IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *dveli*

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *dveli*, THE *DROSOPHILA* ORTHOLOG OF *C. ELEGANS lin-7*

by

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ABSTRACT

Receptors and signal transduction complexes are assembled in a precise manner at specific subdomains of the plasma membrane. Recent research has implicated scaffolding proteins in organizing these receptor and signaling complexes. One well characterized example is the *C. elegans* LIN-2/LIN-7/LIN-10 complex. This complex is essential in the proper localization of LET-23, the EGFR ortholog, to the basolateral membrane surface of vulval epithelial cells.

The mammalian orthologs of the LIN-2/LIN-7/LIN-10 complex have been identified. CASK/VELI/Mint1/X11alpha function as a tripartite complex in neurons, presynaptically and postsynaptically. Presynaptically, the multiprotein complex aids in linking cell adhesion to ion influx, synaptic vesicle fusion with the presynaptic membrane, and subsequent neurotransmitter release. At the post-synaptic membrane, the CASK/VELI/Mint1/X11alpha complex is hypothesized to function in the sorting and proper localization of the NMDA type glutamate receptor, reflecting the function of the *C. elegans* orthologs in receptor localization.

We have identified the *Drosophila* orthologs of LIN-2/CASK, LIN-7/VELI, and LIN-10/Mint1/X11alpha, termed CMG, dVELI and dMINT, respectively. These proteins were found to be highly conserved among species. The *Drosophila* VELI protein was initially identified by the McGlade laboratory, University of Toronto, where it was found to bind phosphorylated *Drosophila* EGFR (DER). We have mapped the chromosomal location of *dveli*, determined RNA transcript distribution and protein localization, and initiated a P-element mutagenesis screen to generate a *dveli* mutant. Furthermore, candidate genes for other proteins known to associate with LIN-7 (PALS) have been identified by sequence analysis.

dVELI expression begins early in the larval stage. It is concentrated mostly in neuropil areas, sites of synaptic connections. This expression pattern continues into adult development. Within the larval CNS, dVELI protein is localized to the neuropil areas of the ventral nerve cord and brain. NMJ staining further localizes dVELI almost exclusively to the post-synaptic density. This post-synaptic localization resembles that of mammalian VELIs, wherein the complex is thought to aid in glutamate receptor sorting and localization. The similarity in structure and expression patterns of dVELI to that of its mammalian orthologs suggests a model in which the *Drosophila* complex aids in the localization of receptors to post-synaptic specializations in neurons.

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LIST OF ABBREVIATIONS

BCIP	5-Bromo-4-chloro-3-indolyl-phosphate, (X-phosphate, 4-toluidine salt)
CAKI	Calcium/calmodulin dependent serine protein kinase (*Drosophila*)
CaMK	Calcium-Calmodulin dependent kinase
CASK	Calcium/calmodulin dependent serine kinase
CMG	Camguk
CNBr	Cyanogen bromide
CNS	Central nervous system
CS-P	Canton S - P-element free
DAB	3, 3'-Diaminobenzidine tetrahydrochloride
DEPC	Diethyl pyrocarbonate
DER	*Drosophila* EGFR
DIG	Digoxigenin
DLG	Discs Large
EGFR	Epidermal growth factor
EM	Electron Microscopy
EMS	Ethylmethane sulfonate
FasII	Fasciclin II
GABA	Gamma-amino butyric acid
Gal4	Galactosidase transgene with 4 binding sites
GST	Glutathione s-transferase
GuK	Guanylate kinase
HRP	Horseradish peroxidase
IPTG	isopropylthio-beta-D-galactoside
L27	LIN-2/LIN-7 heterodimerization domain
LIN	Lineage
MAGUK	Membrane associated guanylate kinase
MDCK	Madin Darby Canine Kidney cells
Mint	Munc18-1-interacting
Munc	Murine uncoordinated
nAChR	nicotinic acetylcholine receptor
NBT	nitroblue tetrazolium chloride
NMDA	N-methyl-D-aspartate type glutamate receptor
NMJ	Neuromuscular junction
NRX	Neurexin
PAGE	Polyacrylamide gel electrophoresis
PALS	Proteins associated with LIN-7
PBS	Phosphate buffered saline
PBT	PBS with Triton
PCR	Polymerase chain reaction

PDZ	Post-synaptic density-95/Discs large A/Zona occludens-1
PID	Phosphotyrosine interacting domain
PSD	Post-synaptic density
PSD-95	Post-synaptic density-95
PTB	Phosphotyrosine binding
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SH3	Src Homology 3
SSC	sodium chloride-sodium citrate
UAS	Upstream activating sequence
VELI	Vertebrate LIN-7
ZO-1	Zona occludens-1

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Dr. J. R. Jacobs dissected and fixed adult *Drosophila* nervous system tissues used for subsequent antibody labeling. Christian Smith, Sick Kids Hospital, Toronto, generously provided the dVELI GST fusion construct used in the generation of a polyclonal antibody. NMJ preparations were performed by Dr. Bryan Stewart at University of Toronto, Sick Kids Hospital.

Chapter 1
Introduction

Reproducibility and efficiency in intercellular communication is essential in maintaining normal cellular function. Countless cellular processes are dependent upon the activation of a unique signal transduction pathway. Receptors on the plasma membrane activate an intracellular signaling cascade that triggers a cellular response to extracellular signals. These receptors and the associated signaling proteins are not distributed randomly on the cell surface, instead there is a precise spatial and temporal organization of such signaling complexes within the cell. Cell polarization determines a defined geometry for the players in signal transduction pathways especially at cell-cell junctions, so that a specific signal may always contact its target site from a defined site within the cell. Polarization of this sort is seen in many cell types, including both epithelial and neuronal cells, and is essential for normal cell function (Woods and Bryant, 1993; Sheng and Wyszynski, 1997).

1.0 Apical-Basolateral Polarization of Epithelial Cells vs. Pre-synaptic-Post-synaptic Polarization of Neuronal Cells

Epithelial cells are arranged in sheets with distinctive and specialized junctions present between adjacent cells within the sheet. These specialized junctions include vertebrate tight and adherens junctions and arthropod septate junctions. They function to provide a physical contact between cells and to limit free diffusion of molecules and ions and contribute to the establishment of a polarized structure, with an apical and a basolateral membrane (Woods and Bryant, 1993). These membranes are biochemically well as morphologically distinct. Receptors for certain growth factors as

well as the ligands that activate such receptors are displayed in a polarized fashion within the epithelial cell. For example, *C. elegans* LET-23, similar to the epidermal growth factor receptor (EGFR), is basolaterally located within vulval epithelial cells (Simske *et al,* 1996) where it can detect its ligand, LIN-3, from adjacent cells. Similarly, the EGFR is found at the basolateral membrane within human gastrointestinal epithelial cells (Playford *et al,* 1996).

Neuronal cells are thought to be polarized in a manner analogous to epithelial cells, wherein the pre-synaptic membrane is the apical membrane, while the post-synaptic membrane is basolateral (Dotti and Simons, 1990). A synapse is an asymmetrically arranged cellular junction consisting of a presynaptic bouton, a synaptic cleft and an electron dense area known as the post-synaptic density (PSD). Neurotransmitters are found within the pre-synaptic bouton where they are released from vesicles into the synaptic cleft, allowing for contact with respective receptors and ion channels at the PSD.

Ion channels and receptors are not distributed randomly on the synapse. Voltage-gated ion channels are found in different post-synaptic sites within the same neuron - excitatory receptors into excitatory synapses and inhibitory receptors into inhibitory synapses. Much of what has been learned about this polarization has been the result of studies on neuromuscular junctions (NMJs). For example, Shaker K^+ channels in the *Drosophila* NMJ are located at the PSD (Tejedor *et al,* 1997). Similarly, mice nicotinic acetylcholine receptors (nAChRs) have also been found clustered at the PSD (Apel *et al,* 1995). Segregation of voltage gated ion channels allows for efficient transmission of neuronal signals.

