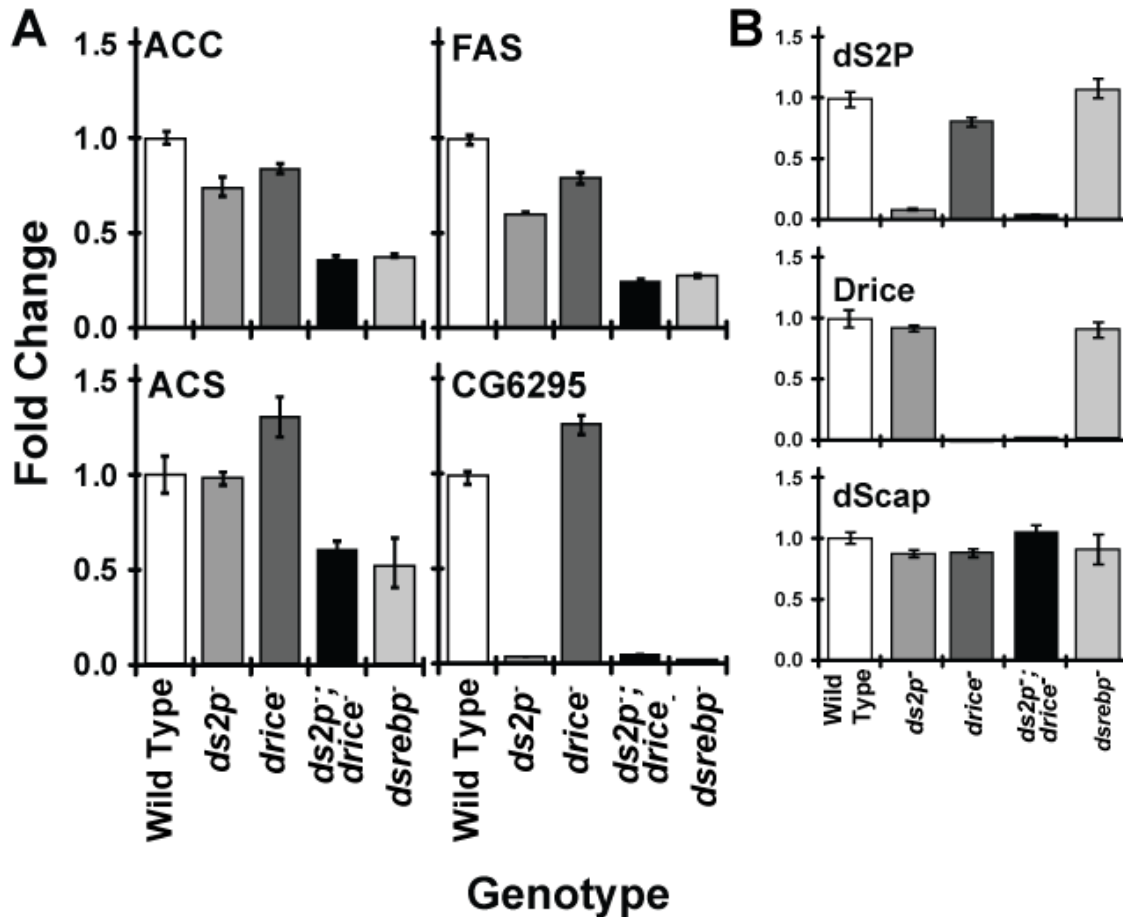


Figure 14. Doubly-mutant *ds2p*; *drice* larvae exhibit the same transcriptional deficit as *dsrebp*⁻ larvae

RNA was prepared from larvae of the indicated genotype collected 60 hrs after egg laying (mid second instar). Transcript abundance was determined by RT-PCR as described (Matthews et al., 2009). For each graph, the transcript measured is indicated at upper left (ACC, acetyl coenzyme A carboxylase; ACS, acetyl coenzyme A synthase; FAS, fatty acid synthase; GC6295, a lipase). (A) Transcriptional targets of dSREBP. (B) Controls for genotype. Data are plotted as fold-change versus wild type. Error bars represent SEM.

Adapted from (Ozdemir and Rawson, 2011)

IS DRICE THE SURVIVAL FACTOR OF *dscap* NULL FLIES?

Drice cleaves the precursor of dSREBP. This is the mechanism by which *ds2p* null flies survive (Amarneh et al., 2009). The cell compartment where Drice cleaves the precursor of dSREBP is not known. If it cleaves dSREBP in the ER membrane, then dScap might be dispensable due to activation of dSREBP by Drice. Figure 15 shows that *dscap* null flies survive by means of another mechanism because *dscap:drice* double mutants have the same survival rate as either single mutant.

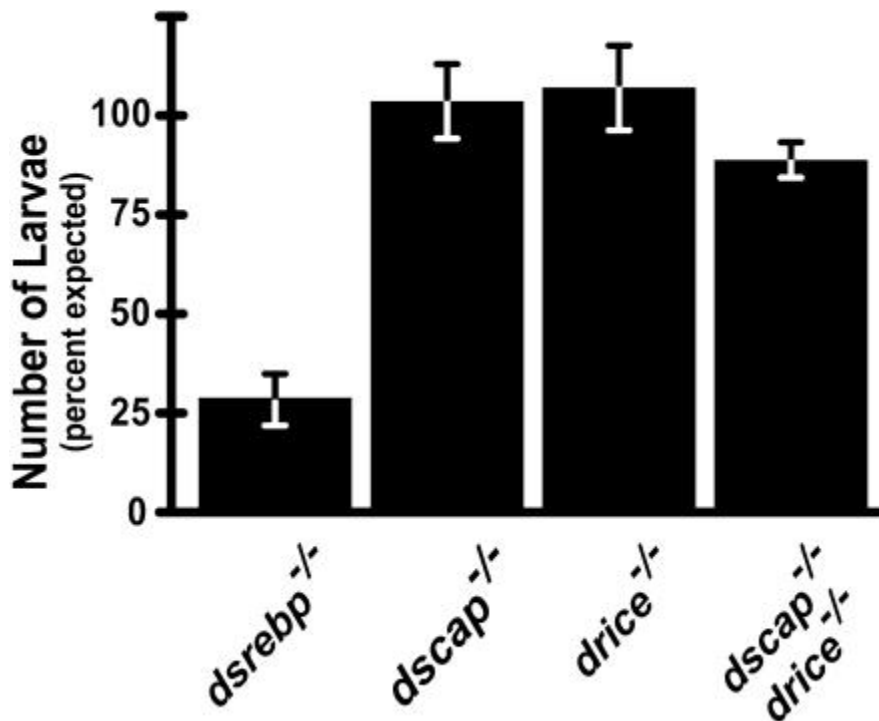


Figure 15. Drice is not the survivor of *dscap* mutants

The caspase Drice is not required for the survival of *dscap* mutants. For the homozygous lethal *dsrebp* and *drice* mutants, crosses were set up using heterozygous males and females balanced with *TM3, ser actin-GFP*. Homozygous *dscap*⁴ females were crossed to *dscap*⁴/CyO, *actin-GFP* males. For the double mutants, *dscap*⁴/*dscap*⁴; *drice*/*TM3, ser, actin-GFP* virgin females were crossed to *dscap*⁴/CyO, *twist-GFP*; *drice*/*TM3, ser actin-GFP* males. Larval survival was assessed as described in MATERIALS AND METHODS.

Data are presented as the mean of the percentage of expected emergence. The emergence of *dscap*; *drice* double mutants does not differ significantly from that of *ds2p* mutants ($P < 0.5$) but is significantly different from the emergence of *dsrebp* homozygotes ($P > 0.05$) by χ^2 tests for independence and goodness of fit. Error bars represent the SEM. From (Matthews, *et al.*, 2010).

ds2p IS DISPENSABLE IN *dscap* MUTANT LARVAE

Doubly-mutant *ds2p*; *dscap* larvae and *ds2p* single-mutant larvae have similar survival rates. Feeding the larvae with fatty acid-supplemented food fully restores the survival rate to wild type levels. The doubly-mutant *ds2p*; *dscap* larvae are no more defective in depositing lipid than are either single mutant of *dscap* or *s2p*.

