國立臺灣大學生命科學院生化科學研究所

碩士論文

Graduate Institute of Biochemical Sciences

College of Life Science

National Taiwan University

Master Thesis

Atg1在果蠅發育上的調控基因篩選分析

A screen for modifiers of Atg1-mediated signaling in Drosophila development

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中華民國 九十九 年 七 月

July 2010

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中文摘要

細胞自噬是具有高度保留性的過程，涉及囊泡分泌及細胞質內蛋白質和胞器的降解。Atg1 是一種絲氨酸/酥氨酸激酶，並且已經知道受到 Tor 訊號傳遞途徑的調控。研究發現在酵母菌中，Atg1 的激酶活性是 CVT 訊息傳遞途徑以及細胞自噬所必須。因此 Atg1 可能是一個可以調控細胞自噬很多步驟的一個調節點。

我利用果蠅為實驗材料，研究結果顯示大量表現 Atg1，在果蠅複眼的發育上會造成細胞死亡和眼睛表面粗糙的表型。儘管目前的研究已經發現一些會與 Atg1 有所關聯的蛋白質，然而要鑑定出其他在細胞自噬過程中可能與 Atg1 有交互作用的受質仍是一個困難的任務。因此為了尋找參與在 Atg1 所調控細胞自噬過程中未知且新穎的基因，我利用表現 Atg1 所造成粗糙眼睛的表型做了果蠅眼睛發育上的調控基因篩選分析。利用能代表果蠅百分之七十基因組的連續染色體缺陷，在測試了 277 株染色體缺陷的果蠅中，我發現了 26 個品系的染色體缺陷果蠅能有效抑制 Atg1 所造成的訊息傳遞途徑。我利用一系列的遺傳分析縮小候選基因的位置，並且找到了三個可能與 Atg1 有關聯且參與在細胞死亡訊息傳遞途徑的基因。

這些能夠與 Atg1 作用的調控蛋白將為細胞死亡與細胞自噬的研究帶來新的發展。我目前正在調查這些調控蛋白是否能與 Atg1 有物理上的交互作用，並且在細胞死亡的過程中扮演什麼樣的生物功能。

Abstracts

Autophagy is a highly conserved cellular process that involves vesicle-mediated sequestration and degradation of cytoplasmic proteins and organelles. Atg1 is a Ser/Thr kinase that is regulated by TOR-dependent signaling. In yeast, studies have found the requirement for Atg1 kinase activity in both CVT and autophagy. Thus, Atg1 is representing a nodal point for controlling multiple steps in autophagic process in response to various stresses. I have examined that overexpression of *Drosophila* Atg1 in the developing compound eye triggers cell death and results in eye roughness. Although a number of proteins have been found to associate with Atg1, the identification of Atg1 substrates important for autophagy remains a difficult task. To identify novel genes involved in the Atg1-mediated pathway, I carried out a dominant modifier screen of the Atg1-induced rough eye phenotype using contiguous chromosomal deficiencies that represent more than 70% of the *Drosophila* genome. Of the 277 deficiencies tested, 26 were identified as suppressors of Atg1 signaling. I characterize a subset of autosomal regions that strongly interact with Atg1. Three novel genes will likely identify Atg1 regulators and should shed some light on how cells are regulated by the balance between cell survival and cell death. I am currently investigating whether these regulators have physical interaction with Atg1 and what is the biological function in cell death.

Introduction

The cell biology of autophagy

Autophagy is a ubiquitous process that involves the bulk degradation of cytoplasmic components through a lysosomal pathway. This process was initially described over 40 years ago, characterized by the engulfment of part of the cytoplasm inside double-membrane vesicles called autophagosome. Autophagosome subsequently fuse with lysosomes to form an autophagolysosome in which the cytoplasmic cargo is degraded (Heymann, 2006; Melendez et al., 2003; Yoshimori, 2004) .

Two major discoveries in the past decade have led to a substantial increase in interest and activity in the field of autophagy research. First, the genetic screens in yeast that led to the discovery of approximately 30 autophagy-related (ATG) genes that are involved in the regulation of this process. Many ATG orthologs have now be identified in higher eukaryotes (Melendez and Neufeld, 2008). Another major finding of this field is the role of autophagy on specific aspects of human health and disease, such as neurondegenerative disease involving formation of intracellular aggregates. Many pathogenic aggregate-prone proteins, including polyglutamine (polyQ)-expanded huntingtin are strongly dependent on autophagy for their degradation, and rapamycin attenuates accumulation of mutated huntingtin proteins and cell death in Huntington's disease (HD) models (King et al., 2008; Ravikumar et al., 2002; Ravikumar et al., 2004;

Sarkar et al., 2009) .

It is also clear that autophagy plays an essential role in ridding cell death in a wide range of cell types and organisms during starvation, stressful conditions, and developmental stages and in some case acting as a cell death effector (Gozuacik and Kimchi, 2004; Levine and Klionsky, 2004; Melendez and Neufeld, 2008) .

The molecular machinery of autophagy

The autophagic process can be divided to several distinct steps: (1)signaling and induction (2)autophagosome nucleation (3)membrane expansion and vesicle completion (4)autophagosome targeting, docking and fusion (5)degradation and re-export of materials to the cytoplasm.

(1) Signaling and induction

Autophagy occurs at basal levels in normal growing conditions, but can be dramatically upregulated by a number of stimuli, including starvation, hypoxia, intracellular stress, high temperature, high culture density, hormones and growth factors deprivation. The best characterized of these stimuli is nutrient starvation, which induces autophagy in part through the inactivation of the protein kinase target of rapamycin (Tor) (Wang and Klionsky, 2003).

In the yeast, more than 31Atg（autophagy-related）genes have been identified to be

required for autophagosome formation. It is currently known that the basic mechanism of autophagy has been well conserved during evolution from the fact that diverse organisms, including yeasts, flies, and mammals, all carry a similar set of Atg genes. Suggesting that autophagy may share an evolutionary regulatory mechanism (Levine and Klionsky, 2004).

The autophagy induction involves Atg1, Atg13, and Atg17 proteins, the complex of these proteins is regulated by TOR-dependent signaling. In yeast, under nutrient rich conditions, Tor phosphorylates Atg13, leading to its dissociation from a protein complex containing Atg1. Atg13 dissociation attenuates Atg1 kinase activity, which correlates with autophagy induction. Under nutrient limitation, Tor activity is blocked, Atg13 is dephosphorylated, and it tightly associates with Atg1. This promotes activation of Atg1, leading to autophagy induction (Chan and Tooze, 2009; Kamada et al., 2010).

However, in Drosophila, Atg13 binds Atg1 in under nutrient-rich conditions, but this interaction is strengthened by nutrient-starvation. TOR also enters into this complex although TOR binding is not nutrient sensitive. Under nutrient-rich conditions, both Atg1 and TOR contribute to Atg1 hyperphosphorylation. In contrast, nutrient starvation promotes hyperphosphorylation of Atg13, primarily via an Atg1-dependent pathway. Thus, nutrient status might control both substrate specificity and kinase activity for Atg1 and TOR within the complex (Chan and Tooze, 2009).

(2) Autophagosome nucleation

A functional complex involved in the vesicle nucleation step consists of the class III phosphatidylinositol 3 kinase Vps34, Atg6/Vps30 and several associated factors. Vps34 is involved in forming two distinct PtdIns 3-kinase complexes (Yorimitsu and Klionsky, 2005). Complex I is consist of Vps34, Vps15 (phosphoinositide-3-kinase, regulatory subunit 4, p150, or simply p150, in mammals), Vps30/Atg6(beclin-1 in mammals), and Atg14(Atg14L or barkor in mammals), and is thought to be an autophagy-specific complex , this complex of proteins recruits other autophagic proteins to the pre-autophagosomal structure or phagophore assembly site (PAS) (Xie and Klionsky, 2007; Yorimitsu and Klionsky, 2005).

However, fundamental questions on the membrane source or dynamics during the formation remain unresolved. Generally assumed to originate from membranes of pre-existing organelles, whereas, the exact source remains unclear. Nevertheless, recent studies suggest the ER as the main source of autophagosomal membrane. Dr. Ktistakis's group reports on the formation of the phosphatidylinositol 3-phosphate (PI3P) enriched omegasome near the ER under the starvation conditions. Autophagy non-selectively engulfs cytoplasm and some organelles making specific identification exceptionally challenging (Hamasaki and Yoshimori, 2010).

(3) Membrane expansion and vesicle completion

Two ubiquitin-like conjugation systems are essential for autophagic vesicle formation, resulting in the formation of an Atg5–Atg12 conjugate, in a noncovalent complex with Atg16, and in the formation of C-terminal lipid-conjugated LC3 (Atg8). These two systems cooperate in isolation membrane expansion allowing it to engulf cytosolic targets, to finally close and form a double membrane organelle, autophagosome. Once an autophagosome is formed, the Atg5–Atg12/Atg16 complex is released, whereas a portion of LC3 remains associated with the autophagosome and is degraded on autophagosome–lysosome fusion to form the lytic organelle called autolysosome in a process referred to as maturation or flux. The degradation of LC3 during autolysosome formation could provide one level of feedback inhibition in the system (Kabeya et al., 2000; Nakatogawa et al., 2007; Suzuki and Ohsumi, 2007).

(4) Autophagosome targeting, docking and fusion

In yeast, once autophagosome completed, the double-membrane autophagosome is transported to the vacuole and the outer membrane of the vesicle fuses with the vacuolar membrane, in a process that is dependent upon Ccz1 and Mon1. Ccz1 and Mon1 form a stable protein complex that binds the vacuole membrane. In the absence of the Ccz1-Mon1 complex, the integrity of vacuole SNARE pairing and the unpaired SNARE class C Vps/HOPS complex interaction were both impaired (Wang et al., 2003). Subsequently, the membrane of the autophagic body is broken down in the vacuole

lumen. The degradation of the autophagic body is dependent on two resident vacuole proteases, Pep4 and Prb1, and acidification of the vacuole lumen. In addition to these factors, the transmembrane protein Atg15 is also required for lysis. Atg15 has a conserved functional motif in esterases and lipases. This protein is transported to the vacuole through the multivesicular body (Mvb) pathway (Yorimitsu and Klionsky, 2005).

In mammalian cells, autophagosomes fuse with lysosomes, in a process similar to that of yeast. It has been suggested that the Rab GTPase Rab7 is targeted to the autophagosome membrane and is involved in a late step, fusion with the lysosome, to allow maturation (Levine and Klionsky, 2004).

Intriguingly, recent studies indicate a causative relationship between autophagosome–lysosome fusion defects and development of neurodegenerative disease. Cells knock down of HDAC6, the ubiquitin- binding deacetylase, histone deacetylase-6, defects in fusion to lysosomes led to a build-up of autophagosomes. EM analysis revealed that many of these autophagosomes showed abnormal morphology and contents. Similar abnormal autophagic structures were also found prominently accumulated in dystrophic axons in Alzheimer's disease (AD) patients. Thus, a defect in lysosome fusion, rather than autophagy activation, might be a key factor contributing to the pathogenesis of certain neurodegenerative diseases (Lee et al., 2010).

(5) Degradation and re-export of materials to the cytoplasm.

After fusion, lytic enzymes inside the lysosome lumen presumably degrade the autophagic body, as in the yeast vacuole. The subsequent breakdown of the internal autophagosomal membrane allows acidic lysosomal hydrolase to access the cytosolic cargo, leading to its degradation and, ultimately, it's recycling.

Atg9, the sole transmembrane protein required in vesicle formation in both bulk and selective autophagy, is absent from the completed autophagosomes. It suggests that the protein is retrieved on or before vesicle completion. In addition, compared with most other Atg proteins, which localize at the PAS, the subcellular localization pattern of Atg9 is unique. Atg9 also localizes to mitochondria, as well as some unidentified peripheral punctate compartments. Atg9 cycles between the PAS and these peripheral sites, suggesting that Atg9 may serve as a membrane carrier and involve in the retrieval materials of autophagosome (He and Klionsky, 2007; Reggiori et al., 2004; Young et al., 2006).

Atg1 plays a critical role in autophagy

(1) The role of Atg1 in yeast

Cumulative evidence has suggested that Atg1 played a critical role in switching between the cvt （cytoplasm-to-vacuole targeting） pathway and autophagy in yeast. In

addition, multiple signaling pathways, including TOR, AMPK, and Ras/PKA, converge on Atg1 to regulate autophagy (Scott et al., 2007).

Atg1 is a Serine/Threonine kinase, interacts with multiple components of the autophagic machinery including Atg11, Atg13, Atg17, Atg21, Atg29, Atg31,Atg8 and Atg18. In yeast, it has been reported that C terminus of Atg1 mediates the interaction with Atg13 and Atg17, thus providing a structural role that is needed to efficiently organize the initial step of PAS assembly. Interactions between Atg13, Atg1, and Atg17 are enhanced by starvation treatment, and both Atg13 and 17 are important for proper regulation of Atg1. Thus, Atg1 may represent a nodal point for controlling multiple steps in the autophagic process in response to various stresses (Cheong et al., 2008; Suzuki and Ohsumi, 2007).

(2) C. elegans ATG1 ortholog unc-51 is dispensable for embryonic development

C.elegans larvae respond to unfavorable environmental conditions (e.g. starvation, high population density, increased temperature) by arresting in an alternative third larval stage, referred to as the dauer diapause. In C.elegans, Atg1 ortholog unc-51 is dispensable for embryonic development, dispite playing an essencial role in autophagy. Mutations in unc-51 result in adults with an uncoordinated phenotype, and axons of the mutant animals display abnormal vesicles and membrane cisternae (Lum et al., 2005; Melendez et al., 2003).

(3) The kinase domain of Atg1 is essential for induction of Autophagy in Drosophila

Recently, it is shown that the Drosophila melanogaster homolog of Atg1 is required for autophagy in the larval fat body, an organ analogous to the vertebrate liver with roles in nutrient storage and metabolism. Atg1 expression is sufficient to affect a full autophagic response, resulting in a marked inhibition of cell growth and a rapid induction of apoptotic cell death (Neufeld and Baehrecke, 2008; Scott et al., 2007). Interestingly, expression of a kinase-negative mutant of Drosophila Atg1 ($Atg1^{K38Q}$) did not lead to increased autophagy in the fat body of normally fed animals, suggesting that the kinase activity of Atg1 is essential for its ability to promote autophagy in Drosophila (Scott et al., 2007).