The requirement for proper localization of signaling membrane complexes within both neuronal and epithelial cells becomes apparent when targeting is disrupted. Apical rather than basolateral localization of *C. elegans* LET-23 results in a vulvaless phenotype

for the nematode, similar to what is seen when LET-23 itself or its ligand, LIN-3, are mutant or missing altogether (Kim and Horvitz, 1990; Hoskins *et al,* 1996; Simske *et al,* 1996). In a similar manner, mislocalization of the human EGFR is seen in several diseases, including polycystic kidney disease (PKD) (Du and Wilson, 1995). In neuronal cells, the gross morphology of synapses is affected by mislocalization. Rapsyn is required for the clustering of nAChrs at the PSD (Apel *et al,* 1995). Targeted disruption of rapsyn in mice results in malformed NMJ and perinatal lethality.

The similarity in polarization of both epithelia and neurons suggests that protein complexes and mechanisms involved in the polarization of receptors and ion channels are shared between the cell types (Dotti and Simons, 1990). In fact, it has been shown that ectopic expression of proteins in neurons that are normally basolaterally located in epithelia localize at the post-synaptic density (Dotti and Simons, 1990). The converse has been found as well. The glutamate receptor (GLR-1), normally situated at the PSD, is localized to the basolateral membrane of epithelial cells when expressed ectopically (Rongo *et al,* 1998).

The shared assembly of differing components at the correct membrane in both epithelial cells and neurons has been the focus of much research. Given the association between improperly localized receptor complexes and human disease, an understanding of the basis of cell polarity is needed. Recent studies have focused on the scaffolding proteins responsible for the assembly of protein complexes believed to be responsible for localizing the receptors to the proper membrane. In particular, what structural domains in scaffolding proteins are essential for assembly? One protein motif that appears to be required in many scaffolding proteins is the PDZ domain (Cho *et al,* 1992; Woods and Bryant, 1991; Itoh *et al,* 1993).

1.1 The PDZ Domain

The PDZ domain is found in a diverse array of proteins seemingly associated with cell junctions, both epithelial and neuronal. It was first identified as a region of three repeats of about 90 amino acids in the post synaptic density protein **P**SD-95, the *Drosophila* tumor suppresser protein **D**iscs Large A (DLG) and the epithelial tight junction protein **Z**ona occludens 1 (ZO-1) (Cho *et al,* 1992; Woods and Bryant, 1991; Itoh *et al,* 1993). The PDZ domain has since been found in a variety of proteins, including transmembrane and scaffolding proteins, implicated in cell-cell junctions.

PDZ domains are typically 90-100 amino acids in length. The domain's secondary structure consist of six ß strands and two αhelices (Doyle *et al,* 1996), with a connecting loop that contains the conserved GLGF repeat (Gly-Leu-Gly-Phe), after which the domain was originally named (Cho *et al,* 1992). The function of PDZ domains in receptor localization is supported by the finding that the N-terminal pair of PDZ domains of PSD-95 binds a specific C-terminal sequence of Shaker type K^+ channels in synapses (Kim *et al,* 1995). PSD-95 has been shown to be concentrated at synapses, where it co-localizes with the Shaker type K^+ channels (Kim *et al,* 1995), as well as the NR2 subunit of the NMDA receptor (Kornau *et al,* 1995), clustering these receptors at both presynaptic and postsynaptic sites. In the absence of PSD-95, Shaker and NR2 proteins do not cluster at the synaptic membranes (Kim *et al,* 1995).

Elucidating the mechanisms by which PDZ proteins function to localize receptors and/or ion channels has been the focus of recent research. Several PDZ proteins have been identified that appear to function in the assembly of multiprotein complexes with transmembrane receptors, adhesion molecules and ion channels. One intensely studied complex that has led to much of our recent understanding of PDZ protein function has

been the *C. elegans* LIN-2/LIN-7/LIN-10 complex, required for the basolateral localization of the LET-23 receptor in vulval epithelial cells (Kaech *et al*, 1998).

1.2 Basolateral Localization of the LET-23 Receptor by the PDZ Protein Complex LIN-2/LIN-7/LIN-10

LET-23 is the *C. elegans* receptor tyrosine kinase (RTK) ortholog of the EGFR (Aroian *et al,* 1990). It is essential for vulval induction during nematode development. LIN-3, the anchor cell signal (Hill and Sternberg, 1992), is the ligand for LET-23, binding the receptor to activate an RTK/Ras signal transduction pathway that determines vulval fate (reviewed in Eisenmann and Kim, 1994).

During the larval stage of development, the vulva is derived from six vulval precursor cells, which are polarized epithelial cells with cell junctions between them (see Sternberg, 1993 for a more in depth review of vulval development). These cells, or Pn.p cells, can possess one of three fates - the 1°, 2° or 3° - leading to the development of the inner part of the vulva, outer part of the vulva or an uninduced fate, respectively. The determination of fate of each of the Pn.p cells is governed by the gonadal anchor cell signal, LIN-3, which activates the RTK/Ras pathway initiating the cell to take on its respective fate.

LET-23 has been found on the basolateral membrane surface, asymmetrically distributed on the surface of vulval epithelial cells (Simske *et al,* 1996). It has been hypothesized that basolateral localization of LET-23 is essential to proper function since LIN-3, the ligand, is found at the basal side of the extracellular matrix (Simske *et al,* 1996). Much research has focused on the mechanism underlying LET-23 localization to the basolateral surface. Genetic screens have identified several genes that result in a vulvaless phenotype. Some of these genes have been implicated in the role of localizing

LET-23 to the proper membrane. *lin-2, lin-7* and *lin-10* were originally identified in a genetic screen for genes involved in the RTK/Ras vulval induction pathway (Ferguson and Horvitz, 1985). Subsequent analysis of these mutations revealed they also result in the mislocalization of LET-23 to the apical surface rather than the basolateral surface (Simske *et al,* 1996; Whitfield *et al,* 1999).

LIN-2, LIN-7 and LIN-10 are all characterized by the presence of various domains known to function in protein-protein interactions. LIN-2 is a member of the membrane associated guanylate kinase (MAGUK) family of proteins, which includes several subfamilies, including the LIN-2-like MAGUKs after the discovery of LIN-2 (reviewed by Dimitratos *et al,* 1999). MAGUKs are thought to participate in the assembly of molecular scaffolds at cell-cell junctions, both epithelial and neuronal. LIN-2, like all the MAGUKs in its subfamily, possesses a PDZ domain, an SH3 domain, a CaM kinase/calmodulin binding domain and a guanylate kinase (GuK) domain (Hoskins *et al,* 1996). LIN-7 is a small protein that contains a single PDZ domain at its carboxy end (Simske *et al,* 1996). LIN-10 was originally thought to be a protein with no known homologies or domain specifications (Kim and Horvitz, 1990), however initial molecular identification of LIN-10 was deemed incorrect (Rongo *et al,* 1998; Whitfield *et al,* 1999). LIN-10 was found to possess two PDZ domains as well as a phosphotyrosine binding (PTB) domain. Through a set of intricate genetic and molecular experiments, LIN-2, LIN-7 and LIN-10 have been shown to form a ternary complex required for the proper localization of LET-23 to the basolateral membrane (Kaech *et al,* 1998).