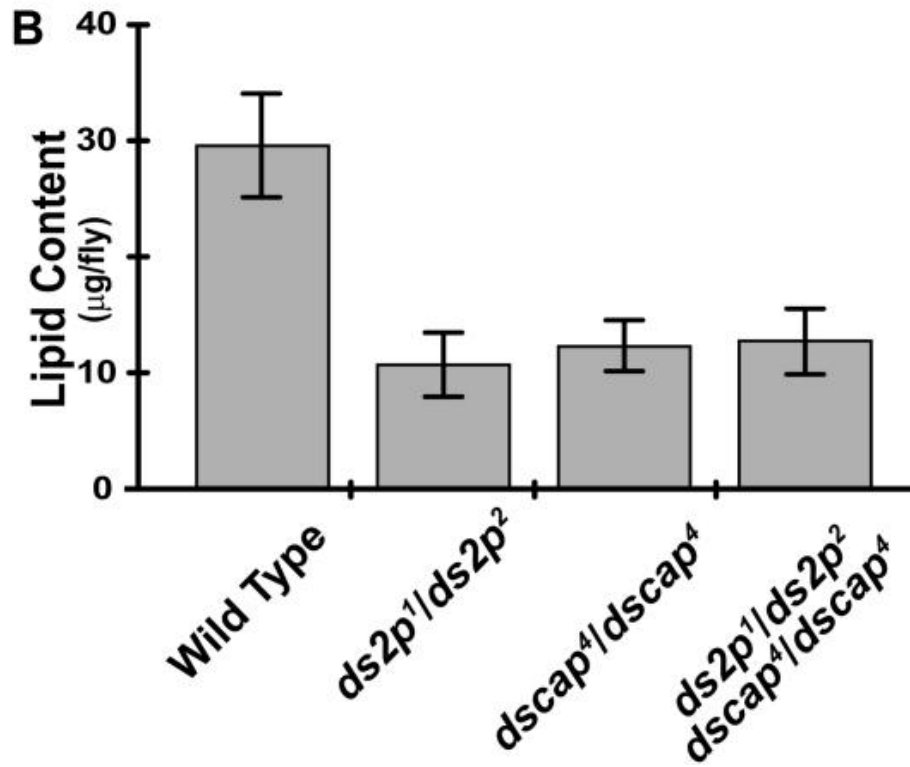
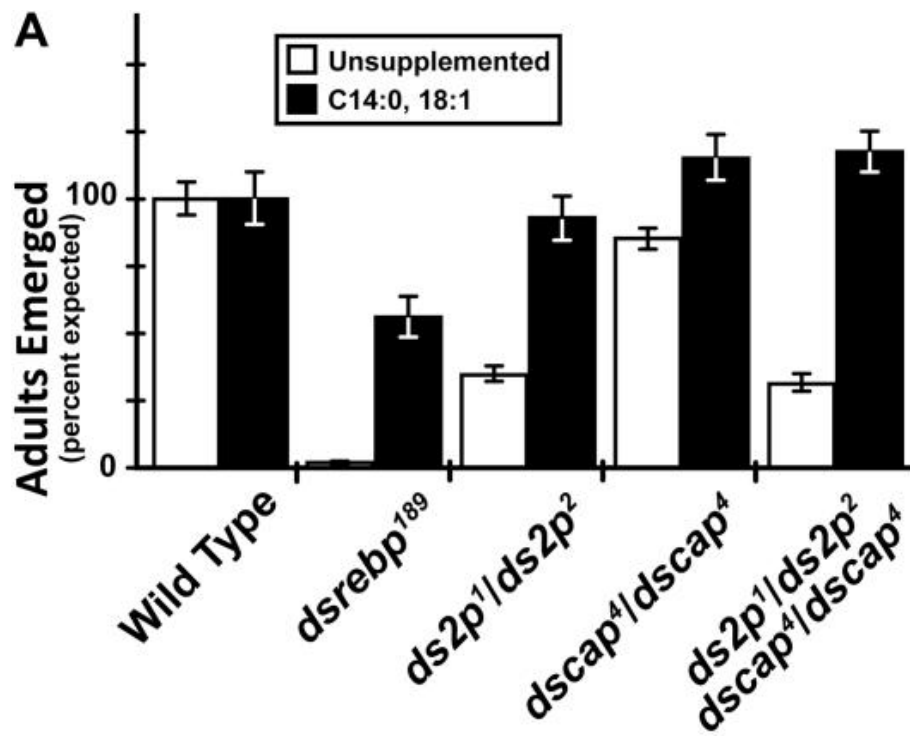


Figure 16. Emergence and lipid content of double mutants

Flies doubly mutant for *ds2p* and *dscap* exhibit phenotypes similar to *ds2p* single mutants. Virgin *ds2p²/ds2p²* females were crossed to *ds2p¹/CyO* males. Virgin *dscap⁴/dscap⁴* females were crossed to *dscap⁴/CyO* males. For the *dscap ds2p* double mutants, virgin *ds2p² dscap⁴/ds2p² dscap⁴* females were crossed to *ds2p¹dscap⁴/CyO*, *actin-GFP* males. Heterozygous *dsrebp¹⁸⁹* males were crossed to females of the same genotype. (A) Rescue of *ds2p*, *dscap* double mutants by dietary supplementation. For each experiment, 10 vials were set up at 1 mg embryos/vial on standard medium and an additional 10 vials of each cross were set up on medium supplemented with 0.075% myristate and 0.15% oleate (C14:0 + C18:1). Shown is the mean of three independent experiments. Error bars represent the SEM. A mean of 1415 adults (range, 872–1973) were scored for each genotype and condition. (B) Lipid content of wild-type and mutant flies reared on standard medium. Lipid mass was determined as described in MATERIALS AND METHODS. The mean of five independent determinations is shown. Error bars represent the SEM. From (Matthews, *et al.*, 2010).

SCAP OVEREXPRESSION

In mammalian cells, Scap is retained in ER membrane via the ER anchor protein, Insig.

Despite all the convergence of the mammalian and fly SREBP pathways, there is not any Insig-like protein in flies (by sequence similarity). We hypothesize that, as in mammalian system, there is an ER resident protein in flies that interacts with the SREBP: Scap complex in ER when the lipid amount is sufficient, keeping it in the ER (Yang *et al.*, 2000).

If this hypothesis is correct, when dScap is overexpressed under conditions where the dSREBP:dScap complex is normally retained in the ER, the putative ER retention factor should become saturated for binding to Scap. Thus, the retention factor-unbound dScap

should escort dSREBP to the Golgi apparatus even when the cells are treated with palmitate and ethanolamine.

If the hypothesis is wrong and dScap self-sufficiently or intrinsically resides in ER, overexpressing it would not compromise suppression by palmitate and ethanolamine treatment.

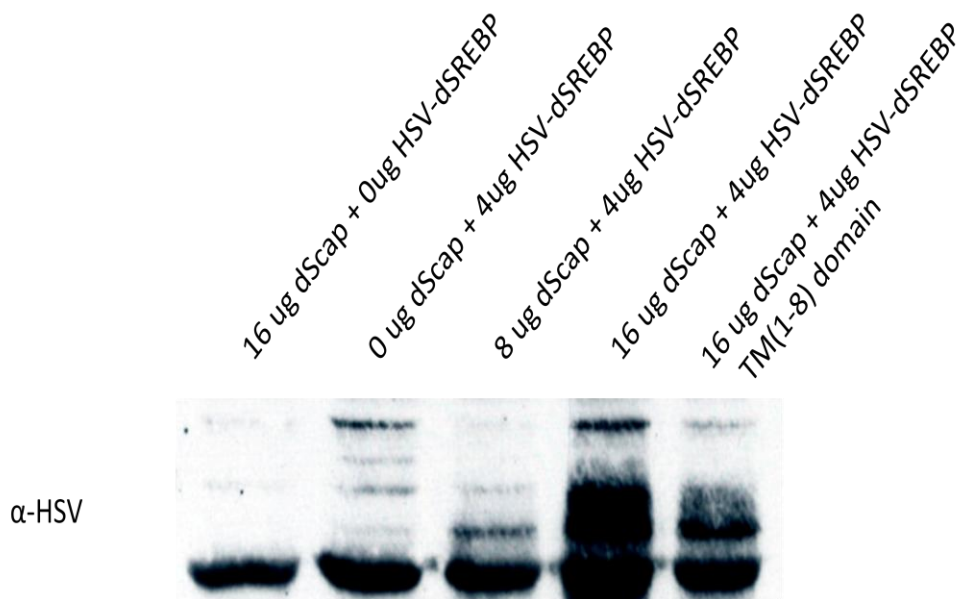


Figure 17. Overexpression of dScap perturbs suppression of dSREBP

S2 cells are transfected with HSV-tagged dSREBP, dScap full-length or TM (1-8) domain plasmids in the amounts shown above the lanes on day 0. After 72 hrs, cells are treated with palmitate and ethanolamine for 4 hrs. Cells are harvested and immunoanalyzed with HSV antibody as explained in Materials&Methods

As shown in the Figure 17, when I overexpressed either full length or transmembrane domain (helices 1-8) of insect Scap with a strong actin promoter, palmitate and ethanolamine treatment cannot suppress dSREBP activation. In mammalian system, the retention factor Insig binds the SSD domain of Scap. In analogy with that, if a putative

retention factor binds to dScap in the transmembrane domain, then overexpression of this fragment would saturate the retention factor. In consequence, dScap-mediated transport of dSREBP to the Golgi would be unregulated by lipid levels.

