Abstract

Determining the role of a candidate gene in Drosophila muscle development

By Chaitali Maity

In Drosophila, 1424 gene was tagged by the enhancer trapping technique, which involves insertion of a modified transposable element. Reporter expression indicated that the gene is expressed in the CNS and developing flight muscles (IFMs) during pupal stage. This thesis investigates a role for the 1424 gene in the development of flight muscles. Forty-two mutant lines that were generated by genetically excising the P-element were tested. Homozygous mutants were generated as P-element insertion has been reported to be lethal in case of 1424 gene. Upon observation of the lethality during the life cycle we found the P-element insertion might be semi-lethal as emergence of homozygous 1424 flies was observed. Complementation further support the semi-lethality of the parent strain as nearly all of the mutant lines were able to generate transheterozygotes, but not all of them were able to generate homozygous mutants. Flight test as well as muscle phenotype analysis of the homozygous mutants confirm that this gene represented as 1424 is involved in IFM development.
Determining the role of a candidate gene in *Drosophila* muscle development

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Overview

Behaviorally and functionally, the life-cycle of holometabolous insects such as *Drosophila* is categorized in two stages- larva and adult. The larva is morphologically and functionally different from the adult. Neuromuscular development occurs twice, once during the embryonic stage, to perform larval specific behavior, and later during the pupal stage to perform adult specific behavior. Metamorphosis occurs during the pupal stage. One of the most interesting behaviors present in the adult is its ability to fly. The flight muscles are located in the thorax and their development occurs during metamorphosis. The indirect flight muscles are the largest and are made of two groups- dorsal longitudinal muscle (DLMs) and dorsoventral muscles (DVMs). During the pupal stage most of the larval muscles are histolyzed followed by the development of new muscles from the adult myoblasts, and in some cases the larval muscles serve as scaffolds for the development of adult muscles. DLMs develop from the persistent larval scaffolds (Fernandes et al., 1991) and DVMs develop de novo using founder cells (Rivlin et al., 2000). The motoneurons that innervate the adult muscles are remodeled larval neurons (Truman et al., 1992). The development of adult muscles as well as remodeling of the motoneurons occurs simultaneously.

The genes that are responsible for bringing about the adult specific changes are mostly unknown. Whether the genes that are responsible for larval neuromuscular development are also responsible for adult neuromuscular development remains mostly unknown.

*Drosophila* has been used as a model genetic system and therefore many tools and techniques are available to identify new genes. Reverse genetic approaches have been used to identify genes based on gene expression. The enhancer gene trapping technique is one of the tools used for identification of novel gene and assaying their function. This thesis deals with the study of a candidate gene, 1424, which was identified by enhancer gene trapping. During early pupal stage, X-gal staining showed the presence of 1424 expression in larval templates that serve as scaffolds for DLM development, larval motoneurons and pupal CNS. Based on the expression pattern in the pupal stage, we hypothesize that 1424 plays a role during adult neuromuscular development. Mutants were generated for studying the behavior and muscle morphology.
Introduction

Flight is one of the most distinct and unique behaviors of the adult *Drosophila melanogaster*. Three major group of muscle fibers responsible for this behavior are located in the adult thorax. They are: Direct flight muscles (DFMs), jump muscles or terger depressor of trochantor (TDT) and indirect flight muscles (IFMs) (Fig. 1A). The DFM s act as hinges for the wing to fine tune the flight and the jump muscles help in the leap before flight. The IFMs are comprised of two groups- dorsal longitudinal muscle (DLMs) and dorsoventral muscles (DVMs). The DLMs act as wing depressors and DVMs cause wing elevation (Miyan et al., 1985).

Synchronization between jump muscle and the IFMs leads to initiation and maintenance of flight during escape behavior (Miyan et al., 1985). DLMs, along with TDT, initiate the flight, which is also known as the escape response. The escape response is mediated by the giant fiber pathway (Tanouye et al., 1980; Thomas et al., 1984). The giant fiber pathway is a small group of neurons that channels the visual input from the head to the thoracic muscles. It forms synapses with DLMs, DVMs and TDT. The giant fiber pathway connects the five motoneurons that innervate the DLMs (Ikeda and Koenig, 1988) via an interneuron (PSI) and the TDT motoneuron directly (King and Wyman, 1980). Mutations, disrupting the synaptic connections in the giant fiber pathway leads to lack in the escape response (Thomas et al., 1982; Thomas et al., 1984) resulting in defective flight ability or flightlessness.

The six pairs of DLMs run along the length of the thorax (Fig. 1B). Each DLM fiber is innervated by one motoneuron, except the two dorsal most fibers. The two dorsal most fibers, DLMa and DLMb (Fig. 1B) are innervated by one motoneuron only (Ikeda and Koenig, 1988). Three pairs of DVMs are the antagonist muscles and connect the dorsal and ventral parts of the thorax. The three DVMs that are present in each hemithorax are named as DVM I, DVM II and DVM III (Fig. 1B). DVM I consists of three muscle fibers, DVM II is composed of two muscle fibers and DVM III consists of two muscle fibers.
Figure 1. (A) Schematic diagram of a wild type fly *D. melanogaster*. (B) Enlarged view of thoracic muscles. Muscles a-f are subsets of DLMs (Dorso-lateral muscles). TDT is the jump muscle.
Development of thoracic muscles

In *Drosophila* adult myogenesis occurs during metamorphosis. During this stage histolysis of most of the larval muscles occurs, followed by the development of head, thorax, leg and abdominal muscles. Myoblasts required for the development of thoracic muscles are set aside during embryogenesis and become associated with the imaginal discs (Lawrence, 1982; Bate *et al*., 1991). The imaginal discs are inconspicuous during early larval stages but become visible during the late larval stages. The myoblasts proliferate inside the discs throughout the larval stages. In the thoracic region of the larva there are three pairs of discs- the wing, leg and haltere. Myoblasts associated with the leg and wing discs are required for the development of the adult thoracic muscles. During metamorphosis the discs evaginate and the myoblasts migrate and fuse with specialized organizer cells (also known as imaginal pioneers-IPs or founder cells) that express the embryonic founder cell gene *dumbfounded* (*duf*) (Dutta *et al*., 2004; Fernandes and Keshishian, 2005). It is believed that the founder cells are initially associated with the imaginal discs (Rivlin *et al*., 2000). The muscles in the thorax are broadly classified as dorsal and ventral muscles (Miller, 1950). The most prominent dorsal muscles are the direct flight muscles (DFMs) and the indirect flight muscles (IFMs). Among the ventral muscles, the jump muscle (TDT) is the most prominent one. Myoblasts that give rise to dorsal muscles are associated with the wing disc, whereas myoblasts that form the ventral muscles are associated with the leg discs (Lawrence, 1982).

The IFMs are the largest set of thoracic muscles. They are classified into two groups- dorsal longitudinal muscles (DLMs) and dorsoventral muscles (DVMs). These two groups of muscle exhibit two very different modes of development. The DLMs develop using persistent larval muscles as templates (Fernandes *et al*., 1991). Fusion of the myoblasts with the persistent larval templates leads to the formation of muscle fibers. These developing muscles thereafter attach to the specific attachment sites on the epidermis. Removal of one or more larval templates by laser ablation results in formation of incorrect number of DLM fibers (Farrell *et al*., 1996). This study proved that presence of larval templates is not necessary for the formation of DLMs but it is necessary for patterning of DLMs and portioning of the myoblasts.
The DVMs do not have larval templates, and myoblasts are patterned using founder cells. These specialized cells were first described by Rivlin et al., through light microscopy and EM studies. They were called imaginal pioneers (IPs) and it was shown that there was one to one correspondence between the number of IPs and DVM fibers (Rivlin et al., 2000). EM studies have shown that IPs are indistinguishable till 2nd instar larval stage but become evident at third instar. During pupal stage, they are easily distinguished from the neighboring cells based on their elongated size with abundant microtubule in the cytoplasm (Rivlin et al., 2000). Recent studies based on the expression of duf- lacZ (an embryonic marker for founder cells) have confirmed one to one correspondence between the number of IPs and DVM fibers (Dutta et al., 2004; Fernandes and Keshishian, 2005).