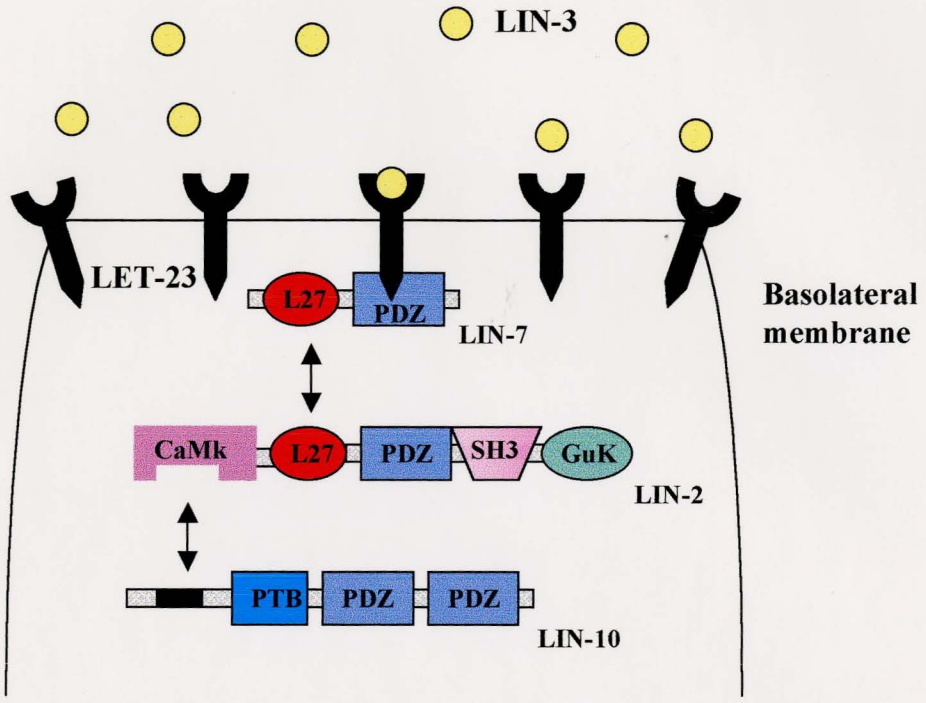
The PDZ domain has been shown to bind to three different types of an amino acid sequence at the carboxy terminal of some receptors and ion channels (Songyang *et al,* 1997; Maximov *et al,* 1999). LET-23 possesses a type I PDZ binding sequence motif (S/T-X-I/L/V-$COOH^-$), with its carboxy end terminating in T-C-L, a classic PDZ binding

motif (Aroian and Sternberg, 1991). A mutation in LET-23 that removes the last six amino acids, and subsequently the PDZ binding motif, results in mislocalization of LET-23 to the apical membrane, similar to what is seen in mutants of LIN-2, LIN-7 and LIN-10 (Kaech *et al,* 1998). The hypothesis that LIN-2/LIN-7/LIN-10 functions as a complex to bind to the carboxy terminus of LET-23 and to localize it to the basolateral membrane surface was verified by co-immunoprecipitation experiments, yeast-two-hybrid screens as well as mutational analysis (Kaech *et al,* 1998).

The protein domain interactions within the ternary complex have been elucidated. Functioning as a complex, LIN-10 binds LIN-2, LIN-2 binds LIN-7 and LIN-7 binds to the C-terminus of LET-23 and localizes it to the basolateral membrane surface. The N-terminal region of LIN-10 binds LIN-2 at its CaM Kinase domain. LIN-2 and LIN-7 interact with each other via a region of LIN-2 between the CaM kinase domain and the PDZ domain and the N-terminal region of LIN-7 prior to its PDZ domain. This region has recently been named the L27 region, indicative of its conservation amongst LIN-7 and LIN-2 MAGUK like proteins (Doerks *et al*, 2000). Finally, the PDZ domain binds to LET-23 through its carboxy sequence, a type I PDZ binding motif
(See figure 1).

The mechanism through which the ternary complex localizes LET-23 to the basolateral membrane surface is not fully understood. The scaffolding complex may act to target LET-23 to the basolateral membrane of epithelial cells or alternatively, the LIN-2/LIN-7/LIN-10 complex may act to selectively retain LET-23 at the basolateral membrane (Kaech *et al,* 1998). Whatever the mechanism, basolateral localization of LET-23 is required for proper signaling.

Figure 1. Basolateral localization of *C. elegans* LET-23 by the tripartite complex of LIN-2/LIN-7/LIN-10. The *C. elegans* LIN-2/LIN-7/LIN-10 complex functions in localizing LET-23 to the basolateral membrane surface of vulval epithelial cells. N-terminal to the PTB domain, LIN-10 is bound to the CaM Kinase domain of LIN-2. LIN-2 and LIN-7 are in turn heterodimerized via their respective L27 domains, a region N-terminal to the PDZ domain. Finally, the PDZ domain of LIN-7 binds the C-terminus of LET-23, which possesses a type I PDZ binding motif (T-C-L). The LIN-2/LIN-7/LIN-10 complex is essential in proper localization of LET-23, with missing or non-functional LIN-2, LIN-7 or LIN-10 resulting in mislocalization of LET-23 and subsequent failure of vulval induction. Basolateral localization of LET-23 allows for contact with its ligand LIN-3, the anchor cell signal. Binding of LIN-3 to LET-23 initiates a signal transduction cascade involving Ras and Raf orthologs, LET-60 and LIN-45 respectively, that determines 1°, 2°, or 3° vulval fates.



LIN-3 (EGF) → LET-23 (EGFR) → LET-60 (Ras) → LIN-45 (Raf) →→→ VULVAL FATE

The identification of the LIN-2/LIN-7/LIN-10 scaffolding complex in *C. elegans* and the resulting phenotype of mutations in genes encoding for these proteins reinforces the importance of the proper targeting of receptors to the respective apical or basolateral surface. With the discovery of the ternary complex, it was noted that both LIN-2 and LIN-10 are similar to known mammalian proteins, CASK and Mints/X11 respectively (Hata *et al,* 1996; Okamoto and Sudhof, 1997). CASK is found in both epithelia and neurons and has been shown to bind neurexin, a cellular adhesion molecule in neurons (Hata *et al,* 1996), while Mints/X11 are a family of proteins that bind Munc18, a protein involved in fusion of vesicles to the presynaptic membrane in neurons (Okamoto and Sudhof, 1997). The similarity of LIN-2 and LIN-10 to mammalian proteins known to function in neurons suggests this complex is a shared component of both epithelia and neurons and has lead to the characterization of the function of the mammalian complex in neurons.

1.3 CASK/VELI/Mint/X11, the Mammalian LIN-2/LIN-7/LIN-10 Complex, Functions in Neurons

CASK is a member of the MAGUK family of proteins, found to be similar to the *C. elegans* LIN-2. It is expressed in synaptic connections where it binds neurexin, a cell adhesion molecule at the pre-synaptic membrane (Hata *et al,* 1996). Some neurexins bind neuroligins, a cell adhesion molecule at the post-synaptic side, forming an intercellular junction (Nguyen and Sudhof, 1997). Intracellularly at the post-synaptic side, PSD-95 is found bound to neuroligin where it acts as a scaffolding molecule to recruit receptors and other molecules to the neurexin-neuroligin synaptic junction (Irie *et al,* 1997). Recent research has now suggested that CASK acts in a similar manner as a scaffolding protein at the pre-synaptic membrane as a member of a tripartite complex

whose other members are VELIs/MALS (LIN-7) and Mints/X11 (LIN-10) (Butz *et al,* 1998; Borg *et al.,* 1998a).