Excess amounts full length dScap abolishes the suppression dSREBP. Although not as strong as full-length dScap, the fragments of dScap containing the transmembrane domain (1-8) can also perturb suppression.

These data suggests that there is, in fact, a retention factor that is overwhelmed by the excessive amount of full length or transmembrane domain of dScap. These data may suggest that the putative retention factor interacts with dScap through the transmembrane domain as Insig does in mammalian system rather than the WD repeat domain at the C terminal end.

CHAPTER THREE

Results

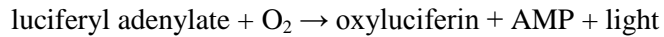
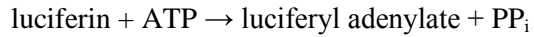
GENOME-WIDE SCREENS FOR MODIFIERS OF THE SREBP PATHWAY

Reporter assays are simple and efficient tools used to evaluate the transcriptional activity *in vivo*, indirectly. The assays are designed with very sensitive reporters. These transgenes are chosen to exhibit very low background levels. The mRNA or protein products of the reporter gene can accumulate inside the cell over time. This accumulation can obscure changes in gene expression, especially over short periods. Thus, destabilizing sequences for mRNA and proteins have been engineered into the reporter constructs in order to study short term responses.

Two of the reporter genes are widely used in research are Green Fluorescent Protein, GFP and luciferase. Research on GFP was awarded by nobel prize in chemistry in 2008. GFP was first cloned from *Aequorea victoria* by Dr Prasher in 1992 (Prasher et al., 1992). Research on self-fluorescent proteins has continued, identifying new proteins and engineering GFP to absorb and emit different spectra of light. Owing to these efforts, now we have palette made of mutant fluorescent proteins from which to choose.

Luciferase is a generic name for the enzymes that catalyze the oxidation of a pigment (luciferin), which produces bioluminescence. The oxidation of luciferin without the enzyme is very slow. That is one of the factors that contribute to the low background in luciferase assays. Among different luciferases from different species, one that is very

often used in research is the firefly luciferase (EC 1.13.12.7) from the firefly *Photinus pyralis*. The reaction has two steps:



The requirement of ATP for this reaction enabled the usage of luciferase as a reporter for cell viability assays. In another type of luciferase assay, activity of an enzyme such as caspases or cytochrome p450 causes their engineered substrates to turn into luciferin.

Luciferase has been expressed in several model systems under different promoter and regulatory sequences. Luciferase functions as a monomer that does not require any post-translational modification for activity. The intensity of the light (and the total light emitted) is directly proportional to the amount of luciferase enzyme.

Dual reporter systems have evolved for better normalization of luciferase signal. The idea is to express dual reporter genes in the same sample. The signal from the “experimental reporter” is changed by experimental conditions. The second reporter, which is expressed under control of a promoter that does not vary under the experimental conditions examined, functions as an internal control for transfection efficiency. For instance, firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*, commonly called sea pansy) luciferases are expressed in the same sample. Firefly luciferase expression is controlled by experimentally relevant promoter and the renilla luciferase is controlled by

a constitutively active promoter. First the signal from the firefly luciferase is measured and then quenched. Then the signal from the renilla luciferase is quantified. Using the latter numbers as a normalizing factor, pipetting errors, differences in cell viability, cell lysis efficiency etc. can be accounted for (see above).

SREBP transcription factors recognize the SRE sequences in the promoter region of lipidogenic genes. Mammalian SRE sequences have been well characterized. The experimentally-verified mammalian SREs show a surprising variation in sequence. This means that knowing the SRE sequence from one gene, such as the gene encoding the low-density lipoprotein receptor (LDLR) is not a useful tool for identifying the SREs in the promoters of other SREBP target genes. This is true even when one separates targets of SREBP-1 from SREBP-2. The mammalian SREs are not clearly present in the promoters of the targets of *Drosophila* SREBPs. Thus, the *Drosophila* SRE sequences in *Drosophila* are not known with precision. The one gene that we know that appears to be solely regulated by dSREBP *in vivo* is a lipase-encoding gene, CG6295. Krista Matthews in our lab cloned the promoter region of CG6295 into GFP and luciferase reporter constructs. GFP is a very stable protein in *Drosophila* (Krista Matthews and Rob Rawson, unpublished data) so that even when she suppressed dSREBP activation, the GFP signal remained significantly elevated. When she used luciferase reporter system, she likewise observed very little suppression. In S2 cells, when dSREBP is treated with 100uM palmitate and ethanolamine, the full suppression lasts only 2 hours. After that dSREBP precursor is proteolytically activated again (Seegmiller et al., 2002).

This is likely because the added palmitate and ethanolamine are metabolized and used for cell growth.

Bilal Amarneh in our lab cloned a putative insect SRE sequence from the promoter of CG6295 (Bilal Amarneh and Rob Rawson, unpublished data). I have employed a luciferase construct harboring three copies of the insect SRE sequence in the promoter region. Treatment of S2 cells with palmitate and ethanolamine did not result in diminished luciferase activity when we used wild type luciferase (see above). I hypothesized that stability of the luciferase enzyme might be masking changes at the level of transcription. I therefore tried an analogous construct made with luciferase that contains a degron box, which destabilizes proteins (Gilon et al., 1998; Rogers et al., 1986). Western blot analysis of those cells transfected with either reporter construct (wild-type or destabilized firefly luciferase) revealed that the dSREBP pathway is suppressed in those cells. Thus we considered the possibility that although the promoter is only regulated by dSREBP in midgut of drosophila larvae; in S2 cells it might require additional transcriptional co-factors that are not present.

To circumvent this limitation, I switched to GAL4-Vp16-dSREBP, UAS-luciferase system. GAL4-Vp16 is an artificial transcription factor that works in S2 cells and *Drosophila* larvae (Kunte et al., 2006). GAL4 is a yeast transcription factor DNA binding domain and Vp16 is a very strong viral transcriptional activator domain. We have a genomic construct in which the transcription factor domain of dSREBP is replaced

by the yeast GAL4 transcription factor. I co-transfected S2 cells with this GAL4-dSREBP and a UAS-luciferase construct. After palmitate and ethanolamine treatment, I initially observed a 5-fold difference in luciferase activity between suppressed and unsuppressed cells. Given the considerations above, I next used GAL4-dSREBP and luciferase constructs each containing a degron box. I introduced the degron box to GAL4 because we have shown that in *Drosophila* S2 cells, the nuclear form of dSREBP has a half-life of less than 30 minutes (Adam Seegmiller and Rob Rawson, unpublished data). With these new, destabilized constructs, I achieved a 20-30 fold dynamic range of luciferase activity in suppressed versus control cells, depending on the duration of palmitate and ethanolamine treatment. As a result of my studies, this assay is now suitable for use in a high through-put RNAi screen in S2 cells for genes that disrupt ER retention of dSREBP.