(4) Homologues of Atg1 in mammal

Atg1 and its mammalian orthologues Ulk1 and Ulk2 (unc-51-like kinases 1 and 2) are serine/threonine kinases. It has been reported that RNAi-mediated suppression of ULK1 expression alone is sufficient to inhibit autophagy and redistribution of mAtg9 from the TGN to endosomes. In addition to the autophagy , the Unc-51 family of serine/threonine kinases was also shown to be involved in axon growth and branching (Nishida et al., 2009; Zhou et al., 2007).

FIP200 (focal adhesion kinase family interacting protein of 200 kDa) has recently been

identified as a mammalian functional counterpart of yeast Atg17. One of the major functions of both Atg13 and FIP200 is to regulate Ulk1 kinase activity; either protein alone can activate Ulk1 kinase activity but both are needed for maximal stimulation of Ulk1 activity. Both Atg13 and FIP200 are required for Ulk1 localization to the preautophagosomal membranes, and the absence of either prevents correct localization of Ulk1. In addition, complex formation appears to be important for Ulk1 protein stability. All three components of the Ulk•Atg13•FIP200 complex are essential for autophagosome formation. Intriguingly, formation of the Atg1•Atg13•Atg17 complex in yeast appears to be altered in response to nutrient conditions, however, recent studies in two independent laboratories have shown that the formation of the mammalian Ulk•Atg13•FIP200 complex is not altered by nutrient conditions (Hara and Mizushima, 2009; Hara et al., 2008; Jung et al., 2009). Recently, a novel, human Atg13 binding protein, Atg101, interacts with Ulk1 is also essential for macroautophagy in mammalian cells (Hosokawa et al., 2009).

Cellular functions of autophagy

(1) Starvation and cancer cell survival

In eukaryotic cells, basal levels of autophagy appear to play a “housekeeping” role to

remove cellular debris such as misfolded aggregate-prone proteins and defective organelles. In this way, autophagy provides a selfrenewal function that may act as an antiaging mechanism and may be especially important in long-lived and highly active cells, such as neurons. However, the best understood and perhaps most fundamental cellular role of autophagy is to provide an internal source of nutrients under starvation conditions. In most cell types, nutrient withdrawal elicits a robust stimulation of autophagy, and this can significantly extend the survival time of yeast and cultured mammalian cells in the absence of nutrients (Melendez and Neufeld, 2008).

Although the autophagic response to nutrient deprivation is generally less pronounced in cancer cell than in normal cells, many cancer cell types are still able to induce autophagy in deprivation of growth factors or nutrient. In addition, autophagy is a cell survival mechanism that can protect cancer cell from cell death either by repressing the the induction of apoptosis, or by maintaining metabolic activity through lysosome recycling of intracellular nutrients (Fingar and Blenis, 2004; Gozuacik and Kimchi, 2006). Recently, in *S. cerevisiae*, the cAMP-dependent protein kinase (PKA) signaling pathway has also been implicated in the control of autophagy. Inhibition of PKA signaling is sufficient to induce robust autophagy activity, and this control appears to occur independently, or in parallel to, that exerted by the Tor pathway. Interestingly, mTOR, which integrates amino acid and growth factor signaling, also senses energy

stress and hypoxia through the activity of AMPK and the hypoxia-inducible gene REDD1.

This suggests that the complexity of signaling circuits that control autophagy cannot always be extrapolated from charts depicting signaling pathway (Stephan et al., 2009). Determining how these pathways influence degradative process, and how these signaling activities are oordinated, are therefore important questions for future work.

(2)Autophagy in programmed cell death

Programmed cell death (PCD) is important for sculpting tissues and destroying harmful cells such as autoreactive immune cells and tumor cells. Excess PCD can be harmful and contributes to various degenerative pathologies, whereas lack of PCD can contribute to the development of proliferative disorders such as cancer. Two types of PCD have been identified according to their morphological criteria ——apoptosis and autophagic cell death (Akdemir et al., 2006; Bursch et al., 2000; Edinger and Thompson, 2004).

Apoptosis is also called the type I PCD that dependent on the activity of caspase proteases and is accompanied by highly conserved morphological changes including chromatin condensation, membrane blebbing and cell shrinkage. Caspases are cysteine proteases that are synthesized as inactive zymogens and activated either by induced proximity within multimeric protein complexes (death-inducing signaling complex and

apoptosome) or by proteolytic cleavage between the large and small catalytic subunits (Bursch et al., 2000; He et al., 2009; Martin and Baehrecke, 2004).

Autophagy belongs to the type II PCD that involves the bulk degradation of cytoplasmic components through a lysosomal pathway (Tsujimoto and Shimizu, 2005). In Drosophila, destruction of larval structures such as the salivary gland and digestive tract during metamorphogenesis is triggered by a sharp rise in the steroid hormone 20-hydroxyecdysone, ecdysone pulses are followed promptly by the expression of the cell-death genes *rpr, Apaf-1 homolog ark*, *hid, caspase dronc and drice,* and the subsequent destruction of these tissues (Brachmann and Cagan, 2003; Martin and Baehrecke, 2004). Interestingly, these tissues display characteristics of both autophagy, such as abundant cytoplasmic vacuolization, as well as apoptosis, including the upregulation of pro-apoptotic genes, caspase activation and DNA cleavage. Loss of function of the Atg genes leads to lethality in the transition from the larval to pupal stages, suggesting that autophagic cell death is also essential for Drosophila puparium formation (Bangs and White, 2000).

In mammalian cells, most reports of the involvement of autophagy in the death execution process are in cells that are defective in the apoptotic pathways. The interaction of Atg5 with the death domain of Fas-associated death domain protein

（FADD）has been shown to play a crucial role in INFγ-induced cell death. Interestingly, the death domain of FADD can active a cell death pathway involving both apoptosis and autophagy that is selectively inactivated at the earliest stage of epithelial cancer development (Codogno and Meijer, 2005; Tourneur and Chiocchia, 2010) . The connection between apoptosis and autophagy is further supported by the recent demonstration that overexpression of the antiapoptotic protein Bcl-2 can inhibit autophagy by interacting with the Atg6 homolog Beclin 1. Beclin 1 mutants that are unable to bind Bcl-2 stimulate autophagy and promote cell death, similar to the effects of Atg1. Recently, it has been shown that induction of autophagy is sufficient to induce cell death. The death resulting from Atg1-induced autophagy is suppressed by caspase inhibition and is associated with caspase activation, DNA fragmentation, and cytoskeletal disruption, suggesting that high levels of autophagy result in apoptotic cell death (Boya et al., 2005).

Other to Beclin 1, Atg4D has been indentified as at the interface between autophagy and apoptosis in recent studies. It is cleaved at DEVD^{63}K by caspase-3 during apoptosis, and further gains GABARAP-L1 priming/delipidation activity following caspase cleavage. Enzymatically active Atg4D regulates autophagosome biogenesis by priming newly synthesized Atg8 to enable covalent attachment of phosphatidylethanolamine, and by delipidating Atg8 at the lysosomal fusion step (Betin and Lane, 2009).

(3) Autophagy in Neuronal development

A role for autophagy in the clustering of neurotransmitter receptors in development has been reported in C. elegans. The clustering of neurotransmitter receptors results from signaling events during development that are initiated when presynaptic terminals are contacted by the postsynaptic cell. In *C.elegans*, body-wall muscles are innervated by both GABA and non- GABA neuron. GABA terminals organize $GABA_A$ receptors into synaptic clusters, which are internalized and degraded, as long as they lack presynaptic input. This degradation of $GABA_A$ receptors is specifically mediated by an autophagic pathway, whereas that of acetylcholine receptors in the same cells is not. In addition, mutation in the Unc51 gene resulted in stalled axon outgrowth, increased axon numbers (all short and stunted), and abnormal accumulation of intracellular membranous structures (Rowland et al., 2006).

In Drosophila, recent studies indicate that autophagy positively regulates development of the larval neuromuscular junction (NMJ). Autophagy induces an NMJ overgrowth phenotype closely resembling that of highwire (hiw), an E3 ubiquitin ligase mutant. autophagy promotes NMJ growth by reducing Hiw levels. Thus, autophagy and the ubiquitin-proteasome system converge in regulating synaptic development (Shen and Ganetzky, 2009). Despite intensive studies focusing on neuron development, UNC-51/ATG1 kinase has been known to regulate axonal transport by mediating

motor-cargo assembly, suggesting that autophagy may also play a role in neuronal function (Toda et al., 2008).

In mammals, Ulk1 was shown to be important for axon formation in cerebellar granule neurons . Furthermore, a yeast two-hybrid screen identified SynGAP and syntenin as binding partners for Ulk1 and Ulk2 proteins in cerebellar granule neurons. Both molecules are modulators of the Rab5-mediated endocytic pathway, indicating a link between endocytosis and axon growth in these neurons. In addition, RNAi-mediated knockdown of Ulk1 and/or Ulk2 resulted in impaired endocytosis of nerve growth factor (NGF), excessive axon arborization, and severely stunted axon elongation. The evidence also indicates that Ulk1/2 mediates a non-clathrin-coated endocytosis in sensory growth cones (Zhou et al., 2007).

(4) Autophagy in cancer

The dysregulation of cell growth, proliferation and apoptosis are hallmarks of tumorigenesis. Autophagy could support survival of cells within a tumour subjected to decreased nutrient availability. Conversely, mice that are haploinsufficient for beclin-1 have decreased levels of autophagy and increased frequency of spontaneous tumours. In addition, autophagy might have other less direct roles in cancer, such as the removal of damaged mitochondria and as a protective response to chemotherapy or

radiation-directed therapies (Yue et al., 2003). Furthermore, several other oncogenes have been implicated in autophagy regulation. The oncoprotein Ras is known to function downstream of growth factor receptor tyrosine kinases. In response to nutrient deprivation, Ras suppresses transformation in a rapamycin-sensitive manner and is counteracted by a dominant-negative Ras. In addition, Ras decreases autophagic protein degradation, which indicates that Ras might prevent autophagy through class-I phosphatidylinositol 3-kinase (PI3K). More recent work has shown that beclin-1 (the homologue of yeast Atg6) is a novel Bcl2-interacting protein. Decreases in beclin-1 levels have been associated with human breast carcinomas, and targeted inactivation of beclin-1 predisposes mice to increased tumour susceptibility (Shaw and Cantley, 2006).

Using Drosophila as a model study autophagy, cell death and development

The *Drosophila* compound eye is particularly suited to the application of genetic approaches to the study of cell proliferation and cell death in the context of organ development (Brachmann and Cagan, 2003; Reme et al., 1999).The adult eye develops from a primordium consisting of approximately 30 cells in the embryo. Cell growth and proliferation occur during all stages of larval development. Most of the cells generated adopt specialized fates (e.g., photoreceptor, pigment cell) during the late larval and pupal stages, leaving approximately 2000 unspecified cells. These excess cells are

subsequently eliminated by a wave of apoptosis. Thus, the final number of cells in the adult eye can be altered by changes in either cell proliferation or cell death. Since over-expression of Atg1, in the developing eye using the Glass Multimer Reporter (GMR) promoter, results in a rough and reduced eye phenotype. Using a genetic approach, I thus screened for dominant modifiers that can suppress the Atg1-induced eye phenotype. Identification and characterization of such modifiers would lead to a better understanding of Atg1-mediated pathways.

Materials and Methods

Drosophila stocks

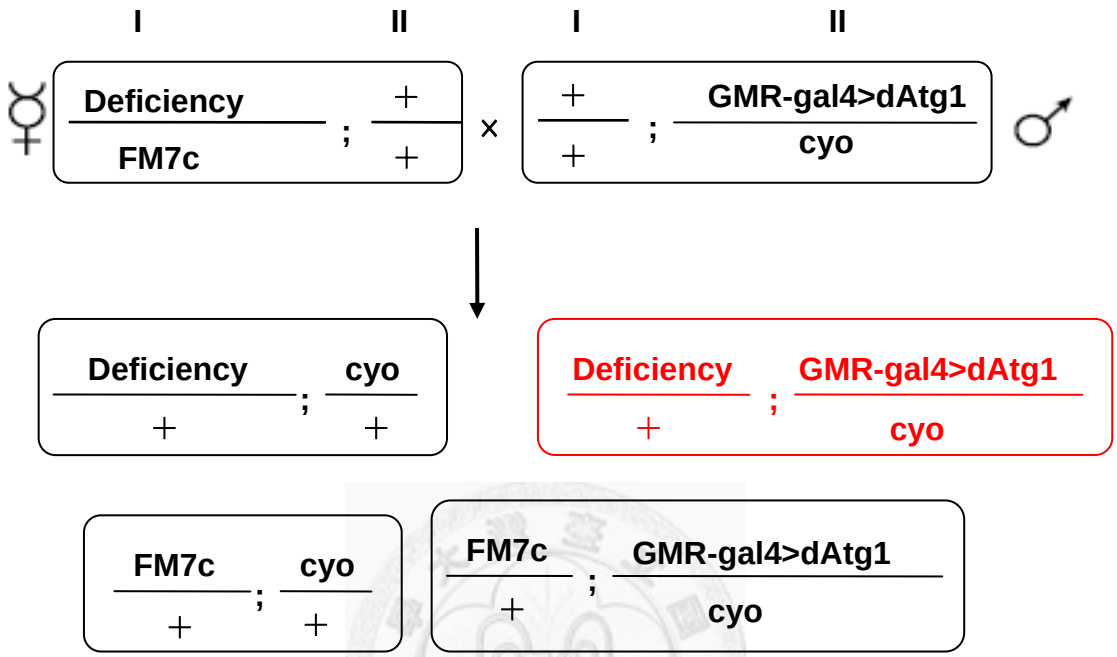
ALL Drosophila stocks are raised on standard medium and maintained at 25℃.