With the identification of the LIN-2 and LIN-10 mammalian orthologs, a LIN-7 ortholog was sought. Through a search of EST data banks, several groups reported the existence of mammalian LIN-7 proteins (Borg *et al,* 1998a; Butz *et al,* 1998; Jo *et al,* 1999; Irie *et al,* 1999). These mammalian LIN-7 proteins, known as VELIs (vertebrate LIN-7), MALS (mammalian LIN-7) or simply mLIN-7, are similar in structure to the *C. elegans* LIN-7 protein. The mammalian orthologs, herein referred to as just VELIs, consist of three isoforms each containing a single PDZ domain as well as the L27 binding domain (Butz *et al,* 1998).

CASK, Mint1/X11alpha and VELI form a tripartite complex that is present in synapses with CASK anchoring the complex to the pre-synaptic membrane through its interaction with neurexin via the PDZ domain (Butz *et al,* 1998). The CaM kinase domain of CASK binds Mint1/X11alpha while the L27 domain of Cask recruits VELI (Butz *et al,* 1998). As with the *C. elegans* LIN-2/LIN-7/LIN-10, this leaves free the remaining C-terminal domains to bind other still unknown proteins within the complex. It is still unknown what VELI binds at the pre-synaptic membrane, analogous to the way LIN-7 in vulval epithelial cells binds LET-23. However, even with this still unknown it is suspected VELI acts to localize either receptors and/or neurotransmitters to the pre-synaptic membrane surface. With this possibility, the tripartite complex of CASK, VELI and Mint1/X11alpha model functions to link cell adhesion (via CASK binding neurexin) to signal transduction (via VELI) and synaptic vesicle exocytosis (Mint1/X11alpha association with Munc 18-1) at the pre-synaptic membrane.

Recent work has suggested that the ternary complex of CASK, VELI and Mint1/X11alpha may have multiple functions at the synapse. One remarkable line of

evidence for this has been the association of VELIs isoforms at the post-synaptic density (Jo *et al,* 1999). VELI-1 and VELI-2 are neuron specific and are enriched at the post-synaptic density in association with PSD-95, while VELI-3 is expressed in peripheral tissues as well (Jo *et al,* 1999). However it has been found that the association with PSD-95 is not direct but secondary through binding of the N-methyl D-aspartate (NMDA) type glutamate receptors. Previous studies have shown that PSD-95 binds the NR2 subunit of NMDA (Kornau *et al,* 1995). VELIs bind the C-terminus of the NR2B subunit of NMDA, which terminates in a typical type I consensus sequence for PDZ domains (Jo *et al,* 1999), suggesting a model in which VELI act to aid in NMDA glutamate receptor sorting. The presence of CASK and Mint1/X11alpha within this VELI complex of NR2 is still unknown, but the possibility of the tripartite complex functioning at both the pre-synaptic and the post-synaptic membrane requires further biochemical characterization.

1.4 VELIs Location in Epithelial Cell-Cell Junctions Suggests a Multi-functional Role for this Novel Protein

The small PDZ protein VELI has been found in several different epithelial tissue types in mammals (Irie *et al,* 1999; Perego *et al,* 1999; Perego *et al,* 2000) suggesting a role in the localization of receptors in the epithelial cells similar to the role of *C. elegans* LIN-7. However, contrary to LIN-7, mammalian VELIs have not been shown to associate with the mammalian EGFR (Borg *et al,* 1998a; Irie *et al,* 1999; Jo *et al,* 1999). VELI in epithelial cells has been shown to associate with the gamma-aminobutyric acid (GABA) transporter BGT-1 (Perego *et al,* 1999). VELI-1 is responsible for the basolateral localization of this transporter in the well characterized Madin-Darby canine kidney (MDCK) cells.

Although VELI has been found to function in epithelial cells as well as neurons, the presence of the tripartite complex in epithelial cells in mammals has yet to be determined. All cells express low levels of CASK (Hata *et al,* 1996). Mints/X11 family of proteins has three known members - Mint1/X11alpha, Mint2/X11beta and Mint3/X11gamma. Mint1/X11alpha and Mint2/X11beta are neuron specific, while Mint 3/X11gamma is expressed in epithelial cells as well (Borg *et al,* 1998b). CASK/VELI interaction in renal epithelial cells has been shown (Straight *et al,* 2000). Co-immunoprecipitation experiments demonstrate that CASK binds VELI and co-localization of both proteins is seen at the basolateral membrane surface of MDCK cells (Straight *et al,* 2000). The proteins were shown to bind one another in a similar manner as in neurons, binding via their L27 regions. However, the presence of the third member of the tripartite complex, Mints/X11 has not been demonstrated. No interaction (via biochemical approaches such as co-immunoprecipitation) has been found between CASK/VELI and the epithelial type Mint/X11, Mint3/X11gamma (Straight *et al,* 2000). Similarly, the localization of Mint3/X11gamma in epithelial cells differs somewhat from that of CASK and VELI distribution. Furthermore, Mint3/X11gamma failed to co-immunoprecipitate with the CASK/VELI complex, even when Mint3/X11gamma was overexpressed in renal epithelia, where normal levels of the protein are relatively low. However, when Mint1/X11alpha, the neuronal type, was misexpressed in epithelial cells it could be co-immunoprecipitated with the CASK/VELI complex (Straight *et al,* 2000). It is possible there is another member of this family that has yet to be detected which functions in the epithelial complex. Further research in this field may shed more light on the function of the Cask/VELI complex in mammalian epithelial cells and the role Mints/X11 have in this epithelial function.

1.5 *Drosophila*- an Excellent Genetic Model Organism for the Study of the Tripartite Complex of LIN-2/CASK, LIN-7/VELI, LIN-10/Mint1/X11alpha

Drosophila melanogaster is an excellent model organism for the study of this scaffolding complex, both genetically and biochemically. The ease of genetic manipulation of *Drosophila* as well as the biochemical tools available for use, including a fully sequenced genome, allows for a wealth of possible experimental approaches in studying protein-protein interactions. With the conservation of the LIN-2/LIN-7/LIN-10 scaffolding complex from *C. elegans* to mammalian species, the question arose as to whether or not this complex functions in *Drosophila*. The only identified *Drosophila* member of this complex to date has been the LIN-2 ortholog, known as CAMGUK (CMG), which appears to function in synapses (Dimitratos, 1999).

cmg was identified by two separate groups, one group naming the gene *caki* (Martin and Ollo, 1996) and the other group referring to the gene as *camguk* (Dimitratos *et al*, 1997). CMG is a member of the MAGUK family of proteins, specifically the LIN-2 like sub-family. It shares domain identities with LIN-2 and CASK, containing a PDZ domain, an SH3 domain, a Guk domain, a CaM kinase domain as well as the LIN-7 binding site domain, L27. When comparing amino acid identity of CMG to LIN-2 and CASK, the *Drosophila* protein is 47% and 61% identical, respectively. Thus, the *Drosophila* protein is more similar to the mammalian protein counterpart, CASK.

Expression patterns of CMG have been studied. Tissue RNA *in situ* hybridization of *cmg* in embryos and larvae detect expression mainly in the central nervous system and optic lobe areas (Martin and Ollo, 1996; Dimitratos, 1999). *CMG* transcripts appear beginning in stage 9-10 embryos, localized to the ventral nerve cord and the brain (Martin and Ollo, 1996). In larval stages, *CMG* transcripts are detected within the ventral ganglion of the nerve cord and throughout the brain, as well as in low levels in all

imaginal discs (Dimitratos, 1999). CMG protein has been detected in the neuropile of the nerve cord and the optic lobe areas of the brain in larvae (Dimitratos, 1999) as well as the neuropile areas of the adult brain, mainly the optic lobe (Martin and Ollo, 1996). Neuropile areas are composed mainly of synapses rather than cell bodies, implying function of CMG in synaptic connections.