Luciferase assay

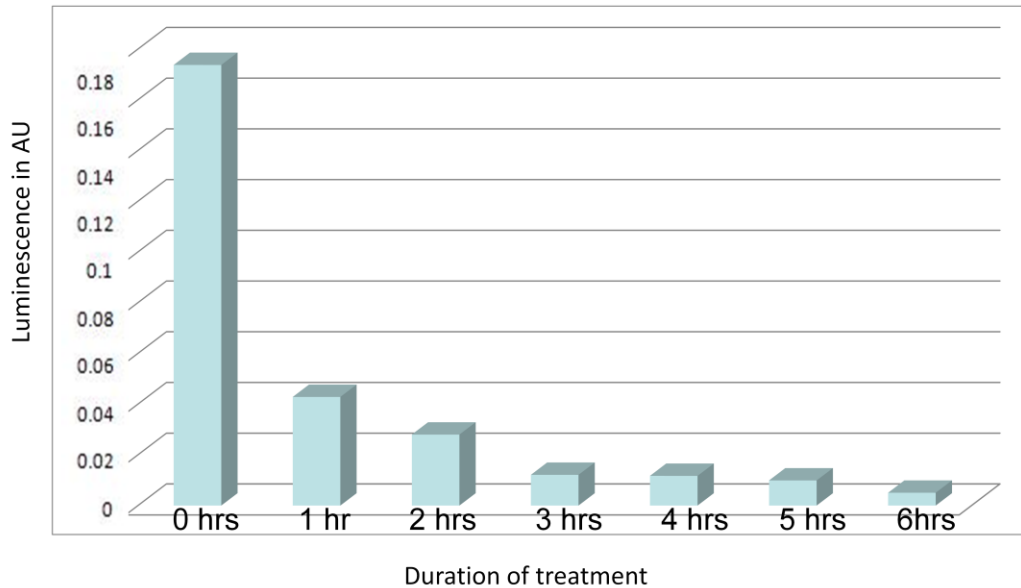


Figure 18. Luciferase assay on S2 cells under suppressing conditions

S2 cells are transfected with pCasper4-GAL4-VP16-dSREBP and p9xUAS-Luciferase-CP plasmids on day 0. On day 3, the cells are treated with 100uM palmitate-BSA and ethanolamine for durations shown on the figure. After the treatment is over, the cells are lysed and luciferase assay is performed as described in Materials& Methods.

ACCUMULATION OF DRICE CLEAVED VS. S2P CLEAVED dSREBP

The significant function of the putative PEST sequence that is present in S2P-cleaved dSREBP but absent in Drice-cleaved dSREBP is confirmed by another experiment. With an undergraduate student, Ali Hadayat, I examined the relative stability of the two different dSREBP cleavage products. We employed two expression vectors: the first encodes amino acids 1-452 of dSREBP, corresponding to the dS2P-cleaved product; the other encodes amino acids 1-386, corresponding to Drice-cleaved dSREBP. Both constructs include a HSV epitope tag. This enables us to detect the resultant proteins

from transfected cells without the background contribution of endogenous dSREBP present in both the transfected and untransfected cells.

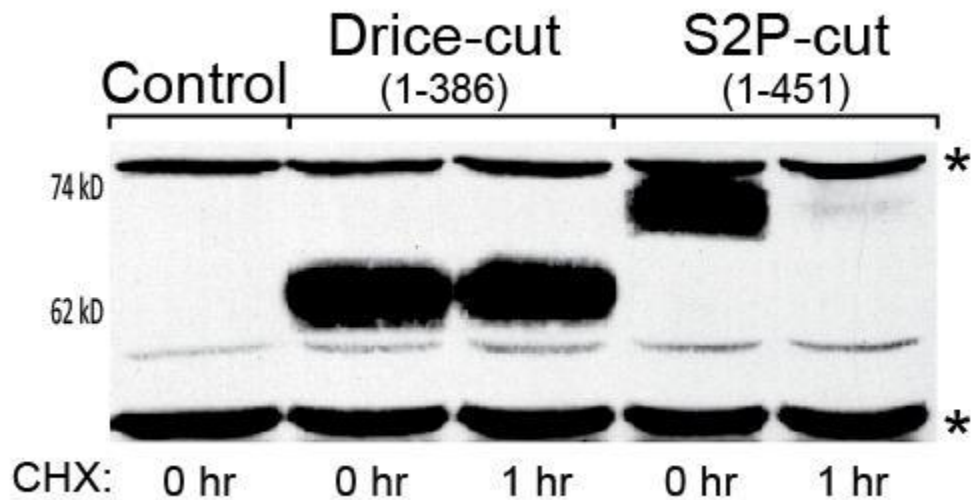


Figure 19. Accumulation of nuclear forms of dSREBP

Drosophila S2 cells were transfected with an empty vector control, two plasmids expressing HSV-tagged dSREBP residues from 1 to 386 and two of 1 to 451. Following transfection, *de novo* translation is inhibited via cycloheximide (CHX) treatment for one hour in order to block the production of additional dSREBP protein. The whole-cell lysates were analyzed by immunoblotting with anti-HSVepitope monoclonal antibody. Asterisks (*) indicate cross-reacting bands.

The fragment corresponding to caspase-cleaved SREBP is much more stable than the dS2P fragment in this assay. We believe that this is because the caspase-cleaved SREBP lack the PEST degradation box as well as a putative phosphorylation site that shorten the half-life of S2P-cleaved dSREBP. This might relate to its physiological function of Drice cleaving dSREBP.

STEARYL DEHYDROGENASE

Fatih Akdemir, a former postdoctoral fellow in our lab, employed GAL4-Vp16-dSREBP/UAS-GFP binary reporter system in combination with a genome-wide fly RNAi library. The idea is that in each fly line different RNAi construct is expressed under UAS promoter. In the tissues that dSREBP pathway is active, GAL4-Vp16 is released from the chimeric precursor and drives the expression of RNAi knockdown hairpin sequence. If the knocked down of the gene has no effect on the dSREBP pathway we do not see any change on GFP signal.

If knocking down the gene even slightly promotes activation of dSREBP pathway, then that forms a positive feedback loop. In other words, that results in more free GAL4-Vp16 and thus better knock-down and more activation of the pathway.

If knocking down the gene inhibits the activation of the dSREBP pathway then it forms a negative feedback loop. As we observe the GFP signal in the 3rd instar larval stage, we observe the effect of the knock down in steady-state.

As the screen is designed in the complexity of an intact organism, rather than cell culture, another possibility is that knocking down the gene, will promote and inhibit the dSREBP simultaneously in different tissues.

Another of the hits from the screen is *seipin*. Knocking down Seipin, decreases the GFP signal in our reporter line. Mutations in human Seipin causes Berardinelli-Seip Congenital Lipodystrophy (BSCL2) type II.

Seipin is found at the junction of Endoplasmic Reticulum and lipid droplets(Szymanski et al., 2007). Yeast mutants which lack Seipin show aberrant lipid droplet morphology(Szymanski et al., 2007). When I knocked down Seipin in *Drosophila* S2 cells I could not detect any change in the suppression of dSREBP pathway via palmitate and ethanolamine treatment. I overexpressed Seipin with a strong actin promoter and could not observe any change either.

Another hit from the genome-wide RNAi screen is stearoyl coenzyme A desaturase 1 (*desat1*). Desat1 is an enzyme that catalyzes the introduction of double bonds into fatty acids with $\Delta 9$ specificity. Interestingly, in lymphocytes of Seipin-deficient patients, the lipid droplets are smaller and the number of lipid droplets is increased as compared to wild-type lymphocytes (Boutet et al., 2009). The analysis of lipid types in these cells revealed that Delta9-desaturase activity is compromised (Boutet et al., 2009). I knocked down *desat1* in *Drosophila* S2 cells, and treated the cells either with palmitate (16:0) and ethanolamine or oleate (18:1) and ethanolamine. As we see in Figure 20, palmitate and ethanolamine treatment cannot suppress dSREBP pathway in *desat1* knocked-down cells. However, oleate and ethanolamine can suppress the pathway. This data suggests that the fatty acids in phosphatidyl ethanolamine that suppress the dSREBP pathway(Dobrosotskaya et al., 2002), should be oleate rather than palmitate. This is

consistent with the typical lipid composition of PE in flies, wherein both the sn1 and sn2 positions are usually occupied by unsaturated fatty acids.