One of the important features of adult specific myogenesis, which is different from embryonic myogenesis is that, it is dependent on innervation (Fernandes and Keshishian, 1998). This resembles vertebrate muscle development where innervation plays an important role (Hamburger et al, 1951; Martin, 1990). Thus, the IFMs can be a useful model for studying myogenesis in vertebrates.
Role of motoneurons in thoracic muscle development

In holometabolous insects, motoneurons that innervate the adult muscle are remodeled larval motoneurons (Truman, 1992). In Drosophila, during the early pupal stage, nerve terminals retract and histolysis of larval muscles takes place, but the nerve remains attached to the persistent muscles (Fernandes et al., 1993) (Fig. 2). As myoblasts fuse and muscles fibers form, adult innervation pattern is simultaneously established. The nerve-muscle interactions are likely to shape neuromuscular pattern. The role of innervation in myogenesis was demonstrated by denervation studies. When the mesothoracic nerve that innervates the DLMs was denervated, the development of IFMs was affected. Although DLM fibers developed, they were thinner in size. However, the development of DVMs was completely abolished (Fernandes and Keshishian, 1998). Denervation also reduced myoblast proliferation and fusion to the larval templates during DLM development indicating that development of adult muscles is nerve dependent. In case of DVMs, denervation led to reduced myoblast proliferation. Additionally, the myoblasts fail to segregate into distinct DVM primordia. The expression of duf was also reduced indicating, that innervation regulates the function of founder cells (Fernandes and Keshishian, 2005). Thus, studies done so far, indicate that motoneurons regulate fiber size, through an effect on proliferation, patterning and organizing the myoblasts into muscle forming units in case of DVMs. Denervation studies in Manduca, also demonstrated similar effects (Duch et al., 2000, Baylies et al., 2001). Studies on development of chick leg and wing muscles and limb development in frog have shown that denervation of motoneurons results in formation of thinner muscles (Fredette et al., 1999; Phillips et al., 1984; Dietz, 1987). Thus, holometabolous insects can be used as a model to study nerve dependent myogenesis.

In addition to motoneuronal influence on muscle development other factors such as ecdysteroids (Consoulas et al., 2005; Sandstrom et al., 1999; Sandstrom et al., 1997) and the epidermis (Fernandes et al., 1996) play an important role during IFM myogenesis.
Figure 2. Schematic diagram of adult myogenesis. (A, B, C) 0-12 hour pupae. (A) Retention of larval nerve and retraction of the peripheral branches. The blue bands represent the persistent larval muscles that serve as templates for development of DLMs. (B) Migration and proliferation of myoblasts (red dots). The myoblast accumulate near the nerve endings (SN, ISN) and over the larval templates. (C) Initiation of adult innervation pattern, fusion of myoblast to the larval templates. (D, E) 12-24 hrs after pupal formation. (D) The formation of the DVMs is initiated, larval templates split to form the six DLMs. (E) The remodeling of neurons are completed, DVMs and DLMs are formed, adult innervation pattern on the DLMs is underway.

Genetic basis of IFM development

The advantage of studying muscle development in *Drosophila* is that, being a genetic model system the genes affecting the process can be identified. Currently, a few genes have been identified that influence IFM development (Fig. 3).

Generating the pool of adult myoblasts

Based on *twist* expression it has been established that myoblast precursor cells for adult thoracic muscles develop during embryogenesis and associate with the imaginal discs (Bate *et al*., 1991). Muscle precursors present in the wing disc proliferate during the larval stages, upon evagination of the disc during metamorphosis the myoblasts fuse with persistent larval templates and IPs (Bate *et al*., 1991; Fernandes *et al*., 1991; Rivlin *et al*., 2000). Decrease in *twist* expression using temperature sensitive *twi* mutation has
exhibited abnormal IFM development in adults (Cripps et al., 1998). Similar results were found using temperature sensitive Notch allele (Anant et al., 1998). Based on the expression pattern of these two mutants it was concluded that Notch as well as twist expression is required for proliferation of the adult myoblasts during the larval stages for formation of correct number and pattern of IFMs.

The vestigial (vg) gene is differentially expressed in the myoblasts of IFMs and DFM s during larval stages. During the late larval stage the expression of vg and cut in IFM specific myoblast is reduced when compared to the DFM specific myoblasts (Sudarsan et al., 2001). Decrease in vg expression during early larval stages resulted in lesser proliferation in IFM specific myoblasts leading to thinner and lesser number of DLMs and complete absence or lesser number of DVMs during metamorphosis (Bernard et al., 2003; Sudarsan et al., 2001). In case of vg null mutants, DFM myogenesis was not affected (Bernard et al., 2003).

Myoblast aggregation and fusion

The IPs, which are required for DVM development, and the persistent larval templates, which are required for DLM development, express the embryonic founder cell marker dumfounded (duf) (Ruiz Gomez et al., 2000). During embryogenesis, duf expression is required in the founder cells for the myoblasts to fuse and develop into larval specific muscles. It serves as an attractant for fusion competent myoblasts.

Similarly, during IFM development, duf expression is required in the larval templates and IPs for myoblasts to fuse to them (Dutta et al., 2004; Fernandes and Keshishian, 2005).

Muscle differentiation and attachment to epidermis

In case of DLM formation, after the fusion of myoblasts to the larval templates, each template splits into two forming six DLMs. twist is required for the splitting of the larval templates as absence of twist expression leads to formation of three DLMs only (Cripps et al., 1998). After splitting of the larval templates, twist is down regulated (Fernandes et al., 1991). Persistent twist expression after splitting of larval templates
resulted in delayed differentiation of the myoblasts (Anant et al., 1998). Similar results were observed in murine myogenesis in mice where persistent twist expression after initiation of myogenesis prevented onset of muscle differentiation (Hebrok et al., 1994), indicating twi has similar function during myogenesis of vertebrates and IFM development in Drosophila.

erect wing (ewg) expression is upregulated just before the fusion of the myoblasts (DeSimone et al., 1996; Roy et al., 1998). It is required for splitting of the larval templates so that correct number of DLMs are formed (DeSimone et al., 1996; Roy et al., 1998; Cripps et al., 1998). During DLM development, it has been established that absence of ewg expression does not affects the fusion of myoblasts but the splitting of larval templates, resulting in fused and lesser number of DLMs (DeSimone et al., 1996). ewg expression is also present in the neurons innervating the IFMs. Introduction of neuron specific ewg minigene in ewg null mutants resulted in formation of fused DLMs with innervation pattern, a composite of that seen in two normal muscles. Introduction of muscle specific ewg minigene rescued both muscle phenotype and innervation pattern (DeSimone et al., 1996).

twist directs the transcription of a regulatory gene D-Mef2 (Drosophila-myocyte enhancer factor-2). In D-Mef2 mutants, abnormal patterning of DLMs (lesser number of DLMs) was observed, a phenotype similar to twist mutants (Cripps et al., 1998). However, unlike twist, D-Mef2 is not downregulated upon myoblast fusion. It is required for the formation of IFMs (Cripps et al., 1998). This suggests that D-Mef2 is regulated by some other gene(s) too. D-Mef2 is a homologue of vertebrate Mef2. In case of vertebrates it is known that Mef2 along with MyoD is necessary for formation of skeletal muscle fiber upon down regulation of twist after myoblast fusion (reviewed in Baylies and Michelson, 2001; Buckingham et al., 2003).

Along with the differentiation of the myoblasts, attachment sites are formed. stripe is a gene that has been extensively studied for its role in determination of the attachment sites for the developing IFMs. stripe mutants show total absence of DLMs (Costello et al., 1986). stripe encodes a Zn\(^{2+}\) protein and is a member of early growth response family of transcription factors (Lee et al., 1995). During larval muscle development, stripe is expressed at the muscle attachment sites (Volk and Vijayraghavan,
1994). A study on the basis of P-element insertion in the *stripe* locus has established that DLM development (fusion and formation of fibers) is not affected. *lacZ* expression was observed at the epidermis, but, after splitting of the larval templates into six fibers, *lacZ* expression was enhanced at the epidermis that serve as attachment sites for the developing muscles (Fernandes *et al*., 1996). Recent studies have shown that *Notch* (*N*) pathway helped in initiation of *stripe* expression (Ghazi *et al*., 2003). This study established the fact that *N* signaling influences proliferation, fusion, patterning as well as attachment of IFMs. This study demonstrated that adult myogenesis is influenced by an array of genes and some of the genes might be involved in more than one specific stage of muscle development.

Apart from the non-structural genes mentioned above, some of the structural genes involved during IFM myogenesis have been identified and their functions have been assessed. Mutations in *heldup*\(^3\) (*hdp*\(^3\)), which is one of the isoforms of *troponinI* (*TnI*) gene, affects the attachment of IFMs and TDT (Barbas *et al*., 1993). Immunofluorescence studies have shown that in case of *TnI* mutants elongation of muscle fibers after being attached to the tendons is severely affected (Nongthomba *et al*., 2004). Both *hdp*\(^3\) and *TnI* mutants have lower levels of *Act88F* (IFM specific gene) and as a result are flightless (Nongthomba *et al*., 2004). Genes like *parkin* and *canB2* are also involved in maintenance of muscle structure. Null mutants of *parkin* gene of *Drosophila* (ortholog of human *parkin*) exhibit defective locomotion, flight and muscle atrophy (Greene *et al*., 2003). Histological analysis revealed severe disruption in flight muscles. Viable *canB2* mutants too exhibit abnormal organization of IFMs (Gajewski *et al*., 2003). When IFM organization was followed using MHC-GFP, it was found that myogenesis was not affected, but, during the stage of attachment of the muscles, retraction of muscle fibers was observed. This phenotype was similar to that observed in *MHC* mutants and an isoform of *TnI* mutants.
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<td></td>
<td>Fusion of myoblasts</td>
<td>twi↑, N↑, duf↑</td>
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<tr>
<td></td>
<td>IPs</td>
<td>ewg↑, twi↓, N↓, duf↑</td>
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<td></td>
<td>Differentiation</td>
<td>sr↑</td>
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<tr>
<td></td>
<td>(Formation of muscle fibers)</td>
<td>twi↓, N↓, duf↓, apt↓</td>
</tr>
<tr>
<td></td>
<td>Attachment to epidermis</td>
<td>sr↑, Tn↑, rbp↑, canB2↑</td>
</tr>
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**Figure 3.** Schematic diagram of the role of various genes in IFM development. ↑ indicates expression of the gene is upregulated and ↓ indicates that the gene preceding this symbol is down regulated.
Genetic screens to identify flightlessness

Genetic screens have been performed to identify novel genes that are involved in flight or identify alleles of a known gene that influence flight muscle development. The screening allows identification of mutants- dominant as well as recessive.

