University of Alberta

A \textit{Drosophila} Member of the UCS Protein Family

by

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in

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Abstract

UCS proteins are a recently recognized family whose members are thought to be co-chaperones specific to the motor region of myosins. Members of the UCS family have been studied in fungi, nematodes, zebrafish, mice and humans. The metazoan and fungal UCS proteins are significantly different, thus the metazoan UCS proteins are grouped into the UNC-45 family. UNC-45 proteins can have a general cellular role, a muscle-cell specific role, or both. My work demonstrates that *Drosophila melanogaster* has a single *unc-45* gene and that the expression pattern suggests a conservation of both roles performed by other UNC-45 homologues. *Dmunc-45* is expressed in the developing mesoderm of the embryo and the one known mutant has a lethal period consistent with muscle aberrations. *Dmunc-45*mRNA is also maternally provided and expressed in tissues other than muscles, supporting a non-muscle role. These results suggest a conservation of UNC-45 function throughout Eukarya.
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<table>
<thead>
<tr>
<th>Abbreviation/Symbols</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>3’</td>
<td>three prime: the end of an RNA region</td>
</tr>
<tr>
<td>5’</td>
<td>five prime: the start of an RNA region</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>CCT</td>
<td>chaperonin containing TCP-1</td>
</tr>
<tr>
<td>Ceunc-45</td>
<td>Caenorhabditis elegans unc-45</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>Def</td>
<td>deficiency, specifically Df(3R)p712, red&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>Dmunc-45</td>
<td>Drosophila melanogaster unc-45</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Drunc-45</td>
<td>Danio rerio unc-45</td>
</tr>
<tr>
<td>F1</td>
<td>The first filial generation of a genetic cross</td>
</tr>
<tr>
<td>GC</td>
<td>General Cell</td>
</tr>
<tr>
<td>gf</td>
<td>gain of function</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>Hsc70</td>
<td>heat shock related protein 70</td>
</tr>
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<td>Hsp90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>IQ motifs</td>
<td>consensus sequence IQxxxRGxxxR found in MHCs</td>
</tr>
</tbody>
</table>
kbp  kilobase pairs
kDa  kilodaltons
lacZ gene encoding beta galactosidase
If  loss of function
MHC myosin heavy chain
min minutes
MLC myosin light chain
N-terminus amino terminus of a protein
P1 parental generation of a genetic cross
Pat paralyzed at the two-fold stage
PBS Phosphate-buffered Saline
PBT Phosphate-buffered Saline with 0.2% Triton X-100
PZ $P\{ry^{+7.2=PZ}\}(3)03692^{03692}$, represents a 3rd chromosome carrying an artificial P-element containing $lacZ$ inserted into the 03692 ($Dm unc-45$) gene region
r rosy gene
RLC myosin regulatory light chain
RNA Ribonucleic acid
RNAi RNA interference
Sb Stubble gene
Ser serrate gene
SM Striated Muscle
TCP-1 t complex polypeptide 1
TPR tetratricopeptide repeats
ts temperature sensitive
UCS UNC/CRO/She domain of a protein, also the name of a protein family that contains this domain.
UTR Untranslated region

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1 Introduction

Myosins are motor proteins that require the aid of chaperones to be functional (Srikakulam and Winkelmann, 1999). The major subunit of the myosin complex is the heavy chain, which contains the motor domain. Studies of muscle myosins, have shown that the motor domain will only fold into a functional form when in a muscle-cell environment (Srikakulam and Winkelmann, 1999). Recent work has implicated four chaperones in the correct folding of the myosin head domain: the chaperonin containing TCP-1 (CCT) (Srikakulam and Winkelmann, 1999), UNC-45 (Barral et al., 2002), heat shock related protein 70 (Hsc70), and heat shock protein 90 (Hsp90) (Srikakulam and Winkelmann, 2004). Three of these, CCT, Hsc70, and Hsp90, have been previously identified as chaperones of multiple substrates (reviewed in Buchner, 1999, Hartl and Hayer-Hartl, 2002, and Valpuesta et al. 2002). UNC-45 is thought to only interact with myosins (see section 1.3 through section 1.5 inclusive). The interaction of UNC-45 and muscle myosin is unique among the known myosin chaperones since UNC-45 appears to remain bound to muscle myosin after the initial folding reaction (section 1.5). In contrast, CCT, Hsc70, and Hsp90 bind only during the folding reaction (Srikakulam and Winkelmann, 1999 and 2004). Thus, a number of chaperones are involved in myosin folding, though only one is known to remain bound to the active myosin.

I am interested in understanding more about how UNC-45 works through the characterization of the Drosophila melanogaster homologue. The founding member of the UNC-45 family was discovered in the invertebrate Caenorhabditis elegans, which has a single UNC-45 gene (Venolia et al., 1999). This single UNC-45 appears to function in a number of myosin-related activities and likely binds more than one type of myosin.
Recent work indicates that vertebrates contain at least two *unc-45* genes with different expression patterns (section 1.6.1 and 1.6.2). While the expression pattern of three vertebrate UNC-45 homologues has been studied; zebrafish (Etheridge *et al.*, 2002), mice, and humans (Price *et al.*, 2002), and UCS protein have been studied in fungi (see section 1.4). *C. elegans* is the only invertebrate in which UNC-45 function has been studied. I am interested in whether or not a single UNC-45 gene is common to invertebrates and if so, is this protein functional in numerous tissues or specialized to a few? To this end, I have studied the expression pattern of the *Drosophila melanogaster* UNC-45 homologue and characterized the phenotype of the one known mutant.

UNC-45 belongs to a larger group of proteins, the UCS family (*UNC-45/CRO1/She4*). UCS proteins have been implicated in a number of myosin-dependent processes throughout Eukarya. UNC-45 binding to multiple myosin types has been seen in *C. elegans* (Ao, 2001). To facilitate the discussion of previous work involving UCS proteins, and to discuss my findings, I will first introduce the different types of myosins and their respective functions. This will be followed by a discussion of previous work on UCS proteins and their possible interactions with different myosins. Finally, I will introduce *Drosophila*, the known roles of the different myosins that are specific to this organism, and what predictions regarding the expression pattern and mutant phenotypes can be made.

1.1 The Myosin Superfamily

Muscle myosin is the founding member of an extremely diverse family of eukaryotic proteins with 15 classes defined to date (Figure 1, Sellers, 2000). *Drosophila* may have two more classes or very divergent members of the identified classes (Sellers,
2000, and Yamashita et al., 2000). Myosins occur in all eukaryotes and are responsible for many types of movement, from trafficking of cytoplasmic components to muscle contraction (see Sellers, 2000, and Berg et al., 2001 for reviews). All functional myosins have at least two different subunits, a myosin heavy chain (MHC) and a light chain (MLC). While myosin classes are defined by the sequence of the MHC, all MHC defined to date all share certain common characteristics; a head (motor domain), a neck region, and a tail (Figure 2). The highly conserved, globular C-terminal head of the myosin heavy chain contains an ATP-dependent actin-binding domain. The neck contains the IQ motifs (regions with the consensus IQxxxRGxxxR), which bind the myosin light chains. There are two known light chain types, the myosin light chains and calmodulin. The light chain, or combination of light chains, that are bound by an MHC varies widely between classes. The neck of the MHC connects the motor domain to the N-terminal tail. The N-terminal tail of different MHC proteins is highly divergent in length, three-dimensional structure, and the sequence motifs that are present. The tail region is often integral to the varied roles of the different myosin classes.

A number of studies have shown binding between UCS proteins and one member of three myosin groups, types I, II and V (Ao, 2001, Barral, et al., 2002, Toi et al., 2003). However, it is also possible that UCS proteins interact with a wider variety of myosins. The type I, II and V are the most common among animals and the best studied (Sellers, 2000). Functional myosin I complexes are very simple, while type V complexes introduce a higher order of protein-protein interaction. Type II myosins form the most complex ordered structures within the cell, and are also the best-characterized myosins.
1.1.1 Type I Myosin

Type I myosins simpler than type II and type V (Figure 2). In addition to an ATP
and actin binding head domain, these myosins have short neck regions and tails of
various lengths. Each MHC I binds one or two light chains. Type I MHC tails will not
form dimers but can carry multiple different domains that bind a variety of clients,
including actin and membranes (reviewed in Coluccio, 1997 and Mermall et al., 1998).

The most common binding partners of the MHC I tail are either actin or membranes
(Coluccio, 1997). Different myosin I complexes have been implicated in cell movement,
cytoskeletal structure, and vesicle transport in many organisms (Coluccio, 1997 and
Mermall et al., 1998). *Saccharomyces cerevisiae* has two functionally redundant type I
myosins (MYO3 and MYO5) (Coluccio, 1997). Yeast cells mutant in both genes show a
number of phenotypes including slow growth, and defects in endocytosis and secretion.
Myosin I has been found in the intestinal microvillus of vertebrates and invertebrates
(Morgan et al., 1995, and Coluccio, 1997). In *Drosophila*, the two known myosin I
complexes (IA and IB) are each localized to specific regions in the developing intestine,
while IB is also localized within the egg chambers of the ovary (Morgan et al., 1995).
The cell types in which *Drosophila* myosin IA and IB are expressed both extend
microvillar arrays, similar to the *brush-border* myosin I of vertebrates (Coluccio, 1997).
Myosin I in the brush-border cells is thought to link the cytoskeleton to the cell
membrane. Thus, myosin I complexes have a variety of roles in different cell types.

1.1.2 Type V Myosin

Type V myosin is slightly more complicated than type I (Figure 2) (reviewed in
Provance and Mercer, 1999, and Reck-Peterson et al., 2000). Type V MHC proteins are
characterized by a classic globular head region, the presence of six IQ motifs in the neck,
and a tail containing globular domains as well as an $\alpha$-helical domain. MHC V proteins dimerize through their $\alpha$-helical domains to produce a two-headed complex with a coil-coiled rod domain and a globular C-terminus. Unlike type II myosin, type V dimers cannot interact with other dimers to form chains (section 1.1.3). The IQ motifs of each MHC V are believed to bind 4 molecules of calmodulin as well as a 17 kDa and a 23 kDa myosin light chain. Thus, type V myosins represent a second level of complexity.

Type V myosins are known to be important in a number of inter and intracellular trafficking events (for review see Reck-Peterson et al., 2000). *Saccharomyces cerevisiae* has two known myosin V complexes. One, Myo4p, is involved in the transport of Ash1 mRNA during budding (Long et al., 1997). The other, Myo2p, is responsible for vacuole inheritance during cell division (Ishikawa et al., 2003). Myo2p seems to have a similar role to mouse *dilute*, a myosin V responsible for membrane trafficking in melanocytes and neurons (reviewed in Provance and Mercer, 1999). The one known type V myosin in *Drosophila melanogaster* is the product of the *didum* gene (Maclver et al., 1998). *Didum* is highly expressed in the maturing oocyte and the RNA is maternally provided to the embryo. During embryogenesis a number of RNAs and proteins are localized to the developing oocyte, thus, it is possible that *didum* is involved in this localization of maternally provided constituents in a manner similar to that of Myo4p, or Myo2p and *dilute*. As discussed in section 1.4.3, a UCS protein is thought to be involved in the function of Myo4p and Myo2p and thus it is possible that UCS homologues are also involved in the function of other myosin V complexes.
1.1.3 Type II Myosin

Type II myosin is structurally more complex than either type I or type V because multiple type II myosins can interact to form a long fiber. One type II myosin complex contains a hexamer of two heavy chains and four light chains (reviewed in Sellers, 2000). MHC II is composed of a conserved motor domain, two IQ motifs, and a long tail. Each MHC II is bound by two light chains; the essential light chain and the regulatory light chain. The regulatory light chain (RLC) of myosin II is required for a functional motor protein and thus deletion or disruption of the RLC prevents myosin activity. The functional myosin II complex is assembled by the dimerization of the tail regions of the two MHC II that each carry two light chains, producing the final hexamer (Cripps et al. 1999). Dimerization of the MHC II tails produces a rod region, which is thought to be very smooth with no unpaired or globular regions. The rods of type II complexes can interact to produce long chains of myosin II motors. This function is thought to be unique to type II myosins.

Type II myosins are divided into two general subgroups based on their sequence; those found in striated muscle (known as muscle myosin), and those found in either smooth muscle or non-muscle cells (Sellers, 2000) (Figure 1). This discussion will be limited to myosin type II from striated muscle cells (muscle myosin II) and myosin type II from non-muscle cells (non-muscle myosin II). Non-muscle myosin II has multiple roles in Drosophila development which will be discussed in section 1.8.

Muscle Myosin II and Muscle Contraction

The structure of striated muscle is shown in Figure 3, and is reviewed in more detail elsewhere (Sherwood, 2001). The contractile unit of the muscle is the sarcomere, a
repeated feature through all striated muscle. The sarcomere is composed of thick
(myosin) and thin (actin) filaments, each of which is anchored to protein complexes (M
or Z lines respectively). In striated muscles, the myosin II hexamer associates with other
hexamers to form the bi-polar thick filament (Figure 2 and Figure 3), which is essential
for muscle contraction. Muscle contraction is achieved when the myosins of the thick
filaments move between the actin molecules of the thin filaments towards the M-line,
causing compression of the sarcomere. The myosins move along the actin by undergoing
a conformational change in the head and neck region.

Although striated muscle structure is comparable between most systems, there are a
few subtleties that are important for this work. *C. elegans* has four myosin heavy
chain genes, *myo-3* (MHC A), *unc-54* (MHC B), *myo-2* (MHC C), and *myo-1* (MHC D)
(reviewed in Wood, 1988). MHC A and MHC B are the major muscle proteins, they are
expressed in all body-wall muscles, while MHC C and D are found solely in the pharynx.
The heavy chains of body wall muscles are composed of MHC A in a small central
region, and MHC B extends from this region to the end of the filament. If MHC B is
removed, the filaments are shorter and the worms are paralyzed, though over expression
of MHC A in this case can significantly improve mobility (Maruyama *et al.*, 1989). In
contrast, *Drosophila* has only one muscle MHC II gene, which is alternatively spliced in
a tissue and developmentally specific manner (George *et al.*, 1989). All known splice
variants of the *Drosophila* MHCII contain a region in the head with high homology to the
region of the *C. elegans* MHCIIIs that is thought to bind UNC-45. Therefore, it is
possible that the *Drosophila* homologue of UNC-45 may bind all isoforms of muscle
MHC II in *Drosophila*.
Non-muscle myosin II and cytokinesis

Non-muscle type II myosin is best known for its role in cytokinesis, although it is also involved in cell structure as well as the movement of macromolecules (Satterwhite et al., 1992). Cytokinesis is the cleavage of the cytoplasm during cell division after the DNA has been separated (reviewed by Guertin et al., 2002). In all eukaryotes except plants, cytokinesis occurs due to the progressive ingress of the cell cortex at the site of division. Actin and non-muscle myosin II co-localize along the division plane at the cell cortex in a structure called a contractile ring. Contraction of the contractile ring by way of the myosin II motor is thought to draw the cell cortex down towards the center of the cell (Figure 4). Thus, non-muscle myosin II is thought to facilitate cytokinesis by the physical constriction of the cell cortex.

Although certain single celled eukaryotes appear to have a secondary mechanism for cytokinesis (reviewed in Gerisch and Weber, 2000), the non-muscle myosin II contractile theory of cytokinesis appears to be an appropriate model in eukaryotes other than plants (reviewed in Guertin et al., 2002). Among yeast, there appears to be both a contractile ring dependent and independent cytokinesis. The budding yeast S. cerevisiae does produce a contractile ring (Lippincott and Li, 1998), though deletion of the single myosin II, Myo1p, produces a highly variable effect (Bi et al., 1998). Certain strains lacking myol show normal growth and division, while others exhibit slowed growth and occasional multinucleated cells, yet all strains fail to show a contractile ring. Thus, certain strains of S. cerevisiae can undergo cell division without type II myosin. Conversely, the fission yeast, S. pombe, is completely dependent on the contractile ring and myosin II function to undergo cytokinesis (Naqvi et al., 1999). Although S. pombe has two non-muscle MHC II chains, Myo2 and Myp2, and both localize to the contractile
ring, only one is essential (Naqvi et al., 1999, and Win et al., 2002). Myo2 localizes to a contractile ring and lack of functional Myo2 produces multinucleate cells (Naqvi et al., 1999). In multicellular organisms the contractile ring is necessary for cytokinesis. The removal of maternal non-muscle myosin regulatory light chain (RLC) from either *C. elegans* or *Drosophila* embryos prevents cytokinesis in the early embryo (Karess et al., 1991, and Shelton et al., 1999). Similarly, the presence of inactive RLC blocks cytokinesis in mice (Simerly et al., 1998). Thus, there is a variable need for the contractile ring in fungi but it is required in metazoans.

1.2 Myosin Motor Domain Requires Chaperones to Fold Properly

The head domain of all myosins binds both actin and ATP, and is the site of force generation and thus central to their function. Conformational changes, due to the exchange of ATP for ADP, cause the motor domain to move in relation to its anchored tail. The result is a translocation of the head along the actin filament (Piazzesi et al., 2002).

The motor domain, is the most highly conserved region in all classes of myosins, and it is believed that all motor domains function in a similar way (Sellers, 2000). For this reason, function of the motor domain is most often studied in muscle myosins as these are abundant and easy to isolate. In order to be functional, the MHC head must be folded into the correct three-dimensional structure. In cell-free or non-muscle cell cultures the head domain will not fold properly, though functional MHC heads do form in cultured muscle cells (Chow et al., 2002). The dependence of the myosin head on the
muscle-specific cell environment suggests specific folding factors, or chaperones, are needed for motor domain folding.

