12-2014

# Investigation of Nuclear and Cytoplasmic Functions of the dLipin Protein of Drosophila Melanogaster

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# Investigation of Nuclear and Cytoplasmic Functions of the dLipin Protein of *Drosophila Melanogaster*

Investigation of Nuclear and Cytoplasmic Functions of the dLipin Protein of *Drosophila Melanogaster*

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by
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Shanghai University
Bachelor of Engineering in Bioengineering, 2009

December 2014
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

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**Abstract**

Lipin family proteins are highly conserved proteins present in species ranging from mammals to yeast. *Lipin 1*, the first *Lipin* gene identified in fatty liver dystrophy (*fld*) mutant mice, encodes the bifunctional protein Lipin 1, which can serve as an $Mg^{2+}$-dependent phosphatidic acid phosphatase (PAP) and transcriptional co-regulator. *dLipin*, the single *Lipin* ortholog of *Drosophila melanogaster*, is required in triglyceride synthesis and fat body development. To study the transcriptional co-regulator activity of dLipin, nuclear receptors were screened to find receptors that interact with dLipin. The genetic interaction data indicated that *Drosophila* hepatic nuclear receptor 4 (HNF4) was a promising candidate for a protein that cooperates with dLipin in gene regulation. To study the importance of the PAP activity without disturbing the co-regulator activity, mutant flies that only express dLipin protein that lacks the PAP enzymatic activity were generated. The mutant flies showed the normal expression level and pattern of dLipin. The delta PAP mutation is lethal to *Drosophila*. Ectopically expressed wild type dLipin or GFP-tagged dLipin protein rescued the flies through the early lethal stage until the pupal and, in some instances, the adult stage. The phenotypes of the fat body cells of the rescued animals showed a correlation with the amount of the ectopically expressed dLipin protein. Fat body cells with low PAP enzymatic activity were round, had lost their polygonal shape and were detached from each other. They also contained very small fat droplets. Sufficient PAP enzymatic activity is needed to ensure a normal developmental rate, too. Starvation resistance was greatly impaired in dLipin-GFP-rescued delta PAP mutant heterozygotes that carried a wild-type *dLipin* allele. In addition, dLipin-GFP proved to be unable to translocate into the cell nucleus and it prevented nuclear translocation of endogenous wild-type dLipin. Together, these data indicate that dLipin-GFP acts in a dominant-negative manner and that nuclear activity of dLipin is required under starvation conditions.

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# I. Introduction

## *1. Lipin gene family*

In both human and mouse, the Lipin gene family encodes three Lipin homologues: Lipin 1, Lipin 2 and Lipin 3. Lipin 1, which is the most extensively studied Lipin protein among the homologues, was first identified in fatty liver dystrophy (*fld*) mutant mice (Péterfy et al., 2001). In 1989, Langner and his colleague found a spontaneous recessive mutation in one of their mouse colony. Although these mice looked normal after birth, their growth rate was slow and they developed fatty liver and hypertriglyceridemia when they began to suckle (Langner et al., 1989). The symptoms were resolved after weaned from milk. However, they still had lifelong tremor and hind limb motor defects caused by peripheral neuropathy, and displayed infertility in males and reduced fertility in females (Langner et al., 1991). These mutant mice had very little adipose tissue, only about 20% of wild type controls, and developed insulin resistance and characteristics similar to lipodystrophy in humans (Garg, 2004; Reue et al., 2000).

The genetic mutation causing fatty liver dystrophy was first identified by positional cloning in 2001 (Péterfy et al., 2001). The null mutation of novel gene encoding a 98 kDa protein was found responsible for the symptoms described above and the gene was named *Lipin1*. In *Lipin1* of *fld* mouse, a deletion of the translation initiation site and its flanking sequence, 0.5 kb duplication in the 3' UTR, and an inversion of more than 40 kb of the genomic sequence were found. *Lipin1* expression occurs in white and brown adipose tissue and is induced during adipocyte cell differentiation. It is also expressed in skeletal muscle and testis (Nadra et al., 2008). In contrast to *fld* mice, ectopic expression of *Lipin1* in white adipose tissue or skeletal muscle in transgenic mouse led to exacerbated obesity induced by high-fat diet. However, even

on a chow diet, transgenic mice which overexpressed *Lipin1* in the musculature became more obese than mice with overexpression in adipose tissue (Phan and Reue, 2005). Therefore, Lipin1 in adipose tissue and in skeletal muscle causes obesity through different mechanisms. In adipocytes, Lipin1 enhances the expression of lipid synthesis and storage genes, while in muscle increased levels of Lipin1 help to decrease the utilization of fatty acids. A change of *Lipin1* expression level is sufficient to induce opposite states of adiposity in mice; a lack of dLipin leads to lipodystrophy while overexpression of *dLipin* leads to obesity (Reue and Zhang, 2008). Other tissues such as liver, brain and kidney also have low level Lipin1 expression. Schwann cells of peripheral nerves also have Lipin1 expression, which explains the peripheral neuropathy in the *fld* mouse (Verheijen et al., 2003).

Lipin 1 has two isoforms, Lipin-1α (891 amino acids) and Lipin-1β (924 amino acids), that result from alternative mRNA splicing. The 33 additional amino acids specific for isoform β may have an influence in its sub-cellular localization. Lipin-1α is mostly nuclear, while Lipin-1β is mostly cytoplasmic (Péterfy et al., 2005; Reue and Brindley, 2008). They also have distinct roles in adipocyte development. Lipin-1α is dominant in early differentiation stage and decreases as development progresses, while Lipin-1β is prominently expressed in mature adipocytes. During adipocyte differentiation, the 10 to 20-hour transient expression of Lipin-1α induces the expression of two important transcription factors peroxisome proliferator-activated receptor γ (PPAγ) and CAAT-enhanced-binding protein α (C/EBPα). In *fld* mouse, the level of adipogenesis inhibitor preadipocyte factor-1 is elevated. Lipin-1β is the dominant isoform in mature adipocytes and is necessary for lipid accumulation (Péterfy et al., 2005). While overexpression of Lipin-1α results in more fat precursor cells, high level of Lipin-1β leads to more lipid storage in each single cell (Phan et al., 2004). In mammals, Lipin2 and Lipin3 are the

other two additional Lipin family members, sharing a 60% amino acid sequence similarity with Lipin1 (Péterfy et al., 2001). The three Lipin proteins have different tissue expression patterns, suggesting distinctive physiological roles (Donkor et al., 2007). Up to now, little is known about Lipin2 and Lipin3. In recent studies, mutations in Lipin2 have been associated with two diseases in humans, an inflammatory disorder called Majeed syndrome, characterizing by recurrent episodes of fever, chronic recurrent multifocal osteomyelitis (CRMO), and a blood disorder called congenital dyserythropoietic anemia (Ferguson et al., 2005; Majeed et al., 2001). The normal expression level and pattern of Lipin1 and Lipin3 in these patients demonstrates that the unique function of Lipin2 cannot be replaced by other Lipin family members.

Lipin family proteins are wildly present in animals ranging from mammals to yeast, playing a fundamental role in metabolism. Different from mammals, *C. elegans* stores fat in intestinal and hypodermal cells. Knockdown the Lipin gene by RNAi in C. *elegans* leads to smaller worms and less amounts of neutral lipids. Not all the mutant animals can survive and about half of them died as embryos. The ones that successfully grow into adulthood have reduced fertility (Golden et al., 2009). SMP2, a *Lipin* homolog, regulates lipid synthesis in yeast. The Nem1-Spo7 phosphatase complex dephosphorylates SMP2 in yeast. Knockdown of SMP2 or the Nem1-Spo7 phosphatase complex results in nuclear expansion (Santos-Rosa et al., 2005).

***2. PAP activity of Lipin***

All the Lipin proteins have two highly conserved regions, one at the amino-terminal end (called N-LIP) and the other at the carboxyl-terminal end (C-LIP), as well as conserved nuclear localization signals. C-LIP contains two motifs, the PAP1 motif, DxDxT and a co activator motif, LxxIL (Finck et al., 2006).

Lipin protein has a dual function: It possesses a phosphatidate phosphatase activity, catalyzing the production of diacylglycerol (DAG) from phosphatidic acid, as well as a transcriptional co-regulator activity, participating in the regulation of lipid metabolism (Carman and Han, 2009; Finck et al., 2006). In mammals, triacyglycerol (TAG) is synthesized from glycerol phosphate, by the step-wise addition of acyl groups through the glycerol phosphate pathway (Coleman and Lee, 2004). These steps are catalyzed by different enzymes. Although the enzyme phosphatidate phosphatase 1 (PAP1), which converts phosphatidate (PA) to DAG, the direct precursor for the synthesis of TAG, as well as membrane phospolipids, phosphatidylcholine and phosphatidylethanolmine, has been extensively studied from the 1950s, its molecular identity remained unknown until recently (Kates, 1955; SMITH et al., 1957; Y STEIN, 1957). The isolation of PAP1 proved difficult, due to protein instability, ineffective purification methods and multiple isoforms (Martin et al., 1987). In 2006, Pah1p, the yeast phosphatidate phosphohydrolase was purified for the first time from *S. cerevisiae* (Han et al., 2006). The DxDxT motif in the C-Lip domain of Pah1p was later shown to be responsible for its PAP1 enzymatic activity (Han et al., 2007). As determined by sequence analysis, this $Mg^{2+}$ dependent PAP1 is a homolog of the mammalian Lipin proteins.

All the mammalian Lipin proteins, Lipin-1α, Lipin-1β, Lipin 2, Lipin 3, have PAP1 enzymatic activity (Donkor et al., 2007; Harris et al., 2007). However, since the three *Lipin* genes have different tissue expression patterns, they may have unique physiological roles. *Lipin1* has the highest expression level in adipose tissue, skeletal muscle and testis, but can also be detected in liver, heart and other tissues (Péterfy et al., 2001). Study of *fld* mice showed that Lipin1 is responsible for all the PAP1 activity in adipose tissue, skeletal muscle and heart while other family members may contribute in other tissues such as liver (Donkor et al., 2007; Harris et

al., 2007). *Lipin2* is expressed in many tissues such as liver, brain and kidney. Due to the up regulation of *Lipin2* in the liver of *fld* mouse, the PAP1 activity in the liver is almost normal, different from adipose tissue and skeletal muscle (Donkor et al., 2007). However, although the expression of *Lipin2* is detected in preadipocytes, it cannot rescue lipodystrophy of *fld* mice resulting from lack of Lipin1 protein (Fawcett et al., 2008).

Different from the acyltransferase enzymes in TAG synthesis, which are located on the membranes of the endoplasmic reticulum (ER) of cells, Lipin proteins are cytosolic and only associate transiently with the ER membrane to carry out PAP1 function when the cellular fatty acids levels are high (Cascales et al., 1984). This was revealed by the fact that no DAG and TAG could be detected when measuring glycerolipid synthesis from fatty acids with microsomal membranes. Only the addition of cytosolic fraction could greatly enhance the synthesis. The stimulating factor in the cytosolic fraction was then revealed to be a soluble PAP (Johnston et al., 1967; Smith et al., 1967).

***3. Co-regulator activity of Lipin***

In addition to the PAP1 enzymatic activity, Lipin proteins have a nuclear localization signal, indicating the ability to localize to the nucleus (Bou Khalil et al., 2009; Péterfy et al., 2005). Confirmed by immunocytochemistry, Lipin 1 was found to reside in both nucleus and cytoplasm in adipocytes, suggesting a nuclear function. Mutation of Ned1p, the Lipin ortholog in *S. prombe*, resulted in an abnormal nuclear structure and the presence of enormously overdeveloped ER-like membranes. Ned1p was also shown to interact with three nuclear proteins, crm1, pim1 and dis, which play role in nuclear transport and nuclear envelope formation (Tange et al., 2002). In *S. cerevisiae*, Smp2, the budding yeast homologue of mammalian Lipin, regulates nuclear membrane growth during the cell cycle. Loss of

*SMP2* causes up regulation of key enzymes involved in lipid biosynthesis and results in the expansion of the nucleus. Cell division can be inhibited by constitutive dephosphorylation of Smp2. The regulation is achieved by Smp2 association with the promoters of the genes encoding phospholipid biosynthetic enzymes, where it functions as a transcriptional repressor (Santos-Rosa et al., 2005).

In mammals, Lipin 1 is also very important in the regulation of gene expression. In mouse liver, nuclear receptor PPARα and transcriptional co-activator PPARγ co-activator-1α (PGC-1α) play a vital role in fatty acid oxidation and glucose homeostasis (Rosen et al., 2000). In mice that lack PCG-1α, the expression of Lipin1 and fatty acid oxidation genes are repressed during fasting (Finck et al., 2006). Further studies in mouse liver showed that Lipin 1 is required for the fasting-induced expression of PPARα and its target genes that are related to fatty acid oxidation. Lipin1, PGC-1α and PPARα physically interact with each other to form a complex that can activate the genes involve in fatty acid oxidation. However, Lipin 1 does not have a DNA binding domain. Instead, it has an LxxIL motif to interact with PPARα. This motif, located downstream of the PAP1 active site in the C-LIP domain, is conserved in both Lipin 2 and Lipin 3 (Finck et al., 2006). Lipin-1α can effectively induce the expression of PPARγ and C/EBPα, the essential transcription factors of adipogenesis. It can also interact with PPARγ, which contains a DNA-binding domain. In addition, Lipin 1 is also known to bind to hepatocyte nuclear factor 4α and the glucocorticoid receptor, which indicates that Lipin 1 may have an effect on the expression of the genes regulated by these proteins (Finck et al., 2006).

### *4. Lipin in Drosophila*

Lipins constitute a highly conserved protein family that is found from yeast to mammals. *Drosophila melanogaster* has only one single Lipin orthologue that is designated as *CG8709* in

Flybase. Flybase lists two protein isoforms with different C-termini, the larger dLipinA isoform and the smaller dLipinB isoform. *Drosophila* Lipin (referred to as dLipin hereafter) was first identified based on sequence similarity by Peterfy (Péterfy et al., 2001).

The expression of dLipin is strongly induced in salivary glands following a pulse of the steroid hormone 20-hydroxyecdysone (20-E) during the late-prepupal stage (Yanling Liu and Michael Lehmann, personal communication). Steroid hormones, including 20-E, act by binding to members of a highly conserved family of nuclear receptors. Considering the fact that mouse Lipin 1 has been shown to physically interact with nuclear receptor PPAR and PGC-1α (Finck et al., 2006), the induced expression of dLipin after the prepupal 20-E pulse may suggest the participating of dLipin in steroid-induced signaling pathways through interactions with specific nuclear receptors.

dLipin is expressed in many *Drosophila* tissues, including fat body (adipose tissue), Malpighian tubules, midgut ceca, ring gland and ovary. A previous study has shown that dLipin is required for fat body development, and TAG synthesis was impaired if dLipin was knocked down in fat body cells (Ugrankar et al., 2011). In addition to the function of promoting fat storage, dLipin was also observed to increase *Drosophila* starvation resistance. Under starvation conditions, *dLipin* is transcriptionally up-regulated (Harbison et al., 2005; Ugrankar et al., 2011) and knockdown of dLipin reduces survival under starvation conditions (Ugrankar et al., 2011).

***5. Drosophila nuclear receptors***

The nuclear receptors (referred to as NRs hereafter) constitute a superfamily of metazoan proteins that have modular functional domains. The DNA binding domain (DBD) is the most conserved region in NRs, containing two zinc fingers that are essential for DNA binding. The other domain, the ligand binding domain (LBD), whose secondary structure contains 12 α-

helixes, allows NRs to function as ligand-dependent regulators during transcription (Aranda and Pascual, 2001). Most nuclear receptors reside in the cell nucleus, but some are located in the cytoplasm where they can be activated by the binding of ligand and relocate into the nucleus. Specific ligands are steroid hormones, retinoic acid, thyroid hormones, fatty acids, leukotrienes and prostaglandins (Escriva et al., 2000). In the nucleus, NRs act as transcription factors and regulate gene expression by interacting with specific DNA sequences. Many NRs were identified by sequence similarity with receptors that had known ligands. Most of these NRs have still no known ligand and are, therefore, referred to as orphan nuclear receptors.

Data of the *Drosophila* genome project allowed the identification of 18 canonical *Drosophila* NRs (with both DBDs and LBDs) and of 3 DBD only receptors (Adams et al., 2000). *Drosophila* is an ideal model organism to study NRs, because many vertebrate NRs have fly orthologs. The ecdysteroid receptor (EcR), which binds 20-hydroxyecdyson (20E), is one of the two NRs in *Drosophila* whose ligand is known. It forms a heterodimer with USP, which acts as the functional receptor (Riddiford et al., 2000). The 20E/EcR/USP complex induces the transcription of many other NRs, including *Drosophila* hormone receptor 3 (DHR3), *Drosophila* hormone receptor 4 (DHR4), *Drosophila* hormone receptor 39 (DHR39), E75 and E78, and it plays an important role during the larval-to-adult transition (King-Jones et al., 2005; Yin and Thummel, 2005). DHR3 is the ortholog of the mammalian ROR receptor, which plays a vital role in lipid homeostasis (Escriva et al., 2000). *Drosophila* HNF4 shares very close sequence similarity with mammalian HNF4, which plays a crucial role in hepatocyte differentiation and lipid homeostasis (Hayhurst et al., 2001). HNF4 has been shown to physically interact with mouse Lipin 1 (Finck et al., 2006). The transcription of *Drosophila* HNF4 is also activated by the late-prepupal steroid hormone pulse as dLipin does (Lee et al., 2003). DHR38 receptor is

expressed widely throughout development and may function as a second ecdysteroid receptor in flies. DHR38 can form dimer with USP, just as EcR, and responds to metabolic signals, implicating it in metabolic control (Fisk and Thummel, 1995; Kozlova et al., 1998).

***6. Ends-in gene targeting in Drosophila***

It is highly useful if we can introduce mutations into specific genes in the genome. Since the complete genome sequence of *Drosophila* is known (Adams et al., 2000), it is possible to engineer specific changes anywhere in the genome. There are two forms of gene targeting called ends-in (insertional) or ends-out (replacement) gene targeting (Gong and Golic, 2003; Rong et al., 2002). The efficiency of both forms is similar, but highly variable, with one homologous recombination event in 500 to 30,000 gametes (Gong and Golic, 2003). These events can be detected through genetic screening. Ends-in targeting, which has been used in this study, is explained in more detail in the result section.

***7. Drosophila as a model***

Since discovery of the first eye-color mutant of *Drosophila melangaster* by Morgan in 1910, the fruit fly has become a widely used experimental organism, especially in genetics and, more recently, in developmental biology. *Drosophila* has very short life cycle (about two weeks) and the flies are easy to handle in the laboratory. There are many established methods in fruit fly genetics and large amount of data about the flies has accumulated over the decades. The completely sequenced *Drosophila* genome makes it easy to study specific genes (Adams et al., 2000). The P-element, a transposon naturally present in *Drosophila*, has been wildly used to generate mutant and transgenic flies (Rubin and Spradling, 1982). The UAS/GAL4 system, which takes advantage of a yeast transcription factor and its response element, was developed

specifically to study gene expression and function in *Drosophila* (Brand and Perrimon, 1993). The FLP-FRT system is used to carry out site-specific recombination (Golic and Golic, 1996). There are also thousands of commercially available RNAi fly lines that can be used to knockdown specific *Drosophila* genes (citation).

Many of *Drosophila* genes and human genes are homologous. Some important human metabolic pathways, such as the TOR and insulin pathways, are explained in *Drosophila* (Grönke et al., 2005). The fat body of *Drosophila*, the major organ for storage of triglycerides and glycogen and secretion of lipoproteins and hormones, is analogous to mammalian adipose tissue and liver (Bharucha, 2009; DiAngelo and Birnbaum, 2009). It plays a very important role in the maintenance of metabolic balance. During the 3$^{rd}$ instar stage, the fat body cell size increases about fivefold. Under starvation or stress, *Drosophila* uses the fat reserves in their fat body tissue to provide energy, just as vertebrates.

### *8. Specific aims of the thesis*

Based on the previous studies on Lipin in mouse, yeast and *Drosophila* reviewed above, I formulated three specific aims, trying to elucidate biological roles of the co-regulator and phosphatidate phosphatase functions of the *Drosophila* ortholog of Lipin, *dLipin*.

**Aim1. Identification of nuclear receptors that interact with dLipin in metabolic and growth control**

In mouse, Lipin 1 is induced by glucocorticoids and can activate mitochondrial fatty acid oxidative metabolism by physically interacting with nuclear receptor PPAR and PPARγ co-activator 1α (PGC-1α) (Finck et al., 2006). The *Drosophila* Lipin ortholog, dLipin is strongly induced following the steroid hormone 20E pulse in late-prepupal salivary glands, suggesting that dLipin may interact with *Drosophila* nuclear receptors and participate in steroid-induced

signaling pathways (Y. Liu and M. Lehmann, personal communication). To identify receptors that interact with dLipin in *Drosophila*, I used a ligand sensor assay and a genetic interaction approach. Transgenic flies that lacked or overexpressed *dLipin* were used in the assays. In the genetic interaction assays I determined whether phenotypes caused by changes in the expression of *dLipin* or a specific nuclear receptor where enhanced or suppressed in animals with changed expression of both.

**Aim2. Generation of flies containing mutations in conserved protein motifs of dLipin**

In order to better understand the biological importance of the enzymatic and transcriptional co-regulator activities of dLipin, I attempted to generate flies that carry mutations in conserved sequence motifs required for these activities. I used targeted ends-out mutagenesis with the goal to create *dLipin* alleles with mutations in the PAP1 motif DIDGT and in a nuclear translocation motif that is required for transcriptional co-regulator functions in the nucleus. Analysis of the phenotypes of animals that express dLipin with only enzymatic activity or transcriptional co-regulator activity should yield important information about the importance of these activities for normal physiology and development.

**Aim3. Characterization of mutant flies**

Successful mutagenesis will allow me to study the phenotypes caused by the loss of PAP enzymatic activity of dLipin without disturbing its co-regulator activity. As the equivalent of mammalian adipose tissue and liver, fly fat body is the main organ for fat storage. I will study the role PAP enzyme plays and the phenotype it causes in the fat body first. However, considering the importance of dLipin in *Drosophila* development, lack of PAP enzymatic activity may lead to an early lethality. The fat bodies may still be underdeveloped when the mutant larvae die. Ectopically expressed wild type dLipin may help to rescue the animals

through the early lethal stage, which will allow me to observe phenotypes caused by lack of PAP enzymatic activity at later times of development.