For P element transformation, the cDNA constructs of dAtg1 were subcloned into the pUAST and injected intoΔ2-3 transposase transgenic embroyo prior to cellularization. More than four independent insertion lines harboring each transgene were established. Transgenic lines including GMR-dAtg1-cat, UAS-dAtg1, UAS-dAtg1K38R were generated. These transgenic flies were balanced with standard procedures. GMR-Gal4 lines were used to drive the expression of UAS constructs.

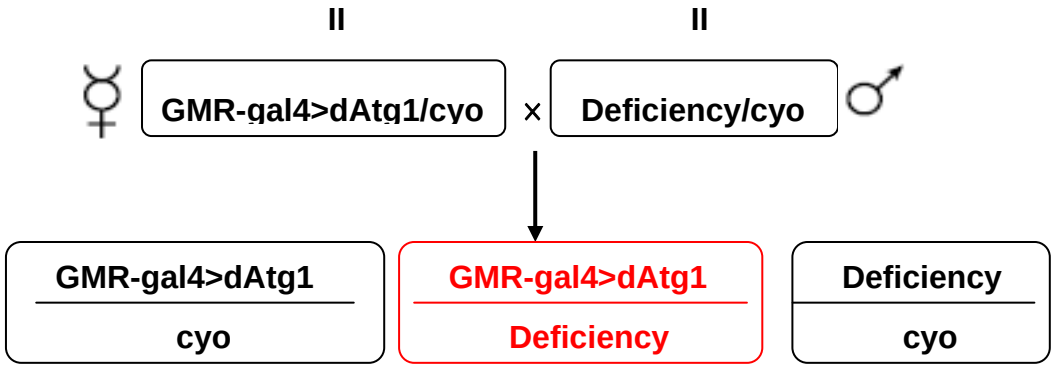
Deficiency kit were obtained from the Indiana University Bloomington Stock Centert, 277 lines used for dAtg1 modifier genetic screen, 74 lines used for further narrowed down the candidate regions. Other stocks utilized including GMR-p35、CG15626（Ei24）/Cyo（from the Bloomington Stock Center）、Lack RNAi lines、Nedd4 RNAi lines and CG15626（Ei24） RNAi lines were obtained from Vienna Drosophila RNAi Center.

Genetic scheme of screening

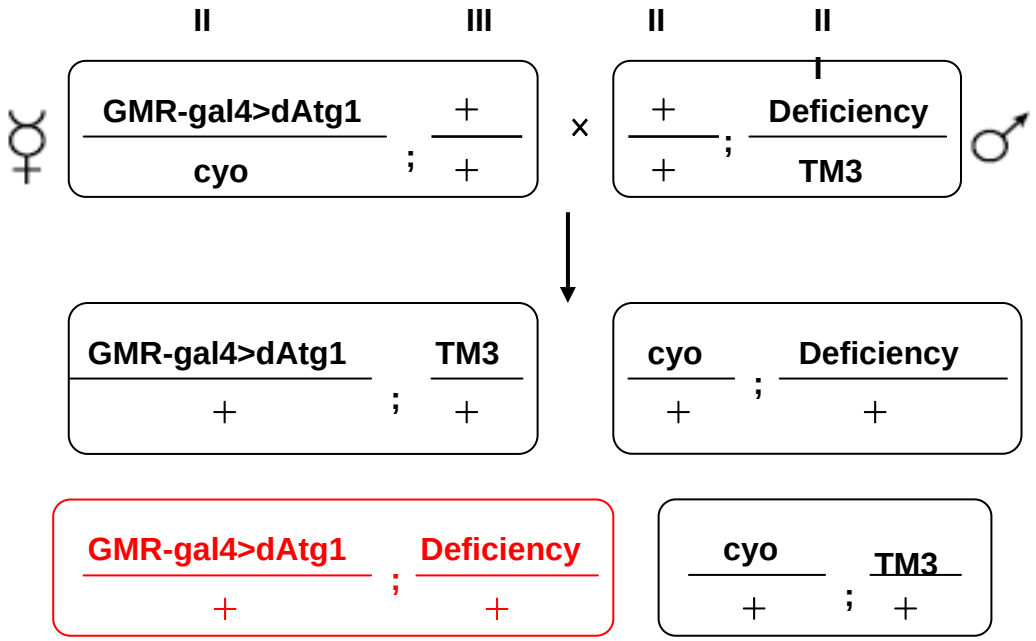
Deficiency on chromosome I



Deficiency on chromosome II



Deficiency on chromosome III



Microscopy

Adult eyes were photographed on Olympus SZX7 disecting microscope with Olympus E330 Live View Digital SLR camera. For analysis by scanning electron microscopy, adult eyes were examined using a Cryo-SEM（Cryo Scanning Electron Microscopy）.

Immunohistochemistry

For imaginal discs, third instar larvae discs were dissect in PBS approximate 72-96 hr after hatching, fixed for about 15 min in 4％ formaldehyde in PBS, and rinsed in PBST （PBS plus 0.1％ Triton-X-100）. Samples were then blocked for 30 min in 5％ normal goat serum in PBS, incubated overnight at 4℃ with primary antibody PBS, washed in PBS, incubated 2hr at RT with secondary antibody in PBS, and washed in

PBS. The eye discs were mounted in DABCO mountant（Sigma）in 90％ glycerol and scanned on a Zeiss LSM510 confocal microscope. Cleaved caspase-3 antibody（cell signaling）was used at 1:200 dilutions. Rhodamine-phalloidin（Sigma）was used at 1:200.

For examined flip-out clone in fat body. Second instar larvae carrying transgenes under the control of hs-Gal4 driver were collected, cultured in vials containing 20% sucrose for starvation 4 hr prior to dissection. After dissection, the samples were then fixed for about 15 min in 4％ formaldehyde in PBS, washed for PBS. DAPI（1μg/ml） was used to stain nuclei, and then mounted in DABCO mountant（Sigma）in 90％ glycerol and scanned on a Zeiss LSM510 confocal microscope.

Results

Induction of apoptosis by Atg1 disrupts the ommatidial structure in a kinase-dependent manner.

To investigate the role of dAtg1 in development, I examined the effects of overexpression of dAtg1 in the Drosophila eye. The Drosophila eye is composed of ~750 precisely patterned units called ommatidia. Previous studies have shown that programmed cell death (PCD) plays a key role in architecture of the compound eye (Brachmann and Cagan, 2003; Reme et al., 1999).

Intriguingly, recent studies have indicated that high levels of autophagy can induce apoptotic cell death in Drosophila (Scott et al., 2007). To examine whether dAtg1 has the ability to regulate programmed cell death in the developing eye, I expressed dAtg1 using the Gal4/UAS system with GMR-Gal4. Since this promoter is expressed in regions posterior to the MF (morphogenetic furrow) of the larval eye imaginal disc, expression of dAtg1 was expected in the posterior region of eye discs.

Comparing to GMR-gal4 controls, Flies overexpress dAtg1 exhibit morphological abnormality of the adult compound eye and cause rough eye phenotypes. To further investigate whether the dAtg1-induced the rough eye phenotype results in apoptosis. I performed immunofluorescence analyses of third instar eye imaginal discs of

GMR-gal4 and GMR-dAtg1 flies with anti-cleaved- caspase-3. Compare to controls, a marked increase of apoptotic cells was found in eye imaginal discs, suggesting that an up-regulation of apoptosis activity. This result reconfirmed by the finding that coexpression of the baculoviral caspase inhibitor p35 in the developing eye can partially suppress the rough eye phenotype caused by dAtg1 excessive activity and high levels of apoptic cell death in third instar eye imaginal discs.（Fig.1）

In Yeast, recent studies show that the kinase domain of Atg1 is essential for induction of autophagy. To determine whether Atg1-induced apoptosis is also dependent on its kinase activity in Drosophila, I expressed Atg1K38D which contains a single Lysine to Aspartate mutation in the catalytic site of kinase domain with GMR-gal4 and investigate GMR-dAtg1K38D flies' eye phenotype. In contrast to the rough eye phenotype caused by active form of dAtg1, flies overexpress dAtg1K38D exhibit a normal eye phenotype. I further confirm this finding by staining of the third instar eye imaginal discs of GMR-dAtg1K38D with cleaved-caspase-3. Comparing to GMR-dAtg1 flies, GMR-dAtg1K38D flies do not lead significant apoptosis in eye imaginal discs. The results indicate the dAtg1-mediated apoptosis dependent on its catalytic activity.

To further confirm the dAtg1 kinase domain is sufficient to induce apoptosis. I placed the dAtg1 kinase domain directly under the control of GMR promoter, GMR-dAtg1-cat, which consist of the isolated kinase region to investigate the eye phenotype. The data show that expression of dAtg1 kinase region is sufficient to induce rough eye phenotype, whereas its effect is milder than overexpression of full length dAtg1 and the disruption region of ommatadia is smaller. To examine the detail of the rough eye phenotypes in GMR-dAtg1-cat flies, I did the retinal tissue sections of wild-type and single-copy GMR-dAtg1-cat flies. It revealed that flies expressing dAtg1 kinase activity resulted in a dramatic disruption of the ommatidial structures. Staining of third instar eye imaginal disc with cleaved-caspase-3 also indicate that expression of dAtg1-cat is enough to induce apoptosis. It means that dAtg1 kinase domain only is sufficient to induce apoptotic cell death and further cause the disruption of ommatidial structure. I reconfirmed the results by coexpression of GMR-dAtg1-cat and GMR-p35. Flies expressing both genes can rescue the dAtg1-cat-induced rough eye effect and apoptosis. Taken together, my data indicate that induction of apoptosis by dAtg1 disrupts the ommatidial structure and is a kinase-dependent manner.（Fig.2）

Genetic screen of the dAtg1-induced rough eye phenotype

To search for other components involved in dAtg1-mediated apoptosis pathway, I

screened for genes that had genetic interaction with dAtg1 and dAtg1-cat. The indentified candidates might involved in the apoptosis pathway and had an effect on *Drosophila* eye development.

I thus performed a dominant modifier screen of a rough eye phenotype caused by expression dAtg1-cat and overexpression dAtg1 full length during eye development. I have screened ~300 contiguous chromosomal deficiency lines obtained from the Bloomington Stock Center which represent more than 70% of the Drosophila genome. Some of deficiency lines could rescue the dAtg1-induced rough eye phenotype and I classified the modifiers into three different groups, strong suppression, moderate suppression and weak suppression, which represents the degree of dAtg1-mediated rough eye phenotype has been rescued（Fig.3）.False positives were excluded by comparing these lines with GMR-gal4 controls.

The whole data of genetic screen is shown in Table.1 and Table.2. In GMR-dAtg1-mediated genetic screen, out of 277 deficiencies screened, 3 suppressors were identified in chromosome I, 26 suppressors were identified in chromosome II, and 15 suppressors were identified in chromosome III. In addition, in GMR-dAtg1-cat-mediated genetic screen, the same of 277 deficiencies were tested and 14 suppressors were identified in chromosome I, 17 suppressors were identified in chromosome II, 30 suppressors were identified in chromosome III. To determine the

precise chromosomal site of these suppressors, I first focused on which had the strongest suppression ability of single-copy GMR-dAtg1. They are DF967 from chromosome I; DF420, DF 693, DF3638, DF6299, DF7441, DF7875, and DF9594 from chromosome II; DF6411 and DF9197 from chromosome III（Fig.4）. According to the cytogenetic map on Flybase, I used ~100 deficiency lines which exhibit small deletion regions corresponding to these deficiency lines and crossed each of them with GMR-dAtg1 flies. Using this approach, I have successfully narrowed down the regions which had genetic interactions with dAtg1. The results were shown in Figure.6, which represent the smallest region.

Candidates of genetic screen inhibits Atg1-induced phenotypes and cell death

Flies carry these deficiency regions can rescue dAtg1-mediate rough eye phenotype. To further confirm whether these deficiency mutants have the ability to inhibit dAtg1-induced apoptosis, I stained third instar eye imaginal discs of each strong suppressor with cleaved-caspase-3 to label apoptotic cells, as shown in Fig.5, my data indicate that these deficiency lines also have the ability to suppress dAtg1-mediated cell death, suggesting that these candidate genes may involve in dAtg1-caused apoptosis pathway.

According to the dAtg1 modifiers screen, I selected three candidate genes include Lack,

Nedd4 and CG15626 from region DF7441（2R:54C8-54D1）,DF693（2L:24C2-25C9）and DF6411(3L:74D3-75B1) respectively.

Lack（Lethal with a check point kinase or dSmurf1）, which locates on the Drosophila chromosome 2R:54C12-54D1, was originally identified as an E3 ubiquitin ligase that involved in the BMP(bone marrow protein)-triggered TGF-β signaling pathway. Lack is known to have specific interaction with Smad1 and Smad5, and induces degradation of Smads. In addition to it's role in regulating the degradation of R-Smads, it also can facilitate the inhibitory activities of I-Smads, which plays a negative role in TGF-β signaling pathway (Izzi and Attisano, 2004; Podos et al., 2001).

Other to Smad, Traf4（Tumor Necrosis Factor receptor-associated factor 4）is also a substrate of Lack ubiquitin ligase. Moreover, recent studies demonstrate that Traf4 protein levels is regulated by Lack, suggesting that Lack not only play a role in TGF-β signaling pathway, but in TNF-α signaling pathway (Fukunaga et al., 2008; Kalkan et al., 2009; Liang et al., 2003; Podos et al., 2001).

Nedd4（neural precursor cells-expressed developmentally down-regulated 4 ）, the gene which located on the Drosophila chromosome 3L:74D3, is known as a regulator of renal sodium channel. It share highly similarity with Lack and function as an E3 ubiquitin

ligase but specific target on Smad2/Smad3. Intriguingly, recent studies have shown that serum/glucocorticoid regulated kinase 1 (SGK1) phosphorylates Nedd4 and reduces Nedd4 interaction with phospho-Smad3, Nedd4 also limits TGF-β and Activin /Nodal signaling pathway in differentiated cells and stem cells by poly-ubiquitination and degradation Smad2/Smad3 (Gao et al., 2009).