EMS screening on autosomes especially, second and third chromosome have been done for identification of dominant flightless mutants. Most of the studies have identified structural mutants. Mhc gene, which is localized on the second chromosome, has been used as a target to identify dominant flightless mutants (Mogami et al., 1986; Cripps et al., 1994). Mhc alleles identified in these screens were homozygous lethal and showed dominant flightlessness. The myofibrils in these mutants were disrupted and showed split ends in the IFMs. EMS screen on chromosome 3R was targeted for Act88F (Cripps et al., 1994). The mutant alleles showed a wide range of phenotype including dominant flightlessness, but viability was not affected. Upon screening of chromosome 3L, a novel gene laker was identified. Some of the alleles of laker were lethal during embryonic stage, whereas viable mutations showed defective myofibrillar structure of IFMs and dominant flightlessness.

EMS screening performed by Nongthomba et al (1999) on second chromosome was targeted for generating recessive flightless mutants. The mutants showed a wide range of phenotypes- raised wing, normal wing position- but defective myofibril structure and disorganized IFMs. Some of the mutants were identified to be the alleles of Mhc and hdp3, while in case of some of the mutants, homology with any known genes could not be established.

All the screening studies mentioned above have identified structural mutants, which are usually dominant for flightless behavior. Our interest is in identifying genes required during myogenesis. Very few such screens have been performed. Flightless screening on X-chromosome is an example. One screen done by Deak et al (1982) identified two recessive mutants: stripe and erect wing. These genes were later identified to be important for the process of myogenesis. Null mutants of stripe were lethal so, heteroallelic mutants were generated to study the muscle phenotype. A later EMS screen identified more alleles of stripe (deLa Pompa et al., 1989).
Beside EMS screening there are other techniques discussed in subsequent section that serve as effective tools for identification of novel genes that are involved in myogenesis.

**Techniques implemented to identify a gene or its function**

Both forward and reverse genetic approaches can be used to identify genes that play a role in the developmental process. In a forward genetic approach, mutations are created randomly and a phenotype of interest is selected. Subsequently, the gene responsible for the phenotype is identified, cloned and mapped. EMS screening mentioned in the earlier section is one of the techniques of forward genetic approach. Reverse genetic approach rely on some prior knowledge of the gene, based on its expression pattern. This approach allows us to understand the cytological and molecular aspect of the gene as well as the functions it is involved in (Leiserson *et al.*, 2000; Chiang *et al.*, 2004). The steps, by which the function of the gene is identified is summarized in Fig. 4.

![Flow chart showing the steps involved in the reverse genetic approach.](image)

**Figure 4.** Flow chart showing the steps involved in the reverse genetic approach.

**Enhancer gene trapping technique**

A well-known technique that has been used to identify genes based on their expression pattern is enhancer gene trapping technique (Wolfner *et al.*, 1994). The technique relies on visualizing tissue specific lacZ gene (reporter gene), which is introduced into the genome. The reporter gene is introduced through a transpososon
called P-element. When P-element is inserted near the promoter of the gene of interest, 
*lacZ* is co-expressed with the gene of interest’s expression. This technique of identifying 
a gene expression is known as enhancer gene trapping (Fig. 5). The P-element is an 
artificial vector that contains-

- A visible marker, that is, mainly an eye color gene (rosy or white). Presence of the 
eye color confirms presence of P-element.
- Bacterial plasmid sequences, that includes a selectable marker like ampicillin 
resistance to facilitate cloning of the gene sequences adjacent to the insertion site 
of the P-element.
- *lacZ* sequences, fused to a weak but general promoter to detect the developmental 
expression pattern of the gene of interest.

![Figure 5](image_url)  
*Figure 5.* Schematic representation of enhancer gene trapping technique. Pr represents the 
promoter of the gene of interest. When the P-element gets inserted near the promoter (Pr), 
*lac Z* expression is found in the regions where the gene is expressed.

The enhancer gene trapping technique also allows generation of new mutations by 
excising the P-element. To mobilize the P-element after being inserted in the germ line, a 
transposase is required. The transposase mobilizes the P-element and allows it to be 
excised out of its original position. Remobilization of P-element often generates flanking 
sequence deletions by imprecise excisions. Imprecise excision allows observation of 
different phenotypes thus, helping in determining the various cellular processes or 
pathways the gene is involved.
Single P-element insertions have been used to identify novel genes (Cooley et al., 1988). To establish a gene library of all the genes in their native and mutated state, the Berkley Drosophila Gene Disruption project (BDGP) was started (Spradling et al., 1999). In this project single P-element insertions in nearly all of the genes (~3600 genes) were performed.

Our candidate gene 1424 was initially identified by Keshishian and his co-workers as it labelled subsets of embryonic motoneurons (Keshishian et al., 1993). It was also identified from the single P-element insert collection generated by BDGP (Spradling et al., 1999). Reporter gene expression showed its presence in larva too. Its expression was found in the CNS, imaginal discs and motoneurons (Meister et al., 1995; Flybase). During metamorphosis, expression of this gene was observed in the larval muscles that serve as templates for DLM development and in regions of the thoracic CNS that corresponds to the sites of DLM neurons (Fernandes, 1998; Yale University) (Fig.6). Based on these observations we hypothesized that 1424 gene has a role to play in adult neuromuscular development.
Figure 6. Reporter gene expression pattern during various stages in the life cycle of *Drosophila*. (A) Embryo-reporter gene expression is seen in the neurons and allover the embryo. (B) Larva-Reporter gene expression (blue) is observed in the CNS. (C, D, E) expression pattern in pupae. (D and E) expression of the 1424 gene in the larval thoracic muscles that serve as templates for the formation of DLMs. APF=after pupa formation. Source: Pictures were taken from Andrew Lee’s poster presentation (2002).
**1424 gene**

Transposon PZ was used for insertion in the 1424 locus (Spradling et al., 1999). The insertion was located at 49E1-49E2 region of chromosome 2R by in situ hybridization (Keshishian et al., 1993, http://flybase.org) (Fig.7). 1424 was identified to be one of the lethal insertions under homozygous conditions by BDGP (Spradling et al., 1999; http://flybase.org, 2000). It was mapped and cloned by BDGP (http://flybase.org; Spradling et al., 1999). It synthesizes two transcripts, 1424-RA and 1424-RB. Both of these transcripts synthesize the same protein (translated product of the transcripts have identical number of amino acids) although they have separate 5’ends. The P-element insertion in 1424 is localized in the 5’ UTR of the 1424-RA transcript. 1424 encodes a product with translation initiation factor activity involved in salivary gland death (Gorski et al., 2003) that is a part of the mRNA cap complex. It has been sequenced and its amino acid sequence contains a middle domain of eIF4G (http://flybase.org; Spradling et al., 1999). Conserved domain search using protein-protein BLAST showed that, the translated sequence of 1424 aligns with MIF4G, MA3 and eIF5C (Fig. 8). MIF4G is the middle domain of eukaryotic translation initiation factor eIF4G, MA3 domains are present in different translation initiation factors. The middle domain of eIF4G (MIF4G) has been shown to be the binding site for 40S ribosome, RNA and eIF4C complex (Hentze, 1997). The MA3 domains have a wide range of function depending on the molecules they are interacting with. Two MA3 domains are present in MIF4G that serve as attachment sites for eIF4A and helps in the initiation of translation. However, in presence of Pdcd genes it is not involved in the binding to eIF4A and thus prevents translation and spreading of tumor (Yang et al., 2004). eIF5C is another translation initiation factor in eIF4C complex. On the basis of the conserved domains 1424 showed homology to NAT1 gene in Homo sapiens (www.NCBI.org). NAT1 is a translational repressor.
Figure 7. Genomic location of 1424 gene on 2R and the insertion site of the P-element (Blue triangle). Map was obtained from Flybase.

Figure 8. Putative conserved domains of 1424 gene that have been detected, by Protein Blast search.
Generating mutants by excision

Being a recessive lethal mutation, 1424 gene stock was maintained in the heterozygous state in form of a balanced stock (1424/CyO). Presence of a balancer (CyO, in our case) prevented meiotic recombination between homologous chromosomes in females (in males, meiotic recombination is absent). As a result, same set of heterozygotes were generated in each generation thus, helping to maintain the stock by mass transfer of adults instead of by mating specific genotypes each generation.