## II. Materials and Methods

### *1. Fly stocks*

*PBac{RB}CG8709*$^{e00680}$ (*dLipin*$^{e00680}$) was obtained from the Exelixis insertion collection at Harvard Medical School. Transgenic flies carrying ligand-sensor constructs for the analysis of *dLipin* interaction with nuclear receptors were obtained from the laboratory of Carl Thummel (Department of Human Genetics; University of Utah). Nuclear receptor RNAi stocks (UAS-dsReceptor[RNAi]/UAS-dsReceptor[RNAi]) were obtained from the Vienna RNAi Center. Transgenic fly lines for heat-inducible expression of wild-type dLipin (*hs-dLipinWT*) and Gal4-driven expression of wild-type *dLipin* and dLipin-GFP (*UAS-dLipinWT* and UAS-dLipin-GFP) were generated by Aimee Jones and Rupali Ugrankar in the Lehmann laboratory. Transgenic flies carrying donor elements for the mutagenesis crosses were generated by Sandra Schmitt in the Lehmann Lab. All the other fly stocks were obtained from the Bloomington Stock Center.

### *2. Fly food and fly maintenance*

To make fly food, 244g cornmeal and 72g dry yeast were added into 400mL tap water. In 4L water, 44g agar, 52mL corn syrup, 328g malt extract, together with the 400mL mix was cooked until boiling. After cool down, 30mL propionic and 40mL Tegosept were added to prevent the growth of bacteria and fungi.

Stocks were kept on fly food at about 16℃ and transferred onto fresh food every 4 to 5 weeks. Flies for experiments were all kept at 25℃.

### *3. Lac Z staining*

Early 3$^{rd}$ instar larvae were collected, heat shocked for 1 hour and transferred to Petri dish with moist black filter paper and kept at 25℃. Six hours later, larvae were dissected in PBS (8 g

NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $K_2PO_4$; pH7.4), and tissue was fixed in 4.0% paraformaldehyde at room temperature for 30 minutes. After three 10-minute washes with PBS, tissue was incubated in 0.2% X-gal staining solution (35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.02% Nonidet P-40, 0.01% Na deoxycholate) overnight. Following the incubation, tissue was then rinsed in PBS several times until the solution was no longer yellow. Tissue was mounted in 1:1 glycerol/PBS mix and observed under bright field optics.

***4. Lipid staining***

Lipid droplets in the fat body cells of *Drosophila* can be stained by dyes such as Nile Red. Wandering 3$^{rd}$ instar larvae were dissected in PBS and their fat bodies were fixed in 4% paraformaldehyde at room temperature for 10 minutes. After rinsed in PBS, fat bodies were stained in 1 μM Nile Red in PBS at 37°C for 20 minutes in the dark. Fat bodies were then washed again in PBS and mounted in SlowFade Gold antifade reagent (Invitrogen # S36938). The mounting medium contains DAPI that stains the nuclei. After 30 minutes to 1 hour incubation in the mounting medium at room temperature, images were taken by a Carl Zeiss AxioVision microscope using TRITC and DAPI filters.

***5. Delta PAP and delta NLS donor element***

The delta PAP and delta NLS donor elements were constructed by Sandra Schmitt. The protocol for plasmid construction is included here to facilitate understanding of the complete mutagenesis experiment. For the delta PAP donor element, 6kb of DNA encoding part of *Drosophila* dLipin (from position +13,087 to + 19,082) was amplified by PCR using primers "lipinfwdnew" and "NLSREV" (table 1) and inserted into the targeting vector pTV2 at the

cloning sites NotI and KpnI. The point mutation that changes the GAC codon into GAG within the DNA encoding the PAP motif was introduced by primers "G-Cmutafwd" and "nonmutarev" (table 1). An I-Scel site was introduced by the primer "GCISceIrevnew" (Table 1) and a SexA1 restriction site was also created. The base change and the presence of the I-Scel site were confirmed by DNA sequencing using the primers "GCseqfwd" and "ISceseqfwd" respectively. This delta PAP donor element was sent out for microinjection to generate transgenic *Drosophila* (Bestgene Inc.)

For the NLS PAP donor element, 6kb of DNA encoding part of the *Drosophila* dLipin (from position +11,030 to + 17,151) was amplified by PCR amplification using primers "lipinfwdnew" and "NLSrev" (Table 1) and inserted into the targeting vector pTarget at the multiple cloning sites NotI and KpnI. The NLS encoding sequences "AAGAAGCGGCGCAAGAAG" was deleted by the primers "NLSmutafwd" (Table 1). An I-Scel site was introduced by the primer "GCISceIrevnew" (Table 1) and a SexA1 restriction site was also created. The presentence of the I-Scel site were confirmed by DNA sequencing using the primers "ISceseqfwd". This delta NLS donor element was sent to BestGene Inc. for microinjection to generate transgenic *Drosophila*.

Table 1. Primers used for donor element

| Primer name | sequence |
|---|---|
| G-Cmutafwd | 5'GGTGGTGATCTCGGAGATTGACGGCACCATCA3' |
| nonmutarev | 5'GCCATTCAGCCGTACGACTAGGTTAGGC3' |
| lipinfwdnew | 5'GCTGCGGCCGCGTTGCTATGGCTGTGGCCAC3' |
| NLSREV | 5'GACTGGGTACCCACCAGCGCCGTCTCCAGCTC3' |

| | |
|---|---|
| GCISceIrevnew | 5’CATCGAACCAGGTATTACCCAGTTATCCCTAGGC GGTCGAACTCCTCGTCCGAGGGTGGT3’ |
| GCseqfwd | 5’GCACCAATGCAAGCTTCAATGC3’ |
| ISceseqfwd | 5’CCCAGGTGCAGCAAAGCGAGC3’ |
| Nlsfwd | 5’ GCTGCGGCCGCTGCGCTCGCGTTCCCTGATCAA 3’ |
| NLSmutafwd | 5’GGTGTCCAAGAGCAAAACCTCGCAAATGAAGAAG A 3’ |

***6. Starvation experiments***

Freshly eclosed adult flies were collected and put into bottles (25 females and 25 males in each bottle) with normal food and yeast paste at 25 ℃ for 5 days. In the starvation vials, normal fly food was replaced by cotton plugs soaked with water. Flies were transferred into starvation vials on the $6^{th}$ day and kept at 25 ℃. Each vial contained 25 flies and males and females flies were kept separately in different vials. Flies were transferred into new vials every day to prevent the growth of molds. Dead flies were counted and removed at the same time every day until all the flies had died. Each experiment was performed in quadruplicate and the survival curves and statistical comparisons of survival curves were made with the log rank test. (online application OASIS [http://sbi.postech.ac.kr/oasis/]).

***7. Mutagenesis crossing scheme --- ends-in targeting***

Site-directed mutagenesis, ends-in mutagenesis, was utilized to generate the delta PAP mutant flies. In these flies, the dLipin protein is believed to have only co-regulator activity while lacking PAP enzyme activity, because the amino acids of its PAP catalytic domain were changed from DIDGT to EIDGT.

Ends-in crosses consist of two steps, targeting and reduction. The first step inserted the fragment (PAP mutant dLipin gene) into fly genome. The second step deleted the wild type copy of *dLipin* and left the PAP mutant copy in the genome by homologous recombination.

1.1 Targeting

The donor PAP mutant fragment can be inserted into *Drosophila dLipin* gene by ends-in mutagenesis crosses.

- PAP mutant donor (on 2nd chromosome) ♂ X $w^{*}$/P{hs-hid}Y; FLP,I-SceI/ P{hs-hid}, $Sb^{1}$ ♀

10 vials were established and every four days adult flies were transferred into fresh vials and larvae in the old vials were heat shocked at 37℃ for one hour on the next day. The heat-induced expression of protein Hid, which can induce programmed cell death, lead to the death of all the males. And in cells of the females, the donor fragment might be excised and integrated into the genome.

- w; w+?, w-?, PAP/+ ♀ X w; FLP/FLP; TM3, Sb/TM6 ♂

Three female flies each were crossed with FLP-expressing flies. A total number of 350 vials of independent crosses were set up. Red-eyed male flies were selected (7 in all) and crossed with the FLP-expressing females again to make sure the red eye color was the result of successful reintegration of the donor fragment into genome instead of no excision of the donor fragment.

- w; w+, PAP/+ ♀ X w; FLP/FLP; TM3,Sb/TM6 ♂

The red-eye progenies were crossed with balancer flies to establish a stock.

- w; w+, PAP/+ ♂ X w[*]; Xa/CyO; TM3, Sb ♀

CyO , Sb progenies with red eyes were picked.

Genomic DNA was collected from these flies. A 3.5kb fragment was amplified with primers ‘PAP high’ (table 2) by PCR to check whether the donor fragment was integrated into the right place. Sequencing of this 3.5kb fragment confirmed the deletion of the I-Scel site and the C to G mutation.

1.2 Reduction

To delete the wild-type *dLipin* gene and leave only the PAP mutant *dLipin* gene in the fly genome, reduction crosses were carried out.

- w; w+, PAP/CyO ♂ X w/w; I-Crel,Sb/TM6 ♀

In each vial, one single red-eye male was crossed with four I-Crel females. 5 vials were established and every four days, adult flies were transferred into fresh vials and larvae in the old vials were heat shocked at 36℃ for one hour on the next day. White-eyed male offspring was selected and individual males were crossed with females carrying balancer chromosomes. Forty vials of crosses were set up.

- w; w+?, w-?, PAP/+ ; I-Crel, Sb/+ ♂ X w[*]; Xa/CyO; TM3, Sb ♀

From each vial, only one white-eyed, Xa male was picked and crossed with balancer line again to establish a stock.

- w; PAP or + /Xa ♂ X w[*]; Xa/CyO; TM3, Sb ♀

White-eyed, CyO males and females were collected and crossed to set up a stock.

Genomic DNA was collected from these flies. A 1.5kb fragment was amplified with primers 'PAP' (Table 2) by PCR. Whether the copy of *dLipin* left in the genome was wild type or PAP mutant could be determined by sequencing this fragment.

Table 2. List of primers used in the mutagenesis confirmation

| Primer | sequence |
|---|---|
| PAP + | 5' AAGGAGGGCATGAATGAAAT 3' |
| PAP - | 5' TTGGTTAGCAGGGGAAAGTA 3' |
| PAP high + | 5' TCGCTGGAGGAAGGTCAGAAGAGTA 3' |
| PAP high - | 5' GGCATAGGTGAGGTTCTCGGCTAG 3' |
| PAP confirm 1 + | 5' TCGGCGGCTATCGTAAGTG 3' |
| PAP confirm 1 - | 5' TGATAATGGAACCAAGGCAATCG 3' |
| PAP confirm 2 + | 5' CTGGCATTGGTAACCGTCGTTGATAA 3' |
| PAP confirm 2- | 5' TCTTCTGCGTGGTGAACTCCTTGA 3' |
| PAP confirm 3 + | 5' GCTGCTTCTGCCACTGCCATT 3' |
| PAP confirm 3 - | 5' ACCACCTTGTCGTTGTGCTTCC 3' |

***8. Quick fly genomic DNA extraction***

In each Eppendorf tube, about 10 adult flies were anesthetized by carbon dioxide and frozen at -80℃ for 20 minutes. Then the frozen flies were ground in 200μL Buffer A (100 mM Tris-HCl, pH 7.5, 100 mM EDTA,and 100 mM NaCl, 1% SDS) with a tissue grinder. After adding an additional 200μL Buffer A, the grinding was continued until only cuticles remained. The solution was then incubated at 65℃ for 30 minutes and then incubated on ice for at least 10

minutes immediately after the addition of 800 µL LiCl/KAc (1:2.5 of 5 M KAc and 6 M LiCl) solution. The solution was centrifuged at room temperature for 15 minutes and 1mL supernatant was transferred into a new Eppendorf tube. 600 µL isopropanol was then mixed with the solution and the solution was centrifuged for 15 minutes at room temperature to precipitate the DNA. Supernatant was discarded and the precipitate was washed with 70% ethanol and air dried. After the wash, genomic DNA was resuspended in 150 µL 10mM Tris pH 7.0 and stored at -20 ℃.

***9. First instar larvae collection***

To encourage the flies to lay eggs, molasses plates (3.5% agar and 14% molasses in distilled water) with dry yeast on the surface were used to replace normal fly food. Adult flies were transferred into bottles (no food) with a hole on the side. The hole was plugged with a cotton ball and the molasses plate was fixed at the opening by tape. Bottles were kept at 25 ℃ overnight to allow the flies to get used to the new food and deposit hold-back eggs. The next morning, old molasses plates were discarded and replaced with new ones. Every three hours, molasses plates were changed and the old ones were continuously incubated at 25 ℃ in storage bags. After 30 hours, larvae were gently picked from the molasses plates by forceps.

***10. Western blot***

The expression level of mutant dLipin protein was tested by Western blot. For each sample, one hundred 1$^{st}$ instar larvae were homogenized in 50µl 2X SDS-PAGE sample buffer and kept at -20°C. 20 µl sample solution each lane was loaded onto an SDS PAGE gel and the gel was run in Electrode Running Buffer at 110 Volts until the blue dye had migrated to the end of the gel. Transfer sandwich (fiber pad, chromatography, the gel and the Nitrocellulose membrane, chromatography paper, and next fiber pad) was made and proteins were transferred from the gel to the nitrocellulose membrane for one hour at 100V in transfer buffer at 4 ℃. Next,

the membrane was blocked in 20% non-fat milk at room temperature for one hour with gentle shaking. dLipin and actin antibody was added at the dilution of 1: 2000 into the milk and incubated at 4℃ overnight with gentle shake. The next morning, the membrane was washed in PBST (PBS with 0.1% Triton X-100) three times (10 minutes each time) and the second antibody was added at a dilution of 1:2500 in TBST. After incubation with the second antibody for 2 hours at room temperature under gentle shaking, the membrane was washed in PBST three times (10 minutes each time) and exposed to the substrate until a band could be seen.

***11. dLipin antibody staining***

All of the 1$^{st}$ instar larval tissues were squeezed out of the cuticle by fine forceps and fixed in 4% formaldehyde for 30 minutes, followed by four 10-minute washes in PBS plus 0.2% Tween 20. Fixed tissues were blocked in PBST with 1% normal donkey serum for 2 hours at room temperature with gentle shaking. Affinity purified dLipin antibody was added into the block solution at a dilution of 1:200. Tissues were incubated overnight at 4℃ with gentle shaking. After four 10-minute washes in PBS plus 0.2% Tween 20, tissues were incubated with Cy3 donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch # 711-165-152) at 1:1000 dilution in PBS plus 0.2% Tween 20 with 1% normal donkey serum at room temperature and wrapped in aluminum foil. Tissues were washed in PBS plus 0.2% Tween 20 four times and mounted in SlowFade Gold antifade reagent (Invitrogen #S36938). Images were taken with a Carl Zeiss AxioVision microscope using TRITC and DAPI filters.

### *12. Rescue of PAP mutant flies by hs-dLipin*

Flies of the genotype PAP/PAP; P *[hs-dLipin]* /+ were generated through crosses. These flies express wild-type dLipin protein from *P[hs-dLipin]* after heat shock and the PAP mutant dLipin protein.

1st instar larvae were collected from molasses plates and transferred onto normal fly food. 50 larvae were placed into each vial. These vials were then incubated at 37℃ for one hour. Heat shock was repeated once a day.

### *13. PAP mutant flies rescued by UAS-dLipinWT and UAS-dLipin-GFP*

dLipin and dLipin-GFP were expressed using the UAS-GAL4 system to rescue the PAP mutant flies. Different driver lines were included and the genotypes of the flies were as follows:

PAP/PAP; UAS-dLipinWT/Tubulin-GAL4

PAP/PAP; UAS-dLipinWT/GAL4-hsp70

PAP/PAP; UAS-dLipinWT/daughterless-GAL4

PAP/PAP; UAS-dLipin-GFP/Tubulin-GAL4

PAP/PAP; UAS-dLipin-GFP/GAL4-hsp70

PAP/PAP; UAS-dLipin-GFP/daughterless-GAL4

Flies were kept at 25℃. Flies carrying the hsp70 (70 kilodalton heat shock protein) driver were heat shocked at 37℃ for 1 hour once a day from embryo or 1st instar larval stage. Larvae that survived until the 3rd instar larval stage were dissected to examine their fat body cells and adult flies were used to carry out the starvation experiments.

### *14. dLipin-GFP localization*

3rd instar wandering larvae were dissected in PBS on ice. The fat bodies were collected and stained with dLipin antibody as described above. dLipin antibody stains both the endogenous wild-type dLipin and dLipin-GFP expressed by the Gal4-UAS system. Images were taken with a Carl Zeiss AxioVision microscope using the TRITC filter (for antibody signal coming from both dLipin and dLipin-GFP) and the GFP filter (for fluorescence signal coming from dLipin-GFP).

## III. Results

### *A. Screening for nuclear receptors that interact with dLipin in metabolic and growth control*

In mouse, Lipin 1 is induced by glucocorticoids and can activate mitochondrial fatty acid oxidative metabolism by physically interacting with nuclear receptor PPARα and PPARγ co-activator 1α (PGC-1α) (Finck et al., 2006). In *Drosophila* the steroid hormone 20E acts by binding to nuclear receptors. The gene encoding the *Drosophila* Lipin ortholog, *dLipin* is strongly induced following a pulse of the steroid hormone 20E in the late-prepupal stage, suggesting that dLipin may interact with *Drosophila* nuclear receptors and participate in steroid-induced signaling pathways (M. Lehmann, personal communication). Identification of nuclear receptors that interact with dLipin will enable us to study how dLipin acts as a transcriptional co-regulator and regulates gene expression in *Drosophila*.

To test whether dLipin interacts with *Drosophila* nuclear receptors, I used two different experimental approaches:

#### 1. Ligand sensor system

A ligand sensor system was used to identify the nuclear receptors cooperating with dLipin.

*Drosophila* has 18 different nuclear receptors and by using a ligand sensor system, the spatial activity patterns of all the nuclear receptors had been studied (Palanker et al., 2006). In this system, there are two main components (Figure 1). One is a heat-inducible fusion protein, which combines the nuclear receptor ligand-binding domain and the GAL4 DNA-binding domain. The second component is a UAS response element, which uses *lacZ* or *eGFP* as a reporter gene. Only when the ligand and the needed co-regulators bind to the fusion protein can the reporter gene be

activated. Combined with an up- or down-regulation of *dLipin*, the assay should be suitable to identify not only the dLipin interaction partner, but also where and when the interaction happens.

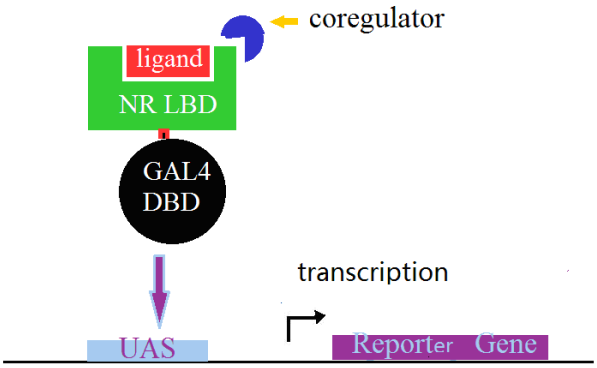



Figure 1. Ligand sensor system. After the binding of the ligand (red) and co-regulator (blue), a fusion protein consisting of a nuclear receptor ligand binding domain (green) and a GAL4 DNA-binding domain (black) can activate the UAS response element (light blue) and start the transcription of the reporter gene, which can be *lacZ* or *eGFP*.

In order to find out the influence of dLipin, I carried out both gain-of-function (flies with over-expressed dLipin) and loss-of-function (flies expressing a reduced amount of dLipin) experiments.

1.1 Loss-of-function

In this experiment, the influence of low levels of dLipin protein on reporter gene activity was examined. If dLipin is needed for the NR activity, no or reduced reporter gene activity should be detected. I used a *lacZ* gene as a reporter whose product is targeted to the nucleus. Therefore, in the control group, the cell nuclei of the tissues where NRs function should turn blue after X-gal staining. And in the experimental group, loss of the blue staining would indicate the participation of dLipin in the NR regulation. Reporter gene activity was examined in a *dLipin* mutant background, *PBac{RB}CG8709*$^{e00680}$/ *Df(2R)Exel7095*. *PBac{RB}CG8709*$^{e00680}$ fly has an insertion of a *PiggyBac* transposon in the 5'UTR of the *dLipin* gene, which disrupts normal expression of *dLipin*, leading to reduced amounts of the protein (Ugrankar et al. 2011).

*Df(2R)Exel7095* is a deficiency that removes the chromosomal region 44B3-44C2, which includes the entire *dLipin* gene. A previous study has shown that a small number of transheterozygotes *PBac{RB}CG8709*$^{e00680}$/*Df(2R)Exel7095* (henceforth referred to as *dLipin*$^{e00680}$/Df) can survive until the 3$^{rd}$ instar larval stage. These animals produce very little dLipin protein and have a severe fat body phenotype (Ugrankar et al., 2011). *dLipin*$^{e00680}$/*Df* animals were used to examine the influence of lack of dLipin on the activity of nuclear receptors DHR96, DHR3, DHR38 and EcR.

As in the gain-of-function experiments (see below), all the salivary gland cells developed blue color in all their nuclei. This staining was caused by a tissue-specific enhancer in the reporter gene construct that drives constitutive expression of *lacZ* independent of the ligand sensor protein (M. Lehmann, personal communication). Therefore, even the salivary glands of w$^{1118}$;UAS-lacZ larvae showed nuclear staining (Figure 2). This staining can be used as an internal control to show that the staining procedure was successful.

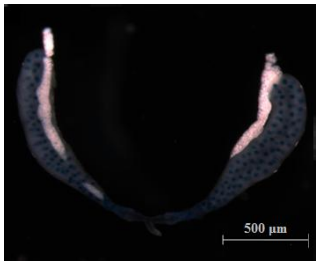



Figure 2. Staining of the salivary glands of *w*$^{1118}$; UAS-lacZ larva

In *dLipin*$^{e00680}$*/Df;* hs-GAL4-DHR96/UAS-lacZ larvae, no blue color developed in the nuclei except the salivary glands (Figure 3A). The staining pattern was not different from the pattern in *dLipin*$^{e00680}$/CyO-GFP; hs-GAL4-DHR96/UAS-lacZ control larvae (Figure 3B). Both *dLipin*$^{e00680}$/CyO-GFP; hs-GAL4-DHR3/UAS-lacZ (Figure 3C) and *dLipin*$^{e00680}$*/Df* ; hs-GAL4-DHR3/UAS-lacZ (Figure 3D) larval tissues showed some blue color in parts of the midgut that

was most likely caused by endogenous beta-galactosidase activity in regions of the midgut. In *dLipin*$^{e00680}$/CyO-GFP; hs-GAL4-DHR38/UAS-lacZ larval tissue, part of the midgut also showed blue color that was not restricted to the nuclei (Figure 3E). The staining pattern was very similar in *dLipin*$^{e00680}$*/Df* ; hs-GAL4-DHR38/UAS-lacZ (Figure3F). Some of the midgut cells of *dLipin*$^{e00680}$*/Df* ; hs-GAL4-EcR/UAS-lacZ larvae also had the staining that was not restricted to the nuclear area (Figure 3G). Meanwhile, no cells were stained in *dLipin*$^{e00680}$/CyO-GFP ; hs-GAL4-EcR/UAS-lacZ larvae (Figure 3H).