In cells, a complex system is devoted to the correct folding of proteins. This system is composed primarily of a general class of proteins called chaperones. Newly synthesized proteins typically interact with a series of chaperones in an assembly-line type manner before assuming their final structure (reviewed in Hartl and Hayer-Hartl, 2002). Myosins are known to interact with the chaperonin CCT, Hsp90, and Hsc70. In reticulocyte cell lysate, skeletal MHC dimerizes and binds its light chains and this complex is then bound by CCT (Srikakulam and Winkelmann, 1999). The MHC II head domain is only partially folded in these lysates, although this folding defect can be relieved if cytoplasmic extract from myotubes is combined with the reticulocyte lysate. The MHC II has also been shown to bind Hsc70 in this system. In cultures of differentiating muscle cell, MHC II co-localizes with Hsp90 and Hsc70 when the myosin head is partially folded, but not after folding is complete (Srikakulam and Winkelmann, 2004). Finally, the UNC-45 protein from C. elegans has been shown to preferentially bind Hsp90 and the MHC II head domain, and demonstrates chaperone activity in vitro (Barral et al., 2002). UNC-45 is also associated with myosins in their functional form (see section 1.5 and its associated subsections). Thus, a model can be proposed where the MHC II is passed through a chain of common chaperones terminating in a chaperone specific to the myosin head alone.
1.3 UCS Proteins are Implicated in Processes that Require Stable Myosin

UNC-45 belongs to the UCS family of proteins that are implicated in myosin-dependent functions, though their designation as chaperones is recent (Barral et al., 2002). This family is named for the first three members discovered, UNC-45 from *Caenorhabditis elegans*, CRO1 from *Podospora anserina* and SHE4 from *Saccharomyces cerevisiae* (Figure 5) (Barral et al., 2002). Homologues have since been identified in *Schizosaccharomyces pombe* (RNG3, Wong et al., 2000), *Danio rerio* (DrUNC-45, Etheridge et al., 2002 and Wohlgemuth, unpublished), mice and humans (MmUNC-45 and HsUNC-45 respectively, Price et al., 2002). Database searches suggested that UCS proteins probably exist in many eukaryotes, including insects and plants (Hutagalung et al., 2002).

Proteins are included in the UCS family based on a common domain within the C-terminal half of the protein (Barral et al., 2002). *C. elegans* UNC-45 and *S. cerevisiae* She4p proteins have shown direct interaction between myosin heads and the UCS domain (Barral et al., 2002, Toi et al., 2003, and Wesche et al., 2003). While research upon the remaining members has not tested a direct interaction, mutations of UCS proteins disrupt processes known to involve myosin.

The UCS family can be divided into fungal and the metazoan members. The fungal members contain an N-terminus with sequence similarity to other fungal UCS proteins, but not to metazoan UCS proteins, nor to any other known protein region. The metazoan UCS’s have an N-terminal extension containing tetratricopeptide repeats (TPR) joined to the UCS domain by a domain conserved between the UNC-45 homologues (Figure 5).
The TPR region of *C. elegans* UNC-45 is known to bind Hsp90 (Barral et al., 2002). Thus, the fungal UCS members may represent an ancestral myosin chaperone in metazoans, the N-terminus may have evolved as myosins developed muscle-related functions. Fungi seem to use UCS proteins in cell division and intracellular trafficking while metazoans use UCS for a muscle-specific function as well as in some general cell functions. (See Hutagalung et al., 2002, and Yu and Bernstein, 2003).

### 1.3.1 TPR Domain and Hsp90

Tetratricopeptide repeat (TPR) containing proteins exist in many organisms and mediate protein-protein interactions (see review Blatch and Lässle 1999). TPR domains are degenerate repeats of 34 amino acids that are often, but not always, seen in tandem. All TPR domains have 8 conserved residues, while different TPR domains that have a common binding partner generally show additional conserved residues. Each repeat contains two anti-parallel α-helices. The TPR domain of *C. elegans* UNC-45 has been shown to bind Hsp90 *in vitro* (Barral et al., 2002) and shows significant similarity with other Hsp90 binding TPR domains.

Hsp90 is an abundant cytosolic protein that accumulates under heat shock and other stress conditions (reviewed in Buchner 1999). Although Hsp90 appears to be a dedicated chaperone, it is expressed at a much greater level than proteins known to require Hsp90 under physiological conditions. Therefore, Hsp90 may fold more clients than those already described. The ability of UNC-45 in *C. elegans* to bind both Hsp90 and myosin II suggests UNC-45 may act as a co-chaperone with Hsp90.

As described above, the fungal members of the UCS family do not have TPR repeats and binding of these proteins to Hsp90 has yet to be tested. Knowledge of the
fungal UCS proteins is limited but does suggest interactions between different myosins and the UCS homologues in each system. Fungal UCS proteins may have myosin chaperone activity as well.

1.4 Fungal UCS Proteins

1.4.1 CRO1 in *Podospora anserina*

In *Podospora anserina*, CRO1 regulates the switch from vegetative growth, where nuclei are in a syncytia, to the cellular stage required for sexual reproduction (Berteaux-Lecellier *et al.*, 1998). *P. anserina* sexual reproduction is described in Figure 6. Once mating has occurred, the male and female nuclei multiply inside syncytial vegetative cells. “Crozier” cells arise containing one nuclei from each mating type. These undergo synchronous division in the same orientation. The position of each mitotic spindle defines the midline at which a septum forms. This produces three cells in a pyramid shape; the top cell contains two nuclei (one of each mating type), while the two lower cells contain one nuclei each. The dikaryotic cell becomes the ascus as its nuclei undergo karyogamy followed by meiosis and a subsequent mitosis to produce four dikaryotic spores. The two lower cells fuse and produce a new crozier. The cycle is repeated and one initial crozier will give rise to many asci. In the *cro1-1* null mutation, the crozier-like cells are multinucleated and spindle position of dividing nuclei is random, although all mitoses are still coordinated.

In the *cro1-1* mutant cells, Actin belts, which should precede septum formation, are often absent and those that are seen are often misplaced. Berteaux-Lecellier *et al.* (1998) postulate that the lack of actin belts and septa in these mutants is due to the random spindle orientation, since factors that align the division plane are believed to travel down
the astral microtubules to the midline of the spindle. In the cro-1-1 crozier, the random orientations of a large number of spindles will spread the division-plane factors throughout the cell. These factors will only occur sporadically in concentrations high enough to initiate septum formation. These multi-nucleated croziers could form for two reasons; either an inability to restrict the number of nuclei originally separated from the syncytial cell, or an inability to form septa between daughter cells after mitosis. A small number of tetra-nucleated croziers were observed, which supports the idea of failed septation. This failure to produce cell walls between nuclei is reminiscent of the failure to undergo cytokinesis in rng3 mutants of *S. pombe*.

### 1.4.2 Rng3p of *Schizosaccharomyces pombe*

The UCS protein Rng3p is thought to be necessary for normal MHC II function in *S. pombe*. Rng3(ts) mutants are unable to undergo cytokinesis at the restrictive temperature (Wong *et al.*, 2000), this is a similar phenotype to mutants of myo2, the type II myosin of *S. pombe*. Yeast doubly mutant for both rng3ts and myo2ts mutations show a more severe phenotype than either single mutant. Rng3p is required for assembly and maintenance of the actomyosin ring, though it is only visibly localized to this structure in the myo2-E1 mutant and not in any other myo2 mutants or mutants of 18 other genes important in cytokinesis (Wong *et al.*, 2000). The myo2-E1 mutation occurs in the motor domain of MHC II and may affect the ability of this region to fold correctly. If this is indeed the case, extended association with Rng3p would support a role for Rng3p in folding the myosin head. Regardless of the lack of cytokinesis in rng3 mutants, myosin II's involvement in cytokinesis and the association of Rng3p and mutant Myo-E1p suggests an interaction between Rng3p and Myo2p, which is consistent with other UCS containing proteins.
1.4.3 She4p of *Saccharomyces cerevisiae*

*S. cerevisiae* carrying *she*4 mutations have impaired receptor-mediated endocytosis, temperature sensitive growth, and poorly organized cortical cytoskeletons (reviewed in Wesche *et al.*, 2003). This is similar to yeast that are double mutants of the two type I myosins, Myo3p and Myo5p. Mutants in She4 also fail to correctly localize bud-specific mRNAs during cell division (Jansen *et al.*, 1996). This is characteristic of mutations of the *MYO4*, the class V myosin of *S. cerevisiae*. She4p may also interact with the type II myosin, Myo1p since *she*4 mutants show a mild cytokinesis defect, which is seen in some *myo1* mutants (Lippincott and Li, 1998). As well, yeast two-hybrid experiments have shown interaction of She4p with class I, II, and V myosins (Wesche *et al.*, 2003 and Toi *et al.*, 2003).

She4p may be a chaperone specific to the myosin head region. She4p interacts specifically with the head domain of myosin V, Myo5p, and exhibits a number of properties consistent with chaperone activity (Toi *et al.*, 2003). Specifically, She4p is required for growth at elevated temperatures, and She4p and myosins only partially co-localize; She4p being both cytoplasmic and specific to myosin structures (Toi *et al.*, 2003). Lastly, myosins bind She4p in a substoichiometric and salt-labile manner, indicating that the association in the cell may be transient (Wesche *et al.*, 2003).

In summary, fungal UCS proteins have proposed roles in cytokinesis and cell trafficking due to their interaction with myosins I, II, and V. Work in *S. cerevisiae* has indicated that these roles are likely due to the chaperone-like function of UCS proteins themselves. While the interactions with type II myosins has been conserved in metazoans, it is unclear that interactions with type I and V myosins have also been
preserved. The metazoan UCS proteins are also termed UNC-45 proteins, since the first
identified metazoan UCS protein was C. elegans UNC-45, and the metazoan UCS
proteins carry N-terminal TPR repeats that are not present in the fungal UCS family
members.

1.5 Chaperone Function is Evident in Metazoans.

1.5.1 UNC-45 in Caenorhabditis elegans

unc-45 has been studied since 1974 when Epstein and Thomson described a
temperature sensitive (ts) allele that causes uncoordinated movement of worms, thus the
designation of “unc” (Epstein and Thomson, 1974). Electron microscopy of muscle
structure revealed that animals at the restrictive temperature produced disorganized
myofibrils, containing fewer thick filaments than wild-type animals. In contrast, mutants
raised at the permissive temperature move well and have almost normal myofibrils with a
wild-type number of thick filaments. This suggested that UNC-45 played a role in thick
filament stability, a prediction strengthened by the subsequent discovery of lethal unc-45
alleles that arrest at the same developmental stage as embryos carrying MHC A loss of
function mutations (Venolia and Waterson 1990). These lethal alleles could be
maternally rescued, suggesting that unc-45 product is present in the oocyte. Finally, a
mechanism of UNC-45 function has been proposed. Barrel et al. (2002) demonstrated
that in vitro the TPR repeats of Ceunc-45 interact with Hsp90 and the UCS domain
interacts with the head domain of muscle myosin II. Ceunc-45 is considered a chaperone
as it prevents the aggregation of myosin II heads in vitro. This work suggested a model
in which UNC-45 acts as a co-chaperone of Hsp90 and aids in folding the myosin head.
1.5.2 Role of unc-45 in C. elegans Muscles

UNC-45 reporter constructs carrying either E. coli lacZ or the GFP gene under control of the unc-45 promoter revealed expression in larval and adult muscles (Venolia et al., 1999). Reporter gene activity was seen in adult body wall, pharyngeal, and anal muscles in all animals, the vulva muscles of hermaphrodites, and the diagonal muscles in the male tail, as well as gonad sheath muscle cells. These reporters also demonstrated expression in embryos coincident with muscle formation (Ao and Pilgrim 2000). Thus, UNC-45 is associated with all muscle types in the worm. UNC-45 immunostaining localizes to the A-bands of muscles in an MHC B dependent manner and this association is not lost in unc-45 mutants at the restrictive temperature, though thick filaments are deformed. In the early embryo at the time that MHC B is assembling into muscle filaments, UNC-45 staining is primarily cytoplasmic. Coupled with previous findings that the amount of MHC B is reduced in unc-45ts mutants (Barral et al. 1998), this suggests that UNC-45 acts to stabilize MHC B once it is assembled into myosin filaments. The lethal phenotype of the null alleles indicates that UNC-45 may also have an earlier role in MHC B folding.

Three lethal alleles of unc-45 have been identified (Venolia and Waterston, 1990). Animals carrying a loss-of-function (If) unc-45 allele are paralyzed at the two-fold stage (Pat) and fail to develop further. These animals also fail to demonstrate pharyngeal pumping. In contrast, individuals that are homozygous for null alleles of unc-54, which codes MHC B, are viable and fertile though paralyzed in their body wall muscles but not in the pharynx. Double mutants of unc-45(If) and unc-54(null) show the unc-45(If) phenotype without any increase in severity. Thus unc-45(If) is epistatic to unc-54(null). If unc-45 were only involved in stabilizing MHC B during thick filament assembly, the
opposite should be true; the unc-54 mutation would be epistatic to the unc-45 gene and rescue the unc-45 embryonic lethality. While the loss of pharyngeal pumping is consistent with UNC-45 having a role in the pharyngeal muscles (as discussed above), it is unlikely that the Pat phenotype is due to lack of pharyngeal pumping as mutants lacking pharyngeal musculature survive to the L1 stage (Venolia and Waterston, 1990). This is strong evidence that unc-45 has a role outside of the one linked to MHC B stability.

The Pat phenotype of unc-45(If) alleles resembles the embryonic arrest of MHC A null mutants (myo-3(0)), and double mutants of the maternally rescuing allele, unc-45(st604) and a myo-3 gain-of-function (gf) allele prevent the maternal rescue of the progeny. This suggests that unc-45 may interact with MHC A in embryonic development (Venolia and Waterston, 1990). Since antibody staining failed to reveal UNC-45 and MHC A co-localization, the role of UNC-45 in MHC A function could occur in the cytoplasm to fold MHC A before assembly into the thick filament. The evidence from UNC-45 staining and mutant analysis suggests that UNC-45 may interact with all muscle myosin heavy chains in the worm.

1.5.3 Role of unc-45 in the C. elegans Embryo
The isolation of a maternally rescuable allele, unc-45(st604), by Venolia and Waterston (1990) indicates that unc-45 is maternally contributed and so is likely to have a role in early embryonic development. In the yeast two-hybrid system, UNC-45 shows an interaction with C. elegans NMY-2 (non-muscle myosin II) and HUM-2 (myosin V) (Ao, 2001). While the function of HUM-2 is unknown (Baker and Titus, 1997), NMY-2 is a non-muscle myosin II involved in embryonic cytokinesis (see section 1.1.3) and in
establishing anterior-posterior polarity of the embryo (Guo and Kemphues, 1996). In wild-type early embryo, UNC-45 co-localizes to the cell boundaries with NMY-2 and (Ao, 2001, and Kachur, unpublished). If NMY-2 function is removed by RNA interference (RNAi), UNC-45 is cytosolic and diffuse, while UNC-45 depleted embryos show a lack of NMY-2 at cell boundaries (Kachur, unpublished). Finally, RNAi against Ceunc-45 produces a phenocopy of embryos treated with RNAi against the regulatory light chain of non-muscle myosin II (mlc-4) (Kachur, unpublished, and Shelton et al., 1999). When embryos are treated with RNAi against either nmy-2 or unc-45, early embryos fail to extrude the polar body, the meiotic products from the maternal division, and cytokinesis fails. The co-localization of UNC-45 and NMY-2 as well as the RNAi experiments suggests that UNC-45 binds NMY-2 and possibly regulates its function.

1.6 Vertebrate UNC-45s

I have discussed the evidence that, at least in C. elegans and possibly S. cerevisiae, UCS proteins are chaperones specific to the MHC, and are likely involved in maintaining the function of the MHC head domain. Chaperones in general are widely expressed and many eukaryotes share a common set of chaperones (reviewed in Hartl and Hayer-Hartl, 2002). Thus, it was predicted that other metazoans are likely to carry homologues of UNC-45. Sequence homologues can be found in many genome databases (Hutagalung et al., 2002 and Ao, 2001). This includes Drosophila melanogaster (this document), Danio rerio (section 1.6.1), Mus musculus (section 1.6.2) and Homo sapiens (section 1.6.2). Only a single UNC-45 homologue has been found in fungal and invertebrate species while all vertebrates examined have two. In the animals where this has been studied in detail, one UNC-45 homologue is expressed ubiquitously (general cell or GC form), the
other is a muscle-specific homologue (striated muscle or SM form) (Price et al., 2002, Wohlgemuth unpublished).

### 1.6.1 The *Danio rerio* unc-45

Two UNC-45 homologues have been identified in *Danio rerio*. As mentioned, the two homologues of vertebrate *unc-45* have been named general cell (GC) UNC-45 and striated muscle (SM) UNC-45, to reflect their expression patterns (Price et al., 2002). GC *Dunc-45* mRNA is ubiquitously expressed in the developing embryo (Wohlgemuth, unpublished), while the SM homologue appears to be confined to the muscles and their precursors (Etheridge et al., 2002, and Wohlgemuth, unpublished), including those of the heart (Etheridge et al., 2002, Wohlgemuth and Crawford, unpublished). In addition, Serene Wohlgemuth has used morpholinos to study the role of Dmunc-45 SM.

Morpholinos are short single stranded antisense oligonucleotides that reduce the translation of their target RNA when injected into the early embryo of zebrafish (Nasevicius and Ekker, 2000). Animals that have been treated with morpholinos are termed morphants. Morpholinos against *Drunc-45* SM have produced paralysis and edema of otherwise normal embryos (Wohlgemuth, unpublished). Electron microscopy has revealed a lack of thick filaments in the body muscles of morphants, which explains the paralysis. The edema observed in Drunc-45 SM morphants is though to be due to a lack of circulation. Although contractions of the heart have been observed in these morphants, there is no observable blood flow. Thus, it appears that Drunc-45 SM has a role in the development or function of *D. rerio* muscles. This is consistent with the role of UNC-45 in *C. elegans*.
1.6.2 Mammalian unc-45

In humans, the general cell (GC) UNC-45 is expressed in 21 diverse tissues as well as over a dozen tumor cell lines, some of which have elevated GC UNC-45 expression (Price et al., 2002). The striated muscle (SM) UNC-45 mRNA is expressed in striated muscle tissue but has not been reported in tissues rich in smooth muscle or in non-muscle tissues (Price et al., 2002).

Work in the mouse has indicated that the GC UNC-45 is involved in cell proliferation as well as fusion of myocytes, possibly through the cytoskeleton, while SM UNC-45 has a sarcomere assembly specific role (Price et al., 2002). In situ localization of SM and GC homologues during embryogenesis reveals that each homologue has a distinct localization pattern. SM UNC-45 is highly expressed in the beating heart. The GC is expressed in all cells of 8-day old embryos (Price et al., 2002). By day 9.75, embryos show the highest GC expression in rapidly developing tissues as well as low ubiquitous expression (Price et al., 2002). This is generally consistent with the staining patterns seen in zebrafish.