This initial data for the LIN-2 ortholog, CAMGUK, suggests function more similar in nature to that of the mammalian complex, functioning in the assembly of components at synaptic densities. However, the identification and characterization of the other members of the complex in *Drosophila,* LIN-7 and LIN-10 orthologs, is needed in order to fully understand the function of this complex in *Drosophila.* This is the focus of this thesis.

The *Drosophila lin-7/veli* cDNA (herein referred to as *dveli*) was isolated in a screen for proteins containing a PDZ domain at Jane McGlade's laboratory, University of Toronto, Canada. dVELI was isolated along with several other proteins. Initial work by the McGlade laboratory found dVELI binds the phosphorylated *Drosophila* EGFR (DER). This cDNA construct was then sent to Roger Jacobs' laboratory at McMaster University where the project was taken over. Because of work on the DER in Roger Jacobs laboratory, this protein was of interest because of its potential association with DER, similar to the way in which LIN-7 associates with LET-23 (Simske *et al,* 1996).

The focus of this thesis will be to characterize *dveli* as well as to identify within the genome candidate genes for proteins likely to associate with dVELI, including LIN-10. *dveli* chromosomal location has been found in the genome and its similarity to other known LIN-7/VELI proteins will be presented. Expression analysis of *dVELI* transcripts has been studied in embryos, larvae and adults leading to further characterization based on protein expression patterns. An antibody to this protein has been generated in order to

study the spatial distribution as well as the temporal distribution of dVELI. Finally, potential mutant alleles of *dveli* will be presented along with P-element mutagenesis of the *dveli* locus.

Other proteins known to be associated with LIN-7/VELIs have been found in the *Drosophila* genome based on sequence similarity. The initial characterization of these proteins will be presented. *Drosophila* LIN-10/Mint1, herein referred to as dMint, has been identified and initially characterized. The *dMint* chromosomal location has been found and dMINT comparisons to other known LIN-10/Mint1/X11alpha proteins to determine amino acid identity will be presented. Initial expression patterns of *dMint* transcripts will also be described.

PALS proteins (**p**roteins **a**ssociated with **L**IN-7), contain the L27 binding domain and have recently been found associated with the amino terminus of mammalian VELIs (Kamberov *et al,* 2000). Two novel members that have been reported to associate with LIN-7 are PALS1 and PALS2. These proteins are both MAGUK protein family members suspected to compete for binding to mammalian VELIs with LIN-2/CASK (Kamberov *et al,* 2000). With the identification of the *Drosophila* LIN-2/CASK protein, CMG and the above mentioned identification of dVELI, the potential for the conservation of other members of this scaffolding complex exists. Therefore, a study of the *Drosophila* genome has been carried out to identify candidate binding partners.

The multi-functional role of the mammalian tripartite complex in both neurons and potentially epithelial cells demonstrates well the idea of conserved scaffolding assembly components working in different contexts to maintain polarity. The localization of CMG to the neuropil suggests the *Drosophila* complex may function in synapses. Its presence in imaginal discs indicates an epithelial role as well. dVELI and dMINT identification and characterization will hopefully bring to light the dual function

of this complex. We hypothesize the complex acts in neurons, functioning to localize receptors to synaptic specializations, and in epithelial cells, localizing receptors to septate junctions.

Chapter 2

Methods

2.0 *Drosophila melanogaster* Strains

Drosophila melanogaster strains used included the following: CS-P (Canton S - P-element free), In(1)LD30/FM7c (*paralytic*), a variety of uncharacterized lethals on the 3rd chromosome referred to as L(3R)96Ba/TM3, L(3R)96Bb/TM3, L(3R)96Bc/TM3, L(3R)96Bd/TM3, L(3R)96Be/TM3, L(3R)96Bf/TM3 and L(3R)96Bg/TM3 as well as a deficiency on the 3rd chromosome, Df(3R96B)/TM3. A P-element inserted within the *toll* gene was used, P{FZ}Tl^{F336}, referred to herein as F336. Finally, P-element mutagenesis crosses were performed making use of several fly lines: Dr/TMSΔ2-3Sb, P{w^{+}=lacW}L(3)OstStt^{J2D9}/TM3Sb and yw^{-}; D/TM3.

CS-P is a wild type control strain known to be P-element free. It was used in all controls and was obtained from the Bloomington Stock Center. In(1)LD30/FM7c is an inversion with a breakpoint disrupting *paralytic* , which encodes a voltage gated sodium channel. It was generously provided by Barry Ganetzky (Ganetzky, 1984). The uncharacterized lethals on the third chromosome were obtained from Bloomington Stock Center. They were generated in an EMS mutagenesis screen by E.B Lewis and were reported to be allelic. All other fly stocks used were obtained from the Bloomington Stock Center.

2.1 Embryo, Larvae and Adult *Drosophila* Collections

Embryos were collected by placing adult flies in 100 ml plastic beakers with holes punched in them for air flow. The open end of the beaker was capped with a 60 X 15 mm petri dish on which apple juice-agar had been placed, along with a dab of yeast paste to encourage egg laying. These "houses" were placed at 25°C for varying times in order to collect a mixture of stages of embryonic development. Plates placed on in the morning were left on at 25°C for about eight hours. These plates were then placed at 18°C overnight to slow development of the embryo. The new plate that was put on in its place stayed on overnight and the next morning was removed and, along with the 18°C plate, was placed at 4°C. Through this method an appropriate mixture of mid stage to late stage embryos were collected. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

1st, 2nd and 3rd instar larvae were collected on yeast food plates or vials. For 1st and 2nd instar larvae, houses were made as explained previously, however this time the plates that were removed were kept at 25°C to age the embryos to the desired larval stage. 1st instar larvae were aged at 25°C for 19-24 hours. 2nd instar larvae were aged for approximately 48-50 hours. For 3rd instar larvae, the wandering stage of *Drosophila* larvae, the larvae were aged for about 80-90 hours in yeast food vials. Those larvae that began to crawl up the sides of the vial were 3rd instar larvae. These larvae were then collected in a larval sieve.

Adult *Drosophila* that were used for different preparations were collected from wild type food vials. Week old adult males and females, which normally would have been disposed of after successful mating and egg laying, were collected in empty food vials to rid the flies of any ingested yeast. After approximately two-three hours, the flies were ready for use.

2.2 Isolation of *dveli* cDNA

Initial isolation of *dveli* cDNA was performed by the McGlade Laboratory at the University of Toronto using a *Drosophila* 22-24 hour embryo EXLOX cDNA library from Novagen. Using a biotinylated peptide that corresponded to the 11 carboxy terminal amino acids of the *Drosophila* Inscuteable protein, which contains the classic PDZ binding motif, several proteins were isolated.

The embryonic cDNA library was plated using the appropriate titer. After plaques formed, the plate was overlaid with IPTG soaked nitrocellulose to induce protein expression. The nitrocellulose was washed, blocked and incubated overnight at 4°C with the appropriate concentration of a mixture of the biotinylated peptide and streptavidin alkaline phosphatase. The next day, the nitrocellulose was washed and reacted with nitroblue tetrazolium chloride (NBT) plus 5-Bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (x-phosphate, 4-toluidine salt)(BCIP) solution. Positives were autosubcloned into vectors and sent off for sequencing. One of the resulting positive clones obtained was the *dveli* cDNA clone (Appendix 1).

2.3a Preparation of Biotin labeled DNA probe for Polytene Chromosome *in situ* Hybrization

Biotin-labeled DNA probes were generated using the BIONICK™ Labeling system (Life Technologies, Inc. cat. # 18247-015). Nick translation produces small (50 - 500 base) biotin-labeled DNA probes through incorporation of biotin-14-dATP.