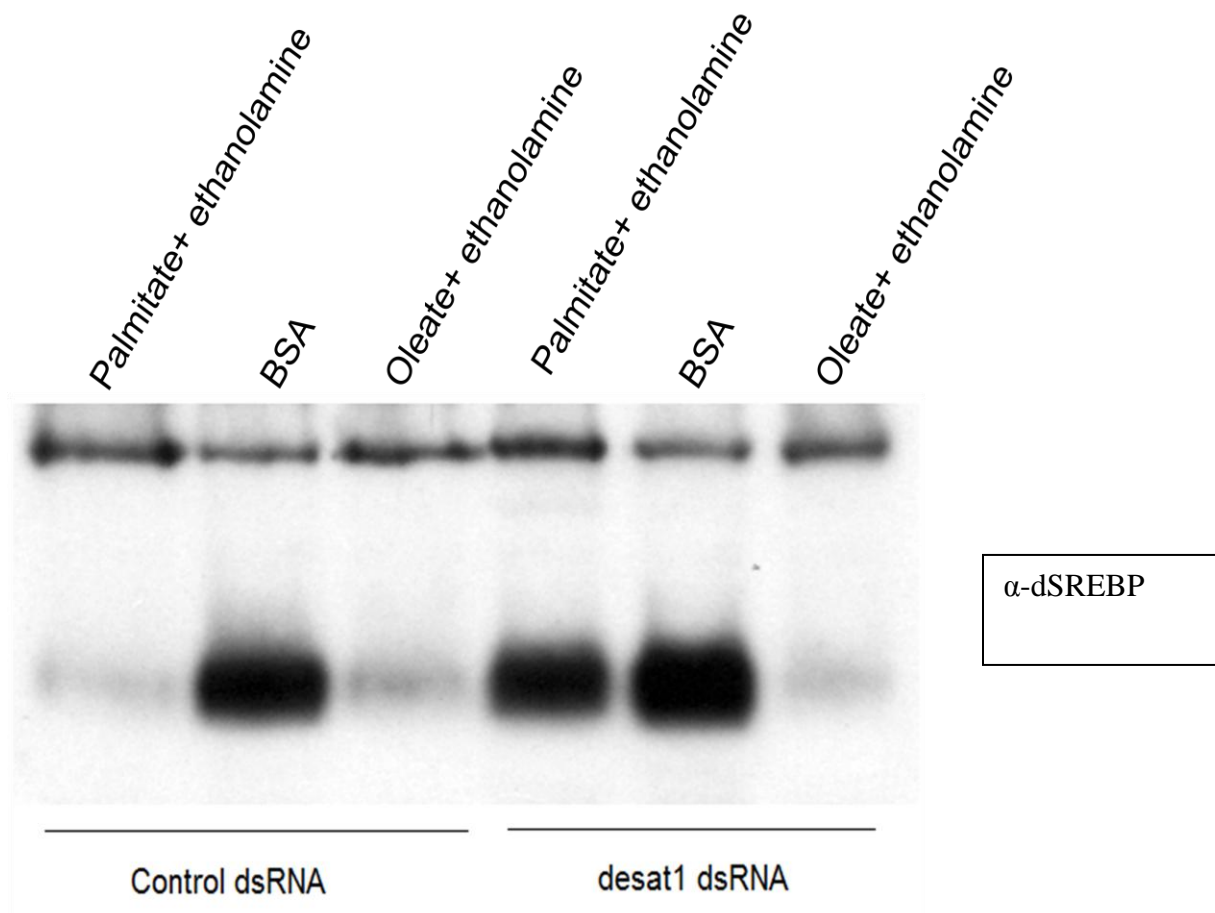


Figure 20. Suppression by palmitate requires *desat1*

As soon as the *Drosophila* S2 cells were seeded, double-strand RNAs (dsRNA) against *desat1* and control dsRNA against mouse Cyp7A1 were added to the culture media. On day 3, the cells were treated with ethanolamine and palmitate or oleate. As palmitate and oleate were conjugated to BSA, we also have BSA treatment control. After 4 hrs of treatment the cells are harvested in RIPA lysis buffer. The immunoblot analysis with dSREBP antibody was performed as described in Materials&Methods.

When the S2 cells are treated with ethanolamine and BSA conjugated palmitate, it is assimilated into phosphatidylethanolamine (Dobrosotskaya et al., 2002). When I knocked down the desat1 in S2 cells BSA-conjugated palmitate and ethanolamine treatment could not suppress SREBP cleavage. However BSA-conjugated oleate could still suppress. My data suggests that palmitate should be reduced to oleate.

CHAPTER FOUR

Methodology

Adapted from Matthews, Ozdemir and Rawson (Matthews et al., 2010)

FLY FOOD

Standard culture medium is cornmeal–molasses–agar (1 liter contains 60 g cornmeal, 15 g dry yeast, 80 ml unsulphured molasses, and 12 g agar, 6 ml propionic acid, and 0.1 g tegosept). Semidefined media is as described (Backhaus, 1984). One liter contains 80 g baker's yeast, 20 g yeast extract, 20 g peptone, 30 g sucrose, 0.5 g $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$, 6 ml propionic acid, and 0.1 g tegosept. Supplemented medium (C14:0 + C18:1) is either of these media, as indicated in the figure legends, to which myristate (0.075%, w/v) and oleate (0.15%, w/v) are added as the sodium salts or to which soy lipids (Avanti Polar Lipids) are added (9% w/v) as indicated.

GENETIC STRAINS

All marker mutations and balancer chromosomes are described and referenced by FlyBase Consortium (2003). Other lines are described in (Amarneh et al., 2009; Kunte et al., 2006; Matthews et al., 2009; Matthews et al., 2010). Crosses were conducted at 25° in vials containing freshly yeasted standard medium. *OreR* flies serve as wild type.

ANALYSIS OF LARVAL GROWTH

Embryos were collected for 2 hr and plated at 10 mg/plate onto semidefined media as previously described (Kunte et al., 2006). Larval stage was determined by mouth hook

and anterior spiracle morphology (Demerec, 1950). Representative larvae were selected at each time point and photographed as described (Kunte et al., 2006)

ADULT EMERGENCE ASSAYS

Embryos were collected overnight and seeded into vials at 1 mg embryo/vial containing standard medium either with no additions or supplemented with 0.075% Na-myristate and 0.15% Na-oleate (Sigma). Larvae were allowed to develop and emerging adults were cleared twice daily from the culture and scored for genotype starting at day 10 after egg laying (AEL).

LIPID MASS DETERMINATION

For each determination, 60 virgin females of the indicated genotype were collected 3 days posteclosion and homogenized in 3.0 ml extraction reagent [CHCl_3 : CH_3OH :PBS (1:2:0.8)] in a 7-ml Dounce homogenizer. The resulting mixture was separated by centrifugation at $1500 \times g$ for 10 min. The aqueous phase was removed and reextracted with 3.0 ml extraction reagent and re-separated. The pooled organic phase was transferred to a preweighed glass tube and dried under anhydrous nitrogen for 30 min. The mass of lipids was measured by weighing the tube on a Metler-Toledo AX105 DeltaRange analytical balance and subtracting the initial mass of the tube.

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). Contrast was adjusted in Adobe Photoshop CS2 using the autocurves function with 50% fading.

WHOLE-LARVAL LYSIS

Larvae of the indicated age and genotype were homogenized in buffer F (125 mM Tris–HCl, pH 6.8, 8 mM urea, and 5% SDS). For first instar larvae, 5 mg of embryos were seeded onto filter paper wet with IPL-41 insect cell culture medium (Life Technologies). Larvae were collected from the paper and homogenized in buffer F. For third instar larvae, larvae were separated from food by salt flotation and homogenized in buffer F. Homogenates were filtered through a 100 μ m Nytex mesh at 1000 g for 1 min. Total protein concentration was determined using BCA protein assay (Promega).