Experiments involving in vitro cultures of mouse muscle cells suggest each UNC-45 homologue has a distinct role in muscle cells (Price et al., 2002). The expression profiles of the GC and SM homologues vary depending on their developmental state. Before myoblast fusion is induced, only the GC homologue is expressed. During fusion of myoblasts, GC expression decreases and SM expression is detected. As the cells begin assembling and remodeling the myofibrils, the expression of SM UNC-45 peaks and GC is weakest. As the myotubes age, the levels of SM decrease slightly, though SM is still present at a higher level than GC. Injecting mouse myoblast cells with either SM or GC
antisense RNA during various stages of differentiation supports divergent roles for the SM and GC homologues (Price et al., 2002). Decreasing the expression of GC UNC-45 during cell division results in significantly reduced cell proliferation, while diminishing SM translation has little effect. During differentiation of the myoblasts into muscles, loss of GC severely inhibits myoblast fusion. Reducing SM at this time results in less fusion of myoblasts, but not to the same extent. Decreasing SM UNC-45 did severely reduce the number of sarcomeres formed in a myotube, though there was no significant loss of MHC II protein in these cells. These in vitro experiments are consistent with the SM UNC-45 homologue having specificity to the muscle MHC II similar to that seen in C. elegans. This work also suggests the GC UNC-45 homologue has specialized towards the interaction with non-muscle myosins. This is similar to the two roles of UNC-45 in C. elegans.

1.7 Using Drosophila as a Model Organism

The Drosophila genome project has identified a single sequence, CG2708, which has sequence similarity to unc-45 (The FlyBase Consortium, 2003). A P-element insertion in or near this locus, which produces homozygous lethality, has been identified (Spradling et al, 1999) and the cDNA has been cloned (Ao, 2001). This preliminary information made Drosophila an attractive model for studying UNC-45 in an invertebrate other than C. elegans.

The purpose of this study was to determine the expression pattern of unc-45 in Drosophila and to determine if it is possible that both muscle and non-muscle functions of the C. elegans unc-45 are conserved in Drosophila. The C. elegans unc-45 mutant phenotypes and staining patterns demonstrate an interaction with both muscle and non-
muscle type II myosins, though interactions with type I and V myosins are also suggested. This preliminary study of unc-45 in Drosophila suggests interactions between UNC-45 and type II myosins, though interactions with type I and V myosins may occur. The limited information regarding the function of type I and V myosins in Drosophila has already been reviewed (sections 1.1.1 and 1.1.2 respectively). While the type II myosins of Drosophila have been introduced, a number of nuances of function must be introduced to facilitate the discussion. The following sections contain a short review of Drosophila development and the roles of type II myosins therein.

1.8 Development of Drosophila and the specific roles of myosin II.

1.8.1 The type II myosins of Drosophila

In Drosophila, non-muscle myosin II has been implicated in multiple processes, including the contractile ring in cytokinesis, morphogenetic movements of several cell types, and attachment of muscle sarcomeres to the cell wall. The non-muscle myosin II heavy chain is encoded by the zipper locus (Young et al., 1993), which is differentially spliced to produce two mRNA isoforms (Ketchum et al., 1990). The differential splicing occurs in the 5' untranslated region (UTR) of the non-muscle myosin heavy chain, thus only one protein product is produced. The function, if any, of the different 5' UTRs is not yet known. As with all type II myosins, the functional non-muscle myosin is a hexamer of two heavy chains and four light chains. Studies of non-muscle myosin function often involve monitoring or inhibition of the regulatory light chain (RLC), which is the product of the spaghetti squash (sqh) gene (Karess et al., 1991).
In *Drosophila*, only one gene encodes the muscle MHC II and this is multiply spliced to produce a variety of stage and muscle-specific isoforms (George *et al.*, 1989). Although there are five groups of alternatively spliced exons, exon 18 is of interest since it is not present in larval myosin isoforms but is present in the adult. This exon encodes an alternate stop site, and thus the adult myosin isoforms are shorter than the embryonic and larval versions. If a fly is engineered to express only an embryonic MHC, resulting adult flies are viable and have apparently normal muscles, although their function is reduced and the flight muscles deteriorate with age (Wells *et al.*, 1996). This suggests the MHC isoform is important for muscle function but not for muscle assembly.

### 1.8.2 The Early Development of *Drosophila melanogaster*

After fertilization of a *Drosophila* embryo, the zygotic nuclei undergo 13 divisions without cytokinesis (Campos-Ortega and Hartenstein, 1985, stages 1 to 4 of Figure 7). The first seven divisions occur in the center of the egg. During these cycles of division, the cloud of nuclei expand along the anterior-posterior (A-P) axis of the egg in a process known as nuclear axial expansion. This expansion is believed to be due, in part, to cortical contraction, which is dependent on non-muscle myosin II recruitment to the cortex during interphase of the nuclear cycle (Royou *et al.*, 2002). The contraction of the cortex would produce a cytoplasmic flux, which moves the nuclei towards the poles.

After nuclear migration, the nuclei undergo three more divisions. During this time, most of the dividing nuclei begin moving towards the cell surface (cortical migration). Two or three nuclei that reach the posterior pole will be separated from the other nuclei into the pole cells (Campos-Ortega and Hartenstein, 1985, stages 3-4 of Figure 7). The remaining nuclei will continue to divide at the cell periphery, forming the syncytial
blastoderm (stage 4, Figure 7). During pole cell formation, both actin and non-muscle MHC II localize to the base of the pole cell where it separates from the egg (Young et al., 1991, and Warn et al., 1985). This co-localization of actin and non-muscle myosin II has been used to hypothesize a cytokinetic mechanism for pole cell separation from the syncytia.

Before gastrulation can occur, the ~6000 syncytial nuclei must be separated into cells by invagination of the plasma membrane (stage 5, Figure 7). This cellularization occurs in two stages; the slow stage, which consists of membrane invagination from the cell cortex to the bottom of the nucleus, and the fast stage, which consists of extension of the cell membrane most of the way around the nucleus. The nucleus will not be completely cut off from the yolk until gastrulation (Campos-Ortega and Hartenstein, 1985). During cellularization, the localization of non-muscle MHC II is dynamic, though it is not thought to be required until the final closure of the base of the cell (Young et al., 1991, Royou et al., 2004). Perturbing the function of this non-muscle MHC II does not prevent the slow phase of invagination, and only slows the fast phase (Royou et al., 2004). Loss of non-muscle myosin II function does prevent basal closure of the cells. Royou et al., (2004) have suggested that the slow phase is primarily based on fusion of cytoplasmic vesicles in a microtubule-dependent manner. The fast phase is only partially dependent on fusion of cytoplasmic vesicles, and may involve the function of non-muscle myosin. Only the final closure of the basal cell membrane is fully dependent on an actomyosin ring similar to that found during cell division.
1.8.3 Gastrulation of the *Drosophila* embryo

After cellularization, the cells divide by conventional cytokinesis, which is dependent on non-muscle myosin II (Royou *et al.*, 2004) (discussed in section 1.1.3). Cell division occurs during gastrulation to produce the different cell layers and types in the embryo. Initially, a mid-ventral band of cells (the ventral furrow) invaginates, followed by germ-band elongation, then retraction of the germ band (Campos-Ortega and Hartenstein, 1985, stage 6 to 11 and images A – D of Figure 7). Germ-band elongation is initiated by a cell plate at the posterior of the cell moving in an anteriodorsal direction, dragging the pole cells along with it (stage 8, Figure 7). This plate will eventually sink into the center of the embryo before being dragged back to its final position by germ-band retraction (stage 9 – 12, Figure 7). Staining patterns of non-muscle MHC II during gastrulation have resulted in the hypothesis that non-muscle myosin II helps drive constriction of the apical ends of cells on the margin of a cell sheet, which allows for cell movement during gastrulation (Young *et al.*, 1991).

1.8.4 Dorsal closure of the *Drosophila* embryo

After the germband has retracted to the posterior of the embryo, it leaves a large portion of the embryo filled by the amnioserosa, which does not form any of the tissues of the final embryo (image E, Figure 7). To form a complete embryo, sheets of cells from the retracted germ band must migrate over the amnioserosa and fuse at the dorsal mid-line. Non-muscle myosin II is known to be crucial to dorsal closure; if the zygote cannot produce a functional *zipper* product, dorsal closure is incomplete and the embryo dies (Young *et al.*, 1993)

Thus, non-muscle myosin II is important at different stages during embryonic development. This involvement is likely due to a common mechanism involving the
actin cytoskeleton of each cell. This cytoskeletal role of non-muscle myosin II is also seen later in development, as part of the development of muscles and neurons. Non-muscle myosin II is also involved in cell proliferation during pupariation, likely due to its role in cytokinesis.

1.9 The Role of Myosin II in Specific tissues

1.9.1 Mesoderm and the Development of Muscles

The cells that will give rise to the mesoderm are defined during the invagination of the ventral furrow and the specific fates of the mesoderm are then defined during germ band elongation and contraction (reviewed in Bate, 1993, light pink in stages 7-10, Figure 7). Initially, ventral furrowing produces a tube of mesoderm cells. The cells of this tube divide and spread to become a single cell layer at the end of germ band extension. During stage 9, the mesoderm divides and produces two cell layers, one group of cells segregates to the interior where it forms the visceral mesoderm, the second group of cells remains associated with the ectoderm and will produce the somatic mesoderm. The visceral mesoderm will give rise to the muscles of the midgut while the somatic mesoderm will produce the body muscles (Bate, 1993, and images E – H, Figure 7). Before germ band retraction, the cells of the somatic mesoderm will form clumps of myoblasts that are roughly centered in relation to the ectodermal segments (stage 11, Figure 7). The mesodermal cells undergo division during stage 11, and these divisions may continue until the end of germ band retraction. The visceral mesoderm forms a continuous band of cells while the somatic mesoderm continues as clumps.

The further development of the visceral mesoderm does not involve complex movement of the mesodermal cells (Bate, 1993). As the germ band retraction pulls the
visceral mesoderm back to the posterior pole, the cells cover the gut and produce two layers of mononucleate striated muscle cells. Generally, staining of these cells becomes more difficult to resolve as the embryo ages due to their interior location and the small width of the cell layer (images E and G, Figure 7).

The development of the somatic mesoderm is significantly more involved than that of the visceral mesoderm. In *Drosophila*, specific mesoderm cells are selected as founder cells and these determine the position of the muscle to be formed (Bate, 1993). Fusion competent myoblasts begin fusing with the founder cells in stage 11 and this continues through germ band retraction. Some large muscle primordia continue recruiting myoblasts into later stages (Bate, 1993). Once the fusion is complete, the muscle cell forms attachments with the extracellular matrix and builds the sarcomeres. In this manner, the striated somatic muscles of the larva are multinucleated cells that are found in well defined positions in each segment (Figure 7, H).

The sarcomere requires both type II myosins to function. Muscle MHC II is expressed beginning in stage 14 (Arbeitman et al., 2002), after most muscle primordia have finished fusing but before dorsal closure is complete. By stage 16 muscle MHC II has been incorporated into the sarcomeres and the larval muscle pattern is evident (image H, Figure 7). Non-muscle myosin II is localized to the Z-lines of the sarcomere and is proposed to act as a contraction-independent actin anchor in the sarcomere (Bloor and Kiehart, 2001). Thus, both types of myosin II are required to produce a functional muscle.
1.9.2 Non-muscle Myosin II in Neural Development

Neurons are a specialized cells since their cell body must be anchored in one place while an extension of the cytoplasm must migrate away from the cell body and find its way to the correct attachment site. Non-muscle MHC II and actin have been observed in fiber tracts and commissures of the ventral nervous system in *Drosophila* embryos (Young *et al.*, 1991, and Young *et al.*, 1993). Also, certain *zip* mutants show defects in axonal patterning. This agrees with the observation that an actomyosin network plays a role in neurite growth and extension (Zhao *et al.*, 1988). Thus, neurons are yet another tissue that relies on the contractile nature of a myosin to cause cell shape change.

1.9.3 Non-muscle Myosin II in the Larval Imaginal Discs

The *Drosophila* larval body plan is degraded during pupation and replaced with entirely new tissue in a new body plan. In *Drosophila*, most of the cells that make the adult structures are located in imaginal discs that form in the larva (reviewed in Held, 2002). These are flat pouches of cells that grow in the larval body cavity until pupation, at which point the discs evaginate (turn inside out) and form the body wall and appendages.

The *zip* allele, *zip*<sup>E(br)</sup> enhances aberrations in imaginal disc elongation and eversion due to mutations in the *Broad-Complex* (BR-C) (Young *et al.*, 1993, and Gothwals and Fristrom, 1991). The E(br) allele of *zip* increases the severity of malformations to the leg and wings due to the *br<sup>1</sup>* allele of BR-C, producing limbs that fail to elongate properly (Gotwals and Fristrom, 1991). Expression of *zipper* is not thought to be regulated by BR-C. The *zip*<sup>E(br)</sup> mutation indirectly implicates non-muscle myosin II in the formation of the wing and leg. Further work with the RLC of *zip*, indicates non-muscle myosin is important for formation of the eyes as well (Edwards and Keihart, 1996). These studies
suggest that the imaginal discs are yet another cell type which harnesses the contractile properties of myosin for proper development.

1.10 Hypothesis

Based upon the sequence similarity between the CG2708 locus and UNC-45, and the lack of any other UCS protein in the Drosophila genome, it is predicted that CG2708 is the only Drosophila homologue of unc-45. Thus, it was predicted that DmUNC-45 should have similar properties to CeUNC-45. DmUNC-45 is likely to bind at least one myosin heavy chain protein and aid in its correct folding. Given that CeUNC-45 interacts with the four C. elegans MHC's along with NMY-2, it is predicted that DmUNC-45 will interact with the Drosophila muscle and non-muscle myosins. Due to the requirement for Drosophila non-muscle MHC II in the early embryo, it is predicted that Dmunc-45 will show a similar expression to that of non-muscle MHC II. Expression concurrent with non-muscle MHC II should include expression of Dmunc-45 in the early embryo as well as in the eye, leg, and wing imaginal discs. Expression of the UNC-45 homologue is expected in both the muscle precursors as well as in developed muscle cells. The prediction that Dmunc-45 will be expressed in muscle precursors is based on the expression of the UNC-45 homologue in D. rerio in cells that will eventually form muscles (Price et al., 2002). Continued expression of Dmunc-45 in developing muscles is predicted based on the expression of the UNC-45 homologues in worms, zebrafish, mice, and humans (Venolia et al., 1999, Etheridge et al., 2002, and Price et al., 2002).
1.11 Statement of Goals

I determined the general expression pattern of \textit{Dmunc-45} and test whether it is possible that the \textit{Drosophila} homologue of UNC-45 interacts with myosins in a similar way to that seen in other organisms. To this end I characterized the RNA expression pattern of the \textit{Dmunc-45} mRNA through RT-PCR of all life stages and \textit{in situ} staining of embryos and larva. I attempted to create a deletion mutant of the locus. I also characterized the P-element insertion 1(3)03692\textsuperscript{03692}, the one known putative mutation of \textit{Dmunc-45}.
2 Material and Methods

2.1 Fly Stocks Used

Oregon R

This stock is considered wild-type. It is described in detail in Lindsley and Grell (1967) and will be referred to as OrR or wild-type throughout the text. This stock was obtained from Ross Hodgetts (Department of Biological Sciences, University of Alberta).

P{ry^{47.2} = PZ}l(3)03692^03692 / TM3, ry^{506} Sb^R K Ser^I

This strain carries a P-element in or near the CG2708 gene region. This insertion was produced for the Berkeley Drosophila Genome Project by Allan Spradling. For brevity, this strain will be referred to as PZ/Sb. The PZ insertion was produced as part of an attempt to cause P-element mediated disruption of all essential Drosophila genes (Spralding et al., 1999). The insertion has been mapped to chromosome position 84E10-11 by in situ hybridization of polytene chromosomes (The FlyBase Consortium, 2003). This insertion produces a homozygous lethal phenotype, which was linked to the P-element by failure to complement a deletion of the region. The disrupted gene (CG2708) was initially identified as tom34 by Spralding et al. (1999), presumably due to similarity between the human TOM34 TPR repeats and the TPR repeats at the N-terminus of the disrupted gene. Please see section 3.1 for the discussion of why CG2708 is not tom34.

Df(3R)p712, red^I e^I / TM3, Sb^I Ser^I

This stock is maintained at the Bloomington Drosophila Stock Center, donated by Thom Kaufman. Df(3R)p712 represents deficiency (3R) pink, which is a deletion in the

\(w^*; Sb^1/TM3, P\{w^{+mc}=ActGFP\}JMR2, Ser^1\)

This stock is maintained at the Bloomington Drosophila Stock Center, donated by Michael Ashburner. A P-element inserted on the 3rd chromosome balancer TM3 carries a rescuing allele of the white gene as well as the S65T version of GFP with a drosophila terminator sequence under the control of the actin 5c promoter (Reichhart and Ferrandon, 1998). The \(w^*\) indicates a mutation in the white gene which results in a loss of eye colour, though the identity of the allele has been lost. The \(w^{+mc}\) allele is a construct of the white gene that lacks the long 5' intron of the native white (The FlyBase Consortium, 2003). This construct produces a range of eye colour from yellow to red depending on where the construct is inserted. In this strain, flies that carry the \(w^*\) and one \(w^{+mc}\) allele have an almost wild-type eye pigmentation.

\(w^*; P\{ry^{+T2}=PZ\}l(3)03692^{03692}ry^{506}/TM3, P\{w^{+mc}=ActGFP\}JMR2, Ser^1\)

This strain was produced in the Hodgetts lab by mating

\[P\{ry^{+T2}=PZ\}l(3)03692^{03692}ry^{506}/TM3, ry^{RK} Sb^1 Ser^1\]

and

\(w^*; Sb^1/TM3, P\{w^{+mc}=ActGFP\}JMR2, Ser^1\)

and selecting progeny with serate wings (Ser) and normal bristles (\(Sb^+\)). These were sibling mated and selected for \(w^*\) in subsequent generations. The \(w^{+mc}\) in a white background produces an eye colour that is not quite wild-type, allowing isolation of
individuals with a mutant white gene on the X-chromosome(s) and the P\textsuperscript{w+mc=ActGFP} balancer. This strain is referred to throughout the text as PZ/GFP

\textbf{\textit{w; Sb A2-3 e/TM6 Ubx e}}

This stock carries the A2-3 allele of the \textit{Drosophila melanogaster} P-element transposase (P/T) linked to the stubble and ebony genes. P/T\textsuperscript{A2-3} is immobile itself but causes excision of other P-elements (Robertson 1996). This allele is expressed in both somatic and germline tissues due to a deletion in the last intron (Ronsseray \textit{et al.}, 1991). This stock was obtained from Dr. John Locke (Department of Biological Sciences, University of Alberta).