One way to obtain viable mutants was through P-element excision. \( \Delta \) 2-3 transposase was used for excising out the P-element to generate excision lines (Andrew Lee, 2002; Undergraduate, Miami University). During excision, it is possible that P-element alone might be excised out (precise excision) or some part of the candidate gene or other neighboring genes might be excised out (imprecise excision). Like precise excisions, imprecise excisions are likely to generate viable mutants (1424*/ 1424*). The possibility that neighboring genes might be excised was ruled out. A neighboring gene vestigial (vg) (CG3830) is located \( \sim \) 30 Kb upstream of 1424 and another CG13319, is located 7kb downstream from the 3’ end of the 1424 gene (Fig. 7). The large distances from 1424 make it unlikely that the sequences from these genes will be deleted in an excision event.
Specific Aims

Overall goal: To identify a role for 1424 gene in flight muscle development by behavior and muscle phenotype analysis.

To achieve this goal, the following specific aims were designed.

Aim 1: To determine the developmental stage, during which 1424 lethality occurs.
Hypothesis: Lethality might be occurring throughout the life cycle or at specific stages.

Aim 2: To examine the viability of flies homozygous for the excision.
Hypothesis: Since 1424 is an insertional lethal, we hypothesize that excision of the P-element will revert lethality and also generate viable mutants.

Aim 3: Examine complementation of 1424 lethality by 1424 excisions.
Hypothesis: Some of the excision lines will generate viable flies when complemented with the parent strain.

Aim 4: To test the behavior of flies homozygous for the excision.
Hypothesis: Viable mutants generated through excisions will show flight defect when compared to wild type.

Aim 5: To examine IFM and innervation phenotypes of homozygous excision lines.
Hypothesis: Excision lines that display flight defects will have muscle defects or innervation pattern alterations, or both.
**Aim 1: To determine the developmental stage during which 1424 lethality occurs.**

**Rationale**

*Drosophila* undergoes four stages of development during its life cycle: embryo, larva, pupa and adult. P-element insertion in 1424 is lethal (Spradling *et al.*, 1999). Determining the stage during which the lethality occurs will indicate that the candidate gene is required at that stage. This information will help us find out a probable role of 1424 gene.

**Hypothesis**

Lethality might be occurring throughout the life cycle or at any specific stages.

**Methods**

**Stocks**

The excision lines were generated by Andrew Lee, Miami University, 2000, by using ∆2-3 transposase. 42 excision lines were generated and named J1-J91 in a random order (Table 1).

<table>
<thead>
<tr>
<th>Excision lines (1424*/CyO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J1</td>
</tr>
<tr>
<td>J31</td>
</tr>
<tr>
<td>J41</td>
</tr>
<tr>
<td>J52</td>
</tr>
<tr>
<td>J86</td>
</tr>
<tr>
<td>J91</td>
</tr>
</tbody>
</table>

**Table 1:** Nomenclature assigned to the forty-two excision lines generated. Parent strain (1424/CyO) was crossed to ∆2-3 transposase to excise out the P-element to generate these lines. The genotype of these lines were denoted as 1424*/CyO.
Generation of 1424/CyO-Act GFP strain

1424/CyO flies were obtained from Keshishian Lab, Yale University. sco/CyO-Act GFP flies were obtained from Dockendorff Lab, Miami University, Ohio. 1424/CyO virgins were crossed to Sco/CyO-Act GFP males. Since this isoform of Actin is expressed in different tissues, Act GFP was used to distinguish between 1424/ CyO embryos and Sco/ CyO embryos. Under a simple fluorescent microscope GFP expressing embryos and larvae were easily distinguishable from non-GFP embryos and larvae. GFP expressing larvae (1424/CyO-Act GFP) were isolated and transferred in vial containing fly food. Adults that emerged after pupal stage were transferred to bottles for expansion. The viable progeny were expected to have two genotypes: 1424/1424 and 1424/ CyO-Act GFP (Table 2).

<table>
<thead>
<tr>
<th>1424/CyO; Act GFP</th>
<th>X</th>
<th>1424/CyO; Act GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓</td>
<td></td>
<td>F1 1424/1424(non- GFP); CyO- Act GFP/ CyO;-Act GFP; 1424/ CyO- Act GFP</td>
</tr>
<tr>
<td>Expected Ratio</td>
<td>1</td>
<td>: 1 : 2</td>
</tr>
</tbody>
</table>

Table 2: Self cross set up to differentiate between embryos heterozygous and homozygous for 1424. The numbers in the bottom row indicates the expected ratio of that genotype. CyO-Act GFP homozygotes are lethal. The expected ratio between 1424 homozygous and 1424 heterozygous flies was 1:2.

Determination of viability of 1424/1424

a. Viability during embryonic stage: 1424/CyO-Act GFP flies were placed in an egg lay chamber for two hours. Under simple fluorescent microscope, total number of embryos GFP and non- GFP were first counted, and thereafter the non- GFP embryos were isolated and transferred to a new apple juice agar plate. The viability of these 1424 homozygous embryos was determined by counting the number of unhatched embryos at the end of 24 hr period, by which time 1st instars are known to emerge. The experiment was repeated eight times. A total of 346 embryos (1424/1424) were analyzed.
b. **Larval viability:** 1\textsuperscript{st} instar larvae (1424/1424) were collected from egg lay chambers and transferred to vials containing fly food. The numbers of larvae reaching the 3rd instar stage were counted. Thereafter, the numbers of 3rd instars reaching 0hr pupal stage were counted. A total of 204 1\textsuperscript{st} instars were collected for analysis.

c. **Viability during pupal phase:** 0hr pupae (P0) were collected on a moist kim-wipe in a petri-dish, and observed through metamorphosis. Based on the external characteristics of the developing pupae, pupal stages were assigned (P0, P1,.....) Staging was done according to Ashburner, 1989 (Table 3). The pupae that ceased to show further development and turned black in color were identified as dead. Approximately one hundred pupae were observed for these viability studies.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0 (0hr APF)</td>
<td>Transparent white colored pupa</td>
</tr>
<tr>
<td>P4 (12 hr APF)</td>
<td>Pupal case is yellowish, head eversion has occurred.</td>
</tr>
<tr>
<td>P5 (13-14 hr APF)</td>
<td>Presence of a green body at the posterior part of the thoracic region. Malphigian tubules are white in color.</td>
</tr>
<tr>
<td>P7-P8 (17-20hr APF)</td>
<td>Malphigian tubules are green in color, the green body is in between the malphigian tubules. Head, thorax, abdomen, legs and wing demarcation are clearly visible.</td>
</tr>
<tr>
<td>P14-P15 (92-95 hr APF)</td>
<td>Bristles present on the head (Antennae), thorax, abdomen and legs. Wing color is black. Development is complete</td>
</tr>
<tr>
<td>P16 (96hr APF)</td>
<td>Adults eclose.</td>
</tr>
</tbody>
</table>

**Table. 3:** External phenotypes of some of the pupal stages (Ashburner, 1989).
Live imaging of 1424/1424 embryos

1424 homozygous flies were placed in an egg laying chamber for two hours. The embryos were then collected and dechorionated in 50% Clorox solution and then rinsed thoroughly in distilled water. The embryos were then mounted in halocarbon oil and observed using DIC optics. A total of 65 embryos were observed till 9.5 hours AEL (after egg lay). In the embryos of wild type, 9.5 hr AEL corresponds to stage 12 (Campos-Ortega and Hartenstein, 1997). During this stage, the germ band shortens and segmentation becomes prominent.

Results

Mating of 1424/CyO siblings did not generate any 1424/1424 flies indicating that the P-element insertion under homozygous condition is lethal. To observe when the lethality was occurring in homozygous condition, siblings of 1424/CyO- Act GFP flies were mated. The expected ratio of GFP (1424/CyO- Act GFP; CyO-Act-GFP/ CyO-Act-GFP) and non- GFP (1424/1424) embryos was 3:1 (Table 2). According to our observation the actual ratio varied between 10%-20% and emergence of 1424/1424 larvae was only 40% (Fig.9A). This result indicates that 1424 is necessary during the embryonic stage but emergence of larvae indicates that the insertion in 1424 locus might be semi-lethal. Since 1424 embryos do hatch into 1\textsuperscript{st} instars, their viability was followed during the larval and the pupal stages. Only 55% of 1\textsuperscript{st} instars were able to reach the 3\textsuperscript{rd} instar stage (Fig. 9B). The 3\textsuperscript{rd} instar stage was easy to identify because during this stage the larvae crawl out of the food, on the wall of the vials. No lethality was observed during the transition from 3\textsuperscript{rd} instar to pupae (0hr). Further analysis showed that viability was affected during metamorphosis as well. Approximately, 88% of pupae were able to complete all the pupal stages (Ashburner, 1989; Table 3) but only 36% of them eclosed (Fig. 9C). Since lethality occurs during embryonic, larval and pupal stages, it can be concluded that 1424 expression is required throughout the life cycle (Fig 10D).