1 µg of the *dveli* cDNA template was pipetted into a 1.5 ml microcentrifuge tube along with 5 µg 10X dNTP mix, 5 µl 10X enzyme mix and distilled water to 45 µl final volume. The mixture was then incubated at 18°C for two hours. The nick translation reaction was terminated through the addition of 5 µl of stop buffer. To separate unincorporated nucleotides from the labeled DNA probe, ethanol precipitation was performed. 5 µl of 3M Sodium Acetate and 100 µl of cold 100% ethanol were added to

the microcentrifuge tube, inverted to mix and placed at -80°C. After 15 minutes, the microcentrifuge tube was centrifuged at 15, 000 xg for ten minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. After centrifuging once again, the supernatant was discarded, the pellet air dried and resuspended in 50 µl of hybridization solution (250 µl formamide, 100 µl 50% dextran sulfate, 40 µl herring sperm DNA, 50 µl 20X SSC, 60 µl ddH_2O). The probe was stored at -20°C until preparation for hybridization to the chromosomal squashes.

2.3b Preparation of Salivary Gland Chromosome Squashes

Salivary glands from 3rd instar larvae were dissected in 45% acetic acid and then transferred into a drop of 1:2:3 solution (1 part lactic acid : 2 parts H_2O : 3 parts acetic acid) on a siliconized coverslip for four-five minutes. Siliconized coverslips were made by dropping coverslips in a solution of 5% dimethyl-dichlorosilane in chloroform and then rinsing off with 100% ethanol. An SSC-Denhart treated slide is placed on top of the coverslip containing the salivary glands, positioning the coverslip in the center of the slide. SSC-Denhart treated slides were made by incubating slides for 2.5 hours at 65°C in SSC-Denhart solution (3X SSC, 0.02% PVP 360, 0.02% Ficoll, 0.02% nuclease free BSA) and then fixing in a 3:1 ethanol : acetic acid fixative for 20 minutes at room temperature and air drying. These SSC-treated slides are stored at 4°C until use.

With the coverslip positioned in the center of an SSC-Denhart slide, the chromosomes were spread by hammering carefully with the blunt end of a pencil. The chromosomes were further spread by making streaks over the coverslip with a needle. The slides were then placed overnight at 4°C. The following day, the coverslips were removed by dipping the slides in liquid nitrogen for 15 seconds and popping the coverslip

off using a razor blade. The chromosomal squashes were then incubated in 95% ethanol for 1.5 hours, air dried and stored at 4°C until used for *in situ* hybridization.

2.3c Polytene chromosome *in situ* label

Slides containing chromosomal squashes were treated with 2X SSC for 30 minutes at 65°C and then dehydrated through an ethanol series at room temperature (70% ethanol for five minutes, 70% ethanol for ten minutes, 95% ethanol for ten minutes). After air drying, the slides were denatured in 0.07 N sodium hydroxide for one-three minutes. The slides were washed sequentially in three baths of 2X SSC for five minutes each at room temperature and again ethanol dehydrated. After air drying, hybridization to the biotin - labeled probe was carried out.

A 1 in 10 dilution of the probe in hybridization solution was heated in boiling water for five minutes to make it single stranded and then immediately placed on ice. 20 µl of the probe was applied to a coverslip, which was then placed on the slide at the position the salivary glands were located. The coverslip was sealed to the slide with rubber cement and the slides placed at 37°C in a moist chamber to hybridize overnight.

The next day, slides were washed in two baths of 2X SSC for ten minutes at 37°C. During these washes, the coverslips were removed. Two more washes with 2X SSC were followed by washing slides for five minutes in 1X PBS. Using the Vectastain® Elite ABC Kit (Vector Laboratories, cat. # PK - 6100) to employ a sensitive method of detection, an avidin and biotinylated horseradish peroxidase macromolecular complex was made by mixing equal volumes (10 µl) of REAGENT A and REAGENT B to 1.25 ml of 50 mM Tris, pH 7.6 containing 4% BSA. 20 µl of this "ABC" solution was incubated with the salivary glands for 1.5 hours at 37°C. After three washes with 1X phosphate buffered saline (PBS), the slides were ready for chromogenic detection.

A solution of 0.33 mg/ml 3, 3'-Diaminobenzidine tetrahydrochloride (DAB) in 1X PBS containing 0.2% Triton (PBT) was added to the slides. After a short incubation period, 2 µl of 30% hydrogen peroxide was added. The slides were then incubated in a moist chamber at 37°C for at least three hours or overnight. After the desired coloration, the reaction was terminated through PBS washes. The slides were then stained for one minute in a solution of 8% Giemsa (Sigma) in 0.05 M Tris buffer, pH 7.6, rinsed in distilled water, air dried and mounted with coverslips using permount.

2.4 Isolation and Mapping of genomic *dveli*

Genomic DNA was isolated from adult CS-P flies by first grinding about 200 flies in liquid nitrogen in a chilled mortar and pestle. The fly parts were then transferred to a 2 ml dounce homogenizer containing homogenization buffer (10 mM Tris-HCl [pH 7.5], 60 mM NaCl, 10 mM EDTA, 0.15 mM spermidine, 0.15 mM spermine) and homogenized with a few strokes. After transferring to a microcentrifuge tube, fly debris was removed by centrifuging at 1000 rpm for approximately one minute. The supernatant was then centrifuged once again at 3000 rpm for five minutes to collect the cell debris. The pellet was collected and resuspended in 0.5 ml homogenization buffer. After the addition of proteinase K to a final concentration of 100 µg/ml and 50 µl of 10% SDS, the mixture was incubated for one hour at 37°C. Following phenol and chloroform extraction of the mixture, the genomic DNA was precipitated through the addition of 1/10 the volume of 3M sodium acetate and 2.5 volumes of cool 100% ethanol and then placed at -80°C for 15 minutes. The pellet was collected through centrifugation, washed with 70% ethanol, air dried and resuspended in the appropriate amount of TE buffer. This genomic DNA was then used in the polymerase chain reaction (PCR).

dveli was isolated from the *Drosophila* genome through amplification of the gene using oligonucleotides designed to pull out the full length genomic sequence (Appendix 2). Restriction sites were also engineered into these primers for subcloning use. After the PCR, the product was subcloned directly from the reaction tube into the pCR2.1 vector using the Original TA Cloning® Kit (Invitrogen, cat.#K200-J10). Potential transformants were analyzed by restriction analysis until the correct transformant was found. The *dveli* pCR2.1 transformant was verified through sequencing using M13 reverse primer and T7. In order to have a more convenient vector containing genomic *dveli* , the product was also subcloned into pBS SKII (Stratagene), where it was again verified by sequencing. To elucidate genomic structure, the full length genomic *dveli* was compared to the cDNA.

2.5 DNA and Protein Sequence Analysis and Alignments

DNA and protein sequences of known *lin-7/velis* and *lin-10/Mint/X11* were obtained and analyzed by the *blastn* and *blastp* programs using the NCBI BLAST search algorithm. Alignments indicating identities among the genes and the domains of the genes were obtained also through the use of Clustal W.

2.6 Preparation of *Drosophila* embryonic, larval and adult total RNA preps

(all solutions are Diethyl Pyrocarbonate (DEPC) treated)

Total RNA from each stage of *Drosophila* development was isolated using TRIzol® Reagent (Life Technologies, cat.# 15596). The embryos, larvae and adults (50-100mg of tissue) were placed in liquid nitrogen and ground in a mortar and pestle. After transfer to a dounce homogenizer containing 2 ml of Trizol reagent, the debris was homogenized with a few strokes. The liquid was transferred to an eppendorf tube,

centrifuged to remove any remaining fly debris and extracted with chloroform (0.2 ml of chloroform per 1 ml of Trizol). The supernatant (which was now clear in color) was removed and the RNA precipitated with 0.75X volumes of isopropanol and centrifuged at 15000 rpm for 30 minutes at 4°C. The pellet was then washed with 70% DEPC treated ethanol, air dried and resuspended in an appropriate volume of DEPC-ddH_2O. The concentration and purity was obtained through OD_{260} and OD_{260}/OD_{280} measurements respectively.