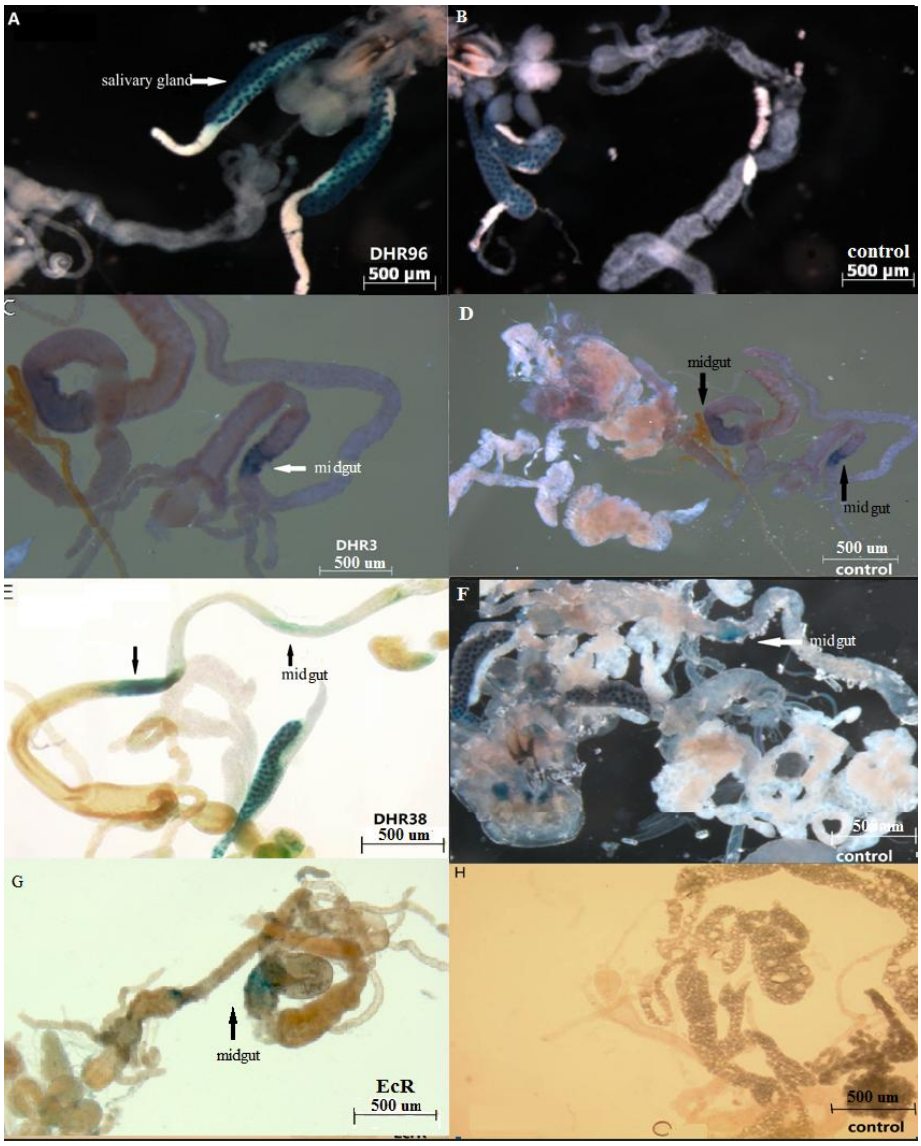



Figure 3. LacZ staining of *dLipin*$^{e00680}$*/Df;* hs-GAL4-NR/UAS-lacZ larvae. 3$^{rd}$ instar larvae were dissected and stained in X-gal solution overnight. Except from the salivary glands, no staining was visible in the *dLipin*$^{e00680}$*/Df* ; hs-GAL4-DHR96/UAS-lacZ larvae (A) or *dLipin*$^{e00680}$*/CyO-GFP* ; hs-GAL4-DHR96/UAS-lacZ larvae (B). Except some unspecific staining in the midgut, no nuclear specific staining was found in *dLipin*$^{e00680}$*/Df*; hs-GAL4-DHR3/UAS-lacZ larvae (C), control *dLipin*$^{e00680}$*/CyO-GFP* ; hs-GAL4-DHR3/UAS-lacZ (D), *dLipin*$^{e00680}$*/Df* ; hs-GAL4-DHR38/UAS-lacZ larvae (E) or control *dLipin*$^{e00680}$*/CyO-GFP* ; hs-GAL4-DHR38/UAS-lacZ larvae (F) and *dLipin*$^{e00680}$*/Df* ; hs-GAL4-DHREcR/UAS-lacZ larvae (G) No blue color developed in any tissue other than salivary glands in *dLipin*$^{e00680}$/ CyO-GFP; hs-GAL4-EcR/UAS-lacZ larvae (H).

No DHR96 activity was detected in either the experimental or the control animals since no blue staining was detected except in the salivary glands. For DHR3 and DHR38, the same

staining patterns were observed. In addition to the salivary glands, some parts of midgut were stained, but the blue color was not restricted in the nuclei. The unspecific staining in these cells was most likely caused by endogenous beta-galactosidase activity. For EcR, no activity was detected in the control animals, although some parts of the midgut of the experimental animals showed blue color. The staining was not restricted in the nuclei either, probably due to the endogenous beta-galactosidase activity. In the loss-of-function experiment, no staining showed specific reporter activity caused by the NR-GAL4 fusion protein. dLipin-NR interaction was not observed.

1.2 Gain-of-function

In the gain-of-function experiments, I expressed *dLipin* in transgenic flies carrying hs-dLipin element, a gene encoding a nuclear receptor LBD/GAL4 fusion protein and a *lacZ* reporter gene. Nuclear receptors DHR3, HNF4 and DHR38 were studied in these experiments. After incubation at 37℃ to induce the heat shock-controlled *dLipin* gene, 3$^{rd}$ instar wandering larvae were dissected and tissues including brain, salivary glands, midgut, and fat body were collected and stained in X-gal solution.

All the salivary glands in these experiments developed blue color quickly in all the cell nuclei. In CyO-GFP/+; hs-GAL4-DHR3/UAS-lacZ larvae, no blue color developed in the nuclei except the salivary glands (Figure 4A). In some fat body cells of P[hs-dLipinWT]/+; hs-GAL4-DHR3/UAS-lacZ larvae, there was blue color in the nuclei. However, the staining pattern was not uniform in the whole fat body and only a few nuclei were stained (Figure 4B). In both CyO-GFP/+; hs-GAL4-DHR38/UAS-lacZ and P[hs-dLipinWT]/+; hs-GAL4-DHR38/UAS-lacZ larvae, the nuclei of some fat body cells and part of midgut showed a blue color ((Figure 3C and 4D1, 4D2). Only the salivary gland cells of CyO-GFP/+; hs-GAL4-HNF4/UAS-lacZ larvae had specific

staining (Figure 4E1 and 4E2), while a few fat body cells of [hs-dLipinWT]/+; hs-GAL4-HNF4/UAS-lacZ larvae showed nuclear staining (Figure 4F).

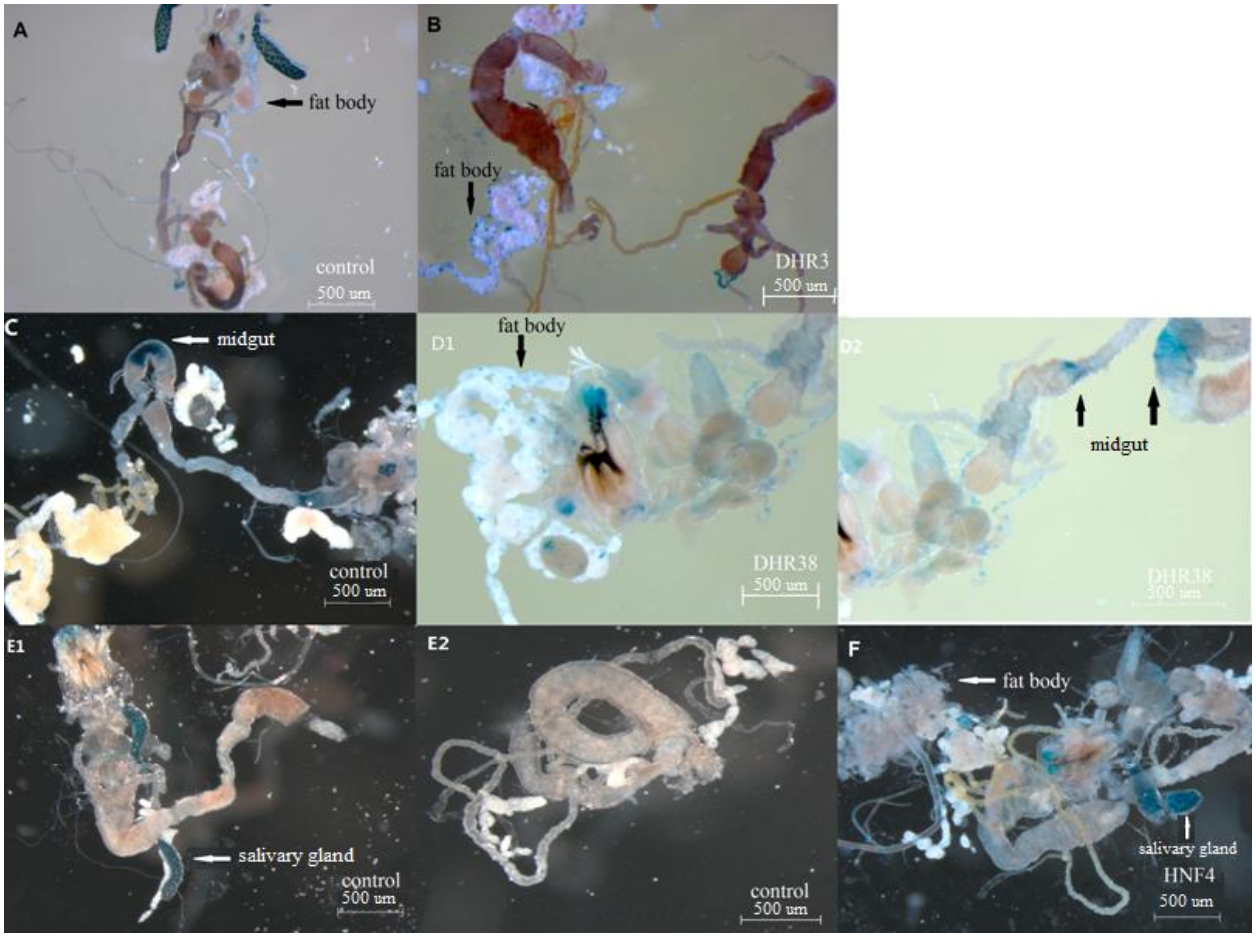



Figure 4. LacZ staining in the tissues of dLipin over expression larvae. No staining in the tissues of CyO-GFP/+; hs-GAL4-DHR3/UAS-lacZ larvae except in the salivary glands (A). In the tissues of p[hs-dLipin]]/+; hs-GAL4-DHR3/UAS-lacZ larvae (B), some of the fat body cells had blue color in the nucleus. In both CyO-GFP/+; hs-GAL4-DHR38/UAS-lacZ (C) and P[hs-dLipinWT]/+; hs-GAL4-DHR38/UAS-lacZ (D1, D2) larvae, the nuclei of some fat body cells and part of midgut showed a blue color. Only the salivary gland cells of CyO-GFP/+; hs-GAL4-HNF4/UAS-lacZ larvae had specific staining (E1, E2), while a few of fat body cells of [hs-dLipinWT]/+; hs-GAL4-HNF4/UAS-lacZ larvae showed nuclear staining (F).

In summary, for nuclear receptors DHR3, DHR38 and HNF4, over expression of *dLipin* resulted in some stained fat body cell nuclei, whereas the control animals did not show nuclear staining in the fat body. However, only a small portion of the fat body cells showed nuclear staining, which is different from the expectation because all the cells were subjected to the same

conditions. Since ectopically expressed *dLipin* did not consistently change reporter activity in the fat body or any other tissue, the data does not provide sufficient evidence of an interaction of dLipin with the receptors tested.

**2. RNAi screen for genetic interactions between nuclear receptors and dLipin**

The second method I used to test whether dLipin interacts with *Drosophila* nuclear receptors is the genetic interaction experiment. I knocked down the expression of *dLipin* and nuclear receptors by RNAi, both alone and in combination. This approach allowed me to look for an enhancement or suppression of phenotypes that would suggest an interaction between dLipin and one or more of the nuclear receptors. Previous experiments have shown that animals that expressed UAS-dLipin[RNAi] activated by a fat body GAL4 driver had small lipid droplets in the larval fat body cells (Ugrankar et al., 2011). If dLipin interacts with a nuclear receptor, it may result in a more or less severe phenotype than the single knockdown of *dLipin*. For instance, the fat body cells may be very small, or contain extremely small lipid droplets, or have almost no lipid droplets.

I observed the phenotypes caused by simultaneous RNAi knockdown of *dLipin* and the nuclear receptor in fat body cells compared to $w^{1118}$ animals and single knockdown animals for dLipin and the nuclear receptor. The nuclear receptors tested were DHR3, DHR4, DHR38, DHR96, HNF4 and EcR. All the nuclear receptors and *dLipin* were knocked down using a fat body-specific driver, Cg-GAL4.

Fat body cells from $w^{1118}$ had big lipid droplets and similar cell size (Figure 5A), while *dLipin* knockdown larvae had variable size of fat body cells and the lipid droplets were very small. The cells were also partially detached from each other (Figure 5B). Nuclear receptor knockdown larvae, including UAS-DHR3[RNAi]/Cg-gal4 (Figure 5C), UAS-DHR4[RNAi]/Cg-

gal4 (Figure 5E), UAS-DHR38[RNAi]/Cg-gal4 (Figure 5G), and UAS-DHR96[RNAi]/Cg-gal4 (Figure 5I) had similar lipid droplet size as cells from $w^{1118}$ larvae. Double knockdowns of both *dLipin* and nuclear receptor *DHR3* (Figure 5D), *DHR4* (Figure 5F), *DHR38* (Figure 5H), DHR96 (Figure 5J) all resulted in small lipid droplets. Most of the UAS-EcR[RNAi]/Cg-gal4 larvae had fat body cells with big lipid droplets and similar size as $w^{1118}$, while a few of them had smaller cell size and small lipid droplets (Figure 5K). All the fat body cells of larvae with knockdown of both *dLipin* and *EcR* had small lipid droplets (Figure 5L). *HNF4* single knockdown larvae had fat body cells with normal size and big fat droplets (Figure 5M). Fat body cells with knockdown of both *dLipin* and *HNF4* had variable size and relatively big lipid droplets (Figure 5N).

For DHR3, DHR4, DHR38, DHR96 and EcR, the double knockdowns (both nuclear receptor and *dLipin*) did not show a more severe phenotype than the single knockdown of *dLipin.* The cell size was not smaller or more variant and the fat droplets were not extremely small. Double knockdowns did not fully or partly rescue the phenotype caused by dLipin single knockdown, either. The phenotypes only showed a simple combination of nuclear receptor single knockdown and dLipin single knockdown. An interaction between dLipin and these NRs cannot be seen from the fat body tissue staining. However, double-knockdown of *dLipin* and *HNF4* seemed to rescue part of the dLipin phenotype. Big fat droplets may be caused by reduced fatty acid oxidation since a previous study has shown that HNF4 can induce the expression of the enzymes that are responsible for fatty acid oxidation (Palanker et al., 2009).

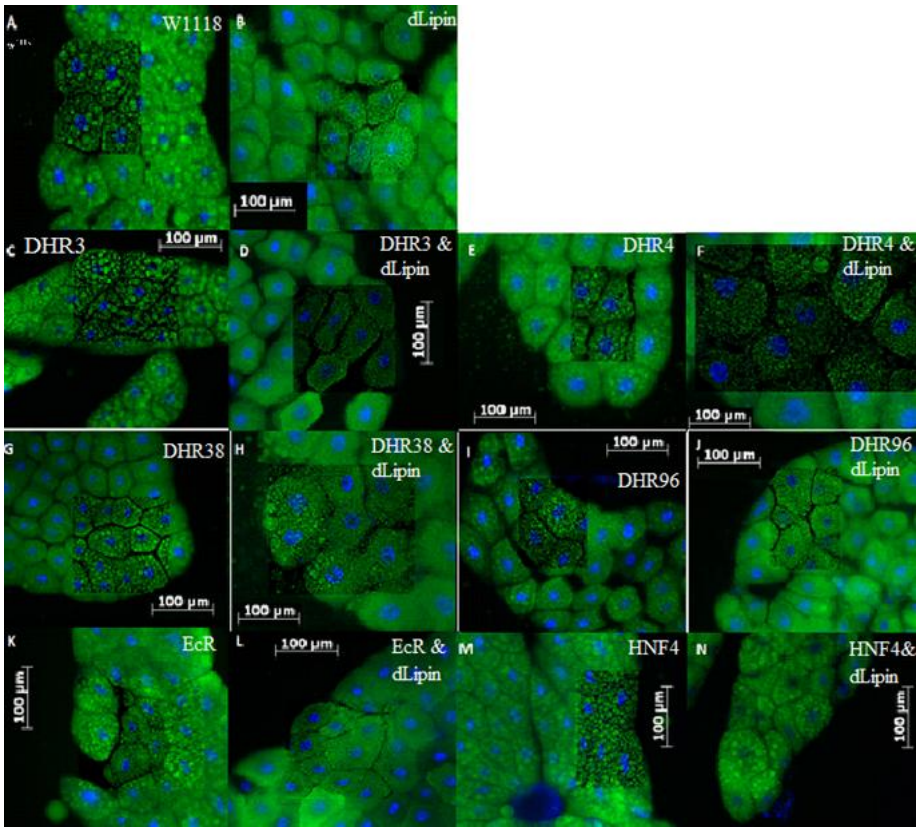



Figure 5. Lipid droplets and nuclear staining in the fat body cells of 3rd instar wandering larvae. Fat droplets were stained with BODIPY (green) and the nuclei were stained with DAPI (blue). Deconvolution was used to create a clearer image (square window in each image). Fat body cells of $w^{1118}$ larvae had big lipid droplets and similar cell size (A) while fat body cells of *dLipin* single knockdown were of variant size and contain small fat droplets(B). Fat body cells of *DHR3* (C), *DHR4* (E), *DHR38* (G) and *DHR96* (I) had similar lipid droplet size as the cells from $w^{1118}$ larvae. Double knockdowns of both *dLipin* and nuclear receptor *DHR3* (D), *DHR4* (F), *DHR38* (H) and *DHR96* (J) all resulted in small lipid droplets. Most of the *EcR* single knockdown larvae had fat body cells with big lipid droplets and similar cell size as $w^{1118}$, while a few of them had smaller cell size and small lipid droplets (K). All the fat body cells of larvae with knockdown both *dLipin* and *EcR* had small lipid droplets (L). *HNF4* single knockdown larvae had fat body cells with normal size and big fat droplets (M). Fat body cells with knockdown of both *dLipin* and *HNF4* had variable size and relatively big lipid droplets (N).

**3. Double knockdowns of both *dLipin* and NR do not largely change starvation resistance**

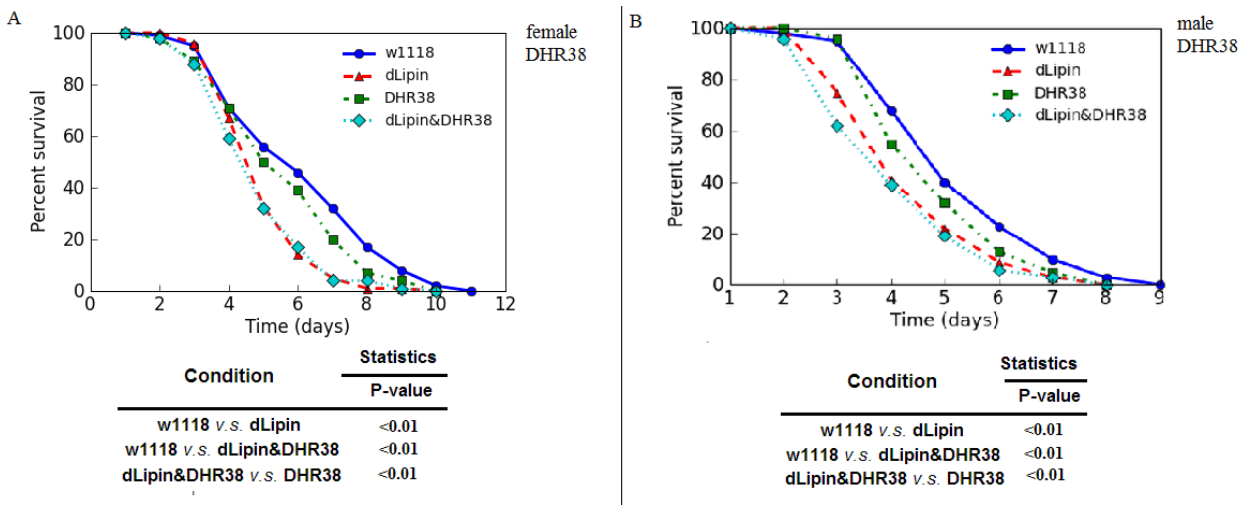
Since for most NRs, except HNF4, the morphology study of fat body cells (*dLipin* single knockdown, NR single knockdown and *dLipin*, NR double knockdowns) did not give me any positive result, I wanted to determine whether an interaction can be seen in another aspect of the *dLipin* phenotype. For HNF4, a possible interaction was observed in the fat body cell

morphology study. If a phenotype indicating HNF4-dLipin interaction can also be seen in another aspect of the dLipin phenotype, the possibility that HNF4 and dLipin interact will greatly increase. *dLipin* is upregulated in *Drosophila* under starvation conditions (Ugrankar et al., 2011) (Harbison et al., 2005). Especially in starved males, *dLipin* levels were much higher than in fed controls. Since RNAi knockdown of *dLipin* leads to decreased starvation resistance (Ugrankar et al., 2011), I examined whether additional knockdown of selected NRs enhances or suppresses this effect. If NR and dLipin do interact and participate in the regulation of starvation resistance, starvation resistance of double knockdowns of both *dLipin* and NR should be different from starvation resistance of knockdown of dLipin alone.