Upon observation of live dechorionated 1424/1424 embryos for nine and half hours AEL (starting from 2hr AEL) we found that 30% of the embryos failed to reach stage 4 i.e. the cellular blastoderm stage (according to Ortega and Hartenstein, 1997). This stage occurs between 2-3hr AEL. At the end of 9.5 hr AEL (stage12- germ band
shortening and prominent germ band segmentation), 25% of the embryos showed the stage specific morphogenetic movement.

**Discussion**

In a genome wide screening of single P-element insertion, the insertion in the 1424 locus has been shown to be lethal (Spradling *et al.*, 1999). It was also known that 1424 gene has 2 transcripts- 1424-RA and 1424-RB (http://flybase.org). The P-element insertion has been localized in the 5’UTR region of 1424. This region is the transcription initiation site of 1424-RA transcript. Our results indicated that P-element insertion affected the viability of 1424 homozygous flies. Based on previous studies, generation of 1424 homozygous flies indicate that one of the transcript has to be functional. Our results showed that viability was affected during embryogenesis and metamorphosis. During the embryonic stage, since most of the embryos failed to reach the cellular blastoderm stage it indicates that 1424 expression is required at early stages of embryogenesis. As lethality rate after stage 12 was not determined, it remains unclear if viability is affected at later stages of embryonic development. Lethality was observed throughout the life cycle in 1424 homozygotes, which strongly suggests that the P-element insertion in 1424 is semi-lethal.

During metamorphosis maximum lethality occurred in P14-P15 stage (Fig. 9 C and D). These were the stages just before eclosion. Studies on *Manduca* have shown that one of the factors that results involved in lethality during late stage pupae is the ecdysteroid. It is known that the ecdysteroid level decreases before eclosion. A drop in ecdysteroid titer is required for release of eclosion hormone and initiation of pupal and adult ecdysis (Schwartz and Truman, 1983). Experimentally increasing the level of ecdysteroid just prior to the emergence of adult resulted in non-eclosion (Weeks, 2003 review). Also studies by Kimura *et al.*, 1990, in *Drosophila* have shown that, proper development of head muscles is required for eclosion. Expression pattern of 1424 in pupae confirms the presence of the gene in CNS (Fig. 6). Since we do not know the exact genes that are regulated by 1424, our results indicate that the candidate gene might be involved in regulation of the hormonal levels necessary for eclosion or the appropriate development and function of head muscles.
**Figure 9:** Viability of 1424/1424 at various developmental stages. (A) Percentage of 1\textsuperscript{st} instar larvae emerging from the embryos. A total of 346 embryos were collected and only 138 1\textsuperscript{st} instar emerged. * denotes a significant p-value (<0.05). (B) Percentage of 1\textsuperscript{st} instar larvae reaching 3\textsuperscript{rd} instar larval stage. Of the 204 1\textsuperscript{st} instar larvae collected, only 113 reached the late 3\textsuperscript{rd} instar stage. All the 3\textsuperscript{rd} instar pupated (0hr pupae). * denotes a significant p-value (<0.05) when compared to 1\textsuperscript{st} instar larvae. (C) Percentage of viable pupae at various pupal stages (stage numbers were assigned according to Ashburner, 1989). 112, 0hr pupae were collected and analyzed. Maximum lethality was observed between P15 and eclosion time. (D) Synopsis of A, B and C. Only 7.9% of adults emerge on average per 100 embryos in 1424/1424.
**Aim II: To examine the viability of the flies homozygous for the excision.**

**Rationale**

P-element insertion in 1424 gene is semi-lethal. Excision lines were generated (Table 1) to serve two purposes. First, to generate viable homozygous mutant flies, which will indicate that presence of P-element caused the lethality. The second purpose was to generate small excisions in the candidate gene and generate viable mutants to test the role of the candidate gene beyond embryonic and larval stages.

**Hypothesis**

Excision of the P-element will revert the lethality and also generate viable mutants.

**Methods**

**Generation of 1424*/1424* flies**

Siblings of each excision line (Table 1) were mated. Twelve virgin heterozygous (1424*/ CyO) flies were crossed to six heterozygous males (1424*/ CyO) (Table 4). They were maintained at two temperatures -18°C and 25°C- to observe if viability of homozygous flies is enhanced at lower temperature. Two day old flies from the F1 generation were used for counting the number of heterozygous and homozygous flies. The fraction of homozygous flies (1424*/1424*) generated was determined for all individual lines. This was calculated by dividing the total number of homozygotes generated with the total number of heterozygotes generated. The expected ratio was 0.5 (Table 4). Two successive rounds of crosses for each temperature were set up. The average of the two results at 25°C and 18°C were compared.
♀(12) 1424*/CyO  X  1424*/ CyO  ♂(6)

F1  1424*/ 1424*  ; 1424*/ CyO  ; CyO / CyO

Phenotype  Straight wing : Curly wing : lethal

Expected Ratio  1 : 2 = 0.5

Table. 4: Self-cross of the excision lines. The homozygous recessive will have straight wings and their expected ratio to the heterozygous will be 0.5 i.e. 50%. CyO=curly. Curly homozygous is not considered in the ratio because they are lethal. The numbers in the parenthesis indicates the number of virgins and males collected for cross set up.

Results

The expected ratio between the homozygous (1424*/1424*) and the heterozygous (1424*/CyO) was 1:2 (Table 4). The excision lines fell into two categories:

a. The lines that did not generated viable homozygous flies. Seventeen lines (J5, J9, J14, J16, J17, J18, J19, J21, J24, J28, J30, J31, J33, J37, J39, J42 and J91), out of forty two excision lines were not able to generate homozygous flies. It is likely that in these lines due to imprecise excision of the candidate gene, the functional ability of the gene might be affected.

b. The lines that generated viable homozygous flies. Homozygous flies were obtained from the remaining twenty-five lines (Fig. 10). Of these, twenty-five lines four lines (J1, J25, J36 and J52) generated the expected ratio (0.5) of homozygous flies both at 18°C and 25°C (maximum difference of 10%). This result indicates that in these lines the viability of the homozygous flies was restored upon excision of the P-element.
Thirteen out of twenty-one lines generated homozygous flies less than the expected ratio at both the temperatures (Fig. 10). Based on results of the self-cross we divided these seventeen lines into three groups (Table 5). These seventeen lines that had generated flies homozygous for excision would be used for behavioral tests and muscle phenotype analysis.

Of the remaining eight lines, four lines- J11, J23, J49, and J55 generated homozygous flies only at 18°C and the other four lines- J2, J22, J40 and J46 generated homozygous flies only at 25°C. In both cases, viability was very low (ratio is less than 5%), they can be referred as escapers. The same conclusion holds true for the lines that had generated homozygous flies at 25°C. These eight lines will not be put through flight test or phenotypic analysis of muscle as the emergence of homozygous flies is very low.

Our hypothesis, that excision of P-element will generate viable mutants was partially supported, as some of the lines were not able to generate viable homozygous mutants.

Discussion

When a P-element is excised, the gene may undergo a precise excision i.e. without taking away neighboring DNA, or an imprecise excision, i.e. some part of the neighboring DNA gets excised along with the P-element. The lines that did not generate any homozygous flies (1424*/1424*) represents a scenario in which the P-element had been excised imprecisely, resulting in loss of function of the candidate gene leading to lethality. These results indirectly confirm that presence of 1424 gene is necessary for viability.

The excision lines that had generated the expected ratio of 1424* homozygous flies (Table 5, 1st row) represent a different scenario wherein either precise excision of the P-element had occurred or that the nature of imprecise excision is small enough to allow for viability as well as normal functional ability of the gene. In the lines that show reduced viability (Table 5, 2nd and 3rd row), it is possible that imprecise excision has occurred or that some secondary lesions might have occurred as a result of the excision. The reason behind the reduced viability remains to be determined.
Figure 10. Viability of 1424* homozygotes at 25°C and 18°C. The ratios are the average of the two rounds.

Table 5. Grouping of the seventeen lines that have generated homozygous flies at both 25°C and 18°C.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio</th>
<th>Excision lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>0.5</td>
<td>J1, J25, J36, J52</td>
</tr>
<tr>
<td>2nd</td>
<td>0.4-0.1</td>
<td>J6, J7, J10, J26, J29, J32, J34, J35, J41</td>
</tr>
<tr>
<td>3rd</td>
<td>&lt;0.1</td>
<td>J13, J20, J27, J86</td>
</tr>
</tbody>
</table>
Aim III: Examine complementation of 1424 lethality by 1424 excisions.

Rationale
Complementation test will be a tool to confirm that excision lines that were generated are capable of generating viable 1424/1424* flies.

Hypothesis
Some of the excision lines (1424*/CyO) will generate viable flies when complemented with the parent strain (1424/ CyO).