CG15626, the gene which located on the Drosophila chromosome 2L:25A8, shared 30% identity with the mammalian Ei24（Fig.6）. Ei24 (etoposide-induced 2.4 kb transcript) , a recently-discovered, p53-induced pro-apoptotic factor, is frequently lost in aggressive breast cancer and appears to play a role in prevention of tumor spread in invasive breast tumors. Ei24 was first isolated by differential display in 3T3 fibroblasts as an etoposide induced gene associated with apoptosis, and is also known as a p53 response gene. The deduced amino acid sequence yields little or no information about the possible function of this gene. However, the *Ei24* gene is localized on human chromosome 11q23, a region frequently altered in human cancers. It suggests that *Ei24* may play an important role in negative cell growth control by functioning as an apoptotic effecter (Gu et al., 2000; Lehar et al., 1996; Mork et al., 2007) .

To examine whether Lack have genetic interaction with dAtg1, I crossed the

GMR-dAtg1 flies with Lack RNAi line. Depletion of Lack can rescue the dAtg1-induced rough eye phenotype (Fig.8), suggesting that Lack involved in dAtg1-mediated cell death pathway. To further confirm that knock down of Lack have effect on dAtg1-induced apoptosis, I dissected the third instar eye imaginal discs and stain them with cleaved-caspase-3. Upon ablation of Lack expression, it reveals marked decrease of apoptotic cells, whereas activity of apoptosis is obvious in the GMR-dAtg1 flies.

I also examine the relationship between dAtg1 and Nedd4, dAtg1 and Ei24. My results showed that both Nedd4 and Ei24 have the ability to rescue dAtg1-induced rough eye phenotype. Immunostaining data also suggest that Nedd4 and Ei24 can inhibit dAtg1-induced apoptosis, indicating they involved in dAtg1-mediated cell death pathway.

To further confirm these data. I use ptc-gal4 to overexpress dAtg1 with Lack RNAi, Nedd4 RNAi and mutant form of Ei24 separately. Recently studies have shown that high levels of dAtg1 on wing can induce anterior cross vein loss wing phenotype. My data show that expression of Lack RNAi, Nedd4 RNAi and mutant form of Ei24 can partial rescue this wing phenotype（Fig.9）.

Depletion of Ei24 expression inhibits starvation-induced autophagosome formation

Since recent studies have shown that dAtg1-mediated high levels of autophagy can induce apoptotic cell death. In addition, above data indicate that deletion of Lack, Nedd4 and Ei24 can suppress activity of dAtg1-mediated apoptosis. To further examined whether Lack, Nedd4 or Ei24 plays a role in regulating dAtg1-caused apoptosis through the induction of high levels autophagy, I investigated whether these gene played a role in starvation-induced autophagy.

Atg8, which associates with the isolated membrane in an Atg5-dependent manner and remains on the membrane even after autophagosomes are completely formed, can be used a general marker for autophagic membrane. In addition, it has been reported that a robust autophagic response is induced in Drosophila larval fat body in response to nutrient deprivation. This process depends on the down-regulation of Tor signaling and activation of Atg1 kinase. Therefore, I investigate whether expression of Lack-RNAi、Nedd4-RNAi or Ei24-RNAi have effect on starvation-induced autophagy . As shown in Figure.10, cells which knock down Ei24 activity significantly blocked the GFP-Atg8 puncta formation, suggesting that autophagy activity has been down-regulated. However, neither expression Lack-RNAi nor Nedd4-RNAi blocked the autophagosome formation. These results suggest that Ei24 involves in the starvation-induced autophagy pathway and then have an effect on dAtg1-induced apoptosis, whereas Lack and Nedd4 may not （Fig.10）.

Discussion

Recent papers have reported that overexpression of dAtg1 directly induces high level autophagy, and then results in cell death. In this study, I performed a genetic screen for dominant modifiers screen that can suppress the dAtg1-induced rough eye phenotype. I tested a total of 277 deficiency lines and ten deficiency lines which can strongly suppress dAtg1-induced phenotype were further confirmed with their ability to inhibit apoptosis caused by dAtg1.

The ability of dAtg1 to induce cell death

Programmed cell death (PCD) is important for sculpting tissues and destroying harmful cells such as autoreactive immune cells and tumor cells. Morphological criteria have been used to identify two types of PCD --apoptosis and autophagic cell death. Type 1 (apoptotic) cell death and type II (autophagic) cell death are viewed as clearly distinct subroutines of cellular demise. However, since both type I and type II programmed cell death are often observed in the same dying cells, suggesting the type I and type II programmed cell death are probably nonexclusive mechanism (Akdemir et al., 2006; Boya et al., 2005; Bursch et al., 2000).

The connection between apoptosis and autophagy is further supported by the recent demonstration that overexpression of the antiapoptotic protein Bcl-2 can inhibit

autophagy by interacting with the Atg6 homolog Beclin 1. Mutant versions of Beclin 1 that are unable to bind Bcl-2 stimulate autophagy and promote cell death, similar to the effects of Atg1.

It is known that Atg1 plays a critical role on the induction of autophagy. My studies directly indicate that Atg1 not only has an important role in autophagy, but also in apoptosis. In addition, the induction of apoptosis is a kinase dependent manner.

Relationship between autophagy and dAtg1-induced apoptosis

It is well-known that the insulin/IGF-1/TOR pathway has been shown to regulate autophagy.(Vellai et al., 2008) However, recent studies have shown more novel signaling pathways that are involved in the regulation of autophagy.

In Saccharomyces cerevisiae, autophagy is also regulated by cAMP-dependent protein kinase (PKA) pathway. Elevated levels of PKA activity inhibited autophagy and inactivation of the PKA pathway was sufficient to induce a robust autophagy response. (Stephan et al., 2009) In addition, In the endoplasmic reticulum (ER), accumulation of misfolded proteins causes stress and activates the unfolded protein response also cause a new pathway to induce autophagy response (Yorimitsu and Klionsky, 2007; Yorimitsu et al., 2006).

In mammalian, activation the transcription factor E2F1 upregulates the expression of

autophagy genes including Atg1, defined as DNA-damage-induced autophagy response (Polager et al., 2008). Moreover, recent studies have shown that in the filamentous fungus Aspergillus fumigatus, autophagy is linked to the metal ion homeostasis process (Richie and Askew, 2008; Richie et al., 2007).

In Drosophila, studies indicate that high levels of autophagy can induce caspase-dependent apoptosis. However, the molecular mechanism is not clear between the autophagy-induced apoptosis. I performed a dAtg1-modifier screen in Drosophila and identified a number of candidate genes which could suppress dAtg1-induced ectopic expression.

In my studies, I found a number of novel candidate genes which is not belong to the well-known autophagy pathway, suggesting that it might have another signaling pathway involve in the linking between autophagy and apoptosis pathway.

Thus, I classified the results of dAtg1-mediated genetic screen into different groups.

One is DNA-damage related and hydrogen peroxide catabolic signaling gene, including Jafrac1 and wee. In Drosophila, It has been known inactivation of both foxo and reaper promotes long-term adult neurogenesis. In addition, Foxo likely activates autophagic cell death, because simultaneous inhibition of Atg1 (Siegrist et al., 2010). Intriguingly, recent studies indicate that Jafrac1, as a downstream effecter of JNK/FOXO signaling in

neurons that enhances stress resistance and extends life span (Lee et al., 2009). Following the genetic screen, it may indicate the gene has a possibility to regulate autophagy and cell death through Foxo in response to oxidative damage and exterior stress.

Second is apoptosis related gene, including Aac11、rep4、Traf1、Ei24. In mammalian cells, Ei24 is directly regulated by p53 and known as a pro-apoptotic factor which localized on the endoplasmic reticulum. Ei24 contains six putative transmembrane domains and may suppress cell growth by inducing apoptotic cell death through the caspase 9 and mitochondrial pathways. In addition; it is known Ei24 has interaction with Bcl-2 with its N-terminal region (Zhao et al., 2005). In my studies, I used the dAtg1-mediated genetic screen found the Drosophila homologues of Ei24. CG15626, whose shares 30% identity with the mammalian Ei24, can inhibit dAtg1-induced apoptosis and autophagy. My results indicate that this gene not only play a critical role in dAtg1-induced apoptosis pathway, but in autophagy pathway. It was confirmed by using a Flip-out strategy to produce fat body tissues made exclusively from Ei24 RNAi cells, it indicate that loss of Ei24 activity has ability to inhibit starvation-induced autophagy pathway.

Third is TGF-β signaling pathway, including Lack, Nedd4, Snoo, and Tab2. Lack and Nedd4 both belong to E3 ubiquitin ligase involved in TGF-β signaling pathway. Lack is known to have specific interaction with Smad1 and Smad5, whereas Nedd4 has specific interaction with Smad2 and Smad3.

Recent progress studying TGF-β signaling mechanisms has revealed the important role for ubiquitin-dependent proteasomal degradation in regulating TGF-β signaling. As TGF-β signaling is tightly regulated by numerous E3 ubiquitin ligases, dysregulated expression or functionality of such E3 ubiquitin ligases may affect the proper transmission of TGF-β signaling, contributing to cancer development (Inoue and Imamura, 2008). In my studies, I found a series of E3 ubiquitin ligase involved in TGF-β signaling pathway; further experiments suggest that Lack and Nedd4 play a role in dAtg1-mediated apoptosis. However, it do not have apparent differences in Atg8 levels when compared Lack knock down clones and Nedd4 knock down clones to wild-type cells. It is likely that except the starvation-induced autophagy pathway, another signaling pathway may involve in Lack and Nedd4 mediated apoptotic cell death.

Based on my results, I propose the following model, illustrated in Figure.11. According to the dAtg1-mediated genetic screen, I found three novel proteins, Lack, Nedd4 and

CG15626（Ei24）. Loss the activity of Ei24 then inhibit dAtg1-triggered autophagy which in turn leads to apoptotic cell death, however, Lack and Nedd4 are likely through the other linking pathway to involve in dAtg1-induced apoptosis. I am currently investigating the detail mechanism between Ei24 and dAtg1, whether they have direct interaction or any possible regulation process.

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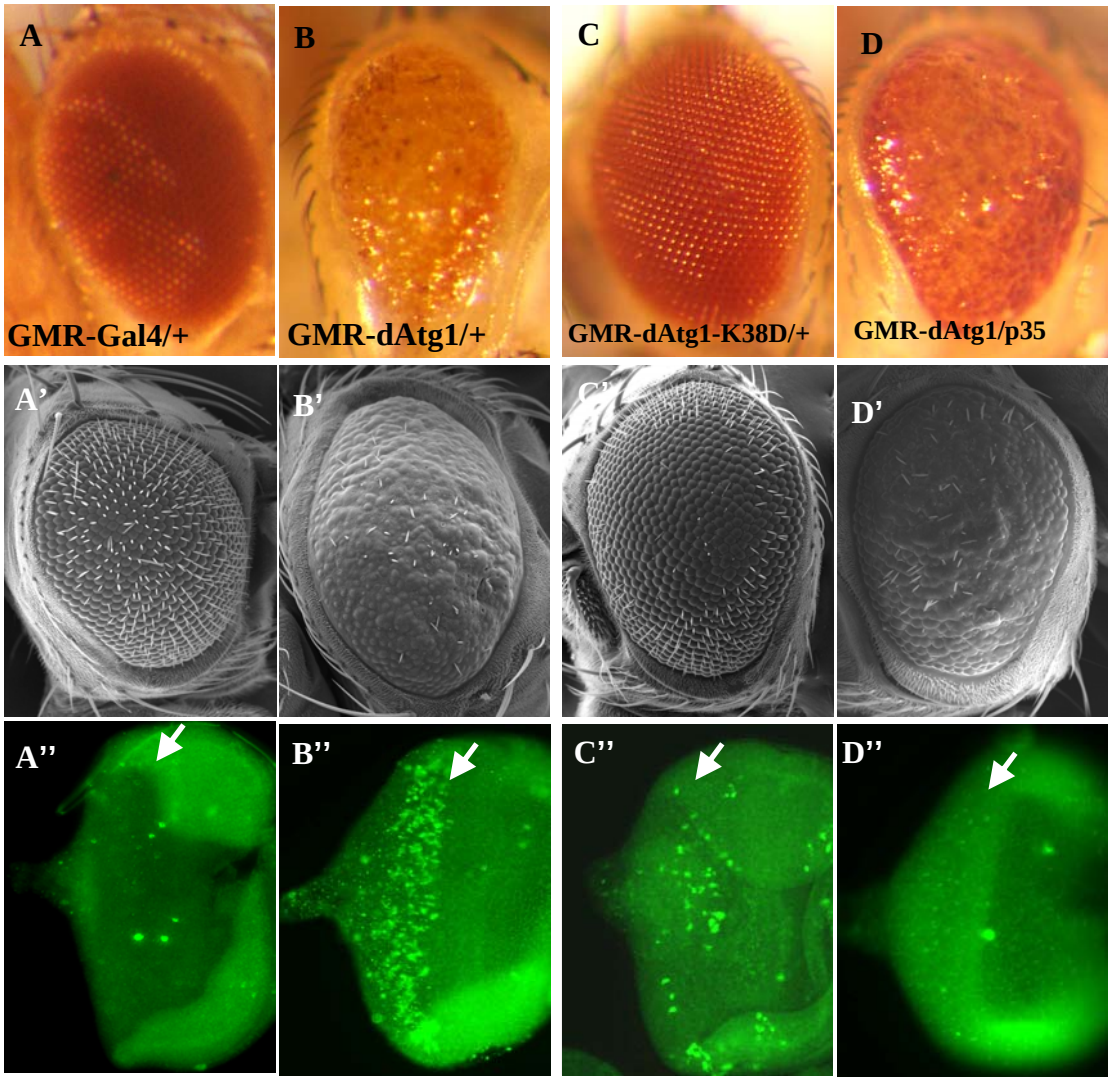


Figure 1. Overexpression of Atg1 induces apoptosis in a kinase-dependent manner

The appearance, scanning electron microscopy photographs and disection of third instar eye immaginal discs of GMR-Gal4 (A,A',A'') or overexpression GMR-dAtg1(B,B',B''),GMR-dAtg1-K38R(C,C',C'') . coxpression of GMR-dAtg1/GMR-p35(D,D',D''), The third instar eye imaginal discs marked the apoptotic cell by staining with cleaved-caspase-3. Arrow indicate the morphogenetic furrow.