Two fat body drivers, Cg-GAL4 and r[4]-GAL4 were used to carry out the RNAi crosses. HNF4 and EcR were knocked down by using the r[4]-GAL4 driver. Since r[4]-GAL4 is a very strong driver, double knockdowns of *dLipin* and NRs such as DHR3, DHR38 and DHR96 were lethal and no adult flies could be obtain. Instead, I used a weaker driver, Cg-GAL4, to carry out the crosses.

Compared with the control group, $w^{1118}$ flies, females with single *dLipin* knockdown by Cg-GAL4 (Figure 6A) showed decreased starvation resistance, especially after day 5. At day 6, the average survival rate of $w^{1118}$ flies was 45% and for *dLipin* knockdown flies, the rate was only 13%. And the maximum life span was reduced from 11 to 10 days. Flies with single knockdown of *DHR38* only showed a very slightly decreased survival rate compared with $w^{1118}$ flies, which was not statistically significant. At day 6, its survival rate was 40% and the maximum life span was reduced from 11 to 10 days. Flies with double knockdowns of both *dLipin* and *DHR38* had a similar survival pattern as those with single knockdown of *dLipin*. The survival pattern of males was similar to females (Figure 6B). The only difference was that the

maximum life span of the control flies was nine days and the reduced life span of all the other knockdown flies was eight days.




| Condition | Statistics P-value |
|---|---|
| w1118 *v.s.* dLipin | <0.01 |
| w1118 *v.s.* dLipin&DHR38 | <0.01 |
| dLipin&DHR38 *v.s.* DHR38 | <0.01 |

| Condition | Statistics P-value |
|---|---|
| w1118 *v.s.* dLipin | <0.01 |
| w1118 *v.s.* dLipin&DHR38 | <0.01 |
| dLipin&DHR38 *v.s.* DHR38 | <0.01 |

Figure 6. Survival rates of the flies with *dLipin* single knockdown, *DHR38* single knockdown and double knockdowns with driver Cg-GAL4. In females (A), compared with $w^{1118}$ (light blue) controls, flies with single knockdown of *dLipin* (red) showed decreased starvation resistance, especially after day 5. Flies with single knockdown of *DHR38* (green) showed a slightly decreased survival rate compared with $w^{1118}$controls, but still did better than flies with *dLipin* knockdown flies. Flies knocking down of both *dLipin* and *DHR38* (light blue) had a similar survival pattern as those with single knockdown of *dLipin*. The survival pattern of males was similar to females (B). The only difference was that the maximum life span of the controls was nine days and the reduced life span of all the other knockdown flies was eight days. The experiment was performed in quadruplicate and the survival curves and statistical comparisons of survival curves were made with the log rank test. Only P-values smaller than 0.05 were listed in the figure.

Compared with $w^{1118}$ controls, female flies with single knockdown of *dLipin*, single knockdown of NR *DHR96* and double knockdowns of both *dLipin* and *DHR96* all showed decreased survival rates after day 4 (Figure 7A). At day 6, the average survival rate for the controls was 45% while the rates of all the other three lines were about 15% to 20%. The maximum life span of all the knockdowns was reduced from 11 to 10 days. Flies with knockdown of *DHR96* had the lowest survival rate before day 5, but became very similar to other two knockdown fly lines after that. In males, the three knockdown fly lines all had

similarly decreased survival rates compared with the controls and their maximum life span was 8 days (Figure 7B), one day shorter than the wild type flies.

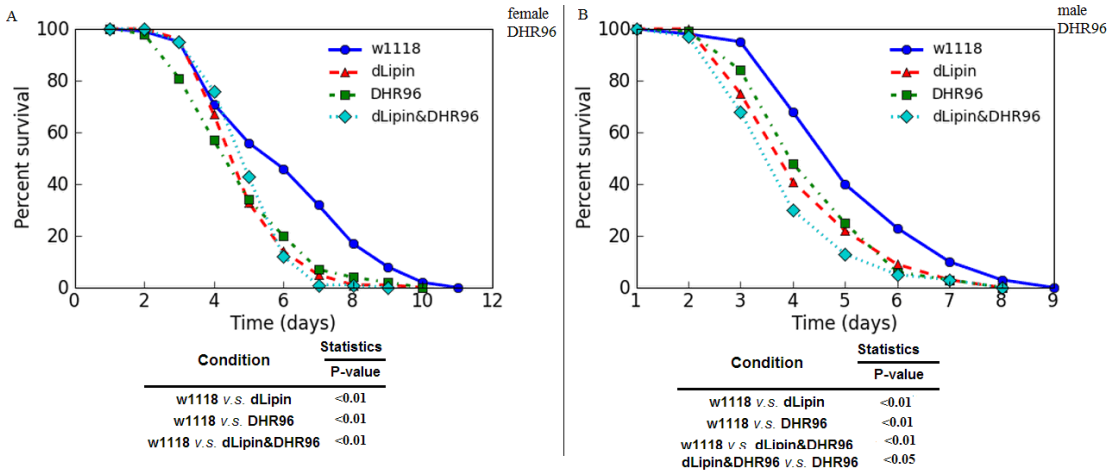
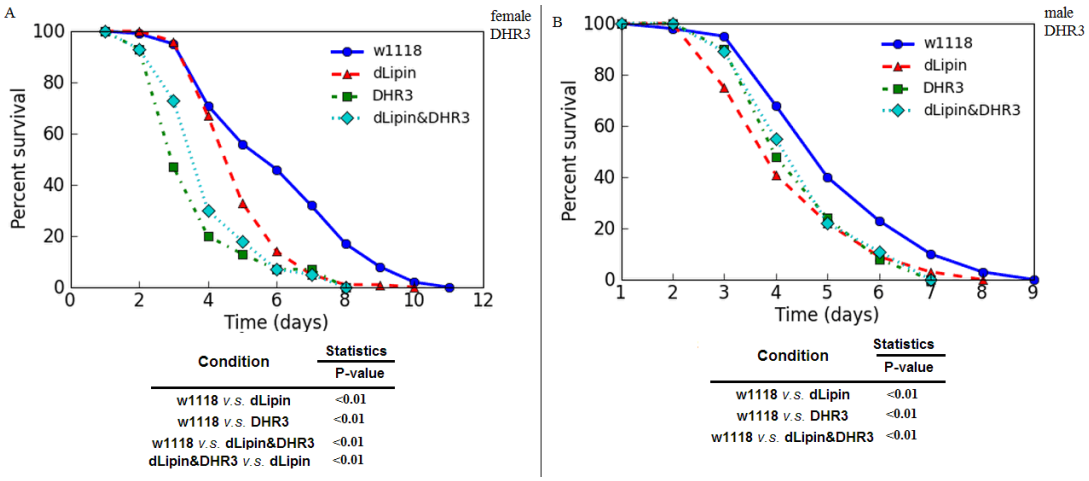



Figure 7. Survival rates of the flies with *dLipin* single knockdown, *DHR96* single knockdown and double knockdowns by driver Cg-GAL4. In females (A), compared with $w^{1118}$ controls (light blue), flies with single knockdown of *dLipin* (red), single knockdown of *DHR96* (green) and double knockdowns of both *dLipin* and *DHR96* (light blue) all showed large decreased survival rates after day 4. In males (B), the three knockdown fly lines all had similar decreased survival rates comparing with the controls and their maximum life span was 8 days, one day shorter than the control flies. The experiment was performed in quadruplicate and the survival curves and statistical comparisons of survival curves were made with the log rank test. Only P-values smaller than 0.05 were listed in the figure.

In females (Figure 8A), compared with $w^{1118}$ flies, the survival rate of the flies with single knockdown of *DHR3* largely decreased and was not a little bit lower than the flies with *dLipin* knockdown at day 4 and day 5. At day 6, the survival rate of the control, $w^{1118}$ flies, was 45% while the flies with *dLipin* single knockdown and *DHR3* single knockdown were both 15%. The maximum life span of the flies with *DHR3* knockdown was reduced from 11 to 9 days. Flies with double knockdowns of both *dLipin* and *DHR3* had a similar survival pattern to the flies with *DHR3* single knockdown. But its maximum life span was 9 days. In males (Figure 8B), the three knockdown fly lines all had similar and decreased survival rates comparing with the controls. At day 6, the survival rate of all the three knockdown lines was 10% while the controls had a

survival rate higher than 20%. The maximum life span of flies with *DHR3* knockdown and double knockdowns was 7 days, two days shorter than the control flies.




| Condition | Statistics P-value |
|---|---|
| w1118 *v.s.* dLipin | <0.01 |
| w1118 *v.s.* DHR3 | <0.01 |
| w1118 *v.s.* dLipin&DHR3 | <0.01 |
| dLipin&DHR3 *v.s.* dLipin | <0.01 |

| Condition | Statistics P-value |
|---|---|
| w1118 *v.s.* dLipin | <0.01 |
| w1118 *v.s.* DHR3 | <0.01 |
| w1118 *v.s.* dLipin&DHR3 | <0.01 |

Figure 8. Survival rates of the flies with *dLipin* single knockdown, *DHR3* single knockdown and double knockdowns by driver Cg-GAL4. In females (A), compared with $w^{1118}$ flies (light blue), the survival rate of the flies with single knockdown of *DHR3*(green) largely decreased and was much lower than the flies with *dLipin* single knockdown at day 4 and day 5. Flies with double knockdowns of both *dLipin* and *DHR3* (light blue) had a similar survival pattern to the flies with *DHR3* single knockdown, but its maximum life span was 9 days. In males (B), the three knockdown fly lines all had similar and decreased survival rates comparing with the controls. The experiment was performed in quadruplicate and the survival curves and statistical comparisons of survival curves were made with the log rank test. Only the P-values smaller than 0.05 were listed in the figure.

In the starvation experiments, a strong fat body specific driver, r[4]-GAL4, was used when studying NR HNF4 and EcR. As shown in Figure 9A, compared with the controls, $w^{1118}$ flies, the survival rate of the flies with single knockdown of *dLipin* by r[4]-GAL4 decreased after day 4.And at day 6, the survival rate of the controls was 45% while the rate of the flies with *dLipin* knockdown was lower than 20%. The maximum life span was reduced from 11 to 10 days. Flies with single knockdown of *HNF4* led to a survival rate similar to the control group before day 4, but a slightly decreased survival rate after that. At day 6, the survival rate of the flies with *HNF4* knockdown was 38%. But after day 8, the survival rate was higher than in the control

group and at day 10 the survival rate was 15% while all the other groups had less than 3% survival rates. The maximum life span was also extended from 11 to 14 days. However, with the number of animals analyzed the difference between *HNF4* single knockdown and the control group was not statistically significant. Flies with knockdowns of both *dLipin* and *HNF4* had a higher survival rate than those with single knockdown of *dLipin*, especially from day 5 to day 7. However, based on the number of observations, the difference was not statistically significant. Similar to the females, single knockdown of *HNF4* in males resulted in a better survival rate than in the control group (Figure 9B). At day 6, the survival rate of the control was 20% while the rate of the flies with *HNF4* knockdown was 30%. The maximum life span of *HNF4* single knockdown animals was extended from 9 to 11 days. There was a tendency of improved starvation resistance in the *HNF4* single knockdown animals. But, the difference was not significant with the number of animals analyzed. The survival rate of the flies with double knockdowns of both *dLipin* and *HNF4* is higher than *dLipin* single knockdown and the difference was statistically significant. In males, the double knockdowns of both *dLipin* and *HNF4* partly rescue the impaired starvation resistance caused by *dLipin* knockdown,.

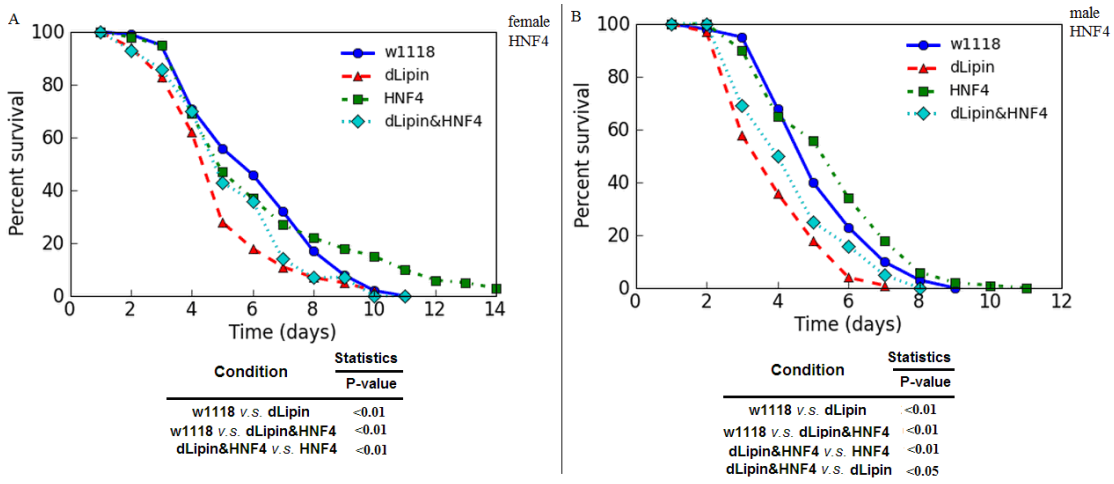
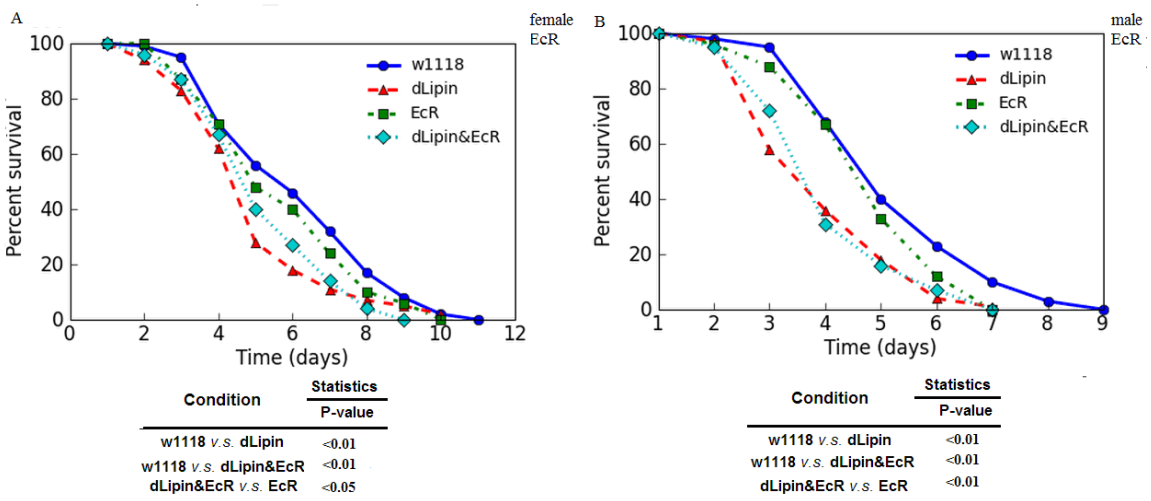



Figure 9. Survival rates of the flies with *dLipin* single knockdown, *HNF4* single knockdown and double knockdowns with the r[4]-GAL4 driver. In females (A), compared with $w^{1118}$ flies (blue),

flies with single knockdown of *dLipin* (red) had a lower survival rate, especially after day 4. Flies with single knockdown of *HNF4* (green) had a similar survival rate to the control before day 4 and a slightly decreased survival rate after that. However, after day 8, the rate came up again. Flies with knockdowns of both *dLipin* and *HNF4* seemed have a higher survival rate than those with single knockdown of *dLipin*, especially from day 5 to day 7. Similar as in females, single knockdown of *HNF4* in males results to a better survival rate than in the control group (B). The survival rate of the flies with double knockdowns of both *dLipin* and *HNF4* is higher than the flies with *dLipin* single knockdown and the difference was statistically significant. The experiment was performed in quadruplicate and the survival curves and statistical comparisons of survival curves were made with the log rank test. Only P-values smaller than 0.05 were listed in the figure.

Female flies with single knockdown of *EcR*, had a similar survival rate to the control group. At day 6, the survival rate of the control and flies with single knockdown of *EcR* was both about 50%. The maximum life span of the flies with single knockdown of *EcR* was reduced from 11 to 10 days. But the slight difference between these two groups were not statistically significant (Figure 10A). Compared with the control group, the maximum life span of the flies with double knockdowns was reduced from 11 to 9 days and at day 6, and the survival rate was only 30%. This survival rate was very similar to the survival rate of the flies with *dLipin* single knockdown. In males (Figure 10B), flies with single knockdown of *EcR* had similar survival rates to the control before day 5. And after day 5, its survival rate decreased, but the difference was not significant. Flies with double knockdowns of both *EcR* and *dLipin* had a very similar survival rate to the flies with *dLipin* single knockdown.




| Condition | Statistics P-value |
|---|---|
| w1118 *v.s.* dLipin | <0.01 |
| w1118 *v.s.* dLipin&EcR | <0.01 |
| dLipin&EcR *v.s.* EcR | <0.05 |

| Condition | Statistics P-value |
|---|---|
| w1118 *v.s.* dLipin | <0.01 |
| w1118 *v.s.* dLipin&EcR | <0.01 |
| dLipin&EcR *v.s.* EcR | <0.01 |

Figure 10. Survival rates of the flies with *dLipin* single knockdown, *EcR* single knockdown and double knockdowns by driver r[4]-GAL4. In females (A), flies with single knockdown of *EcR* (green) had a similar survival pattern to the control flies (blue) while the survival pattern of the flies with double knockdowns (light blue) was similar to the survival pattern of the flies with single knockdown of *dLipin* (red),. In males (B), flies with single knockdown of *EcR* had a similar survival rate to the control before day 5. And after day 5, its survival rate decreased, but the difference was not significant. Flies with double knockdowns of both *EcR* and *dLipin* had a very similar survival rate to the flies with *dLipin* single knockdown. The experiment was performed in quadruplicate and the survival curves and statistical comparisons of survival curves were made with the log rank test. Only P-values smaller than 0.05 were listed in the figure.

Loss of NRs DHR38, DHR96, EcR in both females and males, and loss of DHR3 in males led to a survival rate which was lower than in the control animals but still higher than in *dLipin* single knockdown flies. And compared with the flies with *dLipin* single knockdown, flies with double knockdowns of both dLipin and NR had a similar survival rate to the flies with *dLipin* single knockdown. Double loss of both dLipin and NR did not decrease or improve the survival rate and life span. Loss of DHR3 in female flies resulted in a large decrease in the survival rate, even compared with dLipin single knockdown. And flies with double knockdowns of *DHR3* and *dLipin* had a similar survival pattern to the flies with *DHR3* single knockdown. For NR HNF4, loss of function slightly (not statistically significant) increased the resistance to starvation in both female and male flies. Double knockdowns of *dLipin* and *HNF4* in females leaded to a slightly better survival pattern than dLipin single knockdown. However, compared

with *dLipin* single knockdown, the double knockdowns in male flies showed an increased survival rate which was statistically significant, partly rescued the impaired starvation resistance caused by dLipin single knockdown. So for NRs DHR38, DHR96, EcR and DHR3, no interaction of NR and dLipin was observed from the starvation experiment. For NR HNF4, the starvation result showed a rescue in the starvation resistance ability, which was consistent with the genetic interaction data described above. Although in female flies, the difference between the animals with *dLipin* single knockdown and double knockdowns was not statistically significant, it is still likely that the P value can decrease if more flies are included in the experiment. Further experiments with an increased number of the observations are needed to confirm the interaction.

***B. Generation of flies containing mutations in conserved protein motifs of dLipin***

In mammals, the Lipin 1 gene encodes a 98kDa protein, which acts as phosphatidate phosphatase-1 (PAP1) and transcriptional co-activator (Harris et al., 2007). The protein has highly conserved domains called N-LIP and C-LIP. C-LIP contains two motifs, the PAP1 motif, DxDxT and a co-activator motif, LxxIL. Near the end of the N-LIP domain, there is a nuclear localization signal (NLS) which is required for nuclear translocation (Finck et al., 2006). A previous study has shown that changes in the PAP1 active site can delete the PAP enzymatic activity while the transcriptional co-activator activity is unaffected. However, changes in the co-activator motif can lead to the failure of both PAP1 activity and transcriptional co-activator activity (Finck et al., 2006). In this experiment, I generated flies that only express dLipin protein that lacks the PAP enzymatic activity by changing the PAP1 motif from DIDGT to EIDGT (Figure 11). I also tried to generate flies that only express dLipin protein that lacks the co-regulator activity by deleting the NLS. These mutant flies were generated with the goal to study one specific function of dLipin without disturbing the second function. The delta PAP mutant flies will allow me to

study the importance of the PAP activity of dLipin without disturbing its co-activator activity, while the delta NLS mutant flies will allow me to study the co-regulator function without its PAP activity. Compared with knockdown of *dLipin* by RNAi, PAP activity or co-regulator activity will be completely removed, whereas RNAi flies have reduced, but not completely suppressed activity of dLipin.


Figure 11. Partial amino acid sequence of dLipin. Indicated in the sequence are the putative nuclear localization sequence (NLS, blue), the PAP enzyme activity site (green) and the transcriptional co-activator motif (red). The PAP enzyme catalytic domain was changed by a D (codon: GAC) to E (codon: GAG) exchange at position 812 from DIDGT to EIDGT.

To carry out the targeting, a transgenic donor line, which contains the desired targeting construct flanked by FRT sites (5'GAAGTTCCTATTCtctagaaaGtATAGGAACTTC3'), is needed (Figure 12). If the donor line is crossed with transgenic flies that express FLP recombinase, FRT sites will be recognized and cut by the recombinase, inducing site-directed homologous recombination. One I-Scel restriction site is also inserted into the donor construct. I-Scel is a restriction enzyme that can recognize and cut at an 18bp restriction site. I-Scel sites are not present in the *Drosophila* genome. The site is inserted in the homologous sequence of the donor construct and used to linearize the excised donor construct, which is a prerequisite for homologous recombination. Successful recombination will result in a duplication of the targeted region, separated by the marker gene *white*+. Flies with the dominant white+ allele have red eyes, which indicate the potential targeting event. The tandem duplication of the target gene that is

being produced consists of one copy carrying the mutation and one wild-type copy, separated by a DNA segment that includes the *white+* marker gene, an FRT site, and a I-CreI site. The I-CreI site is specifically recognized and cut by the restriction enzyme I-CreI, which is used in the following "reduction" step.