2.7 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out on embryo, larvae and adult stages of *Drosophila* development with the use of Ready-To-Go™ RT-PCR Beads (amersham pharmacia biotech, cat.# 27-9259-02). Each tube contains one bead that when brought to a final volume of 50 µl will contain approximately 2.0 units of *Taq* DNA polymerase, 10 mM Tris-HCl, 60 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and an RNase inhibitor. Using a one-step protocol, 1 µg of total RNA was added to an RT-PCR bead tube along with 1 µl of first strand primer $pd(T)_{12-18}$, 1 µl each of the forward and reverse primers and distilled water to 50 µl. The reactions were incubated at 42°C for 30 minutes to allow the reverse transcriptase to function and then at 95°C to inactivate the transcriptase before cycling through the thermal cycler. The reactions were run at a typical profile of denaturation at 95°C for one minute, 54°C for one minute and polymerization at 72°C for one minute, repeated for a total of 32 cycles. After cycling, the PCR products were run on a 1% agarose gel, bands were gel extracted and sequenced to confirm the products were cDNA and not genomic.

2.8a Preparation of Dig labeled RNA probes

Sense and anti-sense RNA digoxigenin (DIG) labeled probes were made through *in vitro* transcription with T7 and T3 polymerase, depending upon the orientation, using the DIG RNA Labeling Kit (Boehringer Manheim, Cat.# 1175025). This kit uses a ribonucleotide mix containing digoxigenin labeled uridine triphosphate (UTP) to label the RNA probes.

cDNA plasmid preps purified from overnight bacterial cultures through the Qiagen midi prep kit were linearized (approximately 10 μg) with the appropriate restriction enzymes for two hours at 37°C. After restriction digest, the linearized DNA was purified through phenol/chloroform extraction, precipitated with 3M sodium acetate and chilled 100% ethanol, washed with 70% ethanol and resuspended in the appropriate amount of DEPC treated distilled water to give a working concentration of about 1 μg/μl. To begin the standard labeling reaction, 1 μg of purified linearized template was mixed with 2 μl NTP labeling mixture, 2 μl transcription buffer, 1 μl RNase inhibitor, 2 μl of either T7 or T3 polymerase (depending upon orientation) and DEPC treated dd H_2O to a final volume of 20 μl in an RNase free microcentrifuge tube. This mixture was incubated at 37°C for at least two hours. The labeling reaction was terminated through the addition of 2 μl of 200 mM EDTA, pH 8.0.

2.8b Embryonic and 3rd instar Larval Fixation for RNA *in situ*

CS-P 3rd instar larval brains, nerve cords and imaginal discs were dissected in cold 1X PBS and fixed in 4% formaldehyde in PBS for 30 minutes at room temperature. After fixation, the larval parts were rinsed 4X in methanol and then 5X in ethanol and stored at -20°C until *in situ* label.

CS-P embryos were collected as described in 2.2. When enough plates were collected (usually about four-five plates / reaction), the chorion was removed from the embryos by rinsing with 50% commercial bleach for five minutes. Dechorionated embryos were collected on a nitex sieve and the excess bleach was rinsed away using embryo wash (7% NaCl and 0.05% Triton X-100). The sieve with the embryos was air dried and then dunked into a scintillation vial to remove the embryos. The scintillation vial contained a "fix mix" composed of 2 ml embryo wash, 2 ml 10% formaldehyde, 1 ml 5X fixation buffer (800 mM KCl, 200 mM NaCl, 20 mM Na_3EGTA, 5 mM spermadine HCl, 2 mM spermine, 150 mM pipes, pH7.4) and 5 ml heptane. The vial was shaken well then placed on a rotator for 20 minutes. After the fixation period, the lower aqueous phase was removed (the embryos clumped at the interface). The vitelline membrane of the embryos was then cracked by adding methanol and shaking the mixture vigorously for 20 seconds. The devitellinized embryos that fell to the bottom of the vial were then removed, washed a further three times with methanol, washed 2-4X with 100% ethanol and stored in ethanol at -20°C until *in situ* label.

2.8c RNA *in situ* Hybridization Label

(all solutions are DEPC treated)

Embryo and larval RNA *in situ* labels were performed in a similar manner, with slight variations. After fixation, the embryos and larval parts were washed once again in 100% ethanol and then rinsed in 50% ethanol/50% xylene, followed by shaking in 100% xylene for two hours. After this incubation, the xylene was removed and replaced once again with 50% ethanol/50% xylene and then with 100% ethanol. Both the embryos and larval parts were rinsed several times in 100% ethanol and then once in 50% methanol / 50% PBT. The embryos were then post fixed for ten minutes in PBT with 2%

formaldehyde while the larval parts were post fixed first in 50% methanol / 50% PBT with 5% formaldehyde for five minutes followed by a 30 minute fix in PBT with 5% formaldehyde. Following the post fixes, both the embryos and larval parts were washed for two minutes each in three different PBT washes. The tissues were then incubated with 50 µg/ml Proteinase K in PBT solution, the embryos for three minutes and the larval parts for nine minutes, agitating by hand during the incubation. The Proteinase K reaction was terminated by washing with PBT containing 2 mg/ml glycine. Both the embryos and larvae were then post fixed again in PBT with 5% formaldehyde for ten minutes on a shaker. The post fix was removed and the tissues were washed 4X for two minutes each in PBT and then 3X for two minutes each with 50% PBT / 50% prehybridization solution (50% formamide, 300 mM NaCl, 10 mM Tris-HCl [pH 6.8], 10 mM sodium phosphate [pH 6.8], 1X Denharts solution, 5mM EDTA, 1 mg/ml yeast tRNA). After a single wash in room temperature prehybridization, the embryos and larvae were incubated for 60 minutes at 53˚C with rocking in prehybridization solution that was prewarmed to the temperature. After one hour, the embryos and larvae were incubated with the DIG labeled RNA probes (1:500 to 1:1000 range of dilutions) in hybridization solution (similar to prehybrization solution but without the tRNA and containing 10% w/v dextran sulfate) to hybridize overnight at 53˚C without rocking.

The next morning, the embryos and larvae were washed through a gradient of post hybridization solution (same as prehybridization solution except lacking the tRNA) and PBT (80%, 60%, 40%, 20%) all at 53˚C for 20 minutes each. The tissues were then washed 2X 20 minutes each in just PBT at 53˚C. To remove unbound probe, the embryos and larvae were treated with 20 µg/ml RNase A in a 37˚C water bath for 20 minutes. Following RNase A treatment, the embryos and larvae were washed 3X in PBT at room temperature and then incubated for one hour at room temperature on a shaker

with 1 ml of a 1:2000 dilution of anti-DIG antibody (Anti-digoxigenin-AP, Fab fragments, Boehringer Manheim cat.# 1093 274).

Following antibody incubation, the embryos and larvae were washed 4X for 20 minutes each at room temperature, followed by three five minute washes with detection solution (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris [pH 9.5], 0.1% Tween 20). The solution was then replaced with reaction solution (3.5 µl 5-Bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (x-phosphate, 4-toluidine salt)(BCIP) and 4.5 µl nitroblue tetrazolium chloride (NBT) per 1 ml of detection solution). The embryos and larvae were then placed in the dark for the reaction to proceed.

Once the desired reaction was achieved, the reaction was stopped by removing the reaction solution and then washing 5X with PBT. The embryos and larvae were then washed 2X with PBS and then washed through a gradient of glycerol / PBS to a final storage concentration of 70% glycerol in PBS.