Methods
Complementation test
Twelve virgin 1424/ CyO (parent strain) flies were crossed to six heterozygous males (1424*/ CyO) in a vial containing fly food (Table 6). Crosses were maintained at two temperatures, 18°C and 25°C. Two day old flies of F1 generation were used to determine the proportion of curly winged (1424/CyO; 1424*/CyO) and straight winged (1424/1424*) flies. Two sets of experiments were performed for each temperature.

\[
\begin{array}{c}
\text{♀ (12) 1424/ CyO X 1424*/ CyO ♂ (6)} \\
\downarrow \\
\text{F1 1424*/1424; 1424/ CyO; 1424*/ CyO ; CyO / CyO} \\
\text{Phenotype straight wing : Curly wings : lethal} \\
\text{Expected Ratio 1 : 2 = 0.5}
\end{array}
\]

Table. 6: Complementation test cross. The expected ratio between the curly and straight winged flies will be 50% i.e. 0.5. CyO homozygous flies are lethal. The numbers in the parenthesis indicates the number of virgins and males collected for cross set up.
**Results**

In a cross between a heterozygous excision line and the parental strain, the expected ratio of straight winged flies (1424/1424*) to curly winged flies (1424/ CyO and 1424*/ CyO) was 1:2 (Table 6). Out of 42 lines, eleven lines (J5, J10, J18, J23, J25, J26, J30, J31, J36, J41, and J52) showed the expected ratio (~10% difference). Twenty-eight lines generated 1424/1424* flies lower than the expected ratio (10-15% variation) (Fig. 11) (Table 8), indicating a partial complementation has occurred. This is suggestive of imprecise excision of P-element. Except for J2 and J15, all of the forty excision lines complemented with the parent and generated straight winged flies (1424*/1424) (Table 7).

The J16 line behaved differently. This line generated the expected ratio at 18°C, but not at 25°C. Two lines (J39, J91) generated viable 1424/1424* at 18°C only. Although they didn’t generate the expected ratio, the results indicate that the viability of these lines is enhanced at lower temperature or they might be escapers. Escapers are flies that emerge in very low numbers from a cross that is expected to be lethal. The exact reason behind the emergence of escapers is not fully known.

**Discussion**

The lines that generated the expected ratio of 1424/1424* indicated that the excision of the P-element didn’t affect the functional ability of the candidate gene. When the viability of these lines was compared to the viability of the homozygous mutants, only three of lines (J25, J36, J52) showed the expected ratio in both the crosses (Table 8). It is possible that in these lines either precise excision had occurred or a very small part of the gene was excised as a result the functional ability of the gene was restored (Fig. 12). In the case of J1 line, which showed normal viability (Table 5) but complemented partially (Table 7) indicates that there is a dosage sensitivity. In case of other lines that complemented the parent strain (Table 7) but generated lesser ratio or no viable homozygous flies (1424*/1424*); (Table 8), it is possible that either the extent of excision might have been large as a result the viability of the homozygous flies was affected. However, when complemented to the parent strain, the parent strain being semi-
lethal, the viability of 1424/1424* was not affected or, it might be due to presence of secondary mutations in the excision lines that had affected the viability of the homozygous mutants.

The lines that had generated lesser ratio of 1424/1424* and homozygous flies (1424*/1424*) in the self-cross confirmed that the candidate gene has undergone imprecise excision. Two probable reasons for variability in the viability are:

a. Excision might have occurred in a very small region of 5’that didn’t affect the sequences upstream of the insertion site. As a result the functional ability of 1424-RA might not have been affected to a greater extent. In this case we assume that the viability will not be affected to a greater extent.

b. Excision might have occurred in the 5’ region of the 1424 gene, but the promoter was not affected. As 1424-RB has a separate 5’ UTR it is possible that only 1424-RB was being translated. In this case the translated amount will be lesser than wild type. Decrease in the protein translated might have affected the viability of the flies drastically. In this case we predict that the viability of the homozygous flies will be very low.

The two lines that didn’t complement the parent strain (Table 7, 3rd group) indicated that, either the 5’ end of the 1424 gene might have been excised out or both the promoter and the 5’ end have been excised out resulting in a nonfunctional gene (Fig. 12).

Since our goal was to see if the lines generating homozygous flies (Table 5) were able to complement the parent strain, we found that only three of the lines were able to do so; others did so partially (Table 8). These two results confirm that excision of P-element was able to restore viability to some extent suggesting that functional ability of the candidate gene was at least partially restored in these lines. These seventeen lines will be used for behavior and muscle phenotype analysis. The tests will help us determine the function of gene in flight muscle development.
Figure 11. Complementation of 1424 by the excision lines. Tests were carried out at 18°C and 25°C.

Table 7. Grouping of the excision lines according to the fraction of 1424/1424* flies generated.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio</th>
<th>Excision lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>0.5</td>
<td>J5, J10, J18, J23, J25, J26, J30, J31, J36, J41, J52.</td>
</tr>
<tr>
<td>2nd</td>
<td>0.4-0.1</td>
<td>J1, J6, J7, J10, J11, J13, J14, J17, J19, J20, J21, J22, J27, J28, J29, J32, J33, J34, J35, J37, J40, J42, J46, J49, J55, J86.</td>
</tr>
<tr>
<td>3rd</td>
<td>0.0</td>
<td>J2, J15.</td>
</tr>
</tbody>
</table>
Figure 12. Schematic representation of possible occurrence during excision of P-element followed by the outcome of the complementation test. represents the promoter region of the 1424 gene, represents P-element and represents the ORF (open reading frame.) of the gene.
<table>
<thead>
<tr>
<th>Excision lines</th>
<th>Viability of 1424*/1424*</th>
<th>Viability of 1424/1424*</th>
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<tbody>
<tr>
<td>J1</td>
<td>Normal</td>
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</tr>
<tr>
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<td>Affected</td>
</tr>
<tr>
<td>J10</td>
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</tr>
<tr>
<td>J13</td>
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<td>Affected</td>
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<td>Affected</td>
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<td>J52</td>
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<td>Normal</td>
</tr>
<tr>
<td>J86</td>
<td>Affected</td>
<td>Affected</td>
</tr>
</tbody>
</table>

**Table 8.** Comparison between the viability of the complemented group and homozygous mutant generated by the seventeen excision lines.
**Aim IV: To test the behavior of the flies homozygous for excision.**

**Rationale**

*lacZ* expression in early pupae had indicated that 1424 is expressed in developing DLMs (Fig. 6). The expression pattern suggested that this gene is involved in the development of IFMs. Flight test of the viable mutants (1424*/1424*) will be a means to confirm the role of the gene in flight muscle development.

**Hypothesis**

Viable mutants generated through excisions will show significant flight defect when compared to wild type.

**Methods**

**Stocks and crosses**  
*ry*<sup>506</sup>/*ry*<sup>506</sup> was obtained from Bloomington Stock center. OR, 1424/1424 and 1424*/1424* were generated in the laboratory. 1424/+ was generated by crossing 1424/CyO and OR (Table 9).

**Flight test**

The column flight test (Greene *et al.*, 1986) was performed with a few modifications. The column was divided into eight levels, each level being 5 cm apart from the other. A mineral oil coated transparent sheet was then inserted inside the column so that the inner wall of the column looked oil coated. The bottom of the column was then attached to a funnel, which emptied into a vial. The funnel at the lower end was denoted as level 9 (Fig. 13A). The column was attached to a clamp stand so that it remained vertical. In each round, twenty flies were dropped down through a funnel (present at the top of the column) into the column. The number of flies in each level was noted down. In general, wild type flies tend to fly upwards and get stuck in the upper level of the column. 500 flies (25 rounds) from each of the seventeen excision lines along with OR, 1424/+, *ry*<sup>506</sup>/*ry*<sup>506</sup> and 1424 homozygous were put to flight test. Flight index
for each round was then calculated (Greene et al., 2003) (Fig. 13B). Average flight index was calculated thereafter.

<table>
<thead>
<tr>
<th>♀ 1424/CyO</th>
<th>X</th>
<th>+/+ ♀ (OR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 1424/+</td>
<td>CyO/+</td>
<td>(Straight wing) (curly wing)</td>
</tr>
</tbody>
</table>

Table 9. Generation of 1424/+ flies. The straight winged flies were sorted out and 2- day old flies were used for flight test.

Figure 13. (A) Schematic diagram of the flight test column. The levels are denoted in the descending order i.e. level 1 is the top level whereas the level nine is the bottom level. (B) Calculation of flight index.

B. Calculation of Flight index (FI)

Weighted average = no. of flies stuck in a level X score of that level

<table>
<thead>
<tr>
<th>Level</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tbody>
<tr>
<td>Score</td>
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<td>7</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

FI = Sum of weighted average of each level

total number of flies used X highest score
Results

Of the seventeen excision lines (Table 5) that had generated homozygous flies in the self-cross, flight test were performed on twelve lines. Flight test of the five lines J13, J20, J27, J32 and J86 couldn’t be performed because sufficient number of homozygous adults could not be generated. The controls for flight-test were- \( \text{ry}^{506}/\text{ry}^{506} \) and Oregon R (OR). In our laboratory OR is always used as the wild type. \( \text{ry}^{506}/\text{ry}^{506} \) was used as wild type, as it was the genetic background for P-element insertion. We found that \( \text{ry}^{506}/\text{ry}^{506} \) showed significant defect in flight ability when compared to OR (Fig. 14).