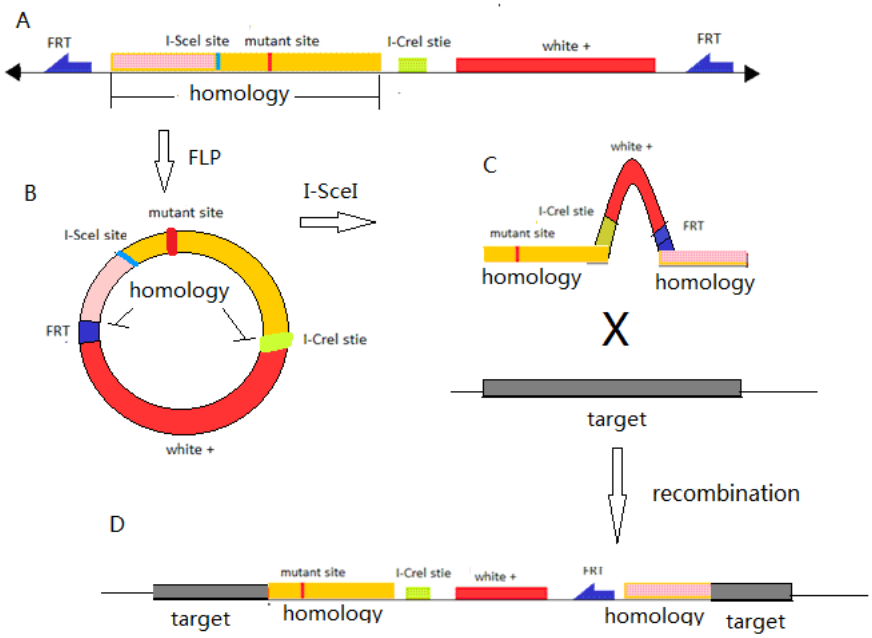



Figure 12. Ends-in homologous recombination. (A) Donor construct contains the homology to the target gene (pink and yellow), I-SceI (light blue) site, I-Crel (green) site and *white+* gene (red), flanked by two FRT sites (blue). (B) FLP recombinase cuts at FRT sites, excising the donor construct from the genome. (C) I-Scel restriction enzyme cuts at specific site and linearizes the donor construct. (D) Homologous recombination results in a tandem duplication of target gene and the insertion of white+, I-Crel site and FRT site.

To obtain flies that only carry the mutant copy of the gene, a "reduction" step is necessary that deletes both the wild-type copy of target gene and the marker gene (Figure 13). I-Crel sites do not exist in the *Drosophila* genome. This site is inserted between the target gene and the marker gene. Cleavage of I-CreI site leads to homologous recombination that deletes one extra copy of target gene and the marker gene *white+*, only leaving the mutant copy of the gene

in the *Drosophila* genome. So ends-in targeting can not only knock out the target gene, but also introduce site-directed mutations -- deletions, insertions and point mutations.

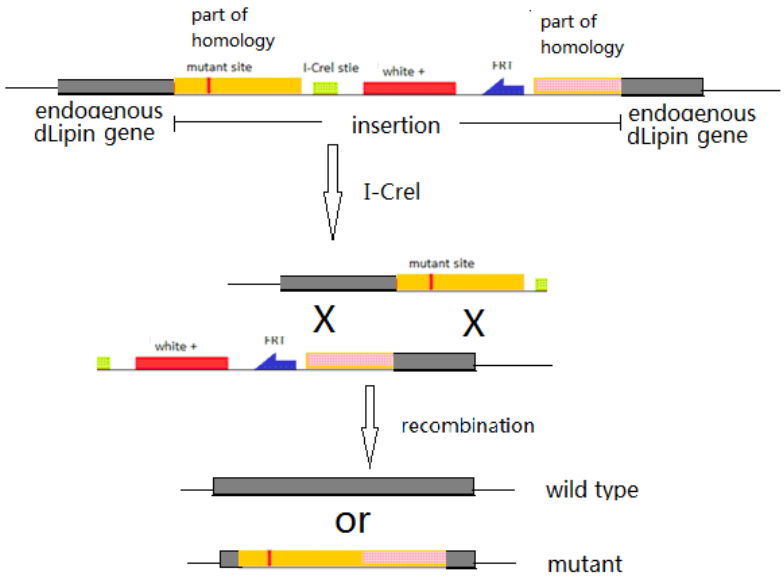



Figure 13. Reduction. The tandem duplication of target gene from homologous recombination contains the two separate parts of the homology to the target gene (pink and yellow), I-Crel (green) site, *white+* (red), and one FRT site (Blue). Cutting at I-Crel site leads to a second homologous recombination event, forming reduced alleles, either mutant or wild type.

**1. delta PAP mutant fragment was inserted into dLipin gene by ends-in targeting**

A transgenic fly line that carries an insertion of a 6kb delta PAP mutant fragment, the *white+* gene, and an I-Crel site, flanked by FRT sites, was used as a donor stock for mutagenesis (Figure 14). Flies of this stock were crossed with flies that expressed flipase (Flp) and the restriction endonuclease I-Scel to excise the fragment flanked by the FRT sites and to accomplish integration of this fragment into the genome by homologous recombination.

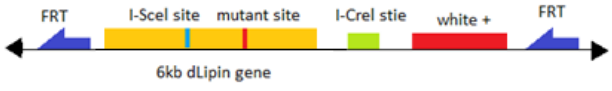



Figure 14. Insertion carried by the donor flies. Insertion includes a 6kb *dLipin* gene (yellow), an I-Crel site (green) and a *white* marker gene (red), and all these elements are flanked by two FRT sites (blue arrows). Inside the 6kb *dLipin* gene, a point mutant (C to G) was made in the

sequence that encodes the PAP catalytic domain to eliminate the PAP enzymatic activity. In front of the mutant site, an I-Scel site was inserted (light blue).

I screened about 300 vials of flies and obtained 12 red-eyed progenies, which indicated the potential targeting event. For further confirmation, I used PCR and DNA sequencing to test whether the excised fragment was inserted at the right place. Unsuccessful excision of the FRT flanked fragment can also result in red eye progenies. Primers "PAP high" (table 2 in methods) were designed to detect the fragment inserted into the *dLipin* gene. Correct insertion would result in a 3.5kb PCR fragment and the I-Scel site would be deleted (Figure 15A and Table 4). A 3.5kb fragment can also be obtained if the FRT-flanked insert was not excised. However, this fragment would contain the I-Scel site (Figure 15B and Table 3). This difference was determined by DNA sequencing. Of the twelve red-eyed flies, four had the correct insert while the other eight flies' red eyes were just due to unexcised FRT flanked fragment (Figure 16). Progenies of the four flies with the correct insert were then independently crossed with balancer lines and kept as balanced stocks.

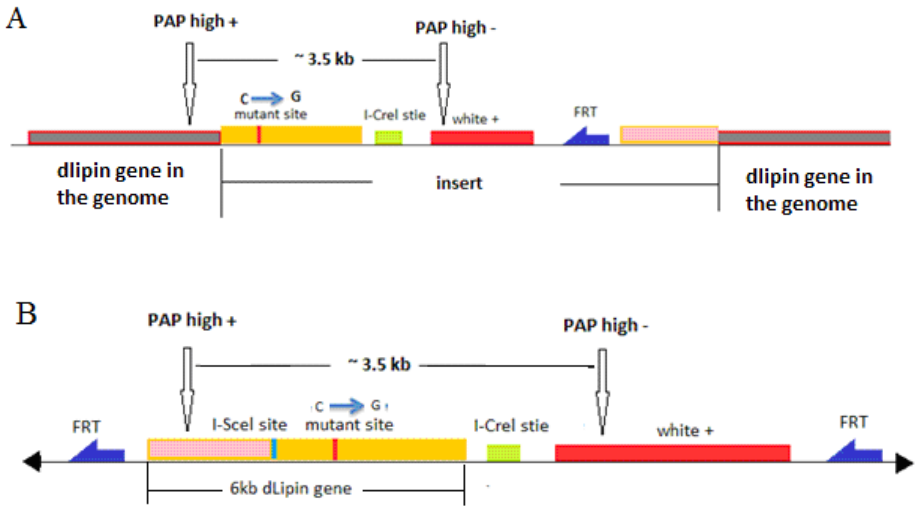



Figure 15. Fragments amplified by PCR for confirmation of target replacement of part of the *dLipin* gene. A 3.5kb fragment can be amplified from flies carrying the right insertion (A) and donor flies (B). In the flies carrying the correct insertion, the I-Scel site (light blue) was cut and

removed and the 6kb *dLipin* gene was divided into two pieces (yellow and pink). The grey color stands for the endogenous *dLipin* gene in the genome. Without excision of the donor element, the 6kb *dLipin* gene and the I-SceI site remain intact.

Table 4. Expected PCR and sequencing results for red-eyed flies obtained after mutagenesis.

| | PCR fragment | Sequence |
|---|---|---|
| W1118 | no product | NA |
| Donor stock | 3.5kb | Contains I-SceI site |
| Insert at the right site | 3.5kb | Does not contain I-SceI site |
| Insert at wrong site | no product | NA |

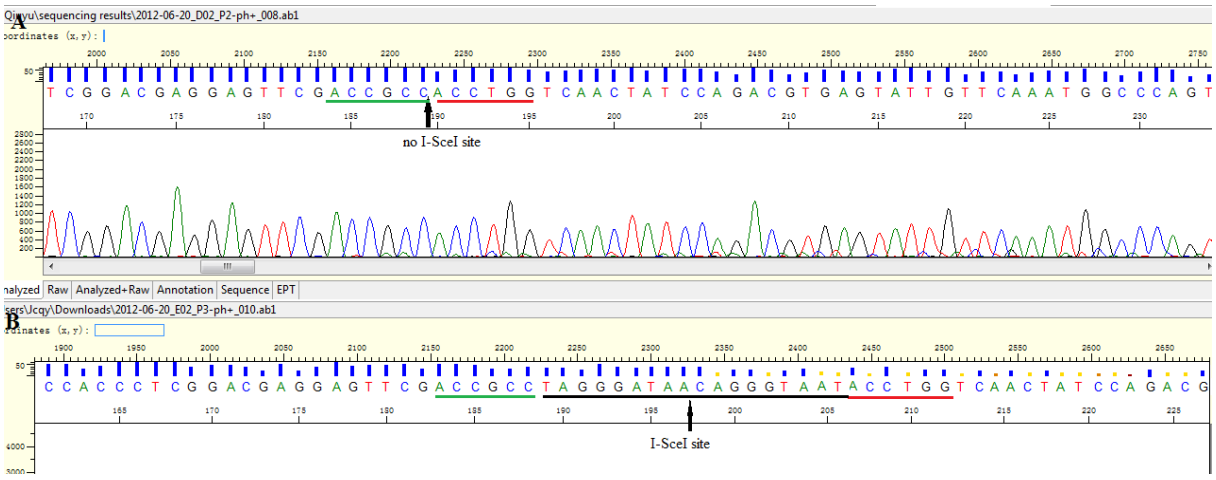



Figure 16. Part of the DNA sequencing results from the flies with right inserted fragment and donor stock. Correct insertion resulted in the deletion of the I-SceI site (A). If the 6kb fragment is not successfully excised, it still contains the I-SceI site (B).

**2. Flies only expressing delta PAP mutant dLipin were obtained by reduction crosses**

Flies that are the products of ends-in targeting still carry the *white* marker gene inserted in their genome and a duplicated copy of the wild-type *dLipin* gene. Reduction crosses allowed me to delete the *white* gene and wild-type *dLipin*, leaving only the PAP mutant *dLipin* gene in the genome. Flies carrying the insert in the correct site were crossed with flies that expressed the restriction endonuclease I-Crel to accomplish the removal of the *white* and wild-type *dLipin*

genes by homologous recombination. These flies would be white-eyed again because of the loss of the *white* gene. I included all the four independent lines and carried out the reduction crosses. White-eyed progenies were obtained and their genomic DNA was collected to carry out a PCR confirmation using primers "PAP" (Table 2 in methods). PCR product which contained the mutant site was sequenced. These four lines all contained the mutant site as indicated by a mixed signal (50% C and 50% G) in the sequencing results (Figure 17).

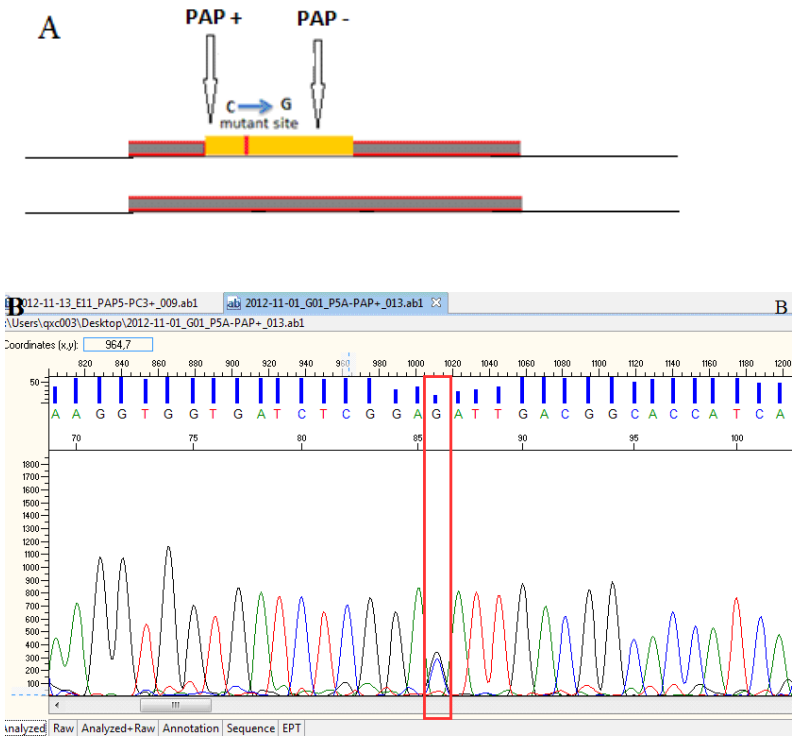



Figure 17. Sequence result for the delta PAP mutant flies. The primers were designed to amplify the fragment containing the point mutation site (A). A mixed signal of C (black) and G (blue) was obtained by sequencing.

To further confirm the right structure of the *dLipin* gene close to the recombination site, three pairs of primers, "PAP confirm1", "PAP confirm2" and "PAP confirm3" (Table 2 in methods) were used (Figure 18). PCR products were sequenced and all the bases were the same as in the wild type *dLipin* gene except the mutant site.

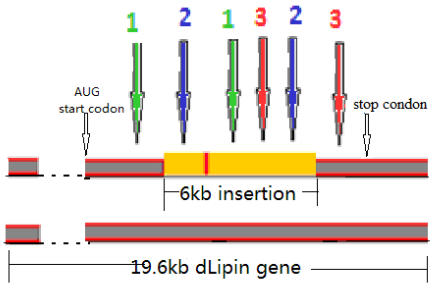



Figure18. Primers designed for confirmation sequencing. 19.6kb endogenous *dLipin* sequences are shown in grey and the 6kb insertion is shown in yellow, which contains the mutant site (red). Three pairs of primers (green, blue and red arrows) were used to confirm the correct structure of the mutant *dLipin* gene. The white arrows indicate the start codon and stop codon. The fragments amplified by these primers were overlapping and covered the whole 6kb inserted fragment and part of the adjacent endogenous *dLipin* sequences in the genome.

Four independent PAP mutant fly stocks were obtained. Only heterozygous flies (delta PAP/CyO) of these stocks could be kept, because the introduced PAP mutation resulted in lethality. These flies were used for the following experiments.

**3. No delta NLS mutant flies were generated by ends-in targeting**

The same method was used in an attempt to generate delta NLS mutant flies. The construct carried by the donor line is showed in Figure 19. In the mutant site, the sequence encoding the NLS (AAGAAGCGGCGCAAGAAG) was deleted.

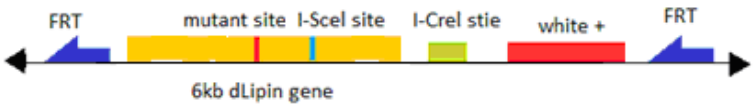



Figure 19. Insertion carried by the donor flies. Insertion includes a 6kb dLipin gene (yellow), an I-Crel site (green) and a *white* marker gene (red), and all these elements are flanked by two FRT sites (blue arrows). Inside the 6kb *dLipin* gene, the sequence encoding the NLS was deleted to interfere with nuclear translocation and, thus, transcriptional co-regulator activity of the protein. Downstream of the mutant site, an I-Scel site was inserted (light blue).

About 500 vials were screened, but no red-eyed fly was found. The delta NLS mutant fragment was not inserted into *dLipin* gene in the flies I screened. Since the efficiency of targeting is highly variable, it is possible that more flies need to be screened to obtain a mutant.

### *C. Characterization of PAP mutant flies*

#### 1. PAP mutation is lethal to *Drosophila*

Since all the delta PAP adult flies were heterozygous (PAP/CyO), I wanted to find out how long homozygous (PAP/PAP) animals can survive and whether I can use these homozygotes to study dLipin protein function. I crossed delta PAP mutant flies with CyO-GFP flies and kept delta PAP/CyO-GFP as a stock. The green fluorescence allowed me to tell homozygous (non-GFP) from heterozygous (GFP) animals. Since both homozygous and heterozygous embryos carry maternal GFP, the difference between these two lines cannot be told until the 1$^{st}$ instar larval stage. Almost all the eggs hatched and developed into 1$^{st}$ instar larvae 24 hours after egg collection, indicating that the mutation is not lethal during embryogenesis. Homozygotes did not show green fluorescence anymore at the 1$^{st}$ instar larval stage. Homozygous and heterozygous larvae were separated and transferred to new molasses plates (25 larvae/each plate), and kept at 25℃. After about 24 hours, most of the GFP larvae developed into 2$^{nd}$ instar larvae while almost all non-GFP homozygous larvae had died (Table 3). The non-GFP larvae alive were all 1$^{st}$ instar larvae. Only heterozygous larvae reached the 2$^{nd}$ instar larval stage and continuously developed to adulthood. All delta PAP mutant homozygotes died as late 1$^{st}$ instar larvae.

Table 3. Number of GFP and non-GFP larvae on the molasses plate at different times

| Plate number | Non-GFP homozygotes (36 hours after hatch) | GFP heterozygotes (36 hours after hatch) | Non-GFP homozygotes (60 hours after hatch) | GFP heterozygotes (60 hours after hatch) | Non-GFP homozygotes (80 hours after hatch) | GFP heterozygotes (80 hours after hatch) |
|---|---|---|---|---|---|---|
| 1 | 20 | 25 | 0 | 25 | 0 | 24 |
| 2 | 23 | 22 | 2 | 20 | 0 | 20 |
| 3 | 19 | 21 | 3 | 19 | 0 | 19 |

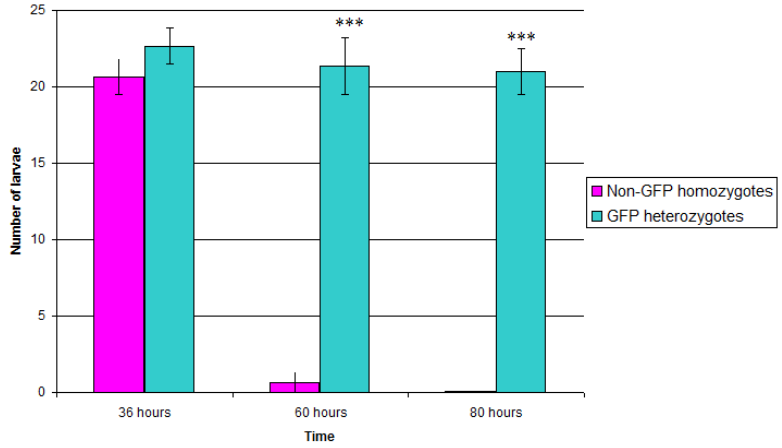



Figure 20. Number of GFP and non-GFP larvae on the molasses plates at different times. 25 1st early instar larvae were used for each group at the beginning of the experiment. Most larvae survived for 36 hours after they had hatched. After 60 hours, only 5 PAP mutant homozygotes were found on the plates while most heterozygotes were still alive (*** P<0.005%). After 80 hours, all the homozygotes had died while 63 heterozygotes were alive (*** P< 0.005%).

**2. Homozygotes of the delta PAP mutant express dLipin protein**

In order to exclude the possibility that the early lethality is due to no or low expression of *dLipin*, I used a dLipin antibody to determine whether homozygous delta PAP mutants express dLipin protein. A Western blot was also included to determine the mutant dLipin protein size and amount.

In a previous study, staining of *Drosophila* tissues with dLipin antibody revealed that *dLipin* is expressed in many tissues including proventriculus, gastric caeca, midgut and fat body (Ugrankar et al., 2011). dLipin antibody detected dLipin protein in tissues of homozygous delta-PAP mutant 1st instar larvae, in particular in the midgut (Figure 21A). The fat body is still

underdeveloped at this early stage in development and, therefore, staining of this tissue could not be unequivocally demonstrated. Overall, expression of dLipin appeared to be very similar to expression in tissues of the control larvae (delta PAP/CyO-GFP). The previous study also showed that dLipin has both cytoplasmic and nuclear localization (Ugrankar et al., 2011). In some cells of delta-PAP mutant larvae, dLipin protein could be detected in the nucleus (Figure 21B), which shows that the delta PAP mutation did not disturb the nuclear translocation ability of dLipin protein.

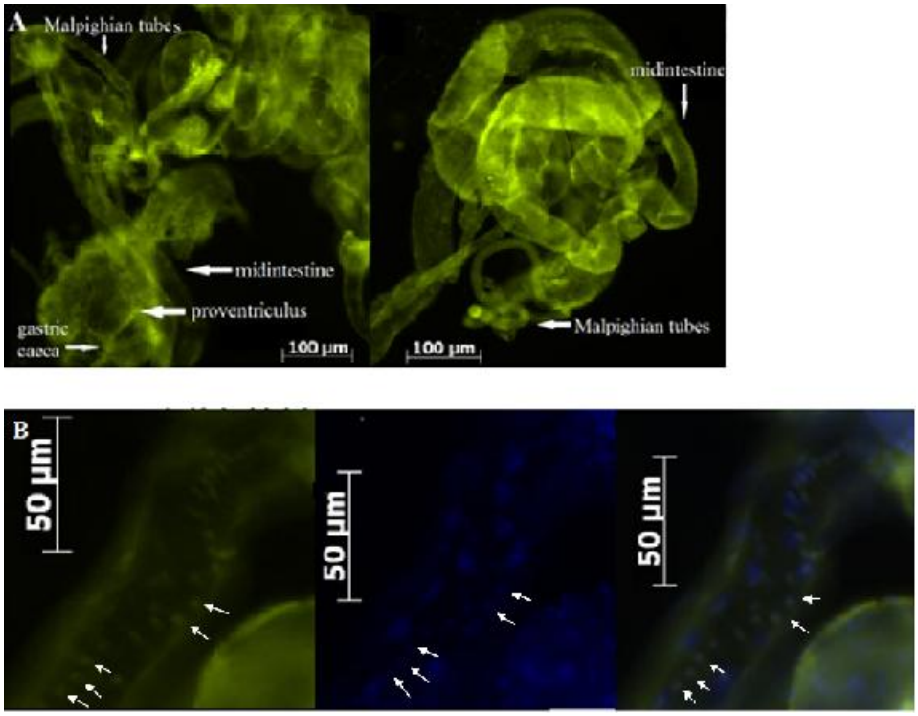



Figure 21. dLipin antibody staining of delta-PAP mutant 1st instar larvae. The proventriculus, gastric caeca, Malpighian tubes and midintestine (midgut) were stained in both control (PAP/CyO-GFP) (A left) and PAP mutant homozygotes (A right). dLipin protein can be detected in cell nuclei (B). dLipin antibody staining of delta-PAP mutant 1st instar larvae (B left), nuclear staining by DAPI (B middle), and nuclear and antibody staining merged (B right). Arrows point to nuclei that contain dLipin protein.