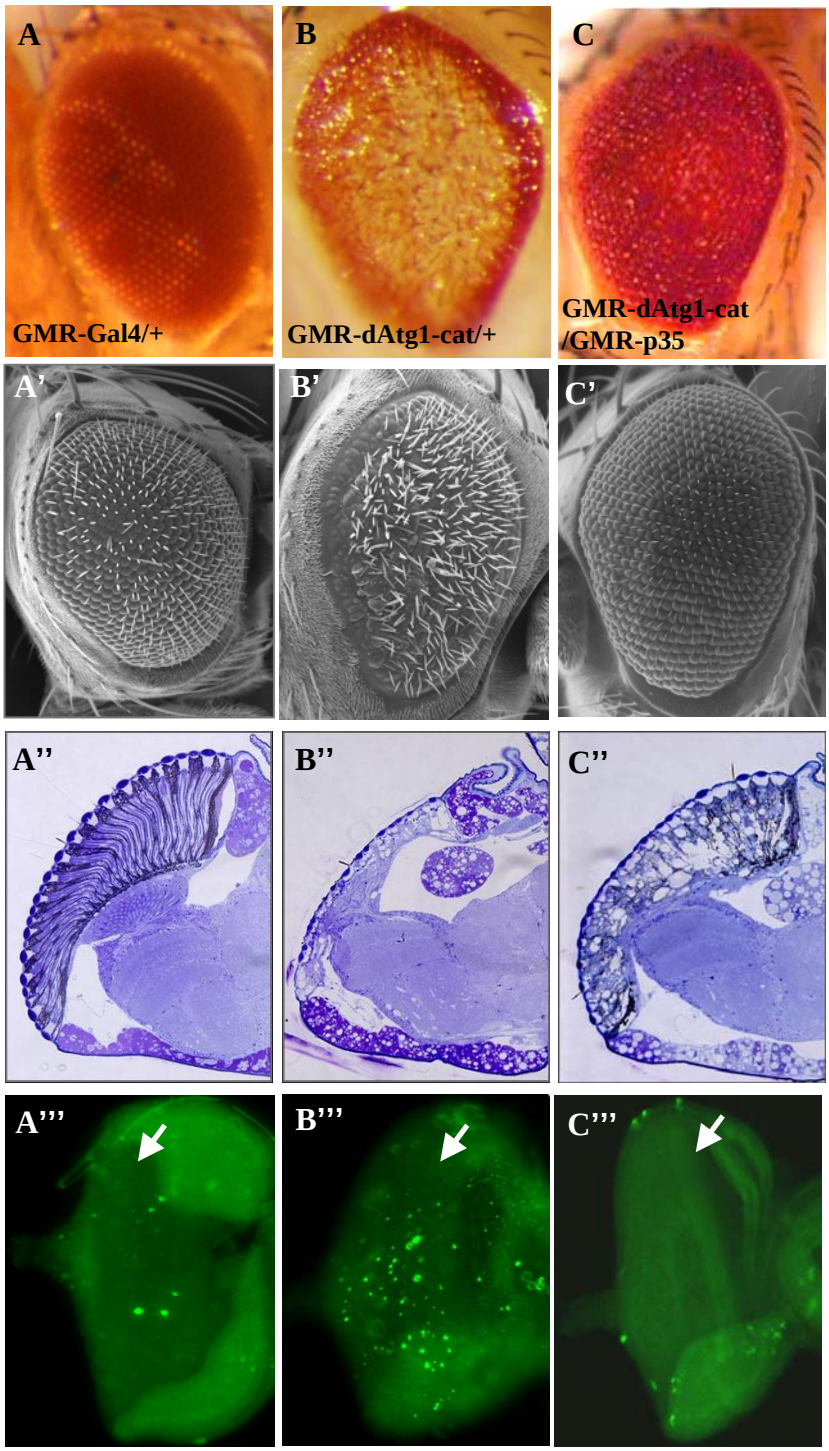


Figure 2. Induction of apoptosis by Atg1 disrupts the ommatidial structure in kinase-dependent manner.

The appearance, scanning electron microscopy photographs, retinal sections and third instar eye immaginal discs sections of GMR-Gal4(A,A',A'',A'''), one-copy of GMR-dAtg1-cat(B,B',B'',B''') and coexpression of GMR-dAtg1-cat/ GMR-p35 (C,C',C'',C'''). In the one-copy-transgenic eye, the underlying retina is severely disrupted, associated with loss of cells. Arrow indicate the morphogenetic furrow.

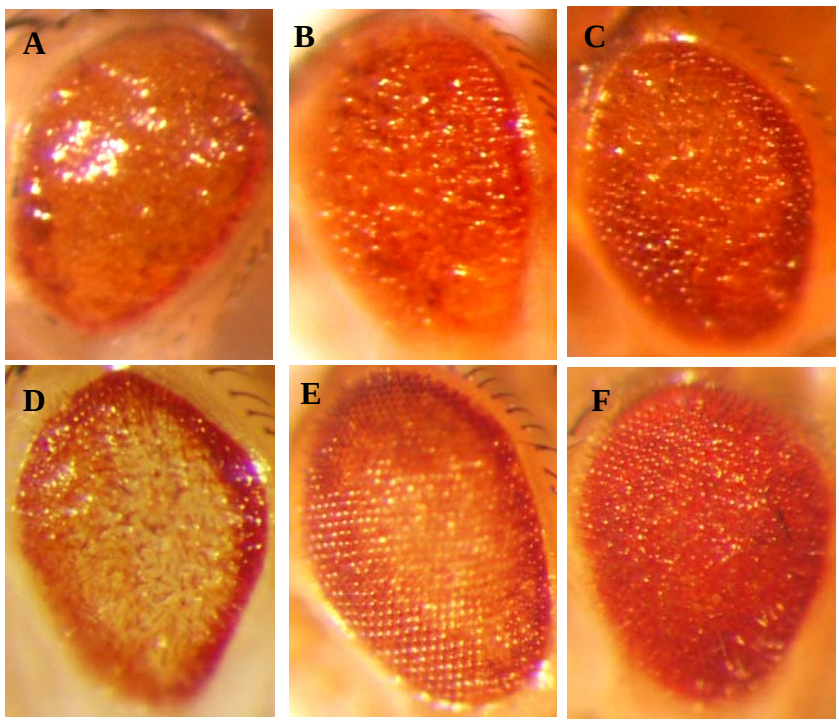


Figure 3. Genetic screen for dominant modifier in Drosophila based on a dAtg1-dependent phenotype.

The weak suppression, moderate suppression and strong suppression of GMR-dAtg1-induced rough eye phenotype (A,B,C). The weak suppression, moderate suppression and strong suppression of GMR-dAtg1-cat induced rough eye phenotype.(E,F,G).

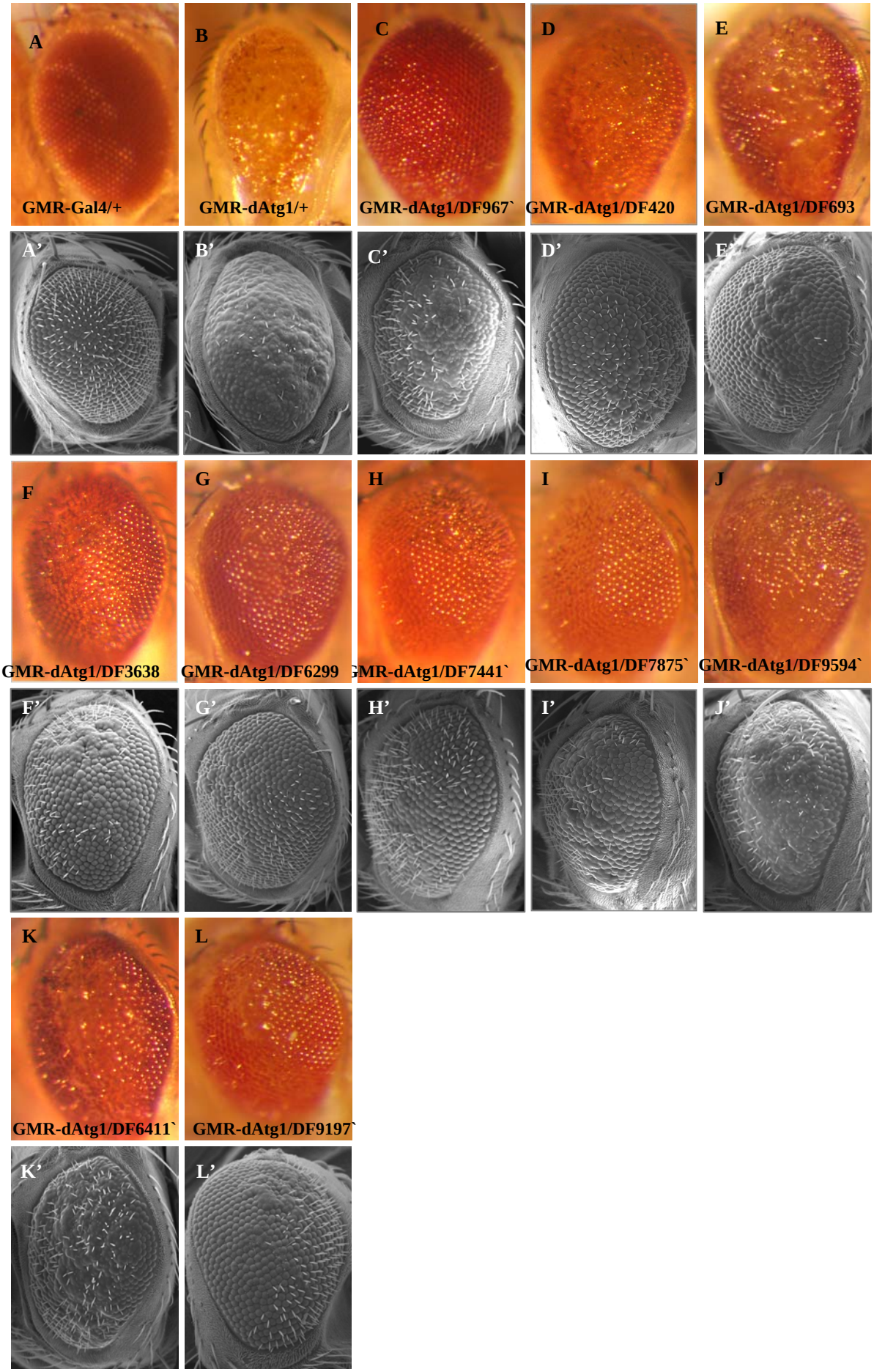


Figure 4. Coexpression of dAtg1 and dificiency line can rescue the rough eye phenotype
Coexpression of the constitutive active form of dAtg1 and the deficiency lines with GMR promoter. Comparing with GMR-Gal4 phenotype（A,A'）,the rough eye phenotype caused by overexpression dAtg1(B,B') can rescue by coexpression of DF967,DF420, DF693, DF3638, DF6299, DF7441, DF7875, DF9594, DF6411, DF9197 (C,C'~L.L')

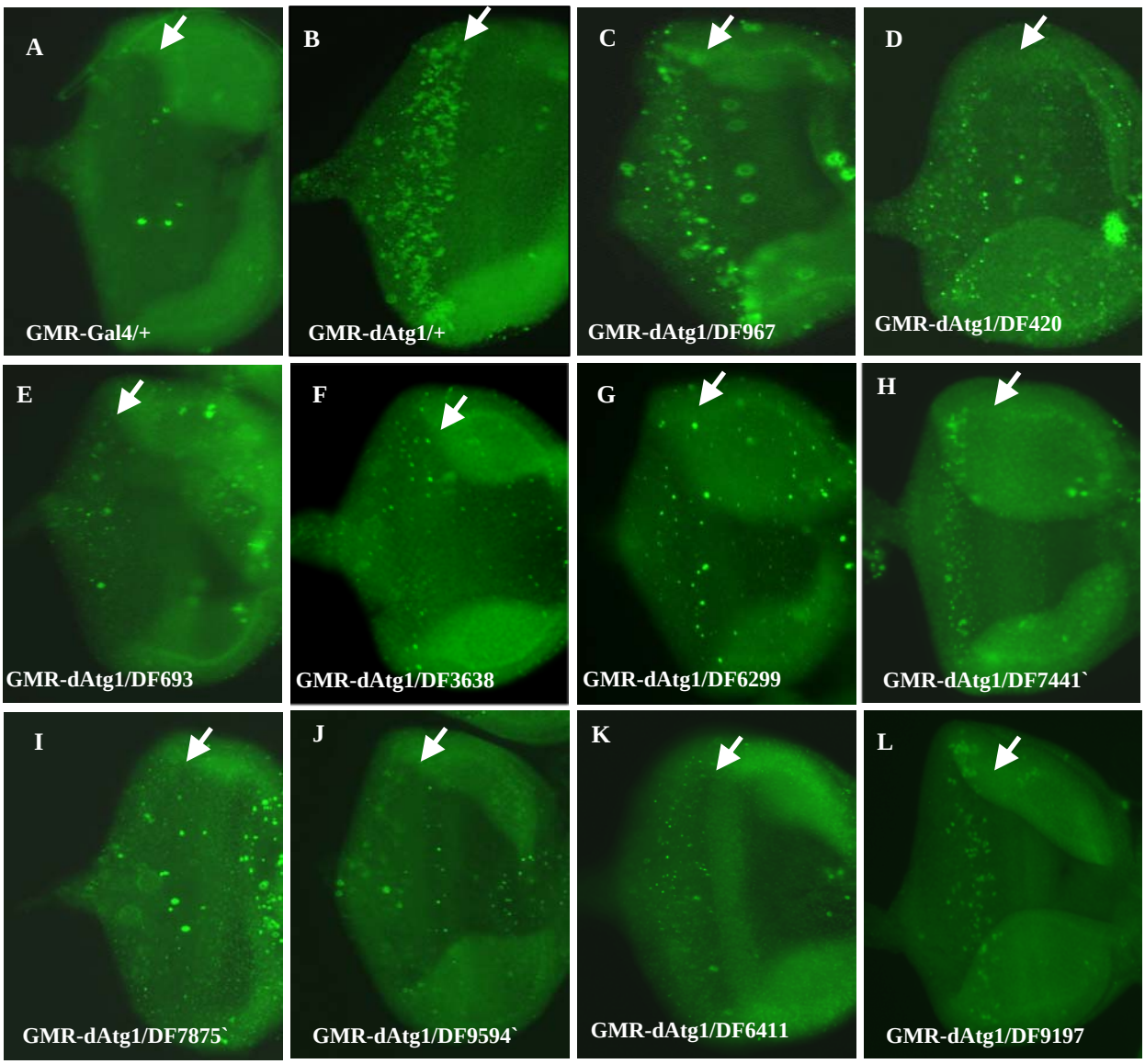
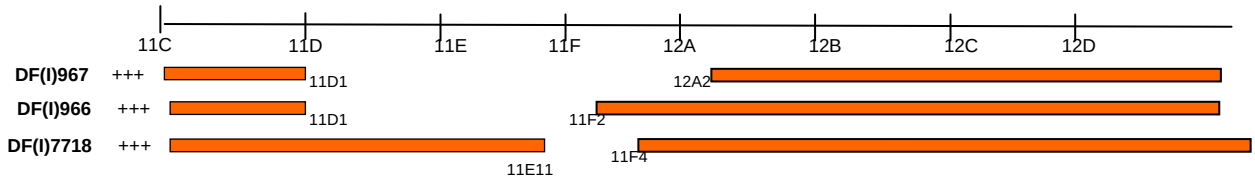