FRACTIONATION OF ADULT FLIES

On day 0, 24- to 48-hr-old males were fed in vials with fresh yeast. On day one, flies were transferred to vials (50 flies/vial) containing 0.25-inch Dacron plugs soaked with “starvation” media (1:3 IPL-41:PBS, made fresh daily). The diluted insect culture medium provides a minimum of nutrition to the adults and greatly improves their survival as compared to starvation on PBS alone. Flies were transferred to vials with fresh starvation media on day three. On day four, dead flies were removed, and the remaining flies were pooled and then divided in two groups. One group was transferred to vials (50 flies/vial) with fresh starvation media, while the second group was transferred to vials (50 flies/vial) with freshly made wet yeast paste. After 24 hr, any dead flies were removed

and the remaining flies were harvested for subcellular fractionation as described (Matthews et al., 2009)

CELL FRACTIONATION

S2 cell are grown in 10% heat inactivated FBS Schneider's media. Cells are harvested and spinned down 1000g for 5 min. the supernatant was discarded. Cells are washed with chilled PBS and spinned down 1000g for 5 min. The supernatant was discarded and the pellet was resuspended in chilled Buffer A. After 15 min incubation in Buffer A the samples are passed through 22.5 g needle syringe 30 times. And elute was spinned down for 7 min at 1000g at 4C. The pellet of this spin is frozen in -80c for 15 min and thawed at room temperature and then agitated in cold room for 1hr and ultraspinned for 30 min at 4c at 100k g of which of the supernatant is the nuclear extract. The supernatant is put in Beckman tubes and ultracentrifuged for 30 min at 100k g. The pellet is denoted as the membrane fraction.

MONOCLONAL ANTIBODIES

IgG1-3B2, against the amino transcription factor domain of dSREBP (1-451) was described in Seegmiller *et al.*, 2002.(Seegmiller et al., 2002) 7A8 anti-dScap antibody is described in (Herz et al., 1990) 9E10 anti-Myc antibody was obtained from Santa Cruz. IgG-611B-1 against acetylated tubulin was from Sigma (St. Louis). HSV monoclonal antibody was from Novagen.

EXPRESSION PLASMIDS

pDS47-HSV-dSREBP was described in Seegmiller *et al.*, 2002.(Seegmiller et al., 2002).

pGAL4-dSREBP was modified from genomic construct that is described in Amet *et al.*(Kunte et al., 2006). pUAST-GFP was cloned by inserting GFP into pUAS vector that is obtained from Promega. pUAS-firefly luciferase-CP was obtained from Promega. pGAL4-CL1-SREBP construct: pGAL4-dSrebp genomic vector (described in Amet *et al.*,(Kunte et al., 2006)) was added a putative PEST sequence and an insect codon optimized CL1 degradation sequence between the VP16 and the dSREBP sequences.

LUCIFERASE REPORTER ASSAY

Drosophila S2 cells were co-transfected with the pGAL4-VP16-CL1-dSrebp construct and p9xUAS-Luciferase-CP on day 0. On day3, the cells were treated with palmitate and ethanolamine for up to 6 hrs. Then the cells were lysed and the luminescence was read in a luminometer.

CELL TRANSFECTION

Fectofly reagent from Polyplus was used as in manufacturer's manual. Briefly, expression plasmids are diluted in NaCl solution, and then Fectofly reagent is diluted in NaCl solution in a separate tube. After quick vortexing, Fectofly dilution is added to the plasmid dilution and the mixture is vortexed for 10 sec and then incubated at room temperature for 30 min. In a drop-wise manner the solution is added into the cell culture media. After 4 hours more tissue culture media is added on top the cells.

dsRNA PRODUCTION AND RNAi

The DNA templates for in vitro double-stranded RNA (dsRNA) synthesis are produced by PCR using primers which have T7 promoter sequence (TAATACGACTCACTATAGGG) on 5' end. PCR products were purified the High Pure PCR Product Purification kit (Boehringer Mannheim). MEGAscript T7 kit (Ambion) as described (Clemens et al., 2000) The dsRNA was kept at -20°C until use. On day 0, S2 cells are seeded 1 million in 1 ml Express 5 SFM media per well in a 6-well plate. Within minutes of seeding 15 micrograms of dsRNA per well is added to the media. Two hours later, 4 ml of Schneider media with 10% FCS is added to each well. On day 3 the cells are harvested and once washed with PBS and then lysed in RIPA buffer with protease inhibitors.

CHAPTER FIVE

Conclusions and Recommendations

Since the development of techniques to grow cells from higher organisms in culture, cell culture has been a fruitful tool for experimental exploration. The study of cultured cells has made possible the understanding of complex biological problems and led to the creation of new paradigms. However particularly for metabolic research, complimentary experiments in model organisms are vital. Much work has been done in rodent models (Grefhorst et al., 2010; Wang et al., 2011b). Our lab uses *Drosophila melanogaster* as one of the model systems in which the enigma of SREBP pathway may be deciphered.

In the course of my dissertation research, I have contributed to the characterization of *Drosophila* Scap. The early life cycle of *Drosophila melanogaster* consists of three larval stages (or stadia), called instars (from the Latin for ‘form, figure, or likeness’). In holometabolous insects (those that change form completely, such as flies), the larval instars are the period when the organism exploits an abundant food source to store energy as fat. The organism uses this stored energy to fuel pupation and metamorphosis as well as the early adult life. During the larval instars, the excess energy from the diet is stored as triglycerides in the fat body. In addition to its role in lipid synthesis and storage, this organ also exhibits other functions that are characteristic of the vertebrate liver (Sondergaard, 1993)

The dSREBP pathway is one of major feedback circuits regulating lipid metabolism in flies. Not surprisingly, *dsrebp* null flies cannot proceed into third instar stage (Kunte et al., 2006). Previously, in all systems studied, SREBP precursors have to travel from ER to Golgi in order to be processed by S1P and S2P. This ER-to-Golgi transport requires Scap. Mammalian cells that lack Scap cannot activate SREBPs and are thus auxotrophic for unsaturated fatty acids (owing to lack of active SREBP-1) and cholesterol (owing to lack of SREBP-2). These cells die unless their culture medium is supplemented with oleate and free cholesterol (Rawson et al., 1999). I wished to determine whether dScap is likewise essential in flies.

dscap IS NOT ESSENTIAL IN THE FLY

Previously our lab has shown that, in *ds2p* null flies, cleavage by Drice produces sufficient dSREBP activity to enable larvae to survive (Amarneh et al., 2009). The data that I present in this dissertation reveals that in the absence of dScap, the dSREBP pathway continues to be activated in subset of the tissues where dSREBP is normally activated and that this activation is sufficient for survival. Using the GAL4-dSREBP binary reporter system, I compared the activation of dSREBP in wild type, *dscap* null, *ds2p* null and *dscap;ds2p* doubly-null flies (Figure 10). In wild type larva, several tissues including fat body, oenocytes and midgut show strong GFP expression. In *dscap* null larva, only oenocytes have strong GFP signal. On the other hand, *ds2p* null larva has GFP signal predominantly in fat body. At this exposure time, *dscap;ds2p* doubly-null

larva does not glow green. These data showing decrease in the number of cells which have active dSREBP is consistent with quantitative RT-PCR data.

As shown in Figure 9, whole larval extracts from wild type larvae has the strongest expression of dSREBP target genes. Although *dscap* null larvae express dSREBP target genes at lower levels than seen in the wild type, the level of expression is significantly greater than seen in *dsrebp* null larvae (Matthews et al., 2010). Furthermore, triglycerides accumulate in the *dscap* null larvae. In *ds2p* null flies, dSREBP continues to be activated in the fat body. This is, presumably, where Drice cleaves the dSREBP precursor. Similar to the case of *dscap* mutant larvae, the expression levels of dSREBP target genes are sufficient for lipid storage and survival through larval life and into adulthood. There is also sufficient lipid to enable adequate provisioning of the oocyte since both males and females of these mutants are fertile. In fact, both *ds2p* and *dscap* null mutants may be easily maintained as homozygous stocks and we have done so for hundreds of generations (Matthews et al., 2009; Matthews et al., 2010).

On the other hand, absence of both *dscap* and *ds2p* cause more dramatic phenotype. In doubly null larva, with the GAL4-dSREBP binary reporter system shows no GFP expression (Figure 10). When assayed at second instar, there is little difference in triglyceride content between wild-type and any of the mutants (Figure 12). This changes dramatically once the larvae reach the third instar. Wild-type larvae exhibit the expected massive increase in triglyceride content but each of the mutants display a substantial, significant deficit. In addition to accumulating less triglyceride than wild-type larvae, the

doubly-mutant larvae are absolutely smaller, at any given time after egg laying. It may be that those larvae have less TG overall but that TG/total mass is the same, or slightly larger when compared to either single mutant. I speculate that larvae evaluate the TG concentration in their body and if the prospect is favorable at certain stage of larval life, they continue their developmental program. Otherwise they quit eating and die so that other larvae which have relatively better chance of survival will propagate.