I also performed a Western blot analysis to determine whether or not the size and amount of dLipin protein in delta PAP larvae was changed. Samples were prepared from whole 1st instar larvae that were homogenized in SDS-PAGE sample buffer. Actin (45kDa) was used as a

loading control. In samples from both PAP/PAP homozygotes and PAP/CyO-GFP heterozygotes, a specific band similar in size to dLipin (114kDa) was detected (Figure 22). The darkness of these two bands was similar which means that delta PAP mutant protein is present in approximately the same amount as wild type protein.

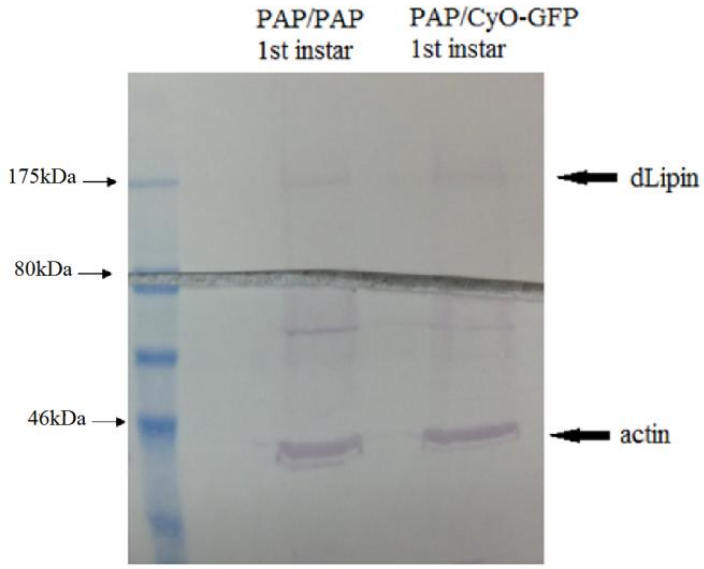



Figure 22. Western blot to detect dLipin in delta PAP mutant 1st instar larvae. In the samples from both PAP/PAP and PAP/CyO-GFP larvae, the dLipin-specific antibody detected similar amounts of a single protein. As previously observed (M. Lehmann, personal communication), the apparent molecular weight of dLipin as determined by SDS-PAGE is somewhat larger than the calculated weight of 114 kDa, probably due to posttranslational modifications.

**3. Delta PAP homozygotes can be rescued by hs-dLipin**

In order to confirm that the lethality is the result of a deficiency in dLipin PAP activity rather than a secondary mutation introduced during the mutagenesis, I attempted to rescue the mutant phenotype by expression of wild-type *dLipin* from a heat-inducible transgene (hs-*dLipin*). If hs-dLipin (isoform A) can rescue the animals through the early lethal stage, this may also make it possible to observe phenotypes caused by lack of PAP enzymatic activity at later times of development.

1st instar larvae were collected from molasses plates and put onto normal food. For heat shock, vials with 1st instar larvae were incubated at 37 ℃ for one hour once a day. The number of heat shock days, non-heat shock days and the dissection time are shown in Figure 23.

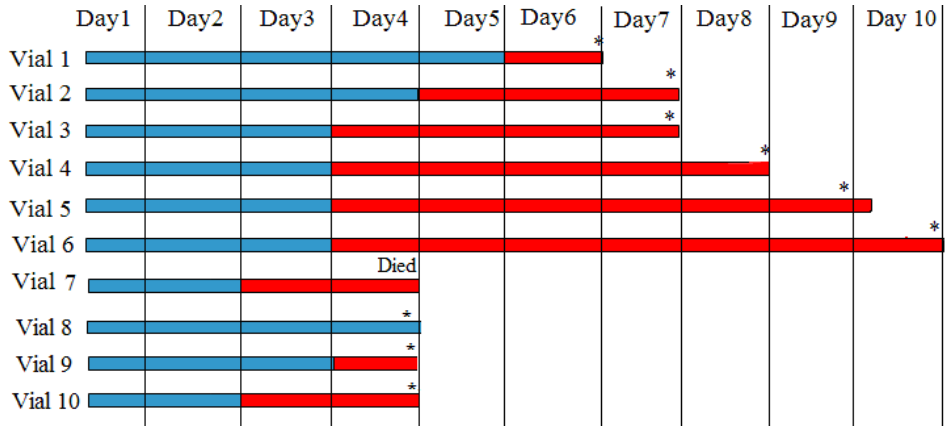



Figure 23. Rescue of delta PAP/delta PAP animals by the expression of hs-*dLipin*. Shown are the time lines for heat shock applications and dissections in different experiments (vials). On heat shock days, larvae were incubated at 37 ℃ for one hour. Blue color indicates heat shock day and red color non-heat shock day. Asterisks indicate the dissection time. All the larvae were dissected at the 3rd larval stage.

Hs-dLipin does rescue the PAP mutant larvae. PAP mutant larvae reached the 3rd instar larval stage after heat shock for one hour once a day for five days (vial 1). Fat droplet staining showed that the fat body cells in these larvae were very similar to those in control animals (Figure 24 A, B). The cells were of normal shape and size and the fat droplets were big. Fat body cells were attached to each other as in the wild type. However, compared to their heterozygous siblings (vial 8 -10), the homozygous larvae came out of food two to three days later. They reached the wandering stage at day 6, while heterozygous larvae came out of food at about day 4.

If the mutant larvae were only heat shocked for four days (vial 2), they still could reach the wandering stage. However, the developmental delay was more severe for these larvae, since they came out of the food at day 7. Fat droplet staining showed that fat body cells of these larvae were of normal size and shape, containing big fat droplets (Figure 24C).

When the number of heat shock days was reduced to three (vial 3, 4, 5 and 6), the larvae could still reach the 3rd instar larval stage, but not the wandering stage. They seemed to be "trapped" at the feeding 3rd instar larval stage. Some of them stayed alive in the food until day 10 before they died, while some died at day 8 or 9. Fat body cells in these larvae were rounded and their sizes were variable. The fat droplets were much smaller, too (Figure 24D, E, F and G). The more time had passed after the last heat shock, the more severe the fat body phenotype became. Three-day was the shortest heat shock time for PAP mutant larvae to reach the 3rd instar larval stage. If only heat shocked for two days, all the larvae died at around day 4 as 2nd instar larvae (vial 7).

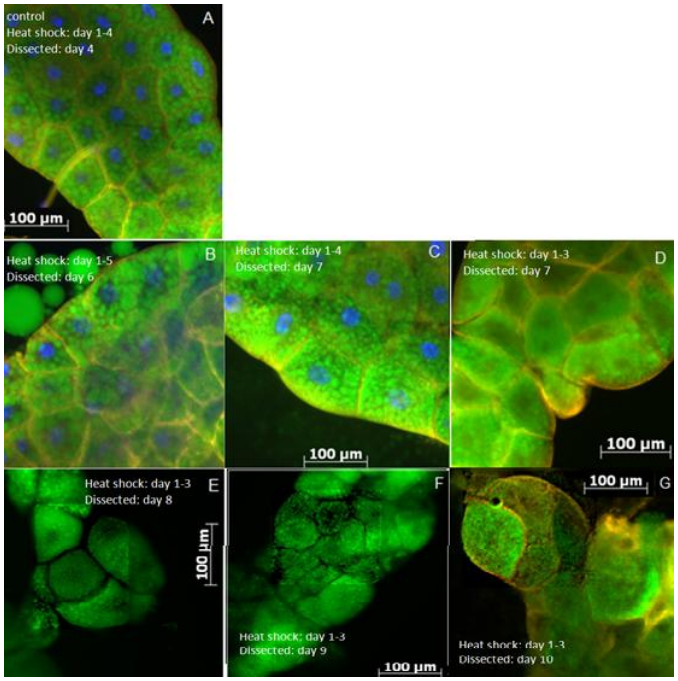



Figure 24. Staining of the fat body from delta PAP/delta PAP and delta PAP/CyO-GFP larvae subjected to heat shock-induced *dLipin* expression. Delta PAP/delta PAP and control animals (delta PAP/CyO-GFP) were heat shocked for different numbers of days and dissected. Fat droplets were stained with BODIPY (green). The cell membrane was stained with CellMask Orange (orange) and the nuclei were stained with DAPI (blue). The fat body cells of control animals (delta PAP/CyO-GFP, heat shocked for four days and dissected) were of similar size and contained big fat droplets. They were closely attached to each other (A). If the larvae were heat shocked for five days and dissected at day 6 (B) or heat shocked for four days and dissected at day 7 (C), cells were still of normal size and shape and fat droplets were still big. If heat shocked for only three days and dissected at day 7, the fat body cells were rounded and fat droplets

became smaller (D). If heat shocked for three days and dissected at day 8 (E), day 9 (F) or day10 (G), the size of rounded cells became variable. The fat droplets were small, too. And the longer time after heat shocked, the more severe the phenotypes were.

If the heat shock continued every day or every other day after the larvae came out of the food, most PAP mutant homozygotes could be rescued until the pupal stage. However, most homozygous pupae died right before or during eclosion. Very few flies successfully eclosed. Successful rescue until the pupal stage indicates that early lethality is due to the elimination of PAP activity of dLipin and not a secondary mutation that was accidentally introduced during mutagenesis.

**4. Ectopically expressed dLipin can be monitored when using dLipin-GFP to rescue the delta PAP mutant flies**

To study the requirement of dLipin in late development, it is important to known how much ectopic dLipin protein is still present in the cells after heat shock. In order to monitor the presence of ectopic dLipin in the mutant flies, I used a GFP-tagged dLipin for rescue experiments which can be expressed through the GAL4-UAS system. Different from the hs-*dLipin* fly line in which *dLipin* expression can be directly induced by heat shock, flies that carry the UAS-*dLipin-GFP* element can only express the GFP-tagged dLipin when combined with proper driver lines. dLipin-GFP can be identified by its green fluorescence. If a heat shock GAL4 driver is used and no more green fluorescence can be detected in larval tissues after the end of heat shock treatment, all the ectopically expressed dLipin protein can be considered as degraded. Larvae in this case only contain delta PAP dLipin and can be used to study the requirement of the PAP activity of dLipin late in development.

However, whether the GFP tag will interfere with the function of dLipin protein remained unknown. In order to test the functionality of dLipin-GFP, I first compared the rescue results of flies carrying UAS-*dLipinWT* and UAS-*dLipin-GFP* elements. The expression of *dLipinWT* and

*dLipin-GFP* can be induced by various GAL4 drivers. UAS-dLipinWT had previously been successfully used in rescue experiments (Ugrankar et al., 2011).

4.1 The GFP tag does not interfere with the PAP activity of dLipin-GFP

A previous study had shown that dLipinWT expressed under control of the *daughterless* driver can rescue $dLipin^{e00680}/Df$ larvae, which express very little endogenous dLipin (Ugrankar et al., 2011). If both dLipinWT and dLipin-GFP were able to rescue the delta PAP mutant flies, the GFP tag does not interfere with the PAP activity of dLipin-GFP.

I was able to confirm the rescue data for dLipinWT. Ectopically expressed dLipinWT rescued delta PAP mutant larvae until the pupal stage and the fat body cells of these animals were normal (Figure 25). However, most of the flies died as pupae and very few reached the adult stage. dLipinWT-rescued flies also had a developmental delay of about three days compared with their heterozygous siblings. With dLipin-GFP, no delta PAP mutant larvae were found in the food. The reason why dLipin-GFP cannot rescue the delta PAP larvae could be interference of the GFP tag with dLipin function or insufficient protein expression. Although the same driver was used to ectopically express *dLipinWT* and *dLipin-GFP*, the expression levels of the proteins may not necessarily be the same, because the expression of UAS-responder elements depends on the genomic integration site.

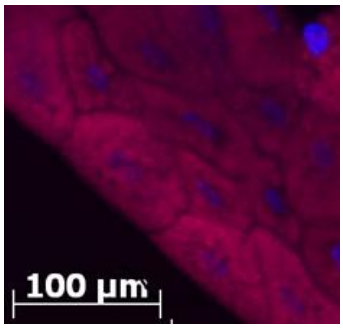



Figure 25. Staining of the fat body from larvae rescued by *daughterless*-GAL4-induced dLipinWT. Fat droplets were stained with Nile Red (red) and the nuclei were stained with DAPI (blue). The cells were intact and contained big fat droplets.

*Tubulin*-GAL4 is a very strong driver that induces protein expression ubiquitously. I wanted to exclude the possibility that the failed rescue when using the *daughterless* driver was caused by an insufficient amount of dLipin protein being ectopically expressed. Using this driver, I found that PAP mutant flies could be rescued until adulthood by either dLipin-GFP or dLipinWT. However, the development of the animals rescued by dLipinWT had a delay of about two days, while animals rescued by dLipin-GFP had a delay of about three days. In both dLipin-GFP and dLipinWT-rescued flies, the fat body looked normal with big fat droplets (Figure 26A and B). However, the size of dLipin-GFP rescued fat body cells was somewhat smaller than the size of dLipinWT-rescued cells, and the rescued flies were infertile, while dLipinWT-rescued flies were able to produce offspring.

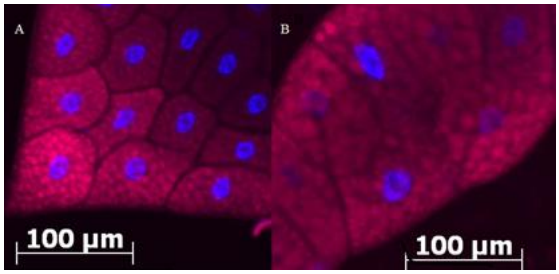



Figure 26. Staining of the fat body from larvae rescued by *tubulin*-GAL4-induced dLipin. Fat droplets were stained with Nile Red (red) and the nuclei were stained with DAPI (blue). Cells looked normal in the fat body of larvae rescued by dLipin-GFP (A). They were well arranged and with big fat droplets. Larvae rescued by dLipinWT had similar fat body cells (B). However, the size of dLipin-GFP-rescued fat body cells appeared to be smaller than that of the dLipinWT-rescued cells.

Snice dLipin-GFP rescued larvae had slightly smaller fat body cells and would develop into infertile adult flies, the possibility remained that activity of dLipin-GFP was somewhat diminished compared to dLipin. However, dLipin-GFP was able to rescue. The finding that dLipin-GFP can rescue delta PAP homozygotes until adulthood indicates that the GFP tag does not disrupt the PAP enzymatic function of dLipin protein and can be used in rescue experiments.

4.2. Rescue experiments with heat shock-induced dLipin-GFP

After making sure that the GFP-tagged dLipin can be used in rescue experiment just as dLipinWT, I proceeded to use heat-shock-induced expression of *dLipin-GFP* to rescue delta PAP. GAL4-hsp70 only induces the expression after heat shock and, as time passes, the expressed protein will degrade, eventually leaving no ectopic protein in the tissue. The GAL4-UAS system, together with the GFP-tag of dLipin-GPF, allows me to know and control the existence of ectopic dLipin in larvae by adjusting the heat shock time and frequency.

Using the GAL4-hsp70 driver, delta PAP mutant flies with either UAS-*dLipinWT* or UAS-*dLipin-GFP* element could be rescued until the pupal stage if heat shocked for one hour once a day. Fat body cell staining also showed normal cell size and shape (Figure 27). The fat droplets were big too. Similar to the results using the *tubulin*-GAL4 driver, these flies also had a developmental delay. Larvae rescued by dLipinWT came out of food one to two days late while dLipin-GFP larvae came out of food about two to three days late.

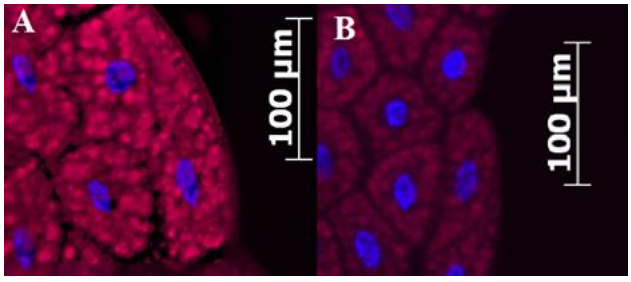


Figure 27. Staining of the fat body of larvae rescued by hsp70-GAL4 driver induced dLipin and dLipin-GFP. Fat droplets were stained with Nile Red (red) and the nuclei were stained with DAPI (blue). Larvae were heat shocked once a day until the 3$^{rd}$ instar larval stage. Cells of larvae rescued by dLipin-GFP (A) and dLipinWT (B) showed similar phenotype. The fat body cells showed normal cell size and shape and the fat droplets were big.

The purpose of using dLipin-GFP was to be able to monitor the stability of the ectopically expressed dLipin. In order to find out the correlation between the stability of expressed dLipin-GFP and the heat shock frequency, I carried out heat shocks as shown in the Figure 28.

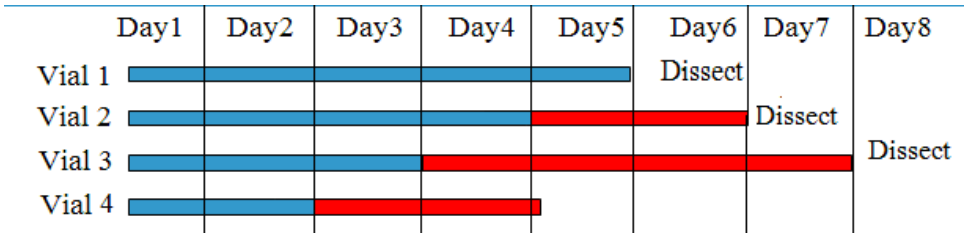



Figure 28. Rescue of delta PAP/delta PAP animals by the expression of *dLipin-GFP*. Shown are the time lines for heat shock applications and dissections in different experiments (vials). On heat shock days, larvae were incubated at 37℃ for one hour. Blue color indicates heat shock days and red color non-heat shock days. All the larvae were dissected at the 3rd instar larval stage.

Thirty 1st instar larvae were put into each vial and observed in the following days, especially their fat bodies. In vial 1, larvae came out of the food on the 5th day and had strong green fluorescence in their fat body cells, indicating the presence of dLipin-GFP. Larvae in vial 2 were heat shocked for four days and, before they entered the wandering stage, they spent another two days in food without heat shock. Their fat body cells were still green, indicating the presence of dLipin-GFP. In vial 3, larvae were only heat shocked for three days and they showed an obvious developmental delay after heat shock stopped. They came out of the food on the 8th day. However, dLipin-GFP still existed in the fat body cells five days after the last heat shock, although the green fluorescence was not as bright as in cells from larvae in vial 1 and vial 2. If only heat shocked for three days, the 8th day was the latest time that live larvae could be found. Larvae in vial 4 were heat shocked for two days and they died on the 4th and 5th day as early 3rd instar larvae. These larvae still exhibited green fluorescence before they died. However, while complete absence of green fluorescence likely indicates total degradation of the protein, the existence of green fluorescence does not necessarily mean the existence of dLipin protein, because GFP is very stable and it is conceivable that the dLipin part of the fusion protein has degraded, while GFP it still intact.

In summary, I could not obtain late 3rd instar larvae or any larvae without green fluorescence, which would have indicated the complete degradation of the ectopically expressed

dLipin before the experimental animals died. This result may indicate that low levels of dLipin are not sufficient to rescue the delta PAP mutants. Alternatively, degradation may affect the dLipin portion of the fusion protein and the GFP tag differently, leading to persistent fluorescence while the ectopically expressed dLipin is already inactive. Further experimentation would be needed to distinguish between these possibilities.

**5. dLipin-GFP localization**

An examination of the intracellular localization of dLipin-GFP did not provide clear evidence that the fusion protein can enter the cell nucleus (Ugrankar et al., 2011), suggesting that the GFP moiety may interfere with nuclear translocation. In order to further test the ability of dLipin-GFP to enter the nucleus, I examined the intracellular distribution of dLipin-GFP in TOR knockdown larvae. Previous studies have shown a robust translocation of dLipin into the nuclei of fat body cells of wandering larvae after RNAi knockdown of TOR using the Cg-GAL4 fat body-specific driver (Sandra Schmitt, personal communication). Therefore, I expressed dLipin-GFP using the Cg-GAL4 driver in TOR knockdown larvae and analyzed the intracellular distribution of dLipin-GFP by fluorescence microscopy. At the same time, I used dLipin antibody staining to detect both the endogenous dLipin and the dLipin-GFP protein.

In the control group, which carried the Cg-GAL4 driver but not UAS-*dLipin-GFP*, there was strong antibody staining in the nucleus, indicating translocation of dLipin protein into the nucleus ((Figure 29A). However, in the experimental group, green fluorescence did not accumulate in the nucleus, suggesting that dLipin-GFP did not enter the nucleus (Figure 29C). Surprisingly, dLipin antibody staining showed that in these animals even the endogenous dLipin did not enter the nucleus (Figure 29D).

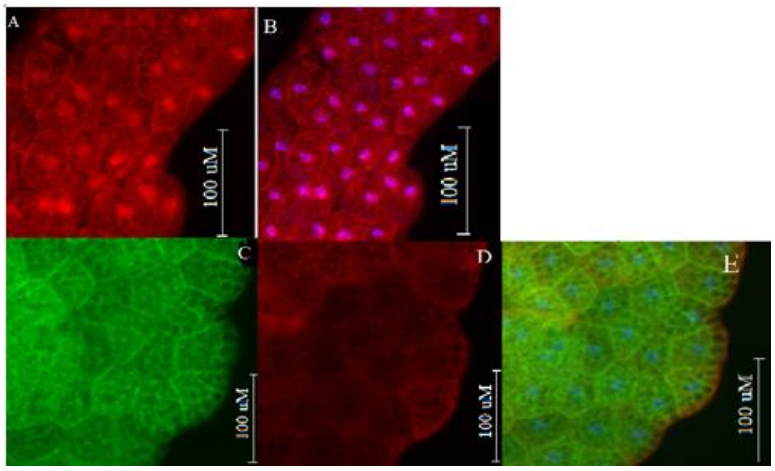



Figure 29. dLipin and dLipin-GFP localization. dLipin and dLipin-GFP proteins were stained with dLipin antibody (orange) and nuclei were stained with DAPI (blue). In the control Cg-GAL4/TOR[RNAi] flies, strong dLipin-antibody staining can be seen in the center area of the cells (A), which overlaps the nuclear area (B). In the Cg-GAL4/TOR[RNAi]; UAS-*dLipin-GFP* flies, green fluorescence showed the distribution of dLipin-GFP, which did not accumulate in the nuclei. (C). dLipin antibody staining showed the distribution of both endogenous dLipin and dLipin-GFP (D). Nuclear staining is shown in blue in E. Neither dLipin nor dLipin-GFP entered the nuclei.