Figure 5. Coexpression of the dificiency lines can rescue dAtg1-induced apoptosis

Coexpression of the constitutive active form of dAtg1 and the deficiency lines with GMR promoter. Staining the third instar eye imaginal discs with cleaved-caspase-3 to label apoptotic cells. The arrow indicate furrow site. Comparing with GMR-Gal4 (A), the marked increase of apoptotic cells caused by overexpression dAtg1(B). The apoptotic cells formation can inhibit by coexpression of DF967,DF420, DF693, DF3638, DF6299, DF7441, DF7875, DF9594, DF6411, DF9197 (C~L). Arrow indicate the morphogenetic furrow.

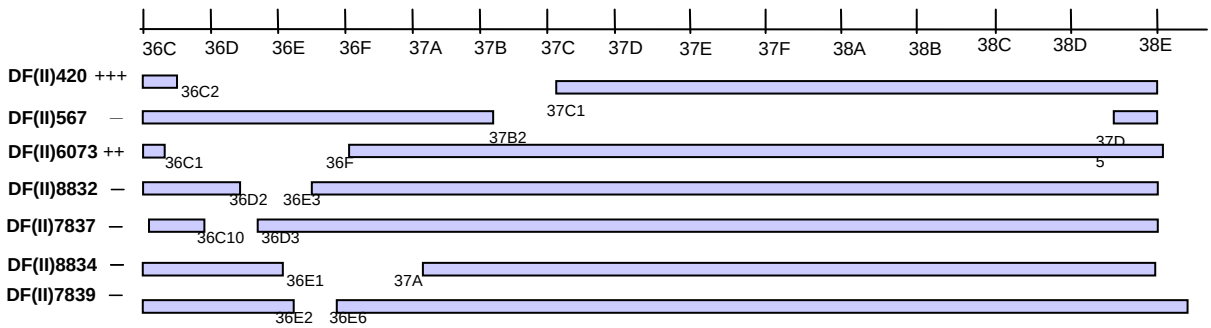
DF(I)967

X



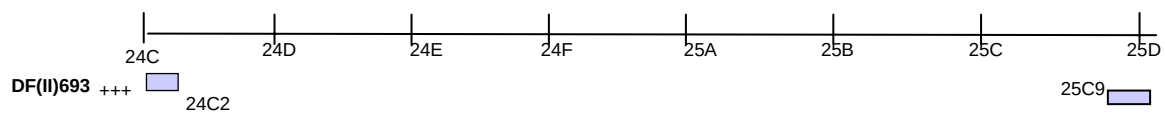
DF(II)420 （36C2~36C10）candidate：Dif、dl、qua

2L



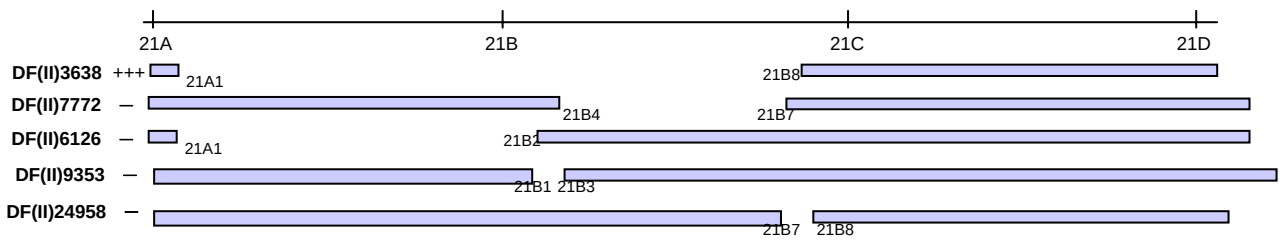
DF(II)693 (24C2~25C9) Candidate:TRAF1,Ei24

2R



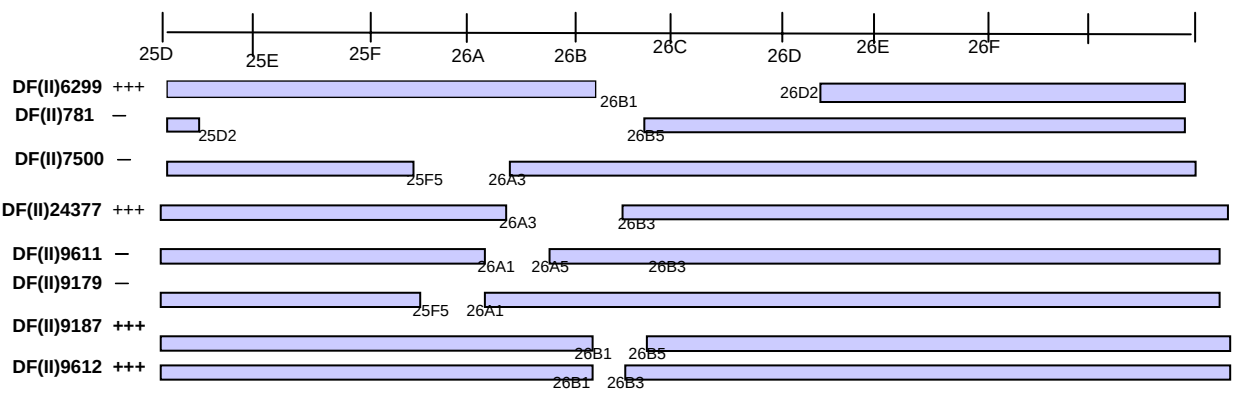
DF(II)3638 (21B3~21B4) candidate：spen

2L



DF(II)6299 (26B1~26B3) candidate：ade2、mtm 、lid

2L



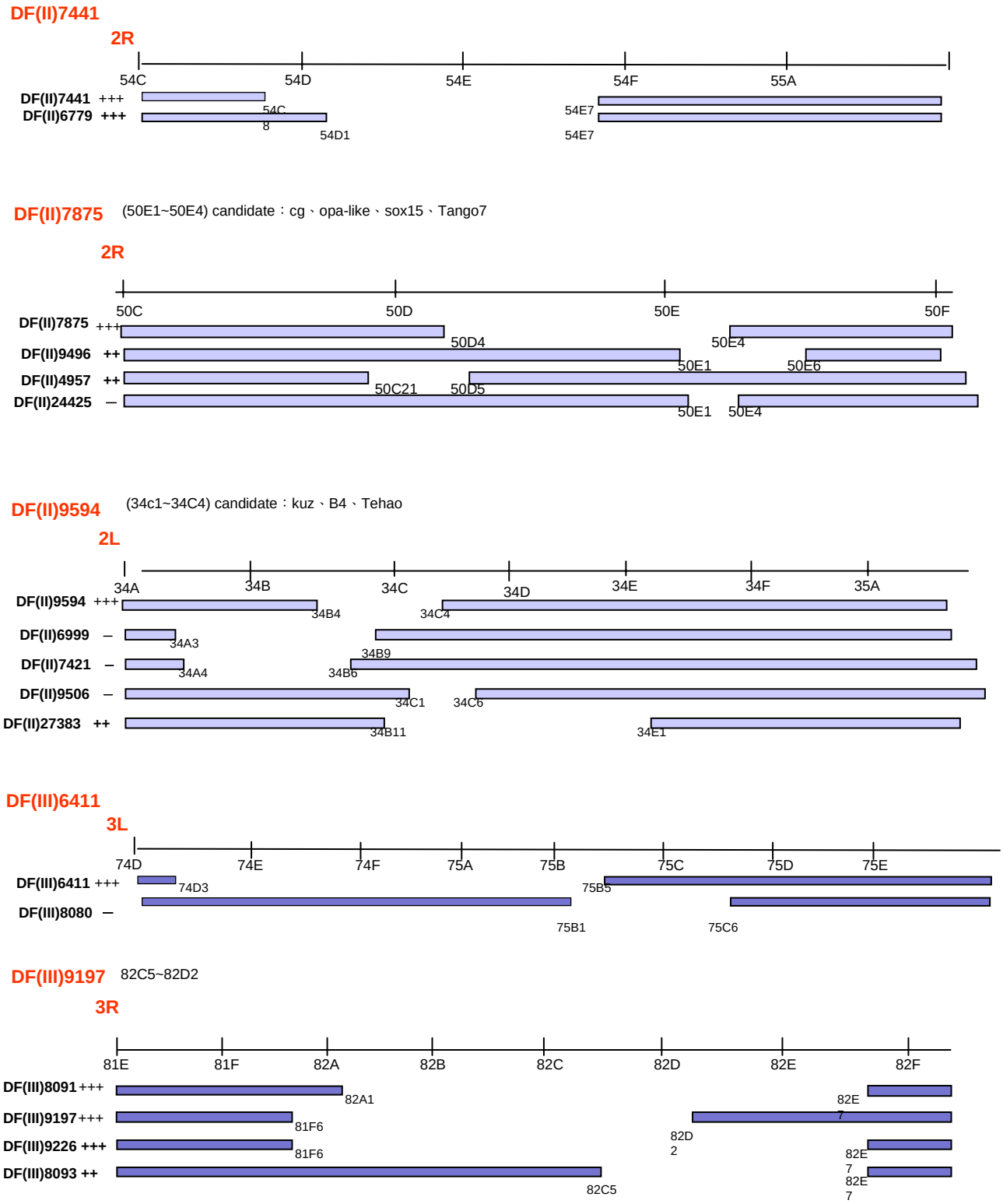


Figure 6. Narrow down the candidate region with its corresponding complementary deficiency lines.

Using the complementary deficiency lines which specific correspond to DF967, DF420, DF693, DF3638, DF6299, DF7441, DF7875, DF9594, DF6411, DF9197 crossed with GMR-dAtg1. According to its eye phenotype narrow down the locating region of candidate gene which has genetic interaction with dAtg1.

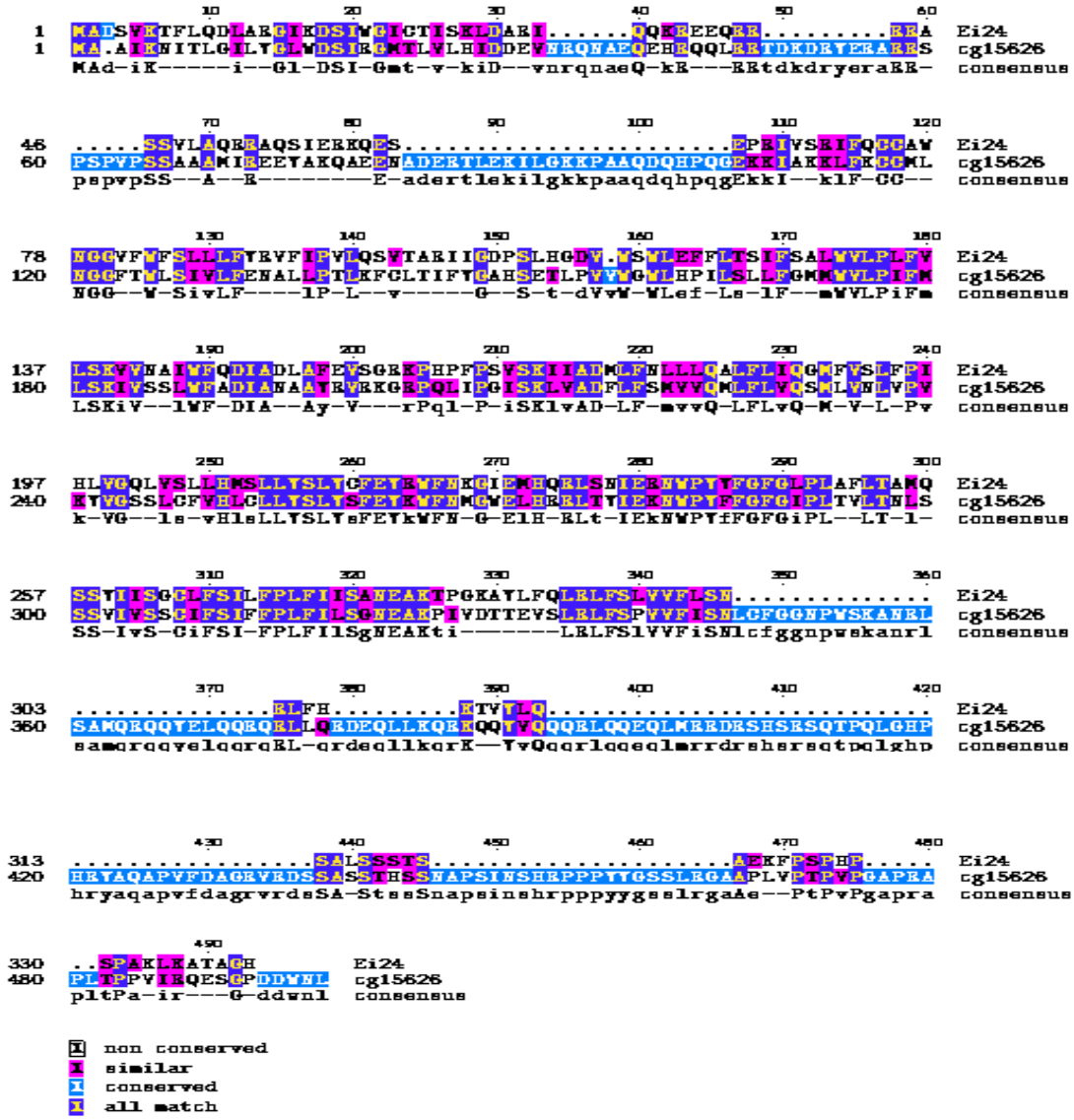


Figure 7. The novel protein, CG15626, which is highly homologous to Ei24.