\textbf{\textit{w; Sco/Cyo ftz-lacZ}}

The \textit{lacZ} gene is expressed under the control of the \textit{Drosophila fushi tarazu} promoter. This stock was obtained from Dr. Shelagh Campbell (Department of Biological Sciences, University of Alberta).

\textbf{\textit{ap lacZ}}

The \textit{lacZ} gene is expressed under the control of the \textit{Drosophila apterous} promoter. This stock was obtained from Dr. John Bell (Department of Biological Sciences, University of Alberta).

\section*{2.2 Care of Drosophila}

\subsection*{2.2.1 Food}

\textit{Standard Cornmeal/Molasses/Yeast media}: 0.8\% w/v agar (Quadra Chemicals), 1.8\% w/v Torula yeast, 7.2\% w/v King yellow cornmeal, 9.6\% v/v Crosby Fancy Molasses
(non-sulfured), 13mM methyl paraben (Sigma), 0.95% EtOH (Brenntag Canada Inc.), and 0.288%v/v propionic acid (Fisher). This was prepared by the University of Alberta Department of Biological Sciences Fly-food kitchen.

_Grape agar: 2.6%w/v bacteriological agar (USBiological), 22.5% v/v Welch’s grape juice, 5% w/v dextrose (Anachemia), 2% w/v sucrose (BDH Inc.)_

_Yeast paste: Safeway brand fast rising instant yeast was dissolved in water in a ratio sufficient to produce a thick paste. This was prepared daily._

### 2.2.2 Handling Drosophila

Stocks were maintained at room temperature (approximately 20°C) in vials containing Standard Cornmeal/Molasses/Yeast media. Adult flies were transferred to fresh media at one-week intervals.

When collecting embryos, two kinds of cages were employed; a 50mL cage and a 650mL cage. The 50mL cages are 50mL polypropylene centrifuge tubes (Corning Incorporated). The tip of the centrifuge tube was cut off with a razor blade and a piece of foam was inserted to allow air exchange. Molten substrate (grape agar, or Standard Cornmeal/Molasses/Yeast media) was poured into the cap of the tube and allowed to set. Anesthetized flies were added to the body of the cage. The cap was screwed in place and the entire apparatus is set on the cap. The assembly was wrapped in tin foil to prevent movement around the tube from disturbing the flies. This cage is ideal for egg collections from a very small number of females (5-20).

The 650mL cages were created from black PVC piping (Bow). A length of pipe, 3-inches (7.62cm) in diameter, was cut into 5.5-inch (14 cm) sections. A square of 625
holes per square inch netting was fixed to one end with Crazy-Glue. Lengths of 3M Electrophoresis and blotting paper (Rose Scientific) 12cm wide and 46cm long were folded accordion-style and fixed around the inside of the cage with lab tape. 3” caps (Bow) with a hole, approximately 4 centimeters in diameter, cut in the center of the cap were used to close the other end of the tube. Fisherbrand 100X15 disposable Petri plates containing substrate were held in place at the bottom of the cage by the cap. This cage is suitable for a moderate number of adult flies (4-6 bottles). This cage was designed by Dan Bushey (Dr. John Locke’s lab, Department of Biological Sciences, University of Alberta).

2.2.3 Microscopy
The dissecting microscope used was a Zeiss Stemi SV II. Transmitted light was provided by a Kramer Scientific Corporation light box. Direct UV light was provided by a Microlites Scientific mercury short ARC photo opti lamp through a GFP 470 filter. Images in Figure 18 were taken with a Nikon CoolPix995 digital camera with Optem microscope eyepiece adaptor mounted on a WildM3 dissecting microscope. Other images were taken with a Zeiss Axioskop compound microscope with a 1.4.0 Spot Diagnostic Instrument digital camera. All images were processed with Spot 3.5.9.1 and Adobe Photoshop 5.0.2.

2.3 Performing In Situ Staining
2.3.1 Cloning
The predicted cDNA of *Dmunc-45* (CG2708) had been previously cloned by Wanyan Ao and is carried in plasmid pDP#WA070 (Ao, 2000).
Plasmid pTC006: The last 1141bp of the cloned cDNA were excised from PDP#WA070 using SmaI and SstII and ligated into a pBluescript SK vector digested with the same enzymes. The sequence orientation of insert was confirmed by restriction digest and sequencing. This clone is confirmed to contain the 3' end of Dmunc-45 cDNA in an orientation such that the T7 promoter transcribes in the antisense direction (Figure 8).

Plasmid pTC007: The same fragment of Dmunc-45 that was used in pTC006 was also inserted into a pBluescript KS vector digested with SmaI and SstII. Identity of the insert was confirmed by restriction digest and sequencing. pTC007 contains 1141bp of the 3' end of the Dmunc-45 cDNA such that the T7 promoter promotes transcription of the sense strand (Figure 8).

Plasmid AJS27: This plasmid was a gift from Dr. Andrew Simmonds (Department of Cell Biology, University of Alberta). The T7 promoter initiates transcription of a partial antisense transcript of wingless (Simmonds et al., 2001).

2.3.2 Production of DIG-Labeled RNA Probe

Plasmids to be used as templates for RNA probes were linearized with enzymes that would cleave the template at the end of the insert opposite the promoter to be used (XhoI for AJS27, SstII for pTC006 and pTC007). The digested DNA was purified using a Sephaglas BandPrep Kit (Amersham Biosciences). Digoxigenin (DIG) labeled RNA was produced as in Hughes and Krause (1999), with the following reagents: DIG RNA labeling mix (Enzo), 5x T3/T7 buffer (Invitrogen), TNT T7Polymerase (Promega) and Protector RNase Inhibitor (Roche). Nima Najand (Dr. Andrew Simmonds Laboratory, Department of Cell Biology, University of Alberta) produced the antisense probe against
the 3' portion of Dmunc-45. The sense transcript of the 3’portion of Dmunc-45 and wingless antisense probe were produced using reagents and equipment in Dr. Andrew Simmonds Laboratory. Probe activity was assessed as described by Grünewald-Janho et al., (1996). DIG labeled RNA of known concentration (Boehringer Mannheim DIG RNA labeling kit (SP6/T7)) was a gift from Dr. Andrew Simmonds.

2.3.3 Staining of Drosophila Embryos and Imaginal Discs

Embryo collection for staining.

Oregon R flies were allowed to lay for 8 h on grape agar plates spread with yeast paste, at room temperature in the dark. Plates were then removed from the cages and aged an appropriate length of time before being fixed as described in Hughes and Krause (1999). Embryos were stored in methanol at –20°C until needed.

Probe annealing and staining.

Fixed embryos were removed from the freezer and processed as in Hughes and Krause (1999). Embryos were digested with Proteinase K for 1 min Hybridization was done overnight (12 to 16 h) with tubes laying on their sides in a box floating in a 56°C water incubator. A 1/3200 dilution of Anti DIG, alkaline phosphatase conjugated antibodies was used to detect RNA hybridization. Antibody binding was done overnight at 7°C on a VWR rocking shaker at speed 2. Unbound antibody was removed over 2.5 h with five changes of hybridization buffer as described in Hughes and Krause (1999). The alkaline-phosphatase reaction was carried out over 3h with 20μL/ml NBT/BCIP Stock Solution (Roche) in 100mM Tris pH9.5, 50mM MgCl₂, 100mM NaCl, 0.1% Tween 20. Embryos were mounted in 80% glycerol in Phosphate-buffered saline with 0.1% Triton X-100 (PBT) and stored at 4°C.
Staining Imaginal Discs.

Larva at the “wandering third” stage (see section 2.4.1) were removed from the sides of the bottle and washed in water to remove food. In Phosphate-buffered Saline (PBS) the larva were ripped apart about 2/3 of the way to the tail and each body part was inverted. These were stored in PBS on ice until enough tissue could be collected (less than 30 min). These were processed as in Hughes and Krause (1999). Hybridization of probe to RNA, and antibody to DIG label on probe, as described above and as in Hughes and Krause (1999).

2.4 RT-PCR

2.4.1 Production of RNA

Unfertilized eggs

Unfertilized eggs were obtained from virgin Oregon R females. Virgin females were raised four days in a vial with unlimited food at room temperature. These were then moved to a 50mL cage and provided with grape agar and yeast paste for food. The cage was stored in the dark at room temperature and agar and yeast were changed every evening and at regular intervals throughout the day. Eggs deposited on the agar were moved with a fine paint-brush to a small mesh basket where they were washed and dried. These were then transferred to a sterile 1.5mL Eppendorf tube and frozen at –20°C until 50 mg of unfertilized eggs could be collected. RNA was isolated using Trizol Reagent (Invitrogen) as directed by the manufacturer.
Staged Larvae, Pupa and Adults

Oregon R larvae were obtained by collecting eggs in a 650mL cage over 12 hours and aging this collection until all individuals were within the desired stage.

Three groups of third instar larvae were collected in order to distinguish between transcription in early and mid 3rd instar as well as the pupation-specific transcripts in the late third instar (Karim and Thummel, 1992). The first group is designated as “3rd s” and is a mixed population of larvae in the 3rd instar, though the majority of larvae are in the early portion of this stage. The second group is designated the “94hr collection” and consists of individuals in the middle of the 3rd instar life stage. The last group is designated the “wandering 3rd s”. These were collected off the walls of maintenance bottles to provide a sample of individuals in the late stage of the 3rd instar life stage.

Brown pupa were collected off the walls of the maintenance bottles. These generally had tanned cuticles but wings and eyes were not yet obvious. Adult male and females of mixed ages were collected and RNA was extracted from samples of each sex separately. Roughly one-half of the liquid volume of each RNA extract was treated with RNase free DNase I (Roche). The other half was not treated.

2.4.2 Production of First Strand cDNA

All cDNA from Oregon R animals was produced using the PowerScriptII kit (BD Biosciences, formally Clontech) following manufacturers instructions. Primers used are 5’CDS and AIINew (Table 2 and Table 3). First strand synthesis of cDNA from homozygous and heterozygous progeny of the PZ/GFP strain was done using the iScript (Bio-Rad) kit as directed in its product insert. All reactions used 1μg of total RNA as
template. 2μL, or 100ng, of first strand cDNA was used as the template for PCR as described in Table 2.

2.5 Performing PCR

PCR reactions consisted of 2mM MgCl₂, 50mM Tris-HCl pH9.2, 16mM (NH₄)SO₄, 0.2mM dNTPs, 5U Taq and 0.1U Pfu and an appropriate amount of template. The amount of template considered appropriate depended on manufacturers instructions if the template was produced by a kit. If no guidelines for template concentration were set, each reaction in an experiment received an approximately equal amount of template. The thermocycler program used varied for each reaction and primer set used (Table 2). All reactions were carried out in a PTC-100 DNA engine (MJ Research) with the heated lid function enabled. Finished reactions were mixed with 4.5 μL 6x gel-loading buffer type II (Sambrook and Russell, 2001) before separation on an agarose gel of appropriate concentration. Buffer type II contains two dyes which migrate at different rates. Certain PCR products migrate at a similar rate to one of the dye fronts. These PCR reactions were loaded with buffer type II that lacked the co-migratory dye. The size of the PCR products was assessed by co-migration with a 100bp ladder (NEBlabs), λHindIII, or λHindIII/EcoRI depending on the size of the product.

2.5.1 Preparing the Template

Single embryo PCR:

This protocol was developed by Dr. Chris Jones and kindly supplied by Dr. Robert F. Clark, (both of the Department of Neurology, Meharry Medical College). Embryos were dechorionated by gently rolling the embryos along a strip of 3M two-sided tape.
until the chorion broke. The embryo was then moved to 20 μL Gloor and Engel’s “squishing buffer” (10mM Tris pH 8.2, 1mM EDTA, 25mM NaCl, freshly diluted 200μg/mL proteinase K), and squished. The samples were then incubated at 37°C for 30min to digest the cells. Finally, each sample was incubated for 5min at 95°C to inactivate the Proteinase K. 1μL of this lysate was used as the template for each 25μL PCR reaction (Table 2 and Table 3).

**Extracting DNA from adult flies**

DNA was extracted from single adult flies as described in Heslip and Hodgetts (1994). Flies were ground in 10 mM Tris-Cl, pH 7.5, 60 mM EDTA, 0.15 mM spermine, and 0.15 mM spermidine and Proteinase K using a sterile pestle and pellet pestle motor (Kontes) and incubated for 15 minutes at 37°C. The homogenate was incubated in 0.2 M Tris-Cl, pH 9.0, 30 mM EDTA, and 2% SDS for 15min before phenol/chloroform extraction and precipitation with NaCl (Sambrook and Russel, 2001). The resulting DNA was dissolved in 50-100μL TE depending on the size of the pellet.

### 2.6 Characterizing the P-Element Mediated Mutation of CG2708.

#### 2.6.1 Location of the P-Element Insertion

DNA was purified from adult PZ/Sb flies and used as a template for PCR using primers CGI 1035 and PzOut5-3 as described in Table 2. The PCR product was purified by electrophoresis on an agarose gel and extracted from agarose using the Sephaglas BandPrep Kit (Amersham Biosciences), as directed by manufacturer. The PCR product
was sequenced using the CG11035 primer and a DyeET kit (Amersham Biosciences) as directed by manufacturer.

The direction in which the P-element is inserted into PZ/Sb was determined using primers specific to either end of the P-element. The primers initiate replication from the P-element towards the surrounding DNA (Figure 9).

Genomic DNA from adult PZ/Sb flies was used as a template for four separate PCR reactions with different primers. Reaction one contained primers CG11035 and KAN2; reaction two contained primers CG11035 and THE3; reaction three contained primers RT2L and KAN2; reaction four contained primers RT2L and THE3. Only two of these reactions should produce products. The orientation of the PZ insert will determine which reactions are successful. One PCR product was sequenced to confirm the direction of the PZ insertion.

2.6.2 Defining Lethal Period

Individuals from stock P{ry^{+7.2}=PZ}l(3)03692^{03692,ry^6}/TM3, ry^{RK} Sb^{1} Ser^{1} and stock Df(3R)p712, red^{1} e^{1}/TM3, Sb^{1} Ser^{1} were crossed and the number of F1 adults hemizygous for the deficiency and the P-element (PZ/Def) were assessed. The results of this cross will help us to test whether the P-element insertion produces an allele which is an amorph (null) or a hypomorph (reduced function). If there is no difference in phenotype (lethality) between individuals homozygotous for the PZ insertion and those hemizygous for the PZ insertion, then this is consistent with the PZ insertion producing a null allele of Dmunc-45 (amorph). If the number of hemizygous adults is significantly lower than the number of surviving homozygotes, then this is consistent with the PZ insertion producing a partially functional allele of Dmunc-45 (hypomorph). It is still
possible that the true result of the PZ insertion is more complex, and more work is required.

Both heterozygous and homozygous progeny of stock

\[w^{[\ast]};\mathcal{P}\{\mathcal{r}^{+7.7} = \mathcal{PZ}\}l(3)03692^{0.3692}\mathcal{r}_{y}506/\text{TM3, \mathcal{P}\{w^{+mc} = \text{ActGFP}\}JMR2, Ser^1}\] (also known as PZ/GFP) were collected. The virgin females and young males were aged separately for 3 days at 25°C. Three crosses were then set up; heterozygous males and females, heterozygous males with homozygous females, and homozygous males and females. The mated flies were held for two days at 25°C with unlimited food before being moved to a 50mL collection cage. Flies were given 24 hours to acclimatize to the new environment before egg collections were taken. Eggs were collected over 12 to 24 h and allowed to hatch at 25°C without manipulation. The eggs were given at least 48 hours to hatch, which is twice the time required for wild-type eggs to hatch at 25°C. When the larva hatched, they were separated based on the presence or absence of detectable GFP. Larvae were transferred to fresh grape agar substrate in a new 50ml cage with yeast paste and returned to their proper temperature. Development of these individuals was monitored until eclosion. Embryos that failed to hatch were dechorionated by rinsing with 50% bleach in water. The stage of development and GFP status were then assessed with a Zeiss Stemi SV II microscope.

Unfertilized eggs were collected from OrR, PZ/GFP virgin females and compared to embryos from PZ/PZ females mated to PZ/GFP males. The virgin females were treated as in section 2.4.1. Eggs from the three groups of females were collected over night and aged 48 hours at room temperature. These were then washed and transferred to
a depression slide (FSC) and covered in medium Halocarbon 200 oil to make the chorion transparent.

2.7 P-Element Excision

2.7.1 Identification of Flies Carrying an Excision Event

To excise the P-element inserted in the CG2708 gene, the strain

\[ P\{\text{ry}^{+7.2=\text{PZ}}\}l(3)03692^{03692}\text{ry}^{506} / \text{TM3, ry}^{\text{RK}}\text{Sb}^{1}\text{Ser}^{1} \]

was crossed to

\[ w; \text{Sb}\Delta2-3e / \text{TM6 Ubx}e. \]

Males of the first filial generation (F1) with genotype

\[ P\{\text{ry}^{+7.2=\text{PZ}}\}l(3)03692^{03692}\text{ry}^{506} / \text{Sb}\Delta2-3e \]

were selected and mated back to

\[ P\{\text{ry}^{+7.2=\text{PZ}}\}l(3)03692^{03692}\text{ry}^{506} / \text{TM3, ry}^{\text{RK}}\text{Sb}^{1}\text{Ser}^{1}. \]

Since male *Drosophila* do not undergo chromosomal crossing-over, the only changes to the genome must be due to excision and re-insertion of the P-element.

Individuals of the F2 generation were then selected for the reappearance of rosy eye phenotype and the Sb marker. A rosy eye individual must have the genotype \( \text{ry}^{506}/\text{TM3, ry}^{\text{RK}}\text{Sb}^{1}\text{Ser}^{1} \) since loss of the P-element is desired, and both chromosomes must carry a mutant rosy allele to produce a rosy eye colour. Only half of the P-element excisions would be detected in this manner since a sperm carrying an excised third chromosome has an equal chance of fertilizing an egg carrying a P-element inserted chromosome (\( \text{ry}^{+} \)) as it has of fusing with an egg carrying a balancer chromosome (\( \text{ry} \)). Virgin females from the F2 were then mated to their male siblings to determine whether or not the excision
was lethal when homozygous. Strains homozygous for the excision were maintained for at least four generations to ensure continued viability.