We do not yet know the mechanism that permits dS1P and dS2P to cleave dSREBP in larvae lacking dScap. It seems likely that these alternative means of activation also operate in wild-type larvae, at least under some circumstances. In the presence of the classical processing machinery, their contribution to overall dSREBP activity appears to be modest. Their contribution does afford enough dSREBP activity, however, to permit survival of the *dscap* mutants. It will be interesting to learn whether these same alternative ways of activating SREBP also occur in mammals.

THE SURVIVAL MECHANISM OF *dscap* NULL FLIES

The mechanism of *dScap* survival may be related to the complex endomembrane system of oenocytes. These enigmatic cells are the site of synthesis for the cuticular hydrocarbons, which are crucial to insect water balance. Oenocytes have also been shown to possess some hepatocyte-like functions as well (Billeter et al., 2009; Clark and Dahm, 1973; Fan et al., 2003; Gutierrez et al., 2007; Locke, 1969; Romer, 1974; Wigglesworth, 1970) .

Bard *et al.*, conducted a genome-wide RNAi screen in order to explore the genes required for the formation of Golgi apparatus in *Drosophila* cell culture (Bard et al., 2006). It is possible that absence of dScap perturbs the organization of the Golgi apparatus. If absence of *dScap* compromises the integrity of ER and Golgi membrane then the dSREBP precursor and the two proteases, dS1P and dS2P, which normally reside in the Golgi, can come together in the same membrane. This would obviate the need for dScap-mediated intracellular transport between compartments.

In *dscap* null flies, feeding with lecithin decreases the GFP signal from GAL4-SREBP/UAS-GFP binary system. These data seem contrary to the above mentioned hypothesis, because if dSREBP precursor and the two proteases are on the same membrane then the regulation of dSREBP activity would be compromised. However, the decrease in GFP signal may be explained, in part, by the 50% reduction of dSREBP mRNA under this condition. If the *dsrebp* promoter is weaker than the GAL4-dSREBP expression also decreases. Thus there is less amount of GAL4-srebp to cleave and release GAL4 which leads to less GFP expression.

Organelle membrane specific fluorescent markers are available for *Drosophila* (LaJeunesse et al., 2004). In order to test whether Golgi mixes with the ER in certain cells and tissues, or at certain times during development, we need to use these marker alleles within the *dscap* mutant background and explore if the ER and Golgi markers co-localize. If the signals merge that would suggest that in the absence of dScap, precursor of dSREBP and the proteases (S1P and S2P) may reside next to each other (*i.e.* in the

same membrane). I have tested the plausibility of this hypothesis via Brefeldin A treatment of S2 cells (see Appendix). Brefeldin A is a toxin that disrupts the distinction between the ER and the Golgi apparatus (Fujiwara et al., 1988). When I treated the *Drosophila* S2 cells with Brefeldin A, even under suppressing conditions (palmitate and ethanolamine treatment), dSREBP precursor is cleaved into nuclear dSREBP (see appendix A). This experiment confirms earlier findings in mammalian cells lacking Scap (Rawson et al., 1999) and demonstrates that mixing of the ER and Golgi in insect cells can also result in constitutive activation of dSREBP.

THE COPI TRANSPORT SYSTEM

Another possibility is that the ER and Golgi apparatus membranes are intact and spatially separated but that active S1P and S2P travel back to the ER membrane. The coatamer protein complex I (CopI) is responsible for the retrograde transport of vesicles from the Golgi apparatus to the ER (Malhotra et al., 1989; Rothman and Wieland, 1996). It also plays an important role in lipid homeostasis (Beller et al., 2008). If the regulation of the CopI transport system is disturbed in *dscap* null flies, it is possible that S1P and S2P proteases can travel back to ER where the dSREBP precursor normally is found.

In order to test whether the CopI transport system non-specifically carries S1P and S2P back to the ER, CopI deficient fly alleles can be recombined with flies of the genotype *dscap*^{-/-}, GAL4-dSREBP,UAS-GFP. If this line viable and if the larvae have GFP signal in oenocytes, that would nullify the hypothesis the CopI vesicle trafficking has some role in dSREBP activation in dScap null flies.

A novel pulse-chase labeling method provides a system to visualize life cycle of proteins (Gaietta et al., 2002; Gaietta et al., 2006). In addition to the fluorescent marker lines from the previous section, we need to generate tetracycline-tagged dSREBP, dS1P, dS2P, and MannII-GFP-4C as Golgi marker fly lines. After isolating the oenocytes, biarsenical labeling of the tetracycline tags will reveal the orientation of these proteins. Orientation of these markers would reveal whether the dSREBP precursor and the proteases, dS1P and dS2P, localize side by side on the same membrane.

COPI transport system was also implicated in the viral pathogenesis in *Drosophila* (Cherry et al., 2006). Formation of aberrant vesicles for viral replication requires COPI trafficking and fatty acid biosynthesis (Cherry et al., 2006). These findings also position COPI trafficking in the intersection of the dSREBP pathway and membrane vesicle formation.

DESATURATION OF FATTY ACIDS

According to systematic suppression analysis of dSREBP pathway by our former lab members Irina Dobrosotskaya (Dobrosotskaya et al., 2002) and Adam C. Seegmiller (Seegmiller et al., 2002), when *Drosophila* S2 cells are treated only with fatty acids only palmitate can suppress dSREBP activation. This is due to the requirement of phosphoethanolamine synthesis through sphingolipid pathway (Dobrosotskaya et al., 2002). However; when the cells are treated both with ethanolamine and fatty acids, oleate can also suppress the dSREBP pathway as strongly as palmitate (Dobrosotskaya et

al., 2002). My data is in line with these previous findings and also suggests that palmitate should be reduced to oleate.

CASPASE AND AUTOPHAGY

Another mechanism of SREBP cleavage is through the Caspase Drice (Pai et al., 1996; Wang et al., 1995; Wang et al., 1996). The physiological relevance of caspase-cleaved SREBP is not known. However, the caspase-cleaved SREBP may be more stable than the S2P-cleaved SREBP because the former lacks the PEST sequence.

We have previously shown that dS2P is dispensable (Matthews et al., 2009). In *ds2p* null flies Drice cleaves the dSREBP precursor right before the first transmembrane helix. Just as seen with *dsrebp* null flies, flies doubly-mutant for *ds2p* and *drice* cannot develop into third instar larvae. Importantly, this lethality can be rescued with fatty acid supplemented food (Amarneh et al., 2009). This tells us that these double mutants die owing to a deficiency in fatty acid accumulation that can be overcome by dietary supplementation. Again, this is precisely what we observe in flies lacking dSREBP. Thus, loss of the two proteases that are known to be able to produce active dSREBP phenocopies loss of dSREBP itself.

Apoptosis-independent functions of caspases have been revealed. Muro *et al.*, found that DIAP1 keeps continuously activated Dronc under control and that this can prevent apoptosis (Muro et al., 2002). Sometimes cells evade apoptosis and activate autophagic pathways (Giansanti et al., 2011). Autophagy is a self-degenerative process of digesting

intrinsic components in lysosomal compartments. The process involves generation of double membrane containing organelles, autophagosomes.

One assay to detect autophagy exploits the localization of a highly conserved autophagosome protein LC3 (apg8 in yeast, Atg8 in *Drosophila*). Newly-synthesized LC3 is diffusely distributed inside the cytoplasm. After processing by Aatg4, LC3 protein becomes LC3-1 which still resides in cytoplasm, however after conjugation of phosphatidylethanolamine to a C-terminal glycine, it is now called LC3-II which locates to the autophagosome membrane with a punctuate distribution (Kabeya et al., 2000). LC3-GFP has been successfully established as an autophagosome marker both in mammalian and *Drosophila* model systems (Kabeya et al., 2000; Rusten et al., 2004; Scott et al., 2004; Yano et al., 2008)

Recently LC3 has also been implicated in the mobilization of internal lipid storage (Singh et al., 2009). It is possible that autophagy plays critical role in oogenesis (Barth et al., 2010). Dry weight of insect oocytes comes from 30-40% lipids (Briegel, 1990; Troy et al., 1975; Vanhandel, 1993) which is the source of energy during embryogenesis (Beenackers et al., 1985; Vanhandel, 1993). During oocytes maturation, in a short period they increase the total lipid amount several fold. Only 1% of the fatty acids are synthesized *de novo* in oocytes; the rest of the lipid deposited in the eggs comes from the fat body and is mobilized to the ovaries via lipophorin (Ziegler and Ibrahim, 2001; Ziegler and Van Antwerpen, 2006). This is consistent with the data that GAL4-dSREBP/UAS-GFP does not show much if any dSREBP activation in ovaries.