Using the complementary deficiency lines which specific correspond to DF967, DF420, DF693, DF3638, DF6299, DF7441, DF7875, DF9594, DF6411, DF9197 crossed with GMR-dAtg1. According to its eye phenotype narrow down the locating region of candidate gene which has genetic interaction with dAtg1.

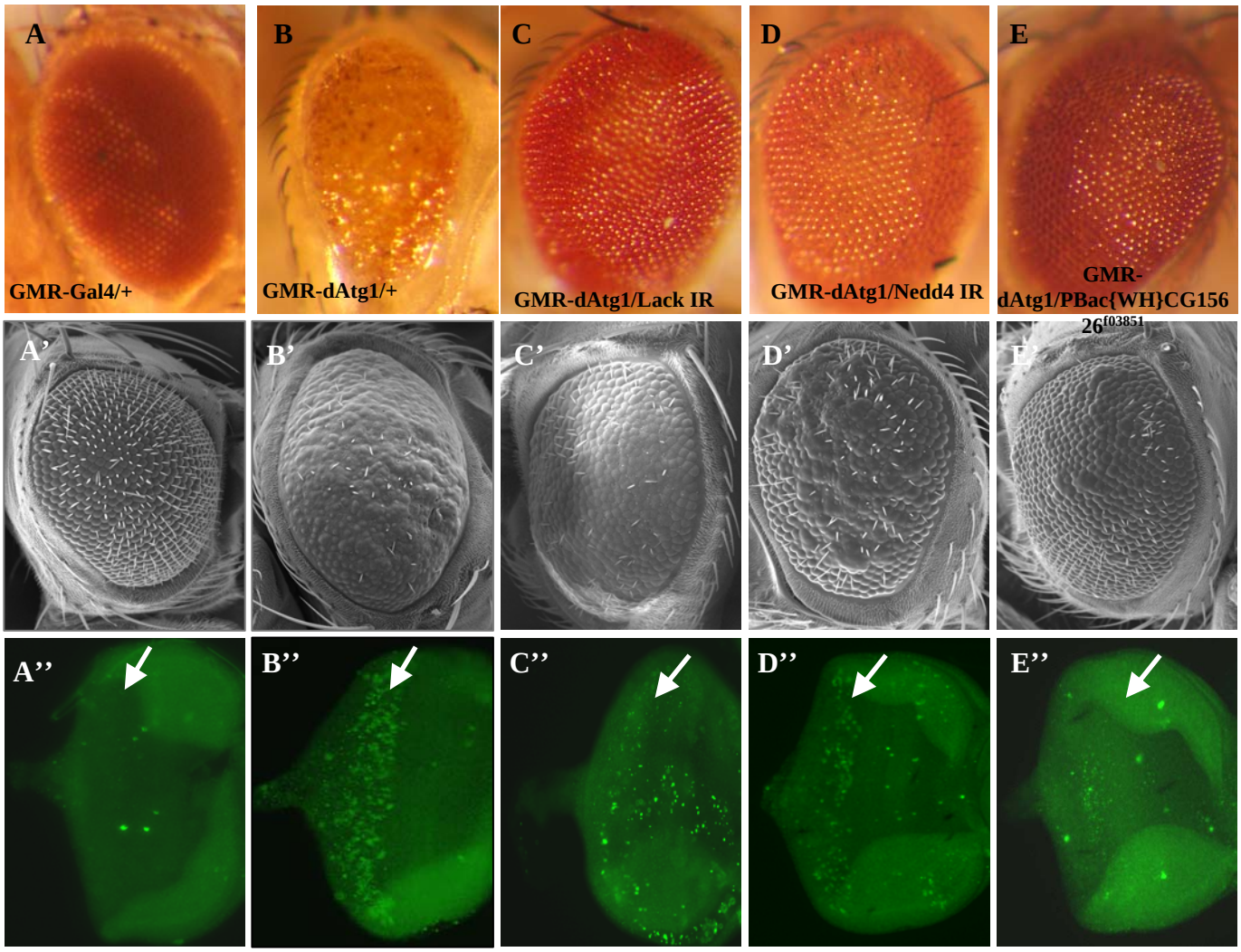


Figure 8. Lack IR, Nedd4 IR, and mutant form of CG15626 (Ei24) can rescue dAtg1-induced eye phenotype and apoptosis.

The GMR-Gal4 control flies eye phenotype and the dissection of eye imaginal discs staining with cleaved-caspase-3 to mark apoptotic cells (A, A', A'') and overexpression of GMR-Atg1 (B,B',B''). Knock down of Lack ,Nedd4 (C,D) and mutant form CG15626 (E) can rescue rough eye phenotype and have inhibition to dAtg1-induced apoptosis. (C''~E''). Arrow indicate the morphogenetic furrow.

	missing one ACV	wild type	missing both ACV	total
ptc-Atg1//PBac{WH}CG15626	7	48	26	81
ptc-Atg1/Lack IR	50	33	70	153
ptc-Atg1/Nedd4 IR	20	3	98	121
ptc-Atg1 control	2	0	99	101
ptc-gal4 control	0	100	0	100

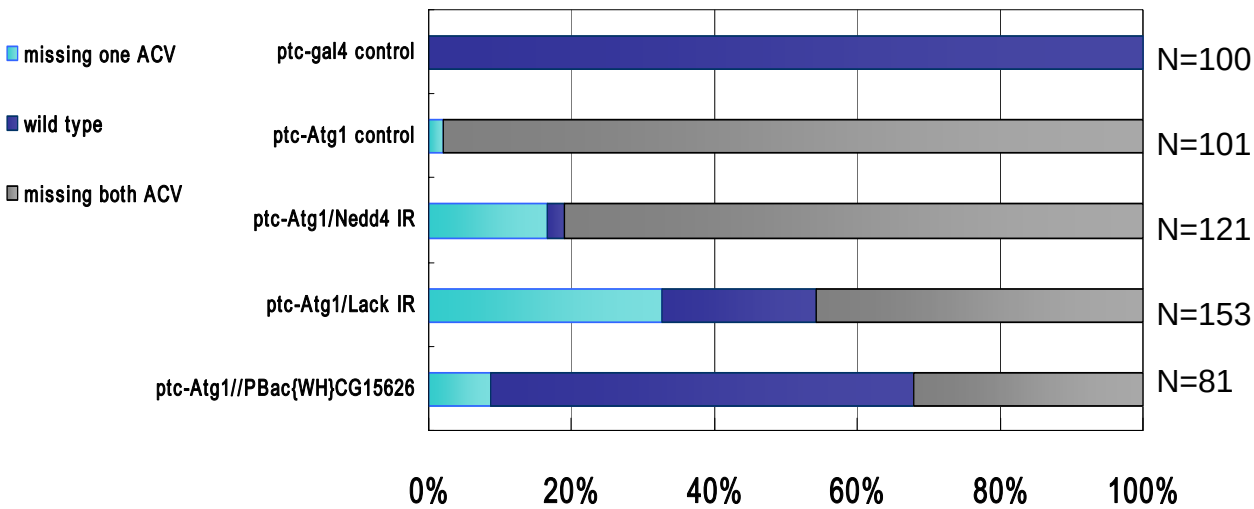


Figure 9. Lack IR, Nedd4 IR, and mutant form of CG15626 (Ei24) can rescue ptc>dAtg1-induced wing phenotype.

The ptc-gal4>dAtg1 control flies loss one or both anterior cross wing vein. Knock down of Lack ,Nedd4 and mutant form CG15626 can partial rescue the wing vein phenotype and have inhibition to dAtg1-inducedwing phenotype.

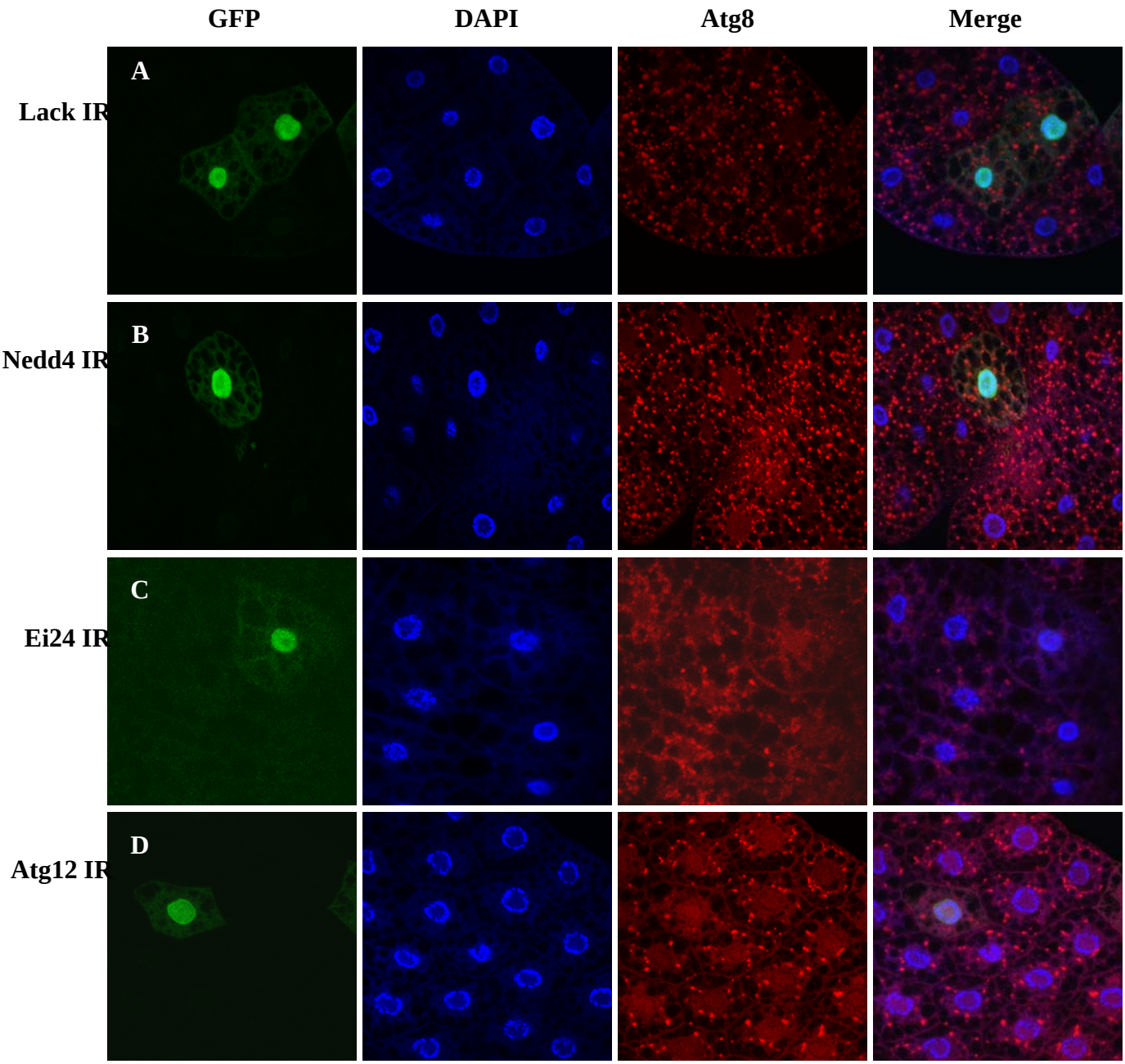


Figure 10. Deletion of Ei24 activity can inhibit starvation-induced autophagy.

Dissection of the second instar larvae fat body under 4hr starvation. The GFP-positive cells indicate knock down of Lack (A), Nedd4 (B), Ei24 (C) and Atg12 (D) (positive control). Autophagy activity was marked by Atg8. The cell which knock down Ei24 show a significant decrease of Atg8, whereas knock down Lack and Nedd4 have not difference.