### 2.7.2 Characterization of P-Element Excisions

To characterize a stock with a homozygous lethal excision, virgin females were selected and mated to Df(3R)p712, red\(^1\) e\(^1\)/TM3, Sb\(^1\) Ser\(^1\) males. These males carry a deletion within the 3\(^{rd}\) chromosome, which includes the CG2708 locus. F1 progeny were counted and a chi-squared test was performed to determine whether or not the hemizygous animals were associated with increased lethality. At the same time, the strain was crossed to strain w\([\ast]\); Sb\(^1\)/TM3, P{\(w^{+me}=\)ActGFP}JMR2, Ser\(^1\) to produce a strain carrying the P-element excision chromosome balanced by the GFP-carrying TM3 balancer (ex/GFP). This stock was crossed to the original P-element stock (P{\(ry^{+7.2}=\)PZ})l(3)03692\(^{03692}\)ry\(^{506}\)/TM3, ry\(^{RK}\) Sb\(^1\) Ser\(^1\),) and lethality was determined.

In a parallel experiment, PCR of a single individual from each excision strain was performed. The primers used amplified the region spanning the site of P-element insertion. Imprecise excisions should produce a PCR product of different size than seen with wild-type DNA.

### 2.8 Beta-Galactosidase Assays

#### 2.8.1 Staining of Embryos

P{\(ry^{+7.2}=\)PZ})l(3)03692\(^{03692}\)ry\(^{506}\)/TM3, ry\(^{RK}\) Sb\(^1\) Ser\(^1\), Oregon R, and w; Sco/Cyo ftz-lacZ were collected in 650mL cages. Embryos were collected over three and a half hours and some collections were aged while others were fixed immediately. Embryos ranging from stage 1 to stage 17 were obtained. Embryos were dechorionated in 50%
bleach and fixed for 10-15 min as described in Harlow and Lane (1999). β-gal staining was carried out overnight at 37°C while rotating 360° on a LabQuake shaker/Rotisserie (Barnstead-Thermolyne). Embryos were mounted in 90% glycerol in PBS and stored at 4°C.

2.8.2 Staining of Imaginal Discs
Wandering third instar larvae of P{ry^{+7,2}=PZ}1(3)03692^{03692}ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1}, wild-type, and AP-<i>lacZ</i> stocks were collected. Larvae were processed as described in section 2.3.3. Larvae carcasses were fixed and stained as described in Chen et al. (2002). Tissue was fixed for 20 minutes at room temperature. AP-lacZ larva required only 2 hours of staining at 37°C, while P{ry^{+7,2}=PZ}1(3)03692^{03692}ry^{506}/TM3, ry^{RK} Sb^{1} Ser^{1} and wild-type larvae were stained overnight at 37°C. Imaginal discs were dissected away from the body tissue in 60% glycerol in PBS and mounted in the same. Mounted discs were kept at 4°C.
3 Results

3.1 The Predicted Gene CG2708 is Dmunc-45.

Database searches for homologues of the *C. elegans* UNC-45 identified a single locus in the *Drosophila* genome, CG2708 (gene bank accession # AAF54185). According to the database of *Drosophila* genes, *FlyBase*, the CG2708 locus is within cytological position 84E9 on the right arm of chromosome 3 (The FlyBase Consortium, 2003). This locus is predicted to produce an mRNA of over 3 kbp, once two introns have been removed. This would encode a protein of just over 105 kDa (947 amino acids), which is translated from a start codon in exon 2 (Figure 9). *FlyBase* curates the CG2708 locus as translocase outer-membrane 34 kDa, also known as *tom34* (FlyBase I.D FBgn0010812, The FlyBase Consortium, 2003). Studies in humans and mice suggest that TOM34 may be an Hsp90 co-chaperone involved in targeting proteins to the mitochondria (reviewed in Young, *et al.*, 2003). Several lines of evidence argue *unc-45* may be a more reasonable homologue. The *tom34* designation was given in Spradling *et al.*, (1999), due to sequence similarity with the human TOM34 (hTOM34). At that time, the Ceunc-45 sequence had not been released (Venolia *et al.*, 1999). A recent analysis reveals that CG2708 has similarity over its entire length to metazoan UNC-45 family members (Figure 10). A large section of the C-terminal region of CG2708 shows similarity to the UCS region of both the fungal and metazoan UCS proteins (Figure 11). The hTOM34 protein shows similarity to CG2708 only in the N-terminal TPR repeats. The remaining portions of these proteins are quite different in size and sequence (Figure 12). These comparisons were made by the BLAST on-line genetic database search.
engine (Altschul et al., 1990). The alignment information suggests CG2708 is more likely to be the *Drosophila* homologue of *C. elegans* UNC-45 than human TOM34. In the remainder of this text, gene CG2708 will be referred to as Dmunc-45.

### 3.2 Characterizing the expression of Dmunc-45

*C. elegans* has a maternal requirement for *unc-45* as well as a role in muscle development (Epstein and Thomson 1974, Venolia et al., 1999, Ao and Pilgrim 2000, and Ao, 2001). Reporter constructs and immunohistochemistry show a muscle-specific expression pattern of *unc-45* in both larval and adult worms. A possible role for Dmunc-45 in muscle development has been suggested by a micro-array study conducted by Furlong et al., (2001). The *Drosophila* gene *twist* is expressed in the early mesoderm and is essential to the formation of mesoderm and therefore of muscles (for review see Baylies et al., 1998). Furlong et al., (2001) used a micro-array to compare the expression of genes in *twist* mutant embryos (which lack mesoderm specific gene expression) to wild-type expression throughout the *Drosophila* life cycle. An EST corresponding to Dmunc-45 was found to have decreased expression in *twist* mutant embryos. Indirect support for the idea that Dmunc-45 is expressed in the mesoderm is found in another micro-array study (Arbeitman et al., 2002). These authors demonstrate a significant increase in the expression of Dmunc-45 coincident with initiation of muscle MHC II expression (Figure 13). The same micro-array data indicates the presence of Dmunc-45 mRNA in the early embryo before zygotic genes are transcribed. Therefore, we predict Dmunc-45 will demonstrate a maternal requirement and muscle specific role in *Drosophila*.
I tested this prediction by characterization of the Dmunc-45 expression pattern by RT-PCR and *in situ* hybridization, as well as characterizing the phenotype of the one known mutant allele of Dmunc-45. The only known allele of Dmunc-45 is a P-element insertion in the 5' UTR, which is expected to reduce the transcription of Dmunc-45. Excision of the P-element failed to produce a deletion mutation thus the phenotype of the P-insertion was characterized.

### 3.2.1 Expression of *unc-45* Throughout *Drosophila* Development

RT-PCR revealed the presence of spliced RNA of Dmunc-45 in RNA prepared from unfertilized oocytes and from each developmental stage of *Drosophila*. This indicates maternal contribution to the oocyte as well as transcription of the mRNA throughout *Drosophila* development (Figure 14). This RT-PCR reaction is not quantitative, showing only the presence or absence of the transcript, not relative levels. DNase treatment of the RNA samples did not reduce the RNA specific band of Dmunc-45, though the genomic DNA specific band is decreased significantly (Figure 14A). This indicates the DNase is not also digesting the RNA. This is important to demonstrate that any lack of an RNA-specific RT-PCR product is not due to removal of RNA during DNA digestion. The control primers amplify the *RP49* DNA or RNA, which is transcribed at a high level in all stages of *Drosophila* development (O'Connell and Rosbash, 1984). The presence of PCR products specific for the DNA and cDNA demonstrate that cDNA is produced. These results were reproduced in three independent trials. Thus, Dmunc-45 transcripts are present in all developmental stages.
3.2.2 Dmunc-45 is Expressed in the Embryonic Mesoderm

*In situ* staining of wild-type embryos revealed the presence of Dmunc-45 transcript in mesodermal tissues. Although staining with Dmunc-45 sense probe produced uniform background staining, the antisense probe showed a much higher signal in the mesoderm (Figure 15). Staining begins in the mesoderm at stage 10 (stages defined by Campos-Ortega and Hartenstein, 1985). Staining of the visceral (bent arrow) and somatic mesoderm (arrow-head) is seen by stage 12. Specific staining of the visceral mesoderm is not seen in later stages, although this is likely masked by high background levels. The visceral mesoderm is very thin and is unlikely to produce a signal above the background level of the underlying tissue. The somatic mesoderm is stained in subsequent stages of the embryo and becomes restricted to the center of each segment by stage 13. This segmental staining is still present in stage 16 embryos, though it is becoming difficult to distinguish from the background. Staining in older embryos becomes impossible to analyze due to increased staining of the trachea (straight arrows) which may be non-specific. The *Drosophila* peripheral nervous system develops in very close proximity to the somatic mesoderm (Hartenstein, 1993). It is not possible to resolve the differences between adjacent cells with this procedure, thus we cannot determine if there is staining in the peripheral nervous system or not using this technique. Thus, Dmunc-45 is expressed in the mesoderm from full germ band extension, and in the somatic mesoderm as late as stage 16, with expression in the visceral mesoderm that may be transient.

3.2.3 Dmunc-45 is Expressed in the Imaginal Discs

Dmunc-45 antisense probes produced ubiquitous staining throughout all imaginal discs, with no specific pattern apparent (Figure 16). The eye and antennal discs seen in Figure 16 show darker staining in some regions. A comparison of all eye and antennal...
discs stained demonstrates this pattern is not consistent, and altered patterns of increased staining are seen in the other eye and antennal discs. Therefore it is likely that these patterns are artifacts rather than an increased concentration of Dmunc-45 RNA in any region. The wing disc shown in Figure 16 stains more heavily than the other discs in the figure, though no patterns are evident. The slightly enhanced staining around the center of the leg disc is an artifact of the imaging software and is not seen in the actual leg disc. The sense probe does not produce ubiquitous staining in any imaginal discs. The dark spot on the wing and leg discs re-occur in other discs with the same treatment, though the positioning and size of the spots are not consistent. The control wingless antisense probe gave the expected staining pattern, though the leg disc is over-stained. Thus, by this assay, Dmunc-45 is transcribed throughout the imaginal discs.

3.3 Characterizing a P-element Disruption of the Dmunc-45 Gene.

3.3.1 Introduction to Strain PZ/Sb

A strain is available through the Bloomington Drosophila Stock Center that contains a P-element insertion very close to Dmunc-45. Stock P{ry^{17.2=PZ}l(3)03692^{03692,506}ry^{506}/TM3,ry^{RK}Sb^{1}Ser^{1} (PZ/Sb) carries a homozygous lethal insertion of the transposable element PZ near the start of Dmunc-45 (Spralding et al., 1999). The position of this P-element affords me a number of useful genetic tools in the study of Dmunc-45. First, the PZ element is 14.5 kbp long and carries two transcribed genes. The insertion of this element is likely to disrupt the expression of Dmunc-45, which is the speculated reason for the lethality reported by Spralding et al., (1999). Secondly, the PZ element is an enhancer trap; it carries the lacZ gene fused to a weak
Drosophila promoter. This weak promoter does not produce high levels of transcript on its own, but when the P-element is inserted into a gene, the enhancers of that gene often act on the P-element promoter as well, expressing lacZ in a similar pattern to the endogenous gene. Finally, the PZ element is a “disarmed” P-element, which is not capable of producing the transposase enzyme required to mobilize the DNA but still possesses the transposase recognition sequences. If an outside source of transposase is introduced into the genome, this P-element can be mobilized. The presence or absence of this element is monitored by the rescue of a recessive eye colour phenotype by the rosy gene carried by the P-element. Since not all P-element excision events are precise, one can screen for imprecise excisions and produce a variety of alleles depending on what deletions or insertions occur due to P-element activity. The exact site of P-element insertion, the orientation of the insertion, as well as the phenotype caused by the insertion were characterized as part of this project.

3.3.2 The PZ Promoter is Inserted in the Reverse Orientation to Dmunc-45

Diagnostic PCR was performed with primer pairs that produce a product only if the P-element is in one of the two possible orientations. Primers KAN2 and THE3 bind opposite ends (P3’ and P5’ respectively) of the PZ P-element and promote DNA synthesis towards the surrounding sequence (Figure 9). Primers CG11035 and RT2L anneal within the CG11035 or Dmunc-45 gene region respectively and promote synthesis towards the P-element (Figure 9). The size of the bands expected in each case is based on the imprecise location of the P-element listed in Fly Base (The FlyBase Consortium, 2003). This P-element is reported to be localized just 5’ of the predicted RNA of Dmunc-45, though the exact insertion site was not reported.
Primer pairs CG11035/KAN2 as well as THE3/RT2L produced PCR products of predicted sizes (arrows in Figure 17). The primer pair KAN2.CG11035 produced a number of unexpected bands. Since most of these bands are also produced by primers KAN2/RT2L it is likely that the non-specific bands are due to non-specific binding of the KAN2 primer. Lane C contains one unexpected PCR product that is not seen in lane A. This product is not the correct size to be the result of a specific reaction between RT2L and KAN2. Therefore, it is likely a product of non-specific binding of these two primers (* in Figure 17). Control reactions for each primer pair with no template produced no bands. Although this PCR reaction produced a number of unexpected bands, it is still clear that the P-element is inserted such that the lacZ gene is transcribed in the opposite direction to Dmunc-45.

The PCR products that were of the expected sizes were sequenced to determine the exact insertion site. A portion of this sequence aligned with the P-element while the other portion aligned with the sequence of Dmunc-45, though 8 nucleotides of the Dmunc-45 sequence were duplicated (Figure 9). The presence of the P-element sequence, the classic duplication of 8 nucleotides of the target sequence (O’Hare and Rubin, 1983), and the sequence of Dmunc-45 in the sequence of the PCR products indicates that this sequence represents the exact site of P-element insertion. The genomic region surrounding Dmunc-45, the predicted transcription and translation products, as well as the site of P-element insertion are diagramed in Figure 9.
3.3.3 Eye Phenotype of the PZ/Sb Strain

The P\(^{ry+7.2\text{-PZ}}/\text{TM3, } Sb^1 \text{ Ser}^1\) strain (PZ/Sb) produced a previously unreported phenotype in the eye. A small number of individuals demonstrated a darkening of the eye and a number of these also showed a black plaque that covered a portion of the omatidia. To determine if this phenotype was due to the reported PZ insertion, PZ/Sb individuals were mated to the wild-type strain, Oregon R. The F1 progeny was assessed for presence or absence of markers and eye health. If the eye phenotype occurred in progeny carrying the TM3 balancer chromosome and a wild-type chromosome, then this eye phenotype can be judged to be unrelated to the P-element insertion.

3.3.4 Characterizing the Lethal Phenotype Due to the P-Element Insertion in Dm unc-45.

The PZ/Sb strain should produce heterozygotes that are marked by stubble bristles (Sb) and serrate wings (Ser). Both classes of homozygotes are lethal. This strain differs from the reported phenotype for stock PZ/Sb in that the Ser phenotype is absent and a highly variable, low penetrance eye phenotype was observed (Figure 18). It was also noted that the lethality of the homozygous P-element was not 100%.

A portion of progeny from the PZ/Sb strain demonstrate an abnormal eye phenotype that, to the best of my knowledge, is unlike any reported eye aberration. The least severe eye phenotype is a darkening of the eye, reminiscent of the rosy mutation (Figure 18). The more severe forms present a darker eye colour as well as random ommatidia covered in a black plaque. This can range from a few isolated ommatidia to large patches that cover the entire eye. The pattern of plaque deposition is not consistent between the two eyes of a single affected fly. However, both eyes are affected to similar degrees. This
phenotype does not appear to reduce the eye surface area or volume. Of 336 F1’s from a cross of unaffected male and virgin females heterozygous for the P-element insertion, 37 heterozygous (12% of heterozygotes) and 1 homozygous escaper (3% of homozygotes) showed eyes affected to various degrees.

Crossing affected siblings did not produce a line of affected eye individuals. Crosses of PZ/Sb heterozygotes with wild-type flies produced only 7 affected individuals out of 1,655 F1s. Two affected individuals were heterozygous for the balancer and a wild-type chromosome, while 5 were heterozygous for the P-element and a wild-type chromosome. When the P-element was rebalanced with the GFP containing balancer, this stock lost the eye phenotype. The collective evidence from these crosses suggests that the eye phenotype is not due to the P-element insertion. Therefore, investigation of this aspect of the phenotype was not continued.

The P-element insertion in strain PZ/Sb has been described as homozygous lethal (Spradling et al., 1999). However, the lethality is not fully penetrant in our hands. Of the 2,655 flies examined, 257 (10%) individuals do not show the dominant marker, stubble (Sb), carried on the TM3 balancer chromosome. The genotype of Sb+ flies was tested by PCR to confirm homozygosity of the P-element insertion (Figure 19). DNA from 5 Sb+ individuals showed that 4 were homozygous for the P-element insertion while one produced a pattern of bands consistent with a wild-type genome in the area tested. The wild-type individual could be a contaminating wild-type fly or have arisen due to spontaneous excision of the P-element, though an individual homozygous for a spontaneous excision of the P-element would be extremely rare. Contamination is a far more likely explanation for the PCR results. Given that the number of Sb+ flies from this
stock was low, and the fact that the other $Sb^+$ individuals were P-element homozygotes, it is likely that contamination occurred when the flies were collected, not in the stock.

To further characterize the P-element insertion in Dmunc-45, PZ/$Sb$ individuals were crossed to Df(3R)p712, red$^1$ e$^l$/TM3, Sb$^1$ Ser$^1$ individuals. Of 188 adult F1s collected, 21 (11%) had a phenotype consistent with a genotype hemizygous for the P-element insertion and the deficiency. This is similar to the escaper rate for individuals homozygous for the PZ insertion, thus the P-element insertion behaves in a manner consistent with a null allele rather than a hypomorph, at least for the escaper phenotype.