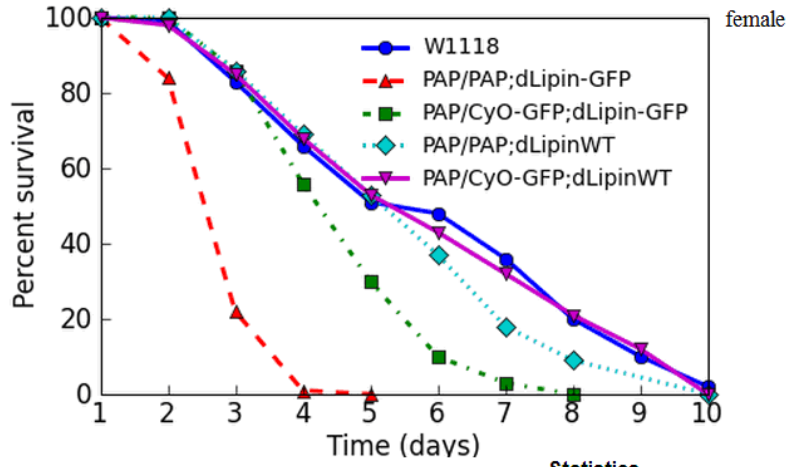
One interesting observation was that, in the control group (Cg-GAL4/TOR[RNAi]), only when female UAS-TOR[RNAi] flies were crossed with male Cg-GAL4/CyO-GFP; UAS-*dLipin-GFP*/+ flies, the progeny showed dLipin translocation into the fat body nuclei. If the cross was carried out the other way, dLipin did not enter the nucleus in the progeny. The results obtained with the TOR knockdown animals strongly suggest that dLipin-GFP cannot translocate into the cell nucleus and that it can even prevent the endogenous dLipin from entering the nucleus.

**6. dLipin-GFP rescued PAP mutant flies showed decreased starvation resistance**

The loss of the nuclear translocation ability of dLipin-GFP does not seem to impair its rescue ability in the delta PAP mutant flies under normal conditions. However, since *dLipin* is upregulated during starvation and knockdown of *dLipin* will decrease the starvation resistance of *Drosophila* (Ugrankar et al., 2011), a rescue experiment under starvation conditions may show a difference between dLipinWT and dLipin-GFP in the PAP mutant flies. The goal of these

experiments was to compare the abilities of dLipinWT and dLipin-GFP to improve starvation resistance. Since with the *tubulin*-GAL4 driver the delta PAP mutant can be rescued until adulthood by either dLipinWT or dLipin-GFP, I used these rescued flies to carry out the starvation experiments.

As expected, in females, delta PAP heterozygotes expressing dLipinWT had a similar survival pattern as the wild type flies. At day 6, the survival rate of the PAP heterozygotes expressing dLipinWT was 50%, while the survival rate of the PAP homozygotes rescued by dLipinWT was 40%. Though the mortality of PAP homozygotes rescued by dLipinWT was slightly higher than that of the $w^{1118}$ group, especially after day 6, the statistical difference was not significant. The maximum survival of $w^{1118}$ flies, delta PAP homozygotes and heterozygotes rescued by dLipinWT was 10 days for all. So, dLipinWT was able to almost fully rescue the reduced starvation resistance.



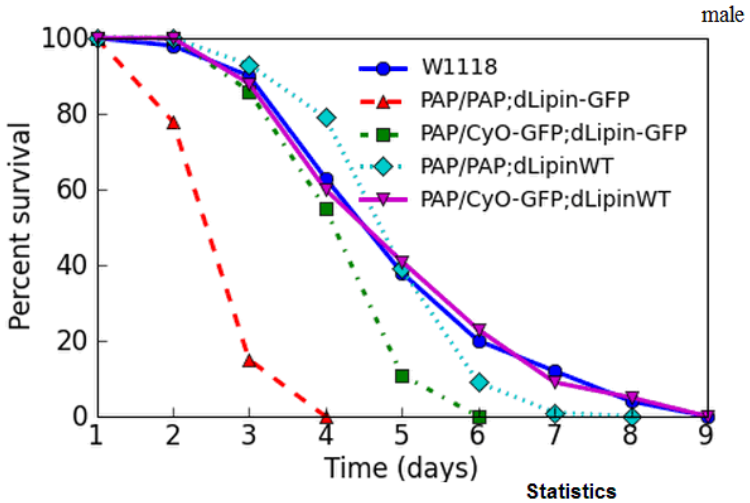

| Condition | Statistics P-value |
|---|---|
| W1118 *v.s.* PAP/PAP;dLipin-GFP | <0.01 |
| W1118 *v.s.* PAP/CyO-GFP;dLipin-GFP | <0.01 |
| PAP/PAP;dLipin-GFP *v.s.* PAP/CyO-GFP;dLipin-GFP | <0.01 |
| PAP/PAP;dLipin-GFP *v.s.* PAP/PAP;dLipinWT | <0.01 |
| PAP/CyO-GFP;dLipin-GFP *v.s.* PAP/CyO-GFP;dLipinWT | <0.01 |

Figure 30. Survival rates of female adult flies rescued by dLipinWT and dLipin-GFP. Heterozygotes with ectopic dLipinWT (purple) showed a similar survival rate as $w^{1118}$ flies (blue). But ectopically expressed dLipin-GFP (green) reduced the maximum life span of

heterozygote from eleven days to eight days. PAP mutant homozygote rescued by dLipinWT (light blue) could live as long as nine days. dLipin-GFP rescued homozygous flies had the shortest maximum life span, which was only five days (red). Survival curves and statistical comparisons of survival curves were made with the log rank test.

PAP heterozygotes expressing dLipin-GFP showed a reduced starvation resistance. At day 6, the survival rate of the PAP heterozygotes rescued by dLipin-GFP was 10%, while all the PAP homozygotes rescued by dLipin-GFP had already died. All the PAP heterozygotes rescued by dLipin-GFP died by the $8^{th}$ day, and the mortality were even higher than the mortality of PAP mutant homozygotes rescued by dLipinWT. Delta PAP homozygotes rescued by dLipin-GFP showed the lowest starvation resistance. At the end of $5^{th}$ day, all the flies had died (Figure 30).

Males had a similar survival pattern as females. The only difference was that males of all genetic backgrounds died earlier than their female counterparts. PAP heterozygotes expressing dLipinWT showed the best survival, which was very similar to the survival of wild type flies. At day 5, both the survival rates of the PAP heterozygotes rescued by dLipinWT and the PAP homozygotes rescued by dLipinWT were 40%. Compared with heterozygotes, PAP mutant homozygotes rescued by dLipinWT showed a maximum survival that was reduced by one day. But the differences between $w^{1118}$ controls, delta PAP mutant homozygotes and heterozygotes rescued by dLipinWT were not statistically significant. As with the females, PAP heterozygotes expressing dLipin-GFP showed reduced starvation resistance. They died even earlier than PAP mutant homozygotes rescued by dLipinWT. At day 5, the survival rate of the PAP heterozygotes rescued by dLipin-GFP was 10% while the all the PAP homozygotes rescued by dLipin-GFP had already died. PAP homozygotes rescued by dLipin-GFP showed the poorest survival and all died within four days (Figure 31).




| Condition | Statistics P-value |
|---|---|
| W1118 *v.s.* PAP/PAP;dLipin-GFP | <0.01 |
| W1118 *v.s.* PAP/CyO-GFP:dLipin-GFP | <0.01 |
| PAP/PAP;dLipin-GFP *v.s.* PAP/CyO-GFP;dLipin-GFP | <0.01 |
| PAP/PAP;dLipin-GFP *v.s.* PAP/PAP;dLipinWT | <0.01 |
| PAP/CyO-GFP;dLipin-GFP *v.s.* PAP/CyO-GFP;dLipinWT | <0.01 |

Figure 31. Survival rate of male adult flies rescued by dLipinWT and dLipin-GFP. Heterozygote with ectopic dLipinWT (purple) showed a similar survival rate as $w^{1118}$ flies (light). But ectopically expressed dLipin-GFP (green) reduced the maximum life span of heterozygote from nine days to seven days. PAP mutant homozygote rescued by dLipinWT (light blue) could live as long as eight days. dLipin-GFP rescued homozygous flies had the shortest maximum life span, which was only four days (red). Survival curves and statistical comparisons of survival curves were made with the log rank test.

Though there seemed to be a tendency, in both males and females, that the starvation resistance of PAP mutant *Drosophila* with ectopically expressed dLipinWT is reduced compared to $w^{1118}$ at later time points, the differences were not statistically significant. Ectopically expressed dLipinWT was able to mostly reinstall the reduced starvation resistance caused by lack of PAP activity. However, ectopically expressed dLipin-GFP was not able to improve the starvation resistance in either PAP mutant heterozygotes or homozygotes. Compared with the control group, dLipin-GFP greatly decreased the survival rate and the life span of both the heterozygotes and homozygotes, although the heterozygotes still survived a few more days than homozygotes. The observation that dLipin-GFP diminishes starvation resistance of heterozygous

animals that carry a wild-type *dLipin* allele strongly suggests that the protein acts in a dominant-negative manner. This conclusion is supported by the observation that dLipin-GFP not only seemed unable to enter the cell nucleus, but also seemed to block the entry of endogenous dLipin into the nucleus (Fig. 29). Since dLipin normally translocates into the nucleus under starvation conditions (Ugrankar et al., 2011), these data further support the idea that dLipin promotes starvation resistance by functioning in the nucleus.

**7. Co-regulator activity alone cannot rescue major phenotypes caused by lack of dLipin under normal feeding condition**

I was not able to generate the delta NLS mutant flies that express dLipin lacking co-regulator activity. However, I was still able to study the nuclear function of dLipin by comparing *dLipin*$^{e00680}$*/Df* with *dLipin*$^{e00680}$/delta PAP. This comparison may tell me if an increased presence of dLipin with a co-regulator motif (which delta PAP still has) makes any difference with respect to the phenotype. A previous study has shown that a small number of *dLipin*$^{e00680}$*/Df* transheterozygotes can survive until the 3$^{rd}$ instar larval stage and contain very little fat body (Ugrankar et al., 2011).

Although most *dLipin*$^{e00680}$/delta PAP larvae died as feeding 3$^{rd}$ instar larvae, a small number of larvae were able to reach the wandering stage. Very few pupae could be obtained and all of them died before eclosion.*dLipin*$^{e00680}$/delta PAP 3$^{rd}$ instar larvae (Figure 32A, middle) were transparent, containing much less fat body than the control, *dLipin*$^{e00680}$/CyO larvae (Figure 32A, right). However, different from *dLipin*$^{e00680}$/delta PAP larvae, which showed a phenotype that was quite consistent, the phenotype of *dLipin*$^{e00680}$*/Df* larvae varied. Some larvae showed a very severe phenotype (Figure 32A, left), while some only showed a slight decrease in the fat body mass (data not shown). *dLipin*$^{e00680}$*/Df* 3$^{rd}$ instar larvae with the most severe phenotype

were more transparent than *dLipin*$^{e00680}$/delta PAP larvae and almost had no fat mass (Figure 32A, left). In order to observe the fat body tissue more closely, 5 *dLipin*$^{e00680}$/delta PAP larvae and 5 *dLipin*$^{e00680}$/*Df* larvae (with severe phenotype) were dissected and the fat body cells were stained with the lipophilic dye BODIPY. Figure 32B shows tissue from one larva that is representative of the specimen I looked at. The size of *dLipin*$^{e00680}$/delta PAP (Figure 32B, middle) fat body cells varied; most of the cells were much smaller than cells in the control group (Figure 32B, right). The cells were round and detached from each other. The fat droplets in these cells were smaller than those in the cells of the control group, and in some extremely small cells there were no fat droplets. The size of *dLipin*$^{e00680}$/*Df* (Figure 32B, left) fat body cells from the larvae with severe phenotype also varied. But the difference among the cells was not as obvious as the difference among the cells of *dLipin*$^{e00680}$/delta PAP larvae. The cells contained small lipid droplets and were also rounded and began to detach from each other. Two independent crosses were carried out and the results were consistent.

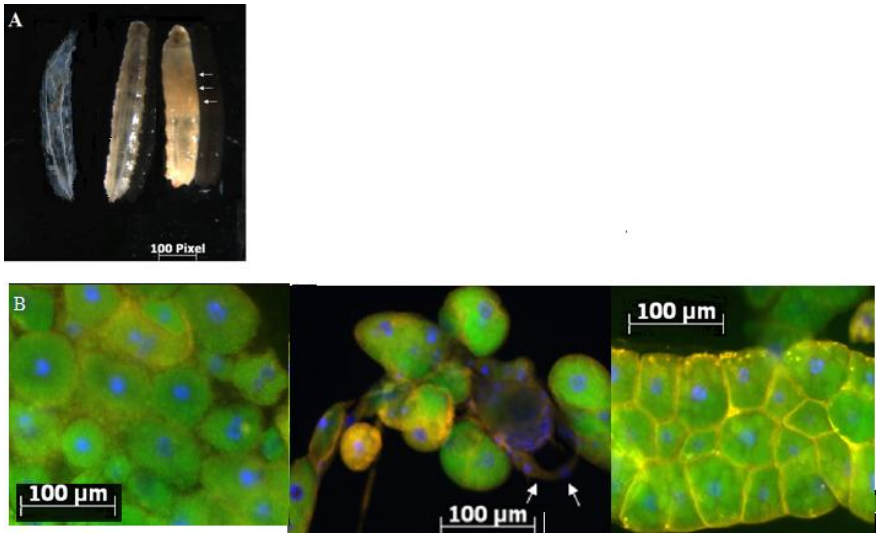



Figure 32. *dLipin*$^{e00680}$/delta PAP 3rd instar feeding larvae and their fat body. *dLipin*$^{e00680}$/delta PAP 3rd instar feeding larvae contained less fat body (A). Compared with the control larva whose fat body resulted in a white color to its appearance (arrows), *dLipin*$^{e00680}$/CyO (right), the *dLipin*$^{e00680}$/delta PAP larva (middle) was transparent, which was the result of reduced fat body mass. *dLipin*$^{e00680}$/*Df* (left) showed a more severe phenotype and contained almost no fat body

mass. The morphology of fat body cells was changed in *dLipin*$^{e00680}$/delta PAP 3$^{rd}$ instar feeding larvae (B). Fat droplets were stained with BODIPY (green). The cell membrane was stained with CellMask Orange (orange) and the nuclei were stained with DAPI (blue). In the fat body of *dLipin*$^{e00680}$/delta PAP larvae (middle), the cells were round and detached from each other. The size was variable and the fat droplets were small. Some extremely small cells even contained no fat droplets (arrows). Cells of *dLipin*$^{e00680}$/CyO larvae are polygonal and of similar size (right). They are closely attached to each other and contain big fat droplets. Morphology of the cells from *dLipin*$^{e00680}$/*Df* larvae (left) showed a less severe phenotype than the cells from *dLipin*$^{e00680}$/delta PAP larvae.

The data indicate that dLipin that is believed to possess co-regulator activity alone cannot rescue lethality caused by lack of dLipin under normal feeding conditions. However, on the whole animal level, the fat body mass of *dLipin*$^{e00680}$/delta PAP larvae seemed increased compared to *dLipin*$^{e00680}$/*Df* larvae. In contrast, on the cytological level the mutant phenotype seemed to be more severe in *dLipin*$^{e00680}$/delta PAP larvae.

## IV. Discussion

Lipin proteins are highly conserved in species from protozoa, plants, yeast to *Drosophila*, fishes and mammalians (Péterfy et al., 2001). In mammal, Lipin1 works as a PAP1 enzyme, which catalyzes the production of DAG from phosphatidic acid. It also behaves as a transcriptional co-regulator, regulating the genes involve in lipid metabolism (Reue and Zhang, 2008). Previous studies have shown that Lipin 1 can be induced by glucocorticoids in mouse and by physically interacting with nuclear receptor PPAR and co-activator PGC-1α, regulating the genes involves in mitochondrial fatty acid oxidative metabolism (Finck et al., 2006). However, for dLipin, the Lipin homolog in *Drosophila*, no research has focused on its transcriptional co-regulator activity. In my research, I tried to reveal the nuclear receptors that can interact with dLipin.

### 1. Interaction of dLipin with nuclear receptors

In *Drosophil*a, dLipin is activated in an apparent response to the steroid hormone 20E in late-prepupal salivary glands, suggesting that dLipin may interact with *Drosophila* nuclear receptors and participate in steroid-induced signaling pathways (M. Lehmann, personal communication). EcR, the NR that directly binds 20E and mediates responses to the hormone (King-Jones et al., 2005), together with other receptors DHR3, DHR38, and HNF4 which respond to metabolic signals (Palanker et al., 2006) were included in my screening. To study the NRs in *Drosophila*, a system called "ligand sensor" system has been developed by Palanker and colleagues in 2006 (Palanker et al., 2006). Both temporal and spatial activity patterns of all the 18 *Drosophila* NRs have been studied by using this system.

To find out the NRs that may interact with dLipin, I first used this system in $dLipin^{e00680}/Df$ flies, which produce very little dLipin protein. If dLipin is needed to carry out the

NR activity, the reporter gene cannot be activated in the flies that lack dLipin. In all the ligand sensor experiments (both loss-of-function and gain-of function), all the salivary gland cells developed blue color in all their nuclei. However, this was not caused by the activation of reporter gene by the ligand sensor protein. Instead, this was due to a tissue-specific enhancer in the reporter gene construct that drives constitutive expression of *lacZ*. Though the staining could not be used to indicate any NR activity in the salivary glands, this staining was a good internal control to show that the staining procedure was carried out successfully. For all the nuclear receptors included in the experiment, the staining pattern was similar between the control and experimental group. For DHR96 and EcR, no reporter activity was observed, which was consistent with a previous report that no DHR96 and EcR activity was detected in any tissue in late 3$^{rd}$ intar larvae (Palanker et al., 2006). DHR3, according to Palanker's study, is greatly activated in the central nervous system (CNS), gut, trachea, Malpighian tubules and fat body. However, my experiment with DHR3 (control) could not repeat this result. In both control and experimental group, part of the midgut was stained, but since the blue color was not restricted to the nuclei, it was most likely caused by endogenous beta-galactosidase activity and not by activation of the reporter gene. Like DHR3, staining to detect DHR38 activity led only to some blue color in part of the midgut. This contradicts the previous observation that DHR38 activity could be greatly detected in CNS and gut, and slightly detected in fat body and oenocytes in late 3$^{rd}$ instar larvae (Palanker et al., 2006).

Since all the salivary glands of the animals described above quickly developed blue color in the nuclei after staining, it can be excluded that unsuccessful repetition of Palanker's results was due to staining failure. I carried out three independent crosses in case some mistakes such as using non-virgin females were made during the crosses. And for each independent cross, the

staining was repeated twice. However, all the results were the same and did not repeat Palanker's results.

To determine if an increase in dLipin can activate receptor activity, I ectopically expressed *dLipin* in larvae carrying the ligand sensor components. For all the NRs that I examined (DHR3, DHR38 and HNF4), the control group did not show any NR activity, which was, again, a contradiction to the previous study (Palanker et al., 2006). However, in the animals of all the three NR groups that over expressed *dLipin*, some of the fat body cells developed blue color in the nuclei. Since Palanker reports activity for all three NRs in the fat body under normal conditions (Palanker et al., 2006), it is difficult to interpret the results of the gain-of-function experiments. My data seem to provide some preliminary evidence that dLipin may interact with the NRs tested, although differences observed between the control and experimental groups were restricted to only few fat body cells.

In both loss-of-function and gain-of function experiments, since data showed in previous study cannot be repeated in most of my control animals, the results from these experiments were not reliable. So, the ligand sensor system did not provide me with evidence of interaction between dLipin and the receptors tested. Because the results of the ligand-sensor assays were inconclusive, I carried out genetic interaction experiments in which the expression of either *dLipin*, the receptor, or both was reduced. However, instead of an enhancement or suppression of the phenotype of dLipin or NR in double knockdown animals, which would have suggested an interaction between dLipin and NR, I only observed additive results for most of the NRs (DHR3, DHR38, DHR4, DHR96 and EcR). Therefore, the interaction experiments did not provide evidence for an interaction of dLipin with most of these NRs tested. The exception was HNF4. The *HNF4* and *dLipin* double knockdowns partly rescued the phenotype caused by *dLipin* single

knockdown. Some of the fat droplets in the fat body cells became normal again. A previous study had shown that dHNF4 can be activated by fatty acids released from TAG and induce the expression of the enzymes for fatty acid oxidation. The phenotype of dHNF4 mutants includes accumulation of TAG and long-chain fatty acids (Palanker et al., 2009). Therefore, the bigger fat droplets may have been due to the impaired ability of breaking down TAG. In mouse, HNF4α is one of nuclear receptors that have been proven to physically interact with Lipin 1. Together with PGC-1α, HNF4 and Lipin 1 amplify PPARα gene expression and PPARα, in turn, regulates the genes that control fatty acid oxidation together with PGC-1α and Lipin1 (Finck et al., 2006). Since dHNF4 is also involved in fatty acid oxidation regulation, it is likely that the observed genetic interaction between dLipin and dHNF4 is indicative of a physical interaction between the two proteins in *Drosophila* as well.

The third approach I used to determine whether dLipin interacts with specific NRs was a test of genetic interaction in the regulation of starvation resistance. A previous study had shown that loss of *dLipin* leads to decreased starvation resistance (Ugrankar et al., 2011). I compared the survival rate of the double knockdowns of both *dLipin* and NR with single knockdown of *dLipin* and single knockdown of NR under starvation conditions.