The flight index is a measure of the flight ability of a particular genotype. Of the twelve excision lines analyzed, all of them exhibited significant difference in flight index when compared to OR (Fig. 14). When compared to \( \text{ry}^{506}/\text{ry}^{506} \), seven excision lines showed significant defect in flight ability \((p < 0.05)\) (Fig. 14). 1424 homozygous and \( \text{ry}^{506}/\text{ry}^{506} \) exhibited no significant difference in flight index \((p=0.18)\) although 1424/1424 showed significant difference when compared to OR. On comparison between 1424/+ and 1424/1424 significant flight defects was observed \((p=0.0001)\). No significant difference in flight index was observed when flight index of 1424/+ was compared to OR and \( \text{ry}^{506}/\text{ry}^{506} \).

Our hypothesis that homozygous mutants \((1424^*/1424^*)\) will show significant flight defect when compared to wild type was partially supported as only seven of the twelve excision lines showed significant flight defects.

Discussion

All the genotypes except 1424/+ showed significant difference in flight ability when compared to the wild type (OR) or \( \text{ry}^{506}/\text{ry}^{506} \). Presence of one copy of wild type gene might have restored the flight ability of 1424/+ flies. 1424 homozygous flies did not exhibit any significant flight defect when compared to \( \text{ry}^{506}/\text{ry}^{506} \). The probable reason might be that they are escapers (viability percentage of 1424/1424 was less than 10%, Fig. 10D) or the P-element insertion being semi-lethal. On comparison with the \( \text{ry}^{506}/\text{ry}^{506} \) some excision lines showed no significant flight defect. In case of J1, that showed normal viability (Table 5) and behavior (Fig. 14) but reduced viability in complementation test (Table 7) a very small imprecise excision might have occurred,
resulting in dosage sensitivity. Some excision lines showed no viability defect (J25, J36 and J52) (Table 5, group 1) and complemented the parent strain but exhibited significant flight defect (Fig. 14). These lines likely represent imprecise excisions; it is also likely the phenotype is a result of secondary mutations. The excision lines, J10, J26 and J41, which show reduced viability, but complemented the parent strain (Table 8) and showed flight defect, presents a scenario that strongly indicates the possibility of secondary lesions due to excision. Excision line, J35 showed reduced viability, didn’t complement the parent strain (Table 7) as well as showed flight defect. This result strongly indicates the possibility of imprecise excision, as well as dosage sensitivity. Excision lines J6, J7, J29 and J34 showed lesser viability as homozygous, but showed no significant flight defect. It is possible that the two transcripts synthesized by 1424 gene, are expressed at two different sites. Excision might have affected one of the transcripts and not the other. In that case, one of the transcripts might be required for CNS development whereas the other might be required for normal flight behavior. The excision lines that show viability defect as well as flight defect strongly indicate that imprecise excision had occurred in those lines. This observation indirectly confirms that 1424 is required for proper flight ability.

The flight test and the viability tests showed that 1424 gene was not only essential for viability but also in the proper neuromuscular development of adult flight muscles. Analysis of flight muscle structure as well as the innervation pattern will help us confirm a role for the candidate gene.
Figure 14. Comparison of flight ability of the excision lines, 1424/1424 and 1424/+ to the controls (ry506/ry506, OR). The * indicates that there is a significant decrease (p<0.05) in the flight ability of that particular genotype when compared to ry506/ry506 flight ability and φ indicates that the flight index is significant to the Oregon R (OR) (p<0.05).
**Aim V: To observe muscle phenotype of the homozygous excision lines.**

**Rationale**

1424 expression was observed in larval templates during metamorphosis, indicating that it might be required for DLM development and in motoneurons. Muscle phenotype analysis will be a means to correlate the expression pattern with flight.

**Hypothesis**

Excision lines that display flight defects will show muscle or innervation pattern alterations or both.

**Methods**

**Muscle phenotype analysis**

Thoraces of two days old adult flies were separated from the head and abdomen and fixed in 4% paraformaldehyde overnight. They were washed in PBS, followed by 0.85%NaCl and 1:1 solution of 0.85%NaCl and 100% EtOH. Thereafter they were dehydrated and embedded in paraffin (protocol was obtained by personal communication with Dr. Robert Schultz from South Western Medical Center- Texas) for either cross-section or longitudinal sections. 10µm sections were taken with a microtome and collected on gelatinized slides. The slides were then kept on a slide warmer overnight. Thereafter, the paraffin attached to the sections was removed by dipping the slides in Xylene (30min). The sections were rehydrated in an ethanol series (100%, 90%, 70%, 50% and 30% for 2min in each concentration) and then stained in 2%methylene blue (1min). Permount was used for mounting the sections. Thoracic sections were viewed under a compound microscope with a 10x objective. An image of one of the sections from each thorax was recorded. Minimum of ten thoracic sections were analyzed for each genotype. The genotypes analyzed were- OR, ry<sup>506</sup>/ry<sup>506</sup>, 1424/1424, 1424/CyO, J1, J6, J7, J10, J20, J25, J26, J29, J34, J35, J36, J41 and J52. Cross-sections were used for analyzing the DLMs and longitudinal sections are used for analyzing the DVMs. The number of IFMs in one hemi-segment of each line was examined and compared with the wild type. Unpaired Student’s t-test was used to analyze significance of any difference.
The fraction of the thoracic area occupied by the DLMs and DVMs was calculated using Image Pro Express software.

**Results**

Sectional analysis of the thorax helps to observe the patterning of DLMs and the DVMs (Fig. 15) and helps to calculate the fraction of area occupied by them in the thorax.

**Figure 15.** Schematic diagram of transverse section of thorax. a-f are the six DLMs. DVM I has three fascicles, DVM II has two fascicles and DVM III has two fascicles. TDT is the jump muscle.
**DLM Phenotype**

There are six pairs of DLM fibers that can be easily identified thoracic cross-sections. In case of OR, all the sections showed 6 pairs of DLMs (Fig. 17A). Similar phenotype was observed in case of ry^{506}/ry^{506} (Fig.16 and Fig. 17A), which was used as a control. 1424 homozygotes showed some aberration in the number of fibers but the difference was not significant when compared to ry^{506}/ry^{506} (Fig 17A). Among the excision lines, the number of DLMs was variable in most of the cases, but only four lines (J25, J26, J41 and J52) showed significant reduction in the number of fibers (Fig. 17A).

Upon analysis of fraction of area occupied by the DLMs in the thorax, only one excision line (J41) showed significant reduction in area (Fig. 17B).

---

**Figure 16.** Cross-sections of the whole thoraces of some of the genotypes analyzed. (A) ry^{506}/ry^{506}. Six DLMs are present in all the thoraces analyzed. (B) 1424/1424. The number of DLMs varies in nearly all of the lines. (C) 1424/CyO. (D) J7, the DLMs on one side are smaller. (E) J41. The numbers of DLM are variable. The bar in each image represents 10µm.
Figure 17. Quantitative analysis of DLMs from the cross-section of whole thoraces. (A) Average number of DLMs per hemithorax. The * indicates p-value<0.05 when compared to ry^{506}/ry^{506}. The numbers in each bar indicates the total number of hemi-thoraces analyzed. (B) Average of ratio of DLM area to thoracic area. The numbers in each bar indicates the number of whole thoraces analyzed.
DVM Phenotype

Three pair of DVMs (DVM I, DVM II, DVM III) is easily identified in longitudinal thoracic sections. In case of OregonR, DVM I consisted of three fascicles, DVM II had two fascicles and DVM III had three fascicles (Fig. 18B). In case of ry\textsuperscript{506}/ry\textsuperscript{506}, average number of fascicles in DVM I and DVM III were same as OR but average number of DVM II fascicles were higher, although, the increase was not significant to OR. Small aberrations in fiber numbers are commonly observed in wild type flies (de la Pompa et al., 1989). 1424 homozygous flies exhibited significant increase in the fascicle numbers of DVM I and DVM III (Fig. 19. A and C). Nearly all of the excision lines exhibited reduction in the fascicle numbers of DVM I; five of them exhibited significant reduction (Fig. 19A). In case of DVM II, eleven excision lines exhibited significant decreases in fascicle numbers (Fig. 19B). The fascicle numbers of DVM III didn’t seem to be affected by excision (except J34, which showed significant increase in fascicle numbers when compared to OR) (Fig. 19C).