To facilitate genotyping of the homozygous escapers, the P-element inserted chromosome was re-balanced with a new TM3 balancer. This TM3 balancer carries a green fluorescent protein (GFP) reporter, transcribed by the actin 5c promoter (Reichhart and Ferrandon, 1998). This cross also replaced the wild-type X-chromosome of PZ/$Sb$ with an X-chromosome carrying a white mutation. The new balancer chromosome contains a rescuing white allele, such that heterozygous adults have an eye colour similar to wild-type. Adults that do not carry the balancer chromosome will have white eyes. This stock, w[*]; P{ry$^{+t7.2}$=PZ}$l$(3)03692$^{03692}$ry$^{506}$/TM3,P{w$^{+me}$=ActGFP} JMR2, Ser$^1$ will be denoted PZ/GFP. This new stock, PZ/GFP, shows a similar escaper rate to stock PZ/$Sb$. Of the 5 escapers tested, all were homozygous for the P-element insertion. Thus, at room temperature (20 – 22°C) approximately 10% of the adult flies from heterozygous parents are homozygous for the P-element, P{ry$^{+t7.2}$=PZ}$l$(3)03692$^{03692}$ry$^{506}$/P{ry$^{+t7.2}$=PZ}$l$(3)03692$^{03692}$ry$^{506}$, which will be denoted by PZ/PZ.
3.3.5 Homozygotes For The P-Element Insertion Show Variable Lethality

Since the GFP status of the offspring from the PZ/GFP stock can be used to indicate the genotype of an individual, the PZ/GFP stock was chosen to determine the lethal period of the PZ/PZ individuals. Both heterozygous and homozygous virgin females were collected and mated to either homozygous or heterozygous siblings. Regardless of the parental genotype, a proportion of the eggs laid failed to undergo any apparent significant development. These appeared to have a normal chorion, though the yolk had a distinct granular appearance. A portion of these eggs showed fluorescence under the UV lamp but this was a yellow colour rather than the distinct green of GFP. The chorion of these eggs does not tan as would be expected from embryos that arrest in stage 17. It is possible these are unfertilized eggs and that the fluorescence is due to yolk proteins or protein degradation rather than the presence of the GFP protein. Unfertilized eggs laid by wild-type or PZ/GFP individuals produced a very similar phenotype to the granular eggs observed in the crosses (Figure 20). 35 unfertilized OrR and 25 unfertilized eggs from PZ/GFP virgin females were compared to 10 undeveloped eggs from a cross of PZ/PZ females and PZ/GFP. All exhibited the same granular phenotype as the eggs that failed to develop in crosses containing the PZ insertion in Dmunc-45. The unfertilized eggs also failed to show tanning of the chorion. Careful observation of eggs produced by parents homozygous for the PZ insertion was also done to determine if the non-descript egg phenotype was due to lack of development or degradation from a developed state. The results of this experiment are detailed in Figure 21. A total of 20 eggs at stage 1 of development (Campos-Ortega and Hartenstein, 1985) were collected and compared. Those eggs that failed to develop were indistinguishable from their siblings when collected, but took on the granular appearance without any obvious attempt to cellularize.
The granular eggs were assumed to be unfertilized due to the lack of any indication of development and the similarity between these eggs and eggs laid by virgin females. Thus, these eggs were removed from the sample of embryos during analysis.

**Analysis of offspring from a heterozygous cross**

484 eggs were collected from heterozygous females (PZ/GFP) mated to their heterozygous siblings (Figure 22). Roughly 28% of these embryos failed to show any development, had a granular phenotype similar to unfertilized eggs, and the chorion failed to tan. These eggs were thought to be unfertilized eggs and are removed from consideration. The sample set is now 350 embryos. These remaining embryos demonstrated the expected Mendelian ratio regarding segregation of the GFP containing balancer chromosome (25% of embryos lack GFP and 75% have GFP expression). The embryos that develop to stage 17 and express GFP are 25% of the total developed embryos. Those embryos that do not express GFP are assumed to be homozygous for the PZ insertion (PZ/PZ). Those individuals that do express GFP and arrest at stage 17 are assumed to be homozygous for the balancer chromosome (GFP/GFP). Those individuals that express GFP and hatch are heterozygous for the PZ insertion and the balancer chromosome (PZ/GFP). Thus, the expected 1:2:1 ratio of homozygotes and heterozygotes is observed.

Of the 164 embryos considered heterozygous for the PZ insertion, 81% were observed as second instar larva, 67% were observed as third instar larva, 54% were observed as pupa, and 82% were collected as adults (Table 4). The GFP expressing larva often burrowed into the agar substrate and were therefore difficult to count, which explains the discrepancy between the number of second instar larva and adults. The
enumeration of first instar larva should be very precise since the first instar larvae were counted as they were moved off the substrate where they hatched and transferred to a new cage. The number of adults is also reliable since the adults were anesthetized and then counted. Therefore, 82% of the heterozygous progeny survived to adulthood. These heterozygotes control for death due to manipulation rather than genotype.

Of the 97 embryos considered to be homozygous for the PZ insertion, 86% hatched, 73% were observed as second instars, 61% were observed as third instars, 41% were seen as pupa, and only 11% eclose to become adults (Table 4). The homozygous larva did not burrow into the agar to the same extent as their heterozygous siblings and are much easier to count, thus the enumeration of individuals at each stage is reasonably accurate. The survival of homozygous embryos to adulthood is significantly different from the 82% survival rate of the heterozygous siblings.

The number of animals achieving the next developmental stage is above 50% for the transitions between first/second instar (76%), second/third instar (81%), and third instar/pupa (66%). The number of individuals that eclose (26%) is much lower than the number of individuals lost at the other boundaries. These results could indicate a requirement for Dmunc-45 in all developmental stages, or that Dmunc-45 is required during pupation. It is possible that Dmunc-45 is important at all life stages and most stringently required during pupation. Thus the loss of unc-45 in the larva reduces their viability but most larva can compensate for this loss. At the same time unc-45 is more important for pupation, resulting in much fewer animals that can compensate for the loss of unc-45 and eclose. It could also be the case that the loss of individuals during the larval stages is due to the method of handling rather than mutation. It should be noted...
that loss of heterozygous siblings occurs between the first and second instars and not at any other stage, thus loss of homozygous larva is less likely to be due to the handling than the genetic background. In either case it appears that individuals homozygous for the P-element have difficulty completing pupation, this suggests *unc-45* is important in the development of the *Drosophila* adult.

*Analysis of offspring from a cross of homozygotes*

109 eggs were collected from homozygous parents (PZ/PZ), and those embryos that demonstrated a granular phenotype were judged to be unfertilized. These were removed from the sample set, leaving 64 embryos, none of which expressed GFP. Of these, 58% arrested at stage 17 and 42% hatched, none expressed GFP. These late stage 17 embryos had mouthparts and looked like first instar larva. Of the individuals that hatched a varying number survived to subsequent larval stages. 30% of the first instars matured to the second instar, 63% of these matured to the third instar, 40% of which pupated, though none eclosed.

The P-element insertion seems to be predominantly lethal at hatching, though those homozygous individuals that do hatch die at a variety of points. The incidence of homozygotes that fail to hatch increases when the maternal genotype is homozygous, though heterozygous offspring of homozygous mothers are spared.

*Analysis of offspring from a homozygous female and a heterozygous male*

A cross of homozygous females with heterozygous males produced 67 eggs (Figure 23). If only those embryos that show some development are considered, the sample size drops to 46 embryos. Of these, 59% lack GFP while 41% have GFP expression. This
data is not significantly different (P=0.05) to the expected Mendelian ratios for inheritance of the balancer chromosome, which carries the GFP construct. Those individuals that do not express GFP are assumed to be homozygous for the PZ insertion (PZ/PZ), while those individuals that do express GFP are assumed to be heterozygous for the PZ insertion and the balancer chromosome (PZ/GFP). Of the heterozygous offspring, 21% arrested at stage 17, while 79% hatched. The heterozygotes that hatched had an 80% survival rate. Of the embryos homozygous for the PZ insertion, one half arrested at stage 17 while the other half hatched. None of the heterozygous larva survived to adulthood. Comparing the number of heterozygotes and homozygotes that hatch when the mother is a heterozygote to when the mother is a homozygote reveals that the number of F1s which hatch is depressed when the mother is homozygous for the PZ insertion. It is interesting to note that a homozygous mother will produce more heterozygous F1 that hatch than homozygous F1s. This suggests that the zygotic genome can partially rescue the maternal effect.

3.3.6 Expression of lacZ from the Enhancer Trap Line

The Embryo

The PZ P-element is an enhancer trap (The FlyBase Consortium, 2003). The P5’ of the P-element contains a weak *Drosophila* promoter that drives expression of the *lacZ* gene from *E. coli*. If the P-element inserts near enhancers of endogenous promoters, then the activity of the P5’ promoter may also be enhanced. Thus, *lacZ* will be expressed in a similar pattern as the gene whose enhancer is “trapped” by the P-element. Since the P-element in the PZ/Sb strain is inserted near the promoter of Dmunc-45, it is possible that a Dmunc-45 enhancer could act on the P-element, and thus the *lacZ* protein should be
present in the same cells as DmUNC-45. Expression of lacZ may not be entirely representative of DmUNC-45 expression. Expression of lacZ from the P-element is not particular to any particular enhancer, thus if DmUNC-45 has multiple enhancers, it is possible certain enhancers will not drive lacZ expression. Conversely, any enhancer near the P-element has the chance to initiate transcription of lacZ, thus enhancers native to the genes surrounding Dmunc-45 may cause expression of X-gal in a tissue that does not express Dmunc-45. For these reasons β-galactosidase staining must be carefully scrutinized.

β-galactosidase staining of embryos collected from PZ/Sb produced two generally reproducible patterns (Figure 25). There was strong staining of what appears to be the invaginating esophagus and staining of muscle precursor tissues in late stage embryos. It is possible that the esophageal staining is not representative of Dmunc-45 expression. The very weak β-gal staining of the mesoderm leads one to believe that the intense β-gal staining in the esophagus is not due to Dmunc-45 related expression of the lacZ gene. This is reinforced by the lack of esophageal staining in the in situ. It is entirely possible that other enhancers in this region are affecting the lacZ expression. However, the three closest open reading frames (Figure 9) have yet to be investigated and ImpE3 localization in the embryo has not yet been characterized (The FlyBase Consortium, 2003). The staining pattern in stage 16 embryos corresponds to the somatic mesoderm in the body segments. This staining is in the correct location to suggest that it is muscle or peripheral nervous tissue. Furthermore this staining is reminiscent of the in situ staining discussed previously (Figure 25).
The Imaginal Discs

Staining of the imaginal discs was also performed. Imaginal discs exposed to X-gal for several hours showed no staining, though control imaginal discs from flies carrying a transgene with lacZ fused to the AP promoter showed the expected staining (Figure 26). When PZ/Sb tissues were stained longer, some pigment deposition occurred. However, the pattern was not consistent between trials and the staining often overlapped with contact points between disc and surrounding tissue. If lacZ is expressed in imaginal discs, the expression is too low to be detected by β-galactosidase staining. It is possible that β-galactosidase staining is less sensitive than in situ hybridization, so lack of β-galactosidase staining does not necessarily indicate a lack of DmUNC-45 at a low level.

3.4 Screening for a Deletion Mutant of Dmunc-45

Although P-element insertion can be very useful in characterizing genes, this particular insertion produced a pleiotropic effect. Therefore, an attempt was made to create a deletion mutant with the intention of producing a more stable phenotype. P-elements can be mobilized and do not always produce a precise excision from their insertion site and thus can be used to created deletions (Salz et al., 1987). Efforts were taken to excise the PZ element from strain PZ/Sb in the hopes of creating a deletion mutation of Dmunc-45.

31 excision events were identified from three separate screens. Of these, 7 were homozygous lethal, 1 (strain 3161) was lethal when hemizygous with the deficiency but was not lethal when heterozygous in the presence of the original P-element insertion. This suggests a lethal disruption has occurred in the region covered by the deletion, but is not due to deletion of the Dmunc-45 gene. Molecular tools were used to be absolutely
certain that the 3161 excision was not altering the sequence of Dmunc-45. PCR spanning the region of P-element insertion showed no change in band length between wild-type and the lethal strain 3161 (Figure 27). Sequencing of the PCR product confirmed that a precise excision had occurred. Therefore, a deletion mutant of Dmunc-45 was not identified.
4 Discussion

Searches for homologues of the *C. elegans* UNC-45 have found only a single locus in the genome of *Drosophila* (Hutagalung *et al.*, 2002, Price *et al.*, 2002, and our lab). Since *Drosophila* appears to have only one *unc-45* gene, it is likely that this gene will exhibit both the muscle-specific and non-muscle roles as seen in *C. elegans*. Despite the fact that both *Drosophila* and *C. elegans* are invertebrates, they have vastly different development programs. Therefore, it is possible that UNC-45 of *Drosophila* may have novel functions.

The results of the experiments presented in this thesis are consistent with the role of UNC-45 in *C. elegans*. A muscle-specific role of Dmunc-45 is supported by the *in situ* hybridization and the P-element lethality in the embryo. *In situ* hybridization shows Dmunc-45 is present in the mesoderm and developing muscles of the embryo (Figure 15) as well as possibly in the larval muscles. A fraction of the embryos homozygous for the P-element insertion in Dmunc-45 arrest at the end of embryogenesis without any obvious morphological defect, though they cannot hatch. This point of arrest has been previously reported when there is disruption of the muscles (Fyrberg *et al.*, 1994). The non-muscle role of Dmunc-45 is supported by the detection of transcript in the egg and by the *in situ* staining of the imaginal discs. Wing and leg imaginal discs have muscle precursor cells associated with them in a defined region while eye and antennal imaginal discs have no associated muscle precursor cells (Held, 2002). The *in situ* staining against Dmunc-45 produces ubiquitous staining throughout all imaginal discs (Figure 16), suggesting Dmunc-45 may have a role in the entire disc rather than specifically in the muscle precursor cells.
The muscle-specific staining of Dmunc-45 is consistent with staining patterns observed in the other metazoan UNC-45 homologues, though the imaginal disc staining is novel. C. elegans, D. rerio, and mice all show UNC-45 expression in both developing and developed muscle cells (Ao, 2001, Etheridge et al., 2002, and Price et al., 2002). Drosophila imaginal discs have no homologous system in the UNC-45 containing organisms studied thus far, though the role in imaginal discs may be similar to the role of Ceunc-45 in the early C. elegans embryo. Thus, it appears that the Dmunc-45 has similar function as that of the other UNC-45 homologues.

4.1 Transcription of Dmunc-45

The results of RT-PCR of wild-type Drosophila RNA demonstrates that Dmunc-45 RNA is maternally provided, and is transcribed during all stages of life. This agrees with microarray data produced by Arbeitman et al., (2002). This group reported a moderate level of Dmunc-45 expression in the first 2.5 hours of embryo development. This time frame corresponds to the initial syncytial divisions of nuclei of the Drosophila embryo. Since zygotic transcription is repressed at this point (Nasiadka et al., 2002), maternal products are required for normal development.

Maternal contribution of UNC-45 is also seen in C. elegans. This was suggested by the maternal rescue of a strong allele of Ceunc-45 (Venolia and Waterston, 1990) and has been confirmed by more recent work (Ao, 2001). In C. elegans, UNC-45 is present at the cell cortex in the earliest cell divisions, before most zygotic expression is observed (Ao, 2001). The maternal contribution of unc-45 has not been assayed in the vertebrate systems. Eight-day-old embryos were the youngest mice embryos investigated for expression of the general cell (GC) or striated muscle (SM) homologues of murine unc-
45 (Price et al., 2002). It is known that maternal RNA is degraded by 46 hours of development in mouse (DePamphilis et al, 1988). Therefore, embryos 8 days old would only show zygotic expression. In Danio rerio, expression of the GC and SM homologues has only been investigated in embryos 9 hours post fertilization or older (Wohlgemuth, unpublished). Zygotic transcription in zebrafish begins at the midblastula transition stage, which is between 2.5 and 5.25 hours after fertilization (Zamir and Yarden, 1997). Thus, the maternal supply of unc-45 has yet to be investigated in vertebrates.

Expression of UNC-45 throughout life is also seen in the other systems studied. In C. elegans, unc-45 mRNA and proteins are ubiquitous throughout the embryo until muscles begin to differentiate, at which point the expression is muscle-specific. This muscle-specific expression is maintained throughout development and adult life (Ao and Pilgrim, 2000, and Ao, 2001). In mice, GC unc-45 is seen in all tissues in both embryos and adults while the SM homologue is seen only in the embryonic heart and in striated muscles of the adult (Price et al., 2002). Although their expression patterns are different, both the GC and the SM unc-45 isoforms in mice are expressed in the embryonic and adult tissues. This is consistent with our finding that Dmunc-45 is expressed in all stages of Drosophila development. The specific expression pattern within these stages is of great interest, though our study was limited to embryonic and larval staining.

4.2 Dmunc-45 in the Early Embryo

The observed maternal contribution, as well as the reported moderate expression of Dmunc-45 in the early embryo (Figure 13, Arbeitman et al., 2002), led us to expect in situ staining in the early embryo. It was also suspected that the lines carrying the P-
element enhancer trap would show β-gal staining in the early embryo. However, neither assay produced staining at early stages of development.

The lack of detectable in situ signal could be due to the high background staining seen in all embryos, including the sense-strand controls. It is possible that the in situ did produce specific staining in the early embryo, but was obscured by the background staining. If Dmunc-45 is present throughout the embryo, a specific ubiquitous staining of the cytoplasm may not produce enough contrast to be distinguished from the background staining. The in situ staining is also less sensitive than RT-PCR. In situ staining relies on the hybridization of a tagged RNA, produced in vitro, with the native RNA. The tag then undergoes a chemical reaction to produce a coloured precipitate. Wherever the precipitate is seen, the probe has bound. The presence of small amounts of tagged RNA cannot be detected because low concentrations of the antibody tag will not produce a visible amount of staining. On the other hand, RT-PCR is based on exponential amplification of a template with specific primers. Only one molecule of template is required for the first reaction after which the PCR products themselves become templates. Thus, RT-PCR can produce a detectable signal from a single template. Therefore, it is possible for the RT-PCR to detect transcripts of Dmunc-45 at much lower levels than in situ staining and it is likely that the RT-PCR demonstrates the expression of Dmunc-45 with greater sensitivity than the in situ staining.

The lack of β-gal staining at early stages of development is consistent with the very faint staining seen in the muscles of stage 16 embryos. Of the PZ/TM3 embryos stained with X-gal, signal was not detected in stage 16 embryos when presented in a lateral aspect. However, when presented in a dorsal aspect, staining could be seen. At stage 16
a dorsal aspect presents the muscle cells stacked in very defined areas of the embryo. Thus, the signal from one cell combines with the signal from its neighbors to produce a darker overall stain than a single stained cell layer. Since this amplified staining is very weak, it is very unlikely that staining in a small population of cells will be detected. It was expected that embryos homozygous for the P-element insertion would transcribe more \( \text{lacZ} \) and thus produce a darker \( \beta \)-gal stain. Though these homozygotes should represent 25% of the eggs observed, I failed to see any embryos with increased \( \beta \)-gal staining.