Compared with both female and male flies of DHR38/DHR96/EcR knockdown and males of DHR3 knockdown, the flies with *dLipin* single knockdown showed a worse survival pattern than the flies with NR single knockdown. And the survival pattern of the flies with double knockdowns was similar to that of the flies with *dLipin* single knockdown. In the NR groups mentioned above, instead of a decrease or improvement in the survival rate compared with the flies with *dLipin* single knockdown, flies with double knockdowns only showed a similar survival rate to the single knockdown with lower survival rate. Female flies with *DHR3*

single knockdown showed a worse survival pattern than the flies with *dLipin* single knockdown. However, the survival rate of the flies with *DHR3* single knockdown was much lower than *dLipin* single knockdown flies or $w^{1118}$ flies even under feeding condition (data not shown). Male flies with *DHR3* single knockdown also had a lower survival rate than *dLipin* single knockdown flies. *DHR3* plays an essential role during embryogenesis and metamorphosis (Carney et al., 1997; Koelle et al., 1992). The dramatic decrease in the survival rate of *DHR3* single knockdown flies may be the result from defects in the fat body tissue due to abnormal development. The double knockdowns of both *dLipin* and *DHR3* showed a survival pattern similar to the *DHR3* single knockdown, not indicating an interaction.

Flies with single knockdown of *HNF4* showed a slightly improved starvation resistance, although it was not statistically significant. Flies with double knockdown of both *dLipin* and *HNF4* had a better survival pattern than the flies with *dLipin* single knockdown. So knocking down of both *dLipin* and *HNF4* could not only partly rescue the phenotype caused by loss of dLipin in fat body cells (the genetic interaction data described above), but also rescue the impaired starvation resistance ability caused by *dLipin* knocking down. The consistency of the rescue ability of *dLipin* and *HNF4* double knockdowns further supports the conclusion that dLipin and HNF4 interact in *Drosophila*. Although differences between some groups (*HNF4* single knockdown flies and control flies, double knockdowns of *dLipin* and *HNF4* female flies and *dLipin* single knockdown female flies) were not statistically significant, the tendency of starvation resistance improvement was obvious. If more flies can be included in the experiment, it is likely that the P value can decrease and show statistical significance.

In summary, I was not able to find evidence that unequivocally shows an interaction between a particular NR and dLipin. However, double knockdown of *dLipin* and *HNF4* did

partly improve the decreased survival rate caused by *dLipin* knockdown under starvation conditions. Together with the result from genetic interaction studies that double knockdowns of *dLipin* and *HNF4* partly rescues the impaired morphology of fat body cells caused by *dLipin* knockdown, the data strongly suggest that HNF4 and dLipin indeed interact. Ffurther experiments such as GST-pull down essay and co-immunoprecipitation are needed to confirm that dLipin and HNF4 physically interact with one another in *Drosophila*.

**2. PAP enzymatic activity is required for *Drosophila* fat body development**

To study one single function of dLipin protein without interfering with the other function, I tried to generate flies containing mutations in conserved protein motifs (PAP enzymatic motif and NLS) of dLipin. The attempt to obtain delta NLS flies with normal PAP activity, but lacking the co-regulator activity, failed. The efficiency of targeting is highly variable and one homologous recombination event happens in 500 to 30,000 gametes (Gong and Golic, 2003). And since delta PAP mutant flies were successfully obtained, which means the method I used was effective, I may have not screened enough flies. The other possibility is that delta NLS dLipin acts in a dominant-negative manner and as a result, the wild type copy of dLipin also cannot function normally and no fly can survive under such circumstance.

Flies with the delta PAP mutation were successfully obtained. The delta PAP mutation of *dLipin* is lethal, indicating that PAP enzymatic activity is essential for survival in *Drosophila* and cannot be provided by another protein. Delta PAP homozygotes can only reach the $1^{st}$ instar larval stage. However, the percentage of larvae that hatch from eggs is almost the same as for the heterozygotes. Previous immunohistochemistry showed that dLipin is strongly localized in the nuclei of ovarian nurse and follicle cells, as well as oocyte cytoplasm (Ugrankar et al, 2011). So despite the fact that the delta PAP homozygotes can only express mutant dLipin protein, they may

still have maternally provided wild type dLipin, which may be the reason why they can complete embryogenesis and survive for a while after hatching.

Since the single amino acid change should only delete the PAP enzymatic activity of dLipin without interfering with its expression pattern, I examined the expression pattern of mutant dLipin in 1$^{st}$ instar larvae of delta PAP homozygotes. Mutant dLipin was dispersed throughout the larval tissues, including proventriculus, gastric caeca and intestine, the same as in the control group (delta PAP/CyO). The 1$^{st}$ instar larval stage is the latest stage that the delta PAP mutants can reach. Since fat body is still underdeveloped at this early stage in development, the presence of dLipin in the fat body is very difficult to determine. In some cells of delta-PAP mutant larvae, dLipin protein could be detected in the nucleus, which shows that the delta PAP mutation did not disturb the nuclear translocation ability of dLipin protein. A Western blot analysis was also included to determine the size and amount of delta PAP dLipin protein in homozygotes. A specific band similar in size to dLipin (114kDa) was detected in both PAP/PAP homozygotes and PAP/CyO-GFP heterozygotes. The expression amount of mutant dLipin was almost the same as in the control. So the mutation that interferes with PAP enzymatic activity did not change the size, translocation ability, expression pattern or amount of dLipin protein.

I confirmed that lethality was the result of delta PAP mutant dLipin rather than any secondary mutation introduced during homologous recombination by successfully rescuing the animals by expressing hs-dLipin. This experiment also allowed me to observe the phenotypes that result from lack of PAP activity in later development since the animals can be rescued beyond the early lethal phase. Ectopic dLipin could be expressed by heat shock and I included different heat shock patterns. First, I noticed a developmental delay and the number of heat shock days influenced the degree of developmental delay. The fewer times the flies were heat

shocked, the less the amount of dLipin was ectopically expressed, and the longer time the larvae needed to reach the 3rd instar wandering larval stage. In *Drosophila*, wandering behavior follows a low titer pulse of 20E, which is triggered by the attainment of critical weight (Beadle et al., 1938). PAP enzyme plays a key role in TAG accumulation and, thus, influences the fat body mass and body weight. If the amount of ectopic dLipin is insufficient, the TAG accumulation rate may decrease and the larvae have to take more time to reach the critical weight. This is consistent with my observation that the fewer the number of heat shocks, the longer the larvae need to begin to wander. If the number of heat shocks was too low, the larvae died before accumulating enough fat body mass. However, compared to the heterozygous controls, the developmental delay could not be avoided regardless of the number of heat shock days. This may be due to the insufficient PAP activity during an early stage. Since I started heat shock about 12 hours after the larvae reached the 1st larval stage, the PAP enzymatic activity level was probably lower than that of their heterozygous siblings before being heat shocked, though they may still have some maternal wild type dLipin protein. Starting the heat shock rescue from the embryo stage may help to answer this question. The other possibility is that the ectopically expressed dLipin may not function as efficiently as endogenous dLipin. Since only one isoform (dLipin A) was ectopically expressed through heat shock, it may be that this isoform cannot fully replace the function of the other dLipin isoform, dLipin B. In mouse, it has been shown that two Lipin 1 isoforms, Lipin-1α and Lipin-1β have distinct functions in the adipocytes (Péterfy et al., 2005; Reue and Brindley, 2008).

The phenotypes of the heat shock-rescued animals also showed a correlation with the number of heat shock days. The longer time passed after heat shock, the more severe phenotypes the flies showed. As the time passed, fat body cells began to lose their polygonal shape and had

variable size and contained smaller fat droplets. Since ectopically expressed dLipin would gradually degrade after heat shock, the longer the time waited to dissect, the less ectopic dLipin existed in the cells and the more severe the phenotypes were. The round shape of the cells may be due to the impaired generation of the phospholipid bilayer which requires the PAP enzymatic activity. The larger size of fat body cells may be due to extra cycles of endoreplication, which was consistent with the longer developmental time. Most PAP mutant homozygotes could be rescued until the pupal stage when hs-dLipin was induced by heat shock every day or every other day. However, most homozygous pupae died right before or during eclosion even when heat shocks were continued after pupation. Very few flies successfully eclosed. This indicates that these flies may lack the energy to emerge out of their case. Since wandering larvae and pupae do not feed anymore, the ectopically expressed dLipin may not have been sufficient in the delta PAP homozyogtes to accumulate enough TAG before reaching the wandering stage. But successful rescue until pupal stage indicates that early lethality is due to the elimination of PAP activity of dLipin.

Although dLipin ectopically expressed after heat shock gradually degrades, as indicated by more severe phenotypes with time passing after the last heat shock, it would be very helpful to know the time course of degradation. Unfortunately, ectopic wild-type dLipin and delta PAP dLipin cannot be distinguished by size or antibody reactivity. Therefore, in order to monitor the ectopically expressed protein in the cells, I used GFP-tagged dLipin instead of untagged wild type dLipin. I first compared ectopically expressed dLipin-GFP and dLipinWT to find out whether the GFP tag would interfere with dLipin function. dLipinWT expressed from UAS-dLipinWT using various GAL4 drivers has been successfully used in rescue experiments (Ugrankar et al., 2011). I started with using the *daughterless*-GAL4 driver to express either

dLipinWT or dLipin-GFP to rescue delta PAP homozygotes. Ectopically expressed dLipinWT was able to rescue delta PAP mutant larvae until the pupal stage, but the animals had a developmental delay for about three days. dLipin-GFP was not able to rescue the mutant animals and no homozygotes were found beyond 1$^{st}$ instar larval stage. But this may not be due to interference introduced by the GFP tag. It may due to an insufficient amount of the protein expressed. The developmental delay in the flies rescued by dLipinWT also may indicate a slight insufficiency of ectopic dLipinWT, which was consistent with the result of the rescue experiment carried out by hs-dLipin. Although the same driver, *daugherless*-GAL4, was used to ectopically express dLipinWT and dLipin-GFP, the expression levels of these two proteins were probably not the same, because the expression of UAS-responder elements depends on the genomic integration site. *Daughterless*-GAL4 is a weak driver and it is very possible that the amount of dLipinWT expressed just reached the threshold to rescue the mutant flies while the amount of dLipin-GFP did not. To exclude this possibility, I next used the *tubulin*-GAL4 driver, a strong driver that induces protein expression ubiquitously, to express dLipinWT or dLipin-GFP in mutant flies. This time, both dLipin-GFP and dLipinWT were able to rescue the delta PAP mutant flies until adulthood. Again, a developmental delay occurred. dLipinWT-rescued flies had a delay of about two days while dLipin-GFP rescued flies had a delay of about three days. This delay may not be due to the expression level of the two proteins, since tubulin-GAL4 is a very strong driver and the expression level should be more than enough. Though it is difficult to explain, this was consistent with my previous observation that even frequent heat shock could not eliminate the developmental delay in the hs-dLipin rescued flies. Ectopically expressed dLipin may do not function as efficient as endogenous dLipin, or the function of dLipin B cannot be fully replaced by dLipinA (the isoform expressed ectopically). In both dLipin-GFP and

dLipinWT-rescued flies, the fat body looked normal with big fat droplets. However, differences still exist between the animals rescued by dLipinWT and dLipin-GFP. The size of dLipin-GFP rescued fat body cells was somewhat smaller than the dLipinWT-rescued cells, and the dLipin-GFP rescued adult flies were infertile, while dLipinWT-rescued flies were able to produce offspring. Though these difference did not exclude the possibility that activity of dLipin-GFP might be somewhat diminished compared to dLipin, the successful rescue until adulthood indicated that dLipin-GFP was indeed suitable to be used in the rescue experiment.

Then, I proceeded to use heat-shock-induced dLipin-GFP to rescue delta PAP mutant flies. The phenotypes of the larvae and developmental delay were very similar to the flies rescued by hs-dLipin (heat shocked every day or every other day). So, dLipin-GFP can replace hs-dLipin in the rescue experiments. In order to find out the correlation between the stability of expressed dLipin-GFP and the heat shock frequency, I carried out different heat shock patterns. The brightness of green fluorescence decreased as time passed after heat shock, which showed that degradation did happen. However, the green fluorescence did not completely fade away before the larvae died.

I could not obtain any larvae without green fluorescence, and the persistence of green fluorescence suggests that the ectopically expressed protein is quite stable and only subject to slow degradation. However, this did not necessarily mean that the dLipin portion of the fusion protein was still intact in the cells. GFP is known to be very stable and may still work when the dLipin part of the fusion protein is already inactive. Therefore, the fat body phenotype should also be studied together with the degradation degree of the dLipin-GFP fusion protein. This could be done by carrying out Western blots using an anti-GFP antibody and determining whether full-sized GFP-tagged proteins are present in late stages. A time course can also be done

to show a degradation profile. It is also possible that an initial decline in dLipin-GFP does not become apparent because of very high and saturating levels of fluorescence shortly after the last heat shock. The latter explanation seems to be very likely, because the activity of the ectopically expressed dLipin obviously declines as indicated by the reduced rescue (fewer heat shocks result in longer developmental delay). If this is the case, additional heat-shock regimes should be tested. Shorter heat shock times such as 15 minutes or 5 minutes could be tried instead of 1 hour, especially for the last heat shock. Shorter heat shocks should reduce the expression level of dLipin-GFP and increase the possibility that the fusion protein has sufficiently degraded when larvae reach the late 3$^{rd}$ instar larval stage. Another possibility would be to give more heat shocks during the larval and prepupal/pupal stages to try to rescue the animals to adulthood. Possibly, a decrease of dLipin-GFP to undetectable levels can be seen in this stage before an onset of lethality. Overall, it should be possible to examine the requirements for dLipin PAP activity at later stages of development using animals rescued by dLipin-GFP using additional modified heat shock regimes.

In summary, the PAP enzymatic activity plays a very important role in *Drosophila* development. Sufficient PAP enzymatic activity is needed to ensure a normal developmental rate and it is also required to keep the normal shape, size and fat droplet formation in the fat body cells.

**3. dLipin-GFP protein has lost nuclear translocation ability**

Since there was a small difference between the dLipinWT and dLipin-GFP rescued animals (smaller fat cells and infertility in adults), I cannot exclude the possibility that activity of dLipin-GFP might be somewhat diminished compared to dLipin. A previous study has examined the intracellular localization of dLipin-GFP, but failed to provide clear evidence that the fusion protein can enter the cell nucleus (Ugrankar et al, 2011). I found that dLipin-GFP cannot enter

the nuclei in the fat bodies of TOR knockdown animals, which otherwise show robust translocation of dLipin into the nuclei of fat body cells in the wandering stage (Sandra Schmitt, personal communication; and Fig. 29). Not only the dLipin-GFP fusion protein, but also the endogenous dLipin protein in these animals could not enter the nuclei anymore. In mouse, Lipin can form homo- or hetero- oligomers. This ability could explain the observed dominant-negative effect. Binding of dLipin-GFP to endogenous dLipin may prevent it from entering the nuclei. Another interesting observation was that in the control group (Cg-GAL4/TOR[RNAi]), only when female UAS-TOR[RNAi] flies were crossed with male Cg-GAL4/CyO-GFP; UAS-dLipin-GFP/+ flies, the progeny showed dLipin translocation into the fat body nuclei. If the cross was carried out the other way, dLipin did not enter the nucleus in the progeny. This may due to the effect of maternal dLipin-GFP left in the progeny. Previous immunohistochemistry showed that dLipin is strongly expressed in the oocyte cytoplasm (Rupali, dissertation). It is very possible that dLipin-GFP also exists in the oocyte cytoplasm. If the female carries both the Cg-GAL4 and UAS-dLipin-GFP elements, it will express dLipin-GFP and the progeny may have some dLipin-GFP proteins maternally from the eggs even though they do not carry the UAS-dLipin-GFP element any more. These maternal dLipin-GFP proteins may be able to keep the endogenous dLipin protein outside the nuclei.

Different from the similar rescue abilities of dLipinWT and dLipin-GFP in PAP mutant flies, the rescue ability of dLipin-GFP was absent under starvation conditions. dLipin-GFP even decreased the starvation resistance in heterozygous control flies. In both female and male flies, dLipinWT-rescued delta PAP mutants did not show much difference compared with heterozygous flies expressing dLipinWT and wild type flies. However, both homozygotes and heterozygotes (both female and male) with ectopically expressed dLipin-GFP showed a large

decrease in the survival rate and life span. These data support the conclusion that nuclear entry and the transcriptional co-activator function of dLipin are critical for its function in starvation resistance. In mice, it has been demonstrated that Lipin1 interacts with transcription factors and, thus, participates in the regulation of genes involved in hepatic fatty acid oxidation and oxidative phosphorylation (Finck et al., 2006). dLipin may also be involved in the regulation of fatty acid oxidation. dLipin-GFP prevents both endogenous wild type dLipin and PAP mutant dLipin from entering the nucleus and the animals, thus, lose the ability to activate genes that promote fatty acid oxidation. These flies will have impaired ability to utilize their fat reserves during starvation, which leads to a low survival rate and short life span. However, there was also a significant difference between the dLipin-GFP-rescued homozygotes and heterozygotes in both female and male. The PAP enzymatic activity in the cytoplasm may also plays a role under starvation since the amount of dLipin with PAP enzymatic activity is lower in the homozygotes than the heterozygotes. The other possibility is that exclusion of the wild-type dLipin from the nucleus in heterozygotes is not 100% effective and the nuclear PAP activity is also important for starvation resistance. In dLipin-GFP rescued heterozygotes, a small amount of wild type dLipin may still be able to enter the nuclei and carry out both PAP and co-regulator activity, while in dLipin-GFP rescued homozygotes there may be no nuclear PAP activity. A third possibility is that homozygotes may accumulate less TAG and fat body mass compared with the heterozygotes. As mentioned above, dLipin-GFP rescued delta PAP mutant homozygotes have a developmental delay and slightly smaller fat body cells. To test this possibility, wild type flies with ectopically expressed dLipin-GFP needed to be included to see whether more wild type dLipin expression can partly rescue the impaired starvation resistance caused by dLipin-GFP.

In summary, dLipin-GFP has lost nuclear translocation ability and can also prevent wild type dLipin from entering the nucleus. The starvation experiments also indicate that nuclear activity of dLipin is required under starvation conditions.

**4. Co-regulator activity alone cannot rescue major phenotypes caused by lack of dLipin**

Comparing the fat body cells of *dLipin*$^{e00680}$/*Df* with the cells of *dLipin*$^{e00680}$/delta PAP allowed me to see if the presence of dLipin with a co-regulator motif (which delta PAP still has) makes any difference with respect to the phenotype. The results were contradictory since the individual cell phenotype of *dLipin*$^{e00680}$/delta PAP larvae was more severe than that of *dLipin*$^{e00680}$/*Df* larvae (with severe phenotype) while whole larvae of *dLipin*$^{e00680}$/delta PAP contained more fat mass than *dLipin*$^{e00680}$/*Df* (with severe phenotype). Although the result was consistent, further quantitative experiments such as cell size and TAG level measurements are needed to substantiate the observation.

If this contradictory observation is true, this may be due to different numbers of fat body cells in the two fly lines. In mouse, Lipin 1 induces the proliferation of preadipocytes through its co-regulator activity (Reue and Zhang, 2008). dLipin's co-regulator activity may have the same function and in *dLipin*$^{e00680}$/delta PAP larvae the fat body cell number may therefore be normal or at least higher than in *dLipin*$^{e00680}$/*Df* larvae. *dLipin*$^{e00680}$/*Df* larvae express dLipin with co-regulator activity as well, but in a lower amount than the *dLipin*$^{e00680}$/delta PAP larvae.

Since co-regulator activity is important during starvation in adult flies, it would be interesting to compare starvation resistance of *dLipin*$^{e00680}$/delta PAP and *dLipin*$^{e00680}$/*Df* larvae. If *dLipin*$^{e00680}$/delta PAP larvae show better starvation resistance, this would indicate that dLipin carrying an intact transcriptional co-regulator motif, but no intact PAP motif, is sufficient to provide the function of the protein in starvation resistance.

## V. Summary

To study the transcriptional co-regulator activity of dLipin, I screened the *Drosophila* nuclear receptors, trying to find out the receptors that interact with dLipin. Though the attempt to use the "ligand sensor system" failed to provide me with reliable results, from the genetic interaction and starvation resistance study, *Drosophila* hepatic nuclear receptor 4 (HNF4) showed a high possibility to interact with dLipin. In both of these two experiments, double loss of HNF4 and dLipin functions slightly rescue the phenotypes caused by *dLipin* single knockdown. However, in the starvation resistance study, the rescue result was not statistically significant, though the tendency was obvious. So, more starvation experiments with increased number of the observations are needed. Further experiments such as co-immunoprecipitation and GST pull down are also required to confirm whether HNF4 physically interacts with dLipin.

In order to study one single function of dLipin without disturbing the other one, I tried to generate the flies containing mutations in conserved protein motifs of dLipin. Mutant flies that only express dLipin protein that lacks the PAP enzymatic activity (delta PAP) were obtained while no flies with dLipin protein that lacks nuclear localization signal (delta NLS) were found. Basing on the finding that dLipin-GFP acts in a dominant-negative manner, delta NLS dLipin protein may also act in the same way and cause death to the embryo or larvae.

Through Western blot and dLipin antibody staining, delta PAP mutant flies showed the normal expression level and pattern of the mutant dLipin protein. Since the PAP enzymatic activity is essential to *Drosophila* development, the delta PAP mutation is lethal and no larvae can survive beyond the 1$^{st}$ instar larval stage. Heat shock induced wild type dLipin protein can rescue the flies through the early lethal stage until pupae or adult stage. The phenotypes of the fat body cells of the rescued animals showed a correlation with the amount of the ectopically

expressed dLipin protein. The longer time passed after heat shock, which means the fewer ectopic dLipin proteins left in the cells, the more severe phenotype the flies showed. Low level of PAP enzymatic activity causes the fat body cells becoming round, losing the polygonal shape and detaching each other. Fat droplets in the cells become smaller, too. Sufficient PAP enzymatic activity is also needed to ensure a normal developmental rate.

GFP-tagged dLipin could also be used to replace heat shock induced wild type dLipin to rescue the delta PAP mutant flies, and the phenotypes of the rescued flies were very similar. The tagged protein not only allowed me to rescue the mutant flies, but also to monitor the ectopic dLipin level. The brightness of green fluorescence decreased as time passed after heat shock, which showed the degradation did happen. Further experiment is needed to build up a time course to show the degradation profile. It can be done by using an anti-GFP antibody in Western blot.

Different from the similar rescue abilities of dLipinWT and dLipin-GFP in PAP mutant flies, the rescue ability of dLipin-GFP was absent under starvation conditions. The starvation resistance was even largely impaired in the dLipin-GFP rescued delta PAP mutant heterozygotes that carry a wild-type *dLipin* allele. dLipin-GFP was found to lose the nuclear translocation ability and act in a dominant-negative manner. The impaired starvation resistance ability caused by the fact that neither ectopic dLipin-GFP nor endogenous wild type dLipin can enter the nuclear indicates that nuclear activity of dLipin is required under starvation conditions.

## VI. References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