Upon analysis of fraction of area occupied by the DVMs in the thorax, no significant difference was observed between ry\textsuperscript{506}/ry\textsuperscript{506} and OR, 1424/1424 and 1424/CyO. When the ratio of DVM area of ry\textsuperscript{506}/ry\textsuperscript{506} was compared to lines homozygous for excision, eleven lines showed significant reduction in the fraction of area occupied by the DVMs in the thorax (Fig. 19D).
Figure 18. Longitudinal sections of the whole thoraces of some of the genotypes analyzed. The three subsets of DVMs were observed. In wild type, three fascicles are present in DVM I, two fascicles in DVM II and two fascicles in DVM III. (A) ry^{506}/ry^{506}. (B) 1424/1424, the number of fascicles differed from that observed in the wild type. (C) 1424/ CyO, DVM I in most of the hemithoraces analyzed showed increased number of fascicles. (D) J35, DVM I is missing and DVM II are smaller in size when compared to wild type. (E) J41, increase in numbers of DVM I and DVM III fascicles are observed, DVM II is sometimes missing. Bar represents 10µm in each image.
A

Average number of DM1 fascicles

<table>
<thead>
<tr>
<th>GENOTYPES</th>
<th>OR</th>
<th>ty50614241424</th>
<th>C v O</th>
</tr>
</thead>
<tbody>
<tr>
<td>J0</td>
<td>20</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>J1</td>
<td>24</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>J2</td>
<td>22</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>J3</td>
<td>24</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>J4</td>
<td>26</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>J5</td>
<td>28</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>J6</td>
<td>26</td>
<td>22</td>
<td>20</td>
</tr>
</tbody>
</table>

B

Average number of DMII fascicles

<table>
<thead>
<tr>
<th>GENOTYPES</th>
<th>OR</th>
<th>ty50614241424</th>
<th>C v O</th>
</tr>
</thead>
<tbody>
<tr>
<td>J0</td>
<td>14</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>J1</td>
<td>24</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>J2</td>
<td>22</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>J3</td>
<td>24</td>
<td>20</td>
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</tr>
<tr>
<td>J4</td>
<td>26</td>
<td>22</td>
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</tr>
<tr>
<td>J5</td>
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</tr>
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<td>J6</td>
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</tbody>
</table>

C

Average number of DMIII fascicles

<table>
<thead>
<tr>
<th>GENOTYPES</th>
<th>OR</th>
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<th>C v O</th>
</tr>
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<tbody>
<tr>
<td>J0</td>
<td>14</td>
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<td>24</td>
</tr>
<tr>
<td>J1</td>
<td>24</td>
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</tr>
<tr>
<td>J2</td>
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<td>28</td>
<td>22</td>
</tr>
<tr>
<td>J3</td>
<td>24</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>J4</td>
<td>26</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>J5</td>
<td>28</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>J6</td>
<td>26</td>
<td>22</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 19. Quantitative analysis of DVMs from the longitudinal-section of whole thoraces. * indicates p-value<0.05 when compared to ry^{506}/ry^{506}. (A-C) numbers in the bar represents the hemithoraces analyzed. (A) Average number of DVM I fascicles per hemithorax. (B) Average number of DVM II fascicles per hemithorax. (C) Average number of DVM III fascicles per hemithorax. (D) Average fraction of DVM area to thoracic area. The numbers in the bars represents the number of thoraces analyzed.
Discussion

DLM and DVM fibers are patterned using distinct modes of development. In case of DLMs, myoblasts derived from the wing disc (Lawrence, 1982) migrate and fuse with three persistent larval templates. As myoblasts fuse, the larval templates split into two to form six DLM fibers (Fernandes et al., 1991). In case of DVM development, each fascicle is prefigured by one founder cell (Rivlin et al., 2000; Dutta et al., 2004; Fernandes and Keshishian, 2005).

Our results exhibit that, presence of P-element didn’t affect the DLM number or fraction of area occupied by the DLMs in the thorax was observed (Fig. 17A, B). In case of 1424/1424 one of the probable reasons might be- the insertion is semi-lethal or the flies emerged are escapers. Some reasons to explain why a subset of the excision lines (Fig. 17A) have lesser number of fibers are:

a. Incorrect splitting- Excision of some part of the gene might have resulted in loss of function of one of the transcript resulting in inadequate translation and thus leading to lack of proper cues that complete the larval template splitting. Thus, fewer fibers are formed.

b. Presence of lesser larval templates- lesser number of templates results in lesser number of DLMs (Farrell et al., 1996).

c. Death of muscle(s) due to improper attachment.

The decrease in the ratio of DVM area indicates reduction in myoblast numbers. This can be due to absence of proper proliferation, resulting in lesser number of myoblasts or due to insufficient cues from the MNs that influence the myoblast fusion to the templates and founder cells. Longitudinal thoracic sections of 1424 homozygous exhibited significant increase in fascicle numbers of DVM I and DVM III indicating, presence of P-element had resulted in increase in number of founder cells. This phenotype was not observed in any of the excision lines instead, most of the excision lines exhibited reduced number of fascicles in DVM I and DVM II. Significant decrease in the DVM areas suggests that myoblast fusing with the founder cells might be affected.

For flight, proper development of IFMs is required. The DLMs act as wing depressor upon contraction whereas DVMs upon contraction causes wing elevation (Miyan et al., 1985). The flight defect observed in 1424 homozygous and in the excision
lines can clearly be correlated with the muscle phenotype observed. Presence of more fascicles in DVMs might have affected the flight ability of 1424 homozygous, whereas the DLM and DVM phenotypes (lesser number of fibers, fascicles numbers or the area occupied by the DVMs) (Fig. 17 and Fig. 19) in excision lines can account for the decrease in flight ability.
Conclusion

A 1999 BDGP project grouped the 1424 insertion into a pool of 25% of insertions that cause lethality (Spradling et al., 1999). Although it has been reported that P-element insertion is recessive lethal, emergence of 1424/1424 flies indicated that the insertion might be semi-lethal. Viability tests of 1424/1424 at different developmental stages indicate that the gene is required throughout the life cycle (Fig. 9).

Excision lines were generated to find out the role of the candidate gene in viability and IFM development. Viability was restored in some of the excision lines (Table 5, 1st row) indicating that the functional ability of the gene in these excision lines was restored upon excision of P-element. Complementation test revealed that 1424* homozygous viability was more affected than that of 1424/ 1424*, as more excision lines complemented the parent strain (Table 5, 1st row; Table 9, 1st row). Behavioral tests (flight test) revealed that 1424/1424 as well as the excision lines exhibited flight defect (Fig. 14). Analysis of muscle phenotype revealed that 1424 homozygotes exhibited an increase in fascicle numbers, whereas excision lines showed either decrease in fascicle numbers or decrease in the IFM area (Fig. 19D) or both. Aberration in IFM numbers or pattern or area can therefore account for flight defects. The observed muscle phenotypes along with the expression pattern of 1424 in early pupae (Fig. 6) strongly suggest that 1424 gene is involved in IFM development.

Based on the results of viability, complementation test, flight test and muscle phenotype analysis the seventeen excision lines were further grouped into seven groups (Table 10).

a. The excision lines in Group 1 exhibit flight defect as well as IFM defect. It seems that upon imprecise excision dosage sensitivity has occurred. In that scenario it is possible that only one of the transcript (1424-RA or 1424-RB) that influences IFM development got affected due to excision.

b. Excision line J1 in Group 2 showed mixed result. This line showed normal viability and flight but, didn’t complement the parent strain and showed IFM phenotype. One of the probable reason for IFM defect might be inadequate
MN influence. The results from the four tests strongly indicate occurrence of imprecise excision resulting in dosage sensitivity.

c. Excision lines in Group 3 indicate the presence of secondary lesion(s) as a result of which only homozygotes exhibited viability and behavioral defect, but complemented the parent strain.

d. In case of Group 4 (J35), the results indicate a strong possibility of imprecise excision, as both viability as well as behavior is affected.

e. Group 5 exhibits result just opposite to Group 1. Excision lines in this group exhibit viability defect but not the behavioral defect. In these cases, it is possible that one of the transcripts of 1424 gene that is expressed in tissues involved in determining viability is affected. These excision lines therefore indicate that dosage sensitivity has resulted upon imprecise excision.

f. Excision line in Group 6 exhibited both viability defect as well as IFM defect. Since we were not able to perform the flight test, the result indicates that imprecise excision had taken away significant portion of the gene.

g. In Group 7, flight test as well as the IFM phenotype analysis couldn’t be performed due to very low emergence of homozygotes. Based on the results of viability and complementation test, the possibility of imprecise excision is very strong.

The results obtained from the viability tests, behavioral tests and muscle phenotype analysis suggests that the excision lines that were put through all of these test have undergone imprecise excision as some variation in the results were always observed (Table 10). This indirectly indicates that our candidate gene is involved determining viability and in the development of IFMs. The variations in the results obtained indicate the possibility of dosage sensitivity or presence of secondary lesions due to excision. The excision lines that show both viability and behavioral indicate that excision had affected both the transcripts of the candidate gene.
<table>
<thead>
<tr>
<th>Group Lines</th>
<th>Excision lines</th>
<th>Viability</th>
<th>Complementation</th>
<th>Flight</th>
<th>IFM Phenotype</th>
<th>DLMs</th>
<th>DVMs</th>
</tr>
</thead>
<tbody>
<tr>
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**Table. 10:** Synopsis of the results obtained from the analysis of excision lines.
Future Directions

a. Determining the extent of excisions in the excision lines- some of the excision lines have exhibited variability in viability as well as in behavior. Determining the extent or position of the excision using PCR will help us determine how much and where the gene has been deleted.

b. Tagging each of the transcripts of 1424 will help us determine if they are co-expressed in the same regions or in different sites. The results from this experiment will help us not only to find their functional specificity but also the genes they are involved in regulating.

c. Examine myogenesis and innervation during pupal development. This experiment will help us find out if, myoblast proliferation is affected due to excision or due to delayed cues from MNs, thus, affecting IFM development.
Literature Cited