### 4.3 Dmunc-45 is Expressed in the Mesoderm

All published work on *C. elegans unc-45* has involved the role of UNC-45 in the muscle. We have shown that transcription of Dmunc-45 in embryonic stages 10 to 16 follows the pattern of the somatic mesoderm and muscles. \( \beta \)-galactosidase staining supports the *in situ* staining of the somatic mesoderm in the later embryonic stage. As mentioned, the earlier expression of Dmunc-45 in the mesoderm is not seen in the \( \beta \)-galactosidase stained embryos, likely a result of the low intensity of this staining reaction. The *in situ* staining in stage 16 embryos is weak and difficult to see. This could be due to high background staining in these embryos, and the fact that the muscle cells are thin. The result is very little contrast between true staining and background. This explains why staining is best seen when a dorsal aspect is presented. The *in situ* staining of the visceral mesoderm is seen only in stage 12 embryos. In earlier embryos, the two mesoderm layers are not sufficiently separated to see this difference. In the later embryos, the visceral mesoderm is likely too thin for staining to be seen against the high background. The visceral muscles of the fly are composed of striated muscles, similar to the somatic
muscles (Bate and Arias, 1993), thus the presence of staining in the viscera is consistent with the expression of Dmunc-45 in the somatic mesoderm.

The \textit{in situ} staining should be a reliable indicator of Dmunc-45 transcription, though the X-gal staining is not necessarily representative of the presence of DmUNC-45. It is also possible that the \( \beta \)-gal gene expression does not reflect the expression pattern of Dmunc-45, or that \( \beta \)-gal represents Dmunc-45 expression only at certain points. \( \beta \)-gal expression is dependent on enhancement of its promoter by elements in the nearby genome. It is possible that the enhancer acting on the \( \beta \)-gal promoter can be any of the enhancers near Dmunc-45. The P-element is inserted in the 5’ UTR of Dmunc-45, which is separated from the start of gene 11035 by only 358bp (Figure 9). It is quite possible for enhancers for both Dmunc-45 and CGI11035 to act on the \( \beta \)-gal promoter. For this reason the X-gal staining is treated as only a suggestion of where the Dmunc-45 transcript may be produced. In comparison to the X-gal staining, the \textit{in situ} staining should reveal the presence of the Dmunc-45 transcript. Although the presence of mRNA does not necessarily indicate the presence of the protein, a protein cannot be produced without an mRNA, so \textit{in situ} indicates tissues where it is possible that the protein is translated. Therefore, Dmunc-45 is transcribed in the mesoderm as early as stage 10, but the protein may not be translated until stage 16, though this is not certain.

The mesoderm expression of Dmunc-45 supports a muscle-specific role implied by Furlong \textit{et al.}, (2001), though the staining in stage 10-11 embryos conflicts with their results. Furlong \textit{et al.}, (2001) used microarray technology to compare levels of gene transcripts in embryos lacking the early mesoderm determining factor, \textit{twist}, compared to pooled wild-type RNA from all stages of life. This indicates that Dmunc-45 transcripts
were significantly reduced only in late stage 11/stage 12 embryos and not in stage 9, 10 or early stage 11 embryos. Furlong et al., (2001) only considered changes of greater than 2-fold to be significant. Since Dmunc-45 is transcribed throughout the Drosophila life cycle, it is likely that the early enrichment in muscle cells was masked by the moderate expression throughout the life cycle

Staining of muscle precursors prior to muscle differentiation is also seen in fish and mice. In D. rerio the striated muscle unc-45 is seen in both precursors to the slow muscles during gastrulation as well as in the somites, which go on to form the trunk muscles (Etheridge et al., 2002). Cultured mouse cells show that SM unc-45 expression was up-regulated when myogenic cells began fusing (Price et al., 2002). This is the first step to producing muscles. Therefore, the expression of Dmunc-45 in muscle primordium is consistent with the SM UNC-45 homologues in zebrafish and mice.

There is also transcription of Dmunc-45 in the larval muscles, though the in situ staining is faint. Because of this, it is not possible to determine if the staining is particular to one portion of the sarcomere or another. Localization of unc-45 to developed muscles is well preceded by the other metazoan UNC-45 homologues. C. elegans UNC-45 localizes to the thick filaments of the adult body wall muscles of wild-type worms. In D. rerio embryos, SM unc-45 is localized to specific developed head muscles as well as the smooth muscle of the swim bladder (Etheridge et al., 2002). In the mouse, the SM unc-45 was seen in Northern blots of skeletal muscle and the heart. In situ of mouse embryos showed high expression of SM unc-45 in the beating heart. In murine cell culture, SM unc-45 expression is present in older myotubes, though it was highest while myofibers were being assembled. Thus, the embryonic mesoderm-specific
expression of Dmunc-45 and the expression in larval muscles is consistent with staining patterns seen for UNC-45 homologues in other systems.

### 4.4 Dmunc-45 is Expressed in Imaginal Discs

We have shown that Dmunc-45 transcripts are uniformly present in the imaginal discs, which form the epidermis of the *Drosophila* head, thorax, and external genitalia. Muscle precursor cells are associated closely with regions of the imaginal discs in the larva but are not derived from the discs themselves. The clusters of muscle precursor cells are found associated with the proximal portion of the wing, haltere, and leg discs, though the imaginal discs do not produce the muscle precursors. The fact that *in situ* staining is ubiquitous throughout the imaginal discs suggest that Dmunc-45 is important in all cells of the discs. Thus, if this is representative of the protein expression, staining of the imaginal discs indicates a role for Dmunc-45 outside of the muscles.

At this point anti-DmUNC-45 antibodies or reporter constructs are necessary to give further insight into the function of unc-45 in *Drosophila*. Although *in situ* hybridization can determine which cells produce moderate to high levels of specific RNA, this procedure fails to recognize regulation through translation, and the localization of the protein product. Antibody staining also reveals the site of antigen function within the cell, which can be used to postulate protein functions. For this reason, antibody staining can allow more insight into the function of the target protein than an *in situ*. The same benefits of antibody staining can be achieved using a reporter construct fused to Dmunc-45. If the reporter is a fluorescent protein, there is the added benefit of monitoring protein localization in living cells. Further studies of Dmunc-45 would be well served by the use of an anti-DmUNC-45 antibody or reporter construct.
Since the imaginal discs of *Drosophila* have no homologous structures outside of insects, this observed expression of Dmunc-45 in imaginal discs is novel in location, though the mechanism of action may be preceident. Recent work in *C. elegans* has suggested an interaction between UNC-45 and the non-muscle myosin type II, NMY-2. The one known *Drosophila* non-muscle myosin II, zipper, is thought to be closely related to the two *C. elegans* NMY proteins (Figure 1, Sellers, 2000). The imaginal discs of *Drosophila* are known to be dependent on zipper for their evagination (section 1.9.3). Therefore, Dmunc-45 in the imaginal discs may be important due to the conserved interaction between UNC-45 and non-muscle myosin II complexes.

### 4.5 Nuances of the P-Element Phenotype

The one known mutant allele of Dmunc-45, a P-element inserted in the 5' untranslated region of the RNA, was characterized. This insertion results in incompletely penetrant lethality in the homozygotes. Because of this, P-element excisions were performed in hopes of obtaining a deletion which may include at least part of the Dmunc-45 gene. Although dozens of excision events were identified, none were imprecise excisions. The P-element insertion is therefore the only extant mutant allele of Dmunc-45 and can provide insights into the function of Dmunc-45 regardless of the incomplete lethality. The survival rate of individuals that are homozygous (PZ/PZ) or hemizygous (PZ/Def) for the P-element is approximately the same. This data suggest that the P-element insertion may act as a Dmunc-45 null, thus characterization of the P-element can be very informative. It is also possible that the insertion allows sporadic expression of the Dmunc-45 gene and that one gene is sufficient to allow a small number of individuals
to survive to adult-hood. The data presented cannot distinguish between these two possibilities, thus both options must be considered.

The P-element insertion appears to produce a maternal effect such that the offspring of a female with at least one copy of Dmunc-45 are more likely to survive embryogenesis than the offspring of a female homozygous for the P-element disruption of Dmunc-45. If the female parent is heterozygous for the PZ insertion, the majority (86%) of homozygous progeny will hatch. Alternatively, if the maternal parent is homozygous for the PZ insertion, regardless of the paternal genome, less than half of the homozygous progeny will hatch. The embryonic lethality can be rescued zygotically by the paternal genome. Of the heterozygous progeny from a homozygous female and a heterozygous male, the majority (79%) will hatch.

A number of embryos from both heterozygous and homozygous mothers arrest at stage 17, though they appear to be morphologically normal. The embryonic lethality at stage 17 has been reported among mutants that have reduced muscle function (Fyrberg, 1994). The observation that some homozygous PZ embryos did move but failed to hatch also suggests a lack of proper muscle function. Although stage 17 is the last stage of larval development, and the zygotic genes are transcribed long before this (Nasiadka, et al., 2002), this lethality appears to be linked to the maternal genotype. If the maternal genotype is PZ/GFP, only 14% of the homozygous progeny arrest at stage 17, though this increases to over 50% F1 arrest at stage 17 if the maternal P1 is homozygous for the PZ-element. Although some zygotic rescue of this arrest is possible it is not complete. Of the heterozygous F1 individuals from a cross of a PZ/PZ female with a PZ/GFP male, 22% of the heterozygotes still arrest at stage 17.
Of the individuals that hatch, the heterozygous progeny have a high survival rate compared to their homozygous siblings. Approximately 80% of heterozygous F1’s that hatch will become adults while only 11% homozygous progeny eclose when the parents are heterozygotes, and none survive when the maternal parent is a homozygote. Analysis of the F1 of heterozygous parents suggests that the limiting stage for homozygous F1 larva is pupal development. More than 60% of homozygous larva that achieve one larval stage will pass into the next larval stage, though only 26% of F1s that pupate will eclose as adults (Table 4). This is also seen in mutations for the regulatory light chain of non-muscle myosin II (sqh), which is required for cytokinesis (Karess et al., 1991, and section 1.1.3). Homozygous sqh mutants from heterozygous mothers develop through the embryonic and larval life stages but arrest during pupation. Perhaps maternal contribution of factors required for cytokinesis will allow embryonic development and the larva will survive because only cells required in adult development divide in the larva. The similarity between the lethal period of sqh and the PZ-insertion in Dmunc-45 gives further support to the postulate that Dmunc-45 interacts with non-muscle myosin II.

The survival of some homozygotes, coupled with the fact that those surviving homozygotes can reproduce, suggests that the PZ insertion has created a leaky mutation. If this is the case, escapers could be due to the P-element not completely preventing expression of DmUNC-45. Variable expression of Dmunc-45 in the presence of the P-element could be a result of the orientation in which the P-element has inserted, or there could be a weak promoter in the 5’ UTR of Dmunc-45.

It is possible that the P-element insertion only reduces the product of the Dmunc-45 locus rather than producing a complete loss of function. Geyer et al., (1991) reported an
instance of a variable phenotype of the yellow locus due to the orientation of a P-element insertion in relation to the transcriptional direction of the yellow gene (Figure 28). Both yellow alleles had the same P-element inserted in the same sequence of the 5' UTR region. The y\textsuperscript{187} allele contains a P-element in the same orientation as the yellow transcript. This produces a cuticle without colour, this is the same phenotype as a null mutation of the yellow gene. The y\textsuperscript{76d28} allele contains the same P-element in the opposite orientation. The cuticle of these flies is tan; an intermediate pigmentation between the wild-type and the null. This difference in phenotype was discovered to be the result of preferential removal of the P-element transcript from the RNA produced by the yellow promoter. This appears to be due to splicing of the mRNA. In the strains used in this thesis, the PZ construct is inserted in the Dmunc-45 locus in the same orientation as the P-element in the y\textsuperscript{76d28} allele. It is possible that preferential removal of the PZ element from Dmunc-45 is occurring by a similar splicing mechanism.

It is possible that RT-PCR could be used to determine if this preferential splicing is truly occurring. RNA from individuals homozygous or hemizygous for the P-element could be treated as the wild-type RNA was for the RT-PCR reactions described in this text. If the Dmunc-45 RNA-specific band is seen, this would indicate that it is possible that DmUNC-45 could be produced. This reaction would require careful control through the testing of co-isolated DNA for the absence of any wild-type Dmunc-45.

It is also possible, though unlikely, that Dmunc-45 contains a second promoter site within the 5' UTR region that produces a low level of transcription of the locus. The 5' UTR does not contain a TATTA box, though this does not exclude the area from acting as a promoter since a TATTA box is absent from some known Drosophila promoters.
(Kutach and Scott, on-line publication). There is only one known full EST of Dmunc-45 (SD10334, The FlyBase Consortium, 2003) and this contains the full 5' UTR. Therefore, there is no direct evidence for a second promoter of Dmunc-45.

4.6 Conclusions

Work with the metazoan homologues of UNC-45 demonstrates a conserved muscle expression, suggesting that UNC-45 is important in muscle development or function. The mesodermal expression of Dmunc-45 seen in this work, and the reduction of Dmunc-45 transcription in embryos lacking a mesoderm (Furlong et al., 2001), suggests this function is conserved in Drosophila as well.

Recent work in C. elegans and the identification of vertebrate UNC-45 homologues has established that UNC-45 family members have important roles outside the muscle. RNAi against UNC-45 in C. elegans embryos indicates involvement of UNC-45 in cytokinesis (Kachur unpublished). This requirement would explain the presence of the vertebrate GC homologue in all cells, as well as provide a possible explanation for the imaginal disc staining seen in Drosophila.

Cells are highly dependent on myosin and actins for cytokinesis. In Drosophila, the non-muscle myosin II is required for basal closure in the cellularizing embryo as well as in cytokinesis (Royou et al., 2004). This non-muscle myosin is also involved in the eversion of imaginal discs (Gotwals and Fristrom, 1991, Edwards and Keihart, 1996, Young et al., 1993). In C. elegans, UNC-45 localizes to the cell cortex of the early embryo in an NMY-2 dependent manner. NMY-2 is a non-muscle myosin II in C. elegans. If the association of UNC-45 and non-muscle myosin II is conserved between
*C. elegans* and *Drosophila*, then Dmunc-45 may interact with zipper in the imaginal discs. This opens a new avenue for exploration of the UNC-45 family of proteins. The zipper gene has been widely studied in *Drosophila* and is involved in a wide number of processes throughout development. In the absence of stable Dmunc-45 mutants, molecular techniques will be useful.

It would be particularly interesting to use RNAi to investigate the role of maternal Dmunc-45 in the embryo, perhaps in an embryo expressing a GFP marker of non-muscle myosin II (produced by Royou et al., 2004). A heat-inducible RNAi construct could also be used to bypass the maternal requirement for Dmunc-45 and investigate its role in the imaginal discs or other areas of development.
### 5 Tables

#### Table 1: Fly Stocks

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name/Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>OrR</td>
<td>Oregon R</td>
<td>This is considered a wild-type strain</td>
</tr>
<tr>
<td>PZ/Sb</td>
<td>( P_{\text{ry}^{+7.2} = \text{PZ}} ) (3) 03692^{03692} \text{ry}^{506} ) ( \text{TM3, ry}^{rk} \text{ Sb}^{1} \text{ Ser}^{1} )</td>
<td>The PZ construct is inserted in the 5' UTR of ( \text{Dmunc-45} ) (CG2708)</td>
</tr>
<tr>
<td>Df(3R)</td>
<td>( \text{Df}(3R)p712, \text{red}^{1} \text{ e}^{1} ) ( \text{TM3, Sb}^{1} \text{ Ser}^{1} )</td>
<td>Carries a deficiency of the third chromosome which deletes ( \text{Dmunc-45} ) and 12 other genes</td>
</tr>
<tr>
<td>Sb/GFP</td>
<td>( w^{+}; \text{ Sb}^{1} ) ( \text{TM3, P}{w^{+mc} = \text{ActGFP}} \text{JMR2, Ser}^{1} )</td>
<td>The GFP gene is driven by the ( \text{Act} ) promoter and marks the presence/absence of the balancer</td>
</tr>
<tr>
<td>PZ/GFP</td>
<td>( w^{+}; \text{ P}{\text{ry}^{+7.2} = \text{PZ}} (3) 03692^{03692} \text{ry}^{506} ) ( \text{TM3, P}{w^{+mc} = \text{ActGFP}} \text{JMR2, Ser}^{1} )</td>
<td>Provides a marked balancer which can be detected in the embryo</td>
</tr>
<tr>
<td>( \Delta 2-3 )</td>
<td>( w; \text{ Sb} \text{ ( \Delta 2-3) e} ) ( \text{TM6, Ubx e} )</td>
<td>Contains the transposase source ( (\Delta 2-3) ) required to mobilize the PZ element</td>
</tr>
<tr>
<td>( \text{fitz-lacZ} )</td>
<td>( w; \text{ Sco} ) ( \text{Cyo fitz-lacZ} )</td>
<td>( \text{lacZ} ) expressed by the ( \text{fitz} ) promoter. This is a control for the embryonic ( \text{lacZ} ) staining</td>
</tr>
<tr>
<td>( \text{ap lacZ} )</td>
<td>( \text{ap lacZ} )</td>
<td>( \text{lacZ} ) expressed by the ( \text{ap} ) promoter. This is a control for the larval ( \text{lacZ} ) staining</td>
</tr>
</tbody>
</table>
**Table 2: Reaction Conditions for PCR Reactions**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Primers used</th>
<th>Thermocycler program*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single embryo PCR</td>
<td>RT2U, RT2L, CG311035, PzOut5-3</td>
<td>94°C for 2 min, 30x(94°C for 30 sec., 58°C or 15 sec., 72°C for 1 min and 30sec.), 72°C for 2 min, 4°C hold.</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>RT2U, RT2L, CHR15 and CHR16</td>
<td>94°C for 3 min, 30x(94°C for 1 min, 55°C for 1 min, 72°C for 2 min), 72°C for 1 min, 4°C hold.</td>
</tr>
<tr>
<td>Determining whether excision caused a deletion</td>
<td>RT2L, CG311035, PzOut5-3</td>
<td>94°C for 1 min, 30x(94°C for 30 sec., 58°C for 15 sec., 68°C for 4 min), 72°C for 1 min, 4°C hold.</td>
</tr>
<tr>
<td>Determining position of P-element insertion in strain 11602</td>
<td>CG11035, PzOut5-3</td>
<td>94°C for 1 min, 29x(94°C for 30 sec., 58°C for 15 sec., 68°C for 30 sec.), 72°C for 1 min, 4°C hold.</td>
</tr>
<tr>
<td>RT-PCR and PCR of escapers</td>
<td>RT2U, RT2L, CG311035, PzOut5-3, CHR15 and CHR16</td>
<td>94°C for 2 min, 30x(94°C for 30 sec., 55°C for 15 sec., 68°C for 2 min and 30 sec.), 72°C for 2 min, 4°C hold.</td>
</tr>
</tbody>
</table>