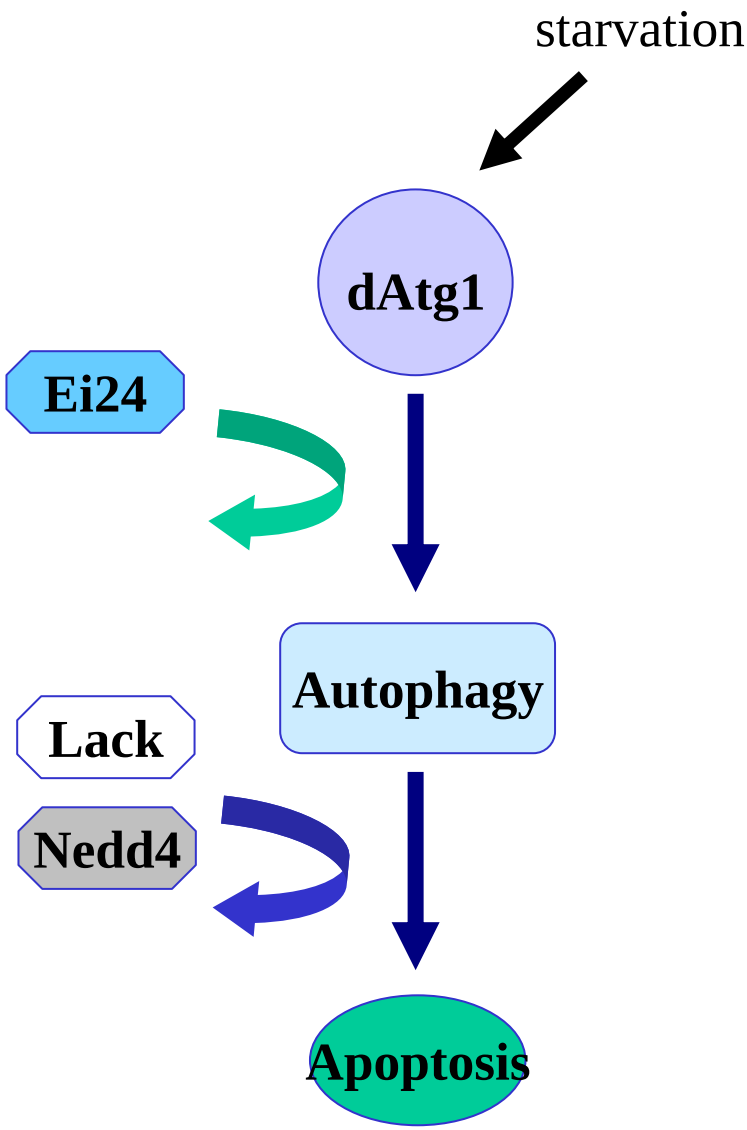


Figure 11. A proposed model for the dAtg1-mediated cell death.

The novel proteins Ei24, Lack, Nedd4, which involved in dAtg1-mediated cell death. Knock down of Ei24 inhibit starvation-induced autophagy, whereas knock of Lack and Nedd4 inhibit dAtg1-induced apoptosis.

Chromosome I

DF kit NO.	Deficiency	Region removed by deficiency	effect on GMR-Atg1	candidate genes in the region
DK1-967	Df(1)C246	11D-12A2	strong suppression	IP3K2、Jafrac1
DK1-8031	Df(1)ED411	3A3-3A8	moderate suppression	giant、boi、wds、sgg
DK1-8032	Df(1)ED418	5C7-5E4	moderate suppression	rux、、mipp2、sqh

Chromosome II

DF kit NO.	Deficiency	Region removed by deficiency	effect on GMR-Atg1	candidate genes in the region
DK2-420	Df(2L)TW137	36C2-37B2	strong suppression	AAC11
DK2-693	Df(2L)sc19-8	24C2-25C9	strong suppression	TRAF1,Ei24
DK2-3638	Df(2L)net-PMF	21A1-21B8	strong suppression	smoothened、PI3K21B
DK2-6299	Df(2L)BSC5	26B1-26D2	strong suppression	Fbw5、Ddr
DK2-7441	Df(2R)BSC45	54C8-54E7	strong suppression	Lack
DK2-7875	Df(2R)Exel7130	50D4-50E4	strong suppression	Opa1-like、O-fut1、cg
DK2-9594	Df(2L)BSC159	34B4-34C4	strong suppression	Rep4、kuz
DK2-781	Df(2L)cl-h3	25F3-26B5	moderate suppression	blue cheese
DK2-2414	Df(2L)spd[j2]	27C1-28A	moderate suppression	WEE
DK2-5420	Df(2L)Dwee1-W05	27C2-27C5	moderate suppression	
DK2-5869	Df(2L)FCK-20	32D1-32F3	moderate suppression	
DK2-6866	Df(2R)BSC26	56C4-56D10	moderate suppression	Tab2、Rgk1、Fak56D
DK2-7147	Df(2L)BSC41	28D1-28D9	moderate suppression	Snoo、Cka
DK2-9496	Df(2R)BSC134	50E1-E6	moderate suppression	O-fut1、Opa1-like、cg
DK2-442	Df(2R)CX1	49C1-50D2	weak suppression	sca
DK2-1547	Df(2R)PC4	55A-55F	weak suppression	
DK2-2471	Df(2R)M60E	60E2-60E12	weak suppression	
DK2-2892	Df(2L)N22-14	29C1-30C9	weak suppression	
DK2-5330	Df(2L)ed1	24A2-24D4	weak suppression	fred、echinoid
DK2-5879	Df(2R)BSC3	48E12-49B6	weak suppression	
DK2-6609	Df(2R)BSC19	56F12-57A4	weak suppression	--
DK2-6965	Df(2L)BSC31	23E5-23F5	weak suppression	Ptpa、Thor
DK2-6999	Df(2L)BSC30	34A3-34B9	weak suppression	Tor、Rep4、
DK2-7143	Df(2L)BSC36	32D1	weak suppression	Samuel
DK2-7531	Df(2L)Exel6049	40B1-40D3	weak suppression	--
DK2-8469	Df(2L)BSC50	30F5-31B1	weak suppression	Bsk、Ror、Pten

Chromosome III

DF kit NO.	Deficiency	Region removed by deficiency	effect on GMR-Atg1	candidate genes in the region
DK3L-6411	Df(3L)BSC8	74D3-75B5	strong suppression	Nedd4、Eip74EF、TORC、krn、Ccn、Eip75B
DK3R-8091	Df(3R)ED5092	82A1-82E7	strong suppression	auxillin、abstrakt、tube、serotonin receptor2
DK3R-9197	Df(3R)ED5046	81F6-82D2	strong suppression	
DK3R-9226	Df(3R)ED5100	81F6-82E7	strong suppression	
DK3R-8093	Df(3R)ED5095	82C5-82E7	moderate suppression	
DK3L-8061	Df(3L)ED210	64B9-64C13	weak suppression	Tie、Srp54k、Uev1A
DK3L-8074	Df(3L)ED217	70F4-71E1	weak suppression	
DK3L-8075	Df(3L)ED218	71B1-71E1	weak suppression	
DK3L-8098	Df(3L)ED4674	73B5-73E5	weak suppression	TSG101、sina、sinah
DK3L-8101	Df(3L)ED4978	78D5-79A2	weak suppression	Pros54
DK3R-8105	Df(3R)ED6232	96F10-97D2	weak suppression	scrib、Toll、Lerp
DK3R-8680	Df(3R)ED5138	82D5-82F8	weak suppression	corto、opa、UdcD6、cno、retinophilin
DK3R-8682	Df(3R)ED5230	84E6-85A5	weak suppression	puc、MAPKK4、pink
DK3R-8967	Df(3R)ED5147	82E7-83A1	weak suppression	corto、cno、retinophilin
DK3R-9228	Df(3R)ED5634	88A4-88B1	weak suppression	rdx、

Table 1. The dificiency lines which have genetic interaction with dAtg1 in Drosophila chromosome.

The Deficieny kit is from the Indiana University Bloomington Stock Center which represent more than 70% of the Drosophila genome. A total of 277 deficiencies were tested and 3 suppressors were identified in chromosome I, 26 suppressors were identified in chromosome II, 15 suppressors were identified in chromosome III.

Chromosome I

DF kit NO.	Deficiency	Region removed by deficiency	effect on GMR-Atg1	candidate genes in the region
DK1-936	Df(I)64c18	2E1-3C2	strong suppression	vinc、phl、giant、boi、wds、sgg
DK1-967	Df(I)C246	11D-12A2	strong suppression	IP3K2、Jafrac1
DK1-970	Df(I)N19	17A1-18A2	strong suppression	upd3、os、psh、wnt5
DK1-8031	Df(I)ED411	3A3-3A8	moderate suppression	giant、boi、wds、sgg
DK1-9171	Df(I)ED7147	10D6-11A1	moderate suppression	Amun、Tango10、p24-1
DK1-9217	Df(I)ED7161	11A1-11B14	moderate suppression	gd、tsg、fw、limk1
DK1-9414	Df(I)ED7265	12F4-13A5	moderate suppression	rut、Eo
DK1-8030	Df(I)ED404	1D2-1E3	weak suppression	TRAM
DK1-8032	Df(I)ED418	5C7-5E4	weak suppression	rux、mipp2、sqh
DK1-8952	Df(I)ED7217	12A9-12C6	weak suppression	garnet、ldd4、Clic
DK1-8953	Df(I)ED7153	11A1-11B1	weak suppression	Amun、Tango10、p24-1
DK1-9054	Df(I)ED6574	2E1-3A2	weak suppression	vinc、phl
DK1-9218	Df(I)ED7261	12F2-12F5	weak suppression	rut
DK1-9625	Df(I)ED6878	6C12-6D8	weak suppression	shf、C3G、Nf-YC

Chromosome II

DF kit NO.	Deficiency	Region removed by deficiency	effect on GMR-Atg1	candidate genes in the region
DK2-282	Df(2R)X58-12	58D1-59A	strong suppression	ari-2
DK2-5869	Df(2L)FCK-20	32D1-32F3	strong suppression	abrupt
DK2-6779	Df(2R)14H10Y-53	54D1-54E7	strong suppression	lack
DK2-6999	Df(2L)BSC30	34A3-34B9	strong suppression	Tor
DK2-567	Df(2L)pr-A16	37B2-38D5	moderate suppression	hook、mib2、hkl、Pax
DK2-693	Df(2L)sc19-8	24C2-25C9	moderate suppression	Traf1、Ei24 、 fred、echinoid
DK2-3518	Df(2R)Jp1	51D3-52F9	moderate suppression	fus、shark、Rho1
DK2-3520	Df(2R)Jp8	52F5-53A1	moderate suppression	shark
DK2-3588	Df(2L)TE35BC-24	35B4-35F7	moderate suppression	Su、cul-3、esg、wek
DK2-3638	Df(2L)net-PMF	21A1-21B8	moderate suppression	smo
DK2-5330	Df(2L)ed1	24A2-24D4	moderate suppression	fred、echinoid
DK2-5420	Df(2L)Dwee1-W05	27C2-27C5	moderate suppression	wee
DK2-1007	Df(2R)nap9	42A1-42F1	weak suppression	EcR、bin3
DK2-2414	Df(2L)spd[j2]	27C1-28A	weak suppression	wee
DK2-3909	Df(2R)59AD	59A1-59D4	weak suppression	synj、arc
Dk2-5246	Df(2R)Egfr5	57D2-58D1	weak suppression	sara、Egfr、ari-2
DK2-6478	Df(2L)BSC17	30C3-30F1	weak suppression	IP3K1

chromosome3

DF kit NO.	Deficiency	Region removed by deficiency	effect on GMR-Atg1	candidate genes in the region
DK3L-8053	Df(3L)ED207	61C9-62A6	strong suppression	emc、bab1、Aplip1、Sac1
DK3L-8074	Df(3L)ED217	70F4-71E1	strong suppression	Trl、mop
DK3L-8098	Df(3L)ED4674	73B5-73E5	strong suppression	zetaCOP、TSG101、sina
DK3L-8079	Df(3L)ED223	73A1-73D5	strong suppression	
DK3L-8080	Df(3L)ED224	75B1-75C6	strong suppression	wrinkle,reaper
DK3L-8087	Df(3L)ED229	76A1-76E1	strong suppression	Bet1、pip、trmpl
DK3R-9210	Df(3R)ED6255	97D2-97F1	strong suppression	
DK3R-9203	Df(3R)ED5331	85C3-85D1	strong suppression	
DK3R-9198	Df(3R)ED5142	82B2-82F8	strong suppression	dip2、tacc
DK3R-9197	Df(3R)ED5046	81F6-82D2	strong suppression	
DK3R-9224	Df(3R)ED5071	81F6-82E4	strong suppression	
DK3R-9226	Df(3R)ED5100	81F6-82E7	strong suppression	
DK3L-8051	Df(3L)ED202	61C9-61F7	moderate suppression	emc、bab1、Aplip、Sac1
DK3L-8052	Df(3L)ED4238	61C9-62A4	moderate suppression	
DK3L-8065	Df(3L)ED4408	66A22-66C5	moderate suppression	nemo
DK3L-8068	Df(3L)ED4470	68A6-68E1	moderate suppression	Sod、chrb
DK3L-8069	Df(3L)ED4475	68C13-69B4	moderate suppression	
DK3L-8070	Df(3L)ED4483	69A5-69D3	moderate suppression	
DK3L-8075	Df(3L)ED218	71B1-71E1	moderate suppression	Eig71E
DK3R-8092	Df(3R)ED5066	82C5-82E4	moderate suppression	
DK3L-8096	Df(3L)ED4287	62B4-62E5	moderate suppression	dlt、Dbx
DK3L-8100	Df(3L)ED4710	74D1-75B11	moderate suppression	
DK3R-8105	Df(3R)ED6232	96F10-97D2	moderate suppression	
DK3L-9071	Df(3L)ED4515	70C6-70C15	moderate suppression	endos
DK3L-9072	Df(3L)ED4528	70C15-70D2	moderate suppression	btl、Fbp1
DK3L-9073	Df(3L)ED4529	70C6-70D2	moderate suppression	
DK3L-9074	Df(3L)ED4534	70C15-70D3	moderate suppression	
DK3R-9280	Df(3R)ED6237	97E4-97E11	moderate suppression	
DK3L-9355	Df(3L)ED4457	67E2-68A7	moderate suppression	simj
DK3R-9478	Df(3R)ED6235	97B9-97D12	moderate suppression	

Table 2. The dificiency lines which have genetic interaction with dAtg-cat in Drosophila chromosome.

The Deficieny kit is from the Indiana University Bloomington Stock Center which represent more than 70% of the Drosophila genome. A total of 277 deficiencies were tested and 14 suppressors were identified in chromosome I, 17 suppressors were identified in chromosome II, 30 suppressors were identified in chromosome III.