If larvae lack sufficient lipid synthesis and therefore exhibit deficient production of lipid that should have been deposited into the fat body, a starvation response is induced. One result of this response is that lipids in fat body are mobilized via autophagy (Scott et al., 2004). Normally, in wild type flies, autophagy is induced in wandering 3rd instar larvae. At this stage, immediately preceding pupariation and metamorphosis, the larvae stop feeding and the animal will not feed again until well after it has emerged as an adult. All its energy needs are met by mobilizing fat stores laid down previously.

In *dscap;ds2p* double mutants, I observed precocious autophagy, starting in the second instar. I dissected the fat body from mutant larvae and stain them with LysoTracker which labels acidic compartments. Observing the tissues under fluorescent microscope showed typical punctuate pattern of autophagy.

In order to sustain lipid homeostasis, dSREBP and autophagy pathway should talk to each other. It is possible that disruption of SREBP pathway mimics starvation and thus induces autophagy (Kunte et al., 2006). It would be interesting to explore the cross talk between autophagy and dSREBP pathway.

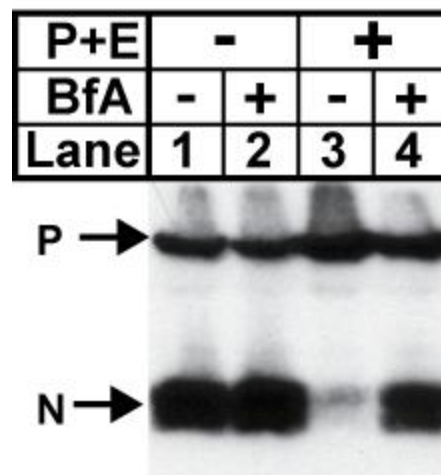
mir-33 IN FATTY ACID METABOLISM

miR-33 is located in the intron regions of SREBF-1 and 2 genes, and it reduces cholesterol export from liver (Najafi-Shoushtari et al., 2010; Rayner et al., 2010). Although *Drosophila*, like all arthropods, are cholesterol auxotrophs, the *dsrebp*

locus also has miR-33. It will also be interesting to explore the role of miR-33 in *Drosophila*. In mammals, miR-33, which is transcribed along with dSREBP, targets transcripts of SREBP target genes (Najafi-Shoushtari et al., 2010). Thus, increased transcription of SREBP itself leads to an increase in one form of translational suppression for its target genes. Such counteracting signals may serve to ‘fine-tune’ the activity of lipid synthetic enzymes when demand increases. Although as yet unproven, it is reasonable to speculate that the conserved miR-33 is playing a homologous role in *Drosophila* lipid metabolism. Exploration of this intriguing possibility is another potentially-productive avenue for future work on lipid metabolism in flies.

APPENDIX A

Brefeldin A treatment of S2 cells

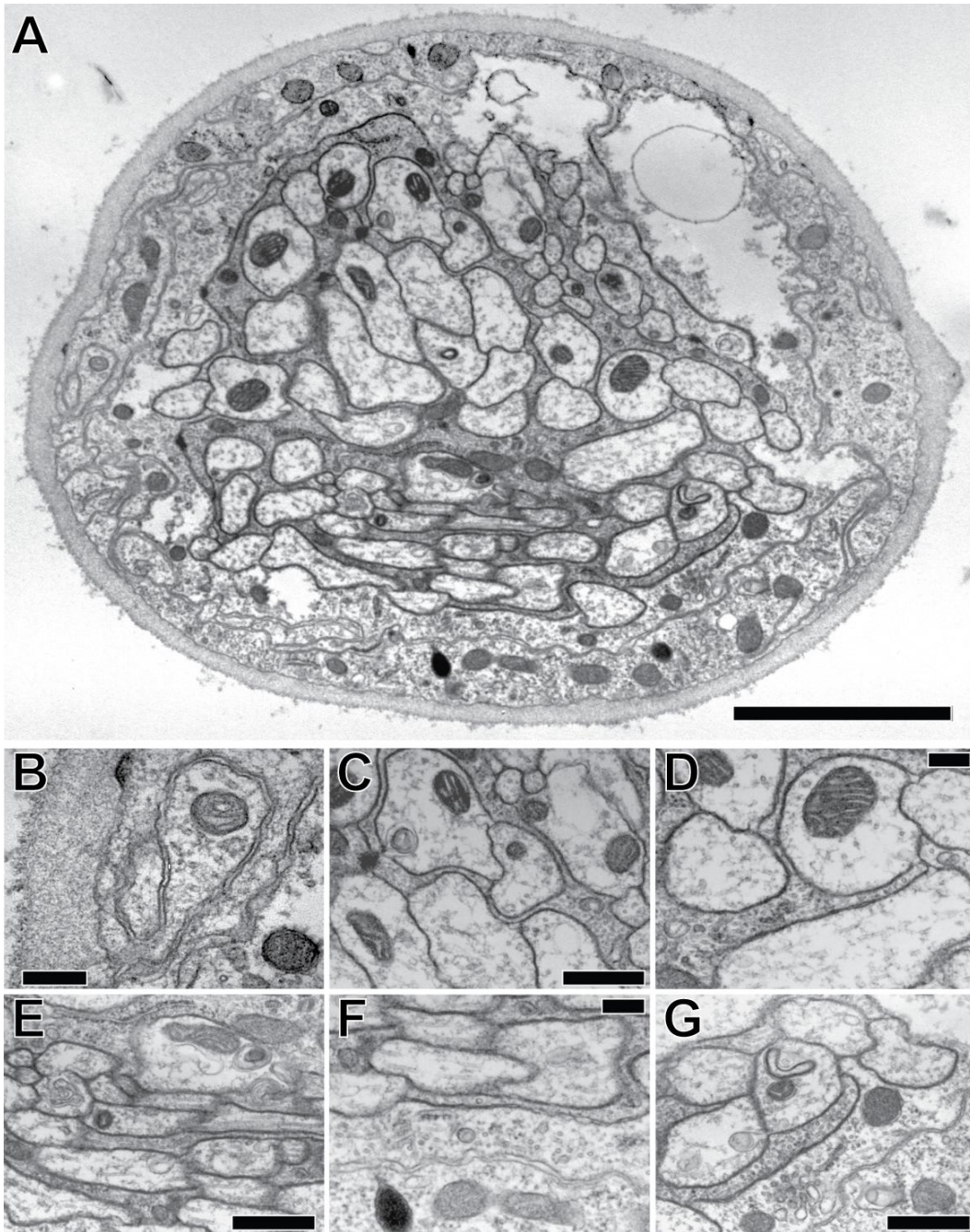


The effect of Brefeldin A treatment on dSREBP suppression

Drosophila S2 cells are treated with Brefeldin A with or without supplementary fatty acid and ethanolamine. Cells are lysed in RIPA buffer and the samples are immunoblotted with dSREBP antibody as described in Materials & Methods.

APPENDIX B

Autophagosomes in oenocytes



A wild-type *Drosophila* oenocyte. The complex endomembrane structure is evident. (A) Overview. Scale bar = 2 μm . The cell is replete with double-membrane-bound vesicles containing mitochondria (i.e. autophagosomes). This is also observed in oenocytes from butterfly and house fly (Clark and Dahm, 1973; Locke, 1969). (B-G) Higher magnification views of regions indicated by the corresponding letters in A. Scale bars = 250 nm. Third-instar larvae were fixed and prepared for electron microscopy, and stained with 3% aqueous uranyl acetate (15 min) and lead acetate (5 min) as described (Michaely et al., 2004). Specimens were imaged on a FEI Tecnai G2 Spirit Biotwin using a SIS Morada 11 mpixel side mount CCD camera.

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