*MJ Research PTC-200 DNA Engine version 3.0*
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5' to 3')</th>
<th>Binding Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT2U</td>
<td>GCAGCCGGTTCAATAGTCTC</td>
<td>Span first intron of Dmunc-45</td>
</tr>
<tr>
<td>RT2L</td>
<td>GTGGCCAAATCAAAAGTAAG</td>
<td>Span first intron of Dmunc-45</td>
</tr>
<tr>
<td>3CG11035</td>
<td>CGCCCGCTTCACCTTGTGGG</td>
<td>Binds end of predicted gene CG11035, directly upstream of Dmunc-45</td>
</tr>
<tr>
<td>PzOut5-3</td>
<td>GACCACCTTATGTTATTTTC</td>
<td>Binds the 5' and 3' inverted repeat sequences of the PZ P-element.</td>
</tr>
<tr>
<td>CHR15</td>
<td>AGCATACAGGCCCAAGATCG</td>
<td>Span single intron of ribosomal protein L32, also known as RP49.</td>
</tr>
<tr>
<td>CHR16</td>
<td>AGTAAACGCGGGTTCTGCAT</td>
<td>Span single intron of ribosomal protein L32, also known as RP49.</td>
</tr>
<tr>
<td>5'CDS</td>
<td>TTTTTTTTTTTTTTTTTTTTN</td>
<td>Binds poly-A tail of mRNA</td>
</tr>
<tr>
<td>All New</td>
<td>CACGGTCTCGGCACAGGTCTGA AATTAGGG</td>
<td>Binds poly-C tracts added by PowerScript reverse transcriptase.</td>
</tr>
<tr>
<td>THE3</td>
<td>TGTACTCCTCAGTGTTATAGC</td>
<td>Binds PZ element approximately 400bp from the 5' end and primes outward</td>
</tr>
<tr>
<td>KAN2</td>
<td>GTCTCTTGCCGACGGGACC</td>
<td>Binds the PZ element approximately 400bp into the 3' end and primes outward.</td>
</tr>
</tbody>
</table>

In the primer 5'CDS, V represents A, C or G while N represents any nucleotide. Primers RT2U, RT2L, 3CG11035, and PzOut5-3 were designed using the Oligo 4.03 primer analysis software (Rychlik, 1992). Primers CHR15 and 16 were a gift from Sandra O'Keefe from Ross Hodgetts Laboratory (Department of Biological Sciences, University of Alberta). Primers 5'CDS and All New were gifts from Wayne Materi from our lab. Primers THE3 and KAN2 were gifts from Kyle Anderson from Ross Hodgetts Laboratory.
Table 4: Survival Rate of the Progeny of the Heterozygous Cross

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Number of GFP-Expressing Individuals Observed</th>
<th>Number of Non-GFP-Expressing Individuals Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Instar</td>
<td>164</td>
<td>83</td>
</tr>
<tr>
<td>Second Instar</td>
<td>133</td>
<td>63</td>
</tr>
<tr>
<td>Third Instar</td>
<td>110</td>
<td>51</td>
</tr>
<tr>
<td>Pupa</td>
<td>88</td>
<td>34</td>
</tr>
<tr>
<td>Adult</td>
<td>134</td>
<td>9</td>
</tr>
</tbody>
</table>
Figure 1: Phylogenetic Tree of Myosins

Myosins of the three most common types are shown. The ovals represent the MHC head and neck regions along with the associated light chains. Myosin Type I motors consist of a single heavy chain and its associated light chains. Type I myosins are implicated in vesicle movement and are known to have actin binding domains in the tail as well as the head. It is possible that these two actin-binding domains allow myosin I to stably bind one actin cable with the tail while the head translocates along another actin filament. Myosin Type II motors consist of two heavy chains which self-associate in the tail region. These coiled-coil tails can associate with the tails of other myosin II molecules and form long chains such as those seen in striated muscles. Four light chains bind the neck regions of this complex. Myosin type V motors consist of two heavy chains, each with six light chains. The heavy chains dimerize through their tails, but the globular C-terminal (black circles) likely prevents multiple myosin V complexes from associating in a chain. These myosin V complexes are thought to function in the transport of vesicles along actin filaments.

Figure 2: Diagram of General Organization and Function of Three Types of Myosins

Myosins of the three most common types are shown. The ovals represent the MHC head and neck regions along with the associated light chains. Myosin Type I motors consist of a single heavy chain and its associated light chains. Type I myosins are implicated in vesicle movement and are known to have actin binding domains in the tail as well as the head. It is possible that these two actin-binding domains allow myosin I to stably bind one actin cable with the tail while the head translocates along another actin filament. Myosin Type II motors consist of two heavy chains which self-associate in the tail region. These coiled-coil tails can associate with the tails of other myosin II molecules and form long chains such as those seen in striated muscles. Four light chains bind the neck regions of this complex. Myosin type V motors consist of two heavy chains, each with six light chains. The heavy chains dimerize through their tails, but the globular C-terminal (black circles) likely prevents multiple myosin V complexes from associating in a chain. These myosin V complexes are thought to function in the transport of vesicles along actin filaments.
Figure 3: Striated Muscle
Figure 4: Cytokinesis in Eukaryotes

Review of the general mechanisms of eukaryote cytokinesis. Cytokinesis is the division of a mother cell into two daughter cells, and the mechanism of cell division can differ between organisms. Yeast and animal cells divide through use of an actomyosin-based contractile ring (orange arrows), while plants are not dependent on an actomyosin ring for cytokinesis. Both yeast and animal cells position the actomyosin ring at the point of cell separation, though this location varies between organisms. In yeast a division septum is synthesized as the actomyosin ring constricts, and it is degradation of this septum which separates the two daughter cells. In animal cells, the constriction of the actomyosin ring restricts the spindle midzone components into the dense midbody before physical separation of the daughter cells. Chromatin is purple, microtubules are green, the actin/myosin ring is orange, division septum is black. Modified from Guertin et al., 2002.
Figure 5: The Three Founding Members of the UCS Family of Proteins

The *C. elegans* unc-45 encodes a protein of 961 amino acids, which contains three tetratricopeptide repeats (TPR) at its N-terminus. *S. cerevisiae* She4p and the *Podospora anserina* CRO1 proteins lack the TPR repeats, although the UCS domains of the three proteins show high similarity. The C-terminal domains of She4p and CRO1 have some similarity to each other though there is little similarity between the fungal C-terminal and the *C. elegans* mid-domain. Modified from Ao, 2001.

Initial steps are normal. Septum does not form. DNA undergoes meiosis but very aberrant spores are formed.

Initial steps are normal. Crozier is multinucleated. DNA undergoes meiosis but very aberrant spores are formed.

FIGURE 6: Sexual Reproduction of Podospora anserina

A) Wild-type crozier formation and the major steps in ascus formation. B) One possible explanation for the abnormal croziers formed by homozygous cro-I mutants. In this case, the nuclei are partitioned correctly but septum formation is sporadic, resulting in multi-nucleated crozier. C) A second possible explanation for the abnormal croziers of homozygous cro-I mutants. In this case the nuclei are not selectively partitioned and the crozier is multinucleated from formation. Modified from Berteaux-Lecellier et al. (1998).
Figure 7: Development of the Drosophila Embryo


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Figure 8: Plasmids pTC006 and pTC007

The position of Dmunc-45 cDNA in the plasmid is denoted by the gray box. The gray arrow indicates the native direction of transcription of this incomplete fragment of cDNA. The arrowheads indicate the positions of the T3 and T7 promoter sites. The sense strand sequence of the insert is shown.
Figure 9: Diagram of the Genomic Region of the Dmunc-45 Gene and P-element Insertion

A) Cytological region 84E9 on the right arm of chromosome 3. The broken arrows indicate genes CG2747 and ImpE3 originate in this region but terminate in cytological region 84E8. The next predicted locus to the right of Dmunc-45 is CG7918 which is 22kbp to the right, in region 84E10. B) Dmunc-45. The shaded boxes represent the exons, the solid gray boxes show the protein regions and the white line and arrow-head indicate the position of the P-element insertion. C) P-element P{PZ} in correct orientation to Dmunc-45. The sequence on either end of the P-element is the DNA duplicated by the insertion. The white boxes marked P3' and P5' respectively, are the tandem repeats that define the P-element. The rosy gene confers wild-type eye colour when expressed in a rosy' background. The lacZ gene is terminated by the translation stop and polyadenylation sites from Hsp70. The pHSS7 region contains a bacterial origin of replication and kanamycin resistance. D) Sequence of Dmunc-45 surrounding the P-element starting from the predicted start of the gene. The bold letters are the Dmunc-45 sequence that has been duplicated and the white boxes represent the P3' and P5' regions of the P-element. Throughout the figure, the arrowheads represent primers used to characterize chromosomes containing the P-element, primer names are shown in italics. All scale bars (line with diamond ends) represent 500bp.

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Figure 10: Comparison of Drosophila UNC-45 with other UCS proteins

Domains of three UCS proteins are compared to the *Drosophila melanogaster* UCS protein, DmUNC-45 (acc. number NP_524796). The fraction X/Y represents the % identity (X) and % similarity (Y). CeUNC-45 is *Caenorhabditis elegans* UNC-45 (acc. number NP_497205), SMAP-1 is the human smooth muscle cell associated protein 1, also known as SM UNC-45 (acc. number NP_061141), and She4p is the *Saccharomyces cerevisiae* SHE4 protein (acc. number CAA63795). The COOH terminal of She4p is not considered to be homologous to the UNC specific domains of the UNC-45 homologues.
Figure 11: Alignment of Drosophila, C. elegans, and human homologues of UNC-45.

Black boxes indicate identical amino acids, gray boxes indicate similar amino acids and the stars indicate the positions of known mutations in C. elegans unc-45. Figure was prepared by ClustalW (Thompson, 1994) and shading was done by Multiple Alignment Show (Stothard, 2000). The accession numbers for each protein are given in Figure 10.
Figure 12: Alignment of CG2708 With hTOM34

The TPR repeats are boxed. The black backgrounds indicate identical amino acids, while the gray boxes indicate similar amino acids. Accession number of hTOM34 is NP_006800, the accession number of CG2708 is NP_524796.
Figure 13: The Expression Profile of CG2708 and Muscle MHC II

The expression profiles of Dmunc-45 and the muscle heavy chain type II (MHCII) from Drosophila melanogaster. These expression profiles were produced from a micro-array comparing expression of a gene at specific time points to that gene's expression throughout the development of Drosophila. The arrows indicate increased expression during embryogenesis. The yellow colour represents high relative levels of expression while blue represents low levels. The brightest colour is at least three-fold different from the reference black (Compiled from Arbeitman et al., 2002).
Figure 14: Expression of Dmunc-45 assayed by RT-PCR

Dmunc-45 mRNA is maternally provided and expressed throughout development. This RT-PCR is not quantitative. The two upper bands, marked with open arrows, are Dmunc-45 DNA/unspliced RNA(upper) and spliced RNA(lower). The lower two bands, marked with filled arrowheads, represent RP49 DNA/unspliced RNA(upper) and spliced RNA(lower). In A, the egg cDNA is used with (w) or without (w/o) DNase treatment to show that DNase treatment does not substantially diminish the presumptive mRNA band, and therefore production of cDNA and subsequent PCR reaction. In B all samples were treated with DNase but DNA digestion was variable in different samples of Dmunc-45. It appears that RP49 is more susceptible to DNase treatment than Dmunc-45. The ladder used was NEBlabs 100bp marker.
Figure 15: In Situ Staining of Wild-type Embryos of Multiple Stages

The developmental stages of each embryo are represented in the lower right corner of each image (Campos-Ortega and Hartenstein, 1985). Arrowheads indicate staining of somatic mesoderm, bent arrow indicates staining in visceral mesoderm, curved arrow indicates staining of head mesoderm and the straight arrows indicates non-specific staining of the developing trachea. Stage 10, 12, and 13 embryos are presented anterior to the left in a lateral aspect. The stage 16 embryos are presented anterior to the left with a dorsal view.
Figure 16: In Situ Staining of Wild-type Imaginal Discs

Hybridization of an antisense RNA probe against Dmunc45 shows uniform staining thought all discs. The negative control is hybridized with a sense RNA of Dmunc45, while the positive control is wingless. Haltere discs show the same pattern as the wing discs in all cases. All discs shown were stained for one hour.
Figure 17: PCR to Determine the Direction of P-Element Insertion.

These four PCR reactions against a heterozygous individual of the PZ/Sb strain indicate that the P-element is inserted opposite to the direction of transcription of Dmunc-45. Lane A is the PCR products of primers KAN2 and CG11035, B is the products of THE3 and CG11035, C is the products of KAN2 and RT2L, and D is the products of THE3 and RT2L. The primer KAN2 binds specifically in the rosyl gene and amplifies towards the P3' of the P-element (Figure 9). Thus, a band will only be expected when KAN2 is paired with a primer which binds outside the P-element and transcribes towards the P3'. The primer THE3 binds in the lacZ gene and amplifies towards the P5' of the P-element. Thus, a band will only be expected when THE3 is paired with a primer which binds outside the P-element and transcribes towards the P5'. The primer CG11035 binds upstream of Dmunc-45 and the primer RT2L binds inside Dmunc-45. The PCR products marked with the arrowheads indicate the bands predicted if the P-element is inserted in the opposite orientation to Dmunc-45. These bands were isolated and sequenced to confirm their identity. Ladder A is a portion of the HindIII/EcoRI ladder. Ladder B is pBluescript digested with HinfI. The ladders are shown at two different brightness levels to highlight the smaller bands. The size of each band in the ladder is given in kbp to the left of the figure.
Figure 18: Variation Within Eye Phenotype of PZ/Sb

These 4 adult flies show the range from mildest to severe versions of this phenotype. The fly in the lower left is wild-type, lower right is slightly affected, upper left is mildly affected and the upper right is severely affected. For any given fly, the pattern of dead omatidia differed between the two eyes but the general level of involvement was the same between each eye. Occasionally, flies with the entire eye involved were found.
**Figure 19: PCR Genotyping of Flies Carrying the P-Element Insertion**

The $r_y^+ Sb$ individuals should be heterozygous for the P-element containing third chromosome and the TM3 balancer chromosome. The $r_y^+ Sb^+$ individuals should be homozygous for the P-element containing third chromosome. Reaction A uses primers CGI1035 and RT2L, which will only amplify in the absence of the P-element. Reaction B uses primers RT2L and PeOut5-3, which only amplifies in the presence of the P-element. Reaction C uses primers RT2U and RT2L, which will amplify either third chromosome. The negative control lacks template, the positive control uses wild-type DNA as the template. The ladder used is pBluescript digested by *Hinfl*, the size of each band in the ladder is given in kbp to the left of the figure.
Figure 20: The Granular Egg Phenotype

A is an unfertilized egg collected from an OrR virgin female. B is representative of the eggs that do not hatch, laid by PZ/PZ females mated to PZ/GFP males. All eggs were collected over 12 hours and aged 24 hours. Anterior is to the left.

<table>
<thead>
<tr>
<th></th>
<th>Total Eggs Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Failed to Hatch</td>
<td>14</td>
</tr>
<tr>
<td>Hatched</td>
<td>6</td>
</tr>
<tr>
<td>Failed to Develop</td>
<td>5</td>
</tr>
<tr>
<td>Arrested at Stage 17</td>
<td>9</td>
</tr>
</tbody>
</table>

Figure 21: Numerical Breakdown of Close Observation of Development
**Figure 22: Numerical Breakdown of Egg Phenotype for Progeny of Heterozygous Cross**

Those eggs which show GFP expression can be either homozygous or heterozygous for the TM3,P[w^act=ActGF)> JMR2, Ser BALancer chromosome. Lack of GFP indicates lack of balancer chromosome.

**Figure 23: Numerical Breakdown of Egg Phenotype for Progeny of Homozygous/Heterozygous Cross**
Figure 24: Numerical Breakdown of Egg Phenotype for Progeny of Homozygous Cross
In situ with antisense Dmunc-45

β-gal staining of PZ/Sb embryo

In situ of Dmunc-45 and the β-gal staining of PZ/Sb embryos shows a similar pattern in body segments. The arrowheads indicate staining in the mesoderm of each segment. The anterior of the embryo is at the top of the page.

Figure 25: β-gal Staining Compared to In Situ Staining of PZ/TM3 Embryos

Imaginal discs were harvested from wandering third larva of the PZ/Sb or Ap-lacZ strains and stained for β-gal activity. Haltere discs show the same pattern as wing discs and leg discs all show the same pattern. Size of each disc relative to each other was determined by comparing size with in situ imaginal discs.

Figure 26: β-gal Staining of Imaginal Discs

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Figure 27: Analysis of Strain 3161

A) Outline of the cross of excision strain 3161 to a strain carrying a deletion of the third chromosome in the region of interest. B) Chi squared test for how well a data set conforms to the expected outcome. The $Sb$Ser and $Sb$ individuals were grouped together under $Sb$. This was tested against the null hypothesis that the excision carried by strain 3161 is not lethal when hemizygous with a deletion of the region. The critical value for this calculation is 3.8. C) Bands produced with CGI 1035 and RT2L primers from either wild-type, $P\{ry^{+}\text{t}7'=\text{PZ}\}1(3)03692\text{ry}^{\text{066}}\text{/TM3, ry}^{\text{066}}\text{Sb}^{\text{3}}\text{Sb}^{\text{1}}\text{Sb}^{\text{2}}$ (PZ/Sb) or P-element excision stock 3161. In PZ/Sb the band produced is from the balancer chromosome, as the insertion of the P-element prevents these primers from amplifying.
Figure 28: Example of Selective Removal of a P-Element Transcript By Splicing

The orientation of different P-element insertions within the 5' region of the yellow gene is indicated. The black boxes indicate the exons while the patterned box represents the first intron. The observed transcripts from each insertion are shown below the figure. The thick black line indicates regions found in the mRNA, dotted line indicates regions spliced out of the mRNA. Modified from Geyer et al., 1991.
7 References


http://flybase.bio.indiana.edu/bin/fbidq.html?FBal0033895&content=ref-data&refdata=FBrf0091438#FBrf0091438


http://flybase.org/