2.9a Preparation of *dveli* GST fusion construct

A *dveli* GST fusion construct was engineered by Christian Smith and sent to McMaster in order to generate an antibody. *dveli* was PCR cloned into pGex 4T (Novagen) at the *Eco*RI and *Sal*I restriction sites. The GST fusion construct was not full length, missing the first 12 amino acids.

2.9b Generation of Polyclonal antiserum

A 21 gauge needle was used to resuspend purified dVELI GST protein in PBS and mix with Freund's complete adjuvant to a total volume of 0.7 ml. This mixture was used to inject subcutaneously into four sites of the species *Rattus norvegicus* , 0.1 cc in each site. The rat received four booster shots, which was co-ordinated with bleeds

following a waiting period for the booster to induce an immune response. Pre-immunization and post-immunization bleeds were taken. Each bleed was approximately 1 ml, except the final bleed which yielded approximately 12 ml. The bleeds were prepared into sera by incubating the blood first at 37°C for one hour to allow maximum clotting. The bleeds were then incubated overnight at 4°C on ice. The next morning, the blood clots were spun down and the sera was removed from the cell pellet. 0.02% sodium azide was added to the serum tubes to prevent bacterial contamination. The sera was stored at -80°C until purification and immunological testing.

2.9c Affinity Purification of anti-dVELI antibodies

dVELI polyclonal anti-serum was purified through affinity purification using CNBr-activated Sepharose® 4B (Pharmacia Biotech, cat.#17-0430-01). Purified dVELI GST protein was bound to the sepharose which was then used to pull down the antibody in a column. The CNBr-activated Sepharose was swollen in 1 mM HCl for 15 minutes. The amount of gel to be made depended on the amount of protein to be conjugated, approximately 5-10 mg of protein per ml of gel (1 g of sepharose material yielded on average 3.5 ml of gel). After 15 minutes, the gel was washed on a sintered glass filter with about 200 ml of 1 mM HCl per gram of dry gel.

Once the gel was prepared, coupling of the antigen to the gel proceeded. The gel was washed 5X in coupling buffer (0.1 M Na_3HCO_3 [pH 8.3], 0.5 M NaCl). Immediately after washing, the gel was incubated with the antigen solution in a 2:1 ratio. This gel-antigen solution was incubated overnight at 4°C with rotation. The following morning, the gel was allowed to settle and excess coupling buffer was removed by aspiration. Unbound antigen was removed from the gel solution by washing 5X with coupling buffer. The gel was then placed in 0.1 M Tris-HCl [pH 8.0] to block remaining

active groups. This was incubated for two hours at room temperature. Following blocking, the beads were poured into the appropriate size column. The column was washed 5-10 column volumes with 1X PBS and then the serum was allowed to flow through the column by gravity flow. The serum was cycled through the column three-four times. The column was then washed of unbound antibody with several column volumes of 1X PBS.

Elution of bound antibody from the antigen within the column was achieved by washing in a stepwise fashion with 0.2 M glycine, pH 2.3. Eluted antibody was neutralized with 1 M Tris (150 µl per ml of eluted antibody) and then dialyzed against PBS overnight at 4°C. The next day, 0.02% sodium azide was added to the purified antibody, which was then stored at -80°C.

2.10 General Antibody Staining of *Drosophila* Embryos

Wild type embryos were collected as previously described in 2.2. After a sufficient number of agar plates were collected (about four-six plates per reaction tube), the embryos were dechorionated with a 50% bleach solution and collected on a nitex sieve by rinsing the embryos onto the sieve with distilled water. The embryos were air dried on the sieve and then dunked in a scintillation vial containing 5 ml of heptane, 4.5 ml of 1X PBS and 0.5 ml of 37% formaldehyde. The scintillation vial containing the embryos was placed on a rotator and the embryos were fixed this way for 30 minutes. After the fixation period, the bottom layer (fixative layer) was removed and the embryos were devitellinized by vigorously spraying with methanol and shaking quickly for 20 seconds. The devitellinized embryos sank to the bottom of the vial. These embryos were removed using a pasteur pipet and placed in a glass test tube. After several methanol

washes the wash was replaced with 1X PBT. Following 3 PBT washes, the embryos were placed on a rotator for 20 minutes in PBT.

The embryos were blocked to remove non-specific binding with a 1:20 dilution of normal goat serum (Jackson Immunoresearch Laboratories, Inc.) in PBT for 40 minutes at room temperature on an orbital shaker. The primary antibody was then added at the appropriate dilution and the embryos were placed on an orbital shaker at 4°C for an overnight incubation. The next day the embryos were washed five times with PBT and then placed on a rotator for four hours at room temperature or overnight at 4°C to remove excess primary. After this extensive washing, the embryos were blocked once again and then incubated on an orbital shaker for two hours at room temperature in the appropriate dilution of horseradish peroxidase labeled secondary antibody. The secondary was washed from the embryos with a two hour washing period using PBT. To react the horseradish peroxidase, the embryos were incubated in a 0.33 mg/ml solution of DAB in PBT for two minutes and then developed through the addition of 3 µl of 0.03% hydrogen peroxide. Once the desired color was observed, the reaction was stopped by washing several times with PBT. The embryos were then dehydrated through an ethanol series and stored in methyl salicylate.

2.11 Immunolabeling of Larval Brains, Nerve Cords and Imaginal Discs

1st, 2nd and 3rd instar larval nervous systems and imaginal discs were dissected in cold 1X PBS and then fixed in 4% paraformaldehyde in sodium phosphate buffer, pH 7.4 for one-two hours at room temperature or overnight at 4°C. The fixative was then removed and the larval tissues were washed in 1X PBS with 0.3% Triton (PBT) for two hours, changing the PBT every half an hour. The tissues were then blocked to remove non-specific binding with a 1:20 dilution of Normal Goat Serum (Jackson

Immunoresearch Laboratories, Inc.) in PBT for 40 minutes. Primary antibody was added at an appropriate dilution based on the antibody and incubated overnight at 4°C on a shaker.

The next day, unbound primary antibody was removed by washing 5X with PBT and then rotating the larvae in PBT for 2 hours, changing the PBT several times over the course of the two hours. The tissues were then blocked as before and incubated in the appropriate dilution of horseradish peroxidase conjugated secondary antibody for two hours at room temperature. The larval parts were then washed of excess secondary with PBT and placed on a rotator in PBT for one-two hours. The peroxidase reaction was performed through the addition of a PBT solution containing 0.33 mg/ml of diaminobenzidine (DAB). After a two minute incubation period in this solution, 3 µl of 0.03% hydrogen peroxide was added to visualize the colourimetric change. After the desired coloration was achieved, the reaction was stopped through PBT washes. The tissues were washed completely of excess DAB with PBT and then 1X PBS and stored in 70% glycerol/PBS.

2.12 Fixation and Antibody Staining of *Drosophila* Adult Heads and Nerve Cords

Adult CS-P heads and nerve cords were dissected in 4% paraformaldehyde in PBS. Immediately after dissection, the tissues were incubated in permeabilizing buffer (1X PBS, 4% paraformaldehyde, 1% Triton, 0.1% deoxycholate) for 30 minutes. Following several washes with PBT, the adult tissues were blocked in a 1:20 dilution of normal goat serum (NGS) in PBT for one hour on a shaker at room temperature. The primary antibody was then added to the tissues at the appropriate dilution and incubated on a shaker at 4°C for 48 hours. After two days, the primary antibody was removed and the tissues were washed of excess primary with PBT over a 10-12 hour period, changing

the PBT solution often. The tissues were then blocked once again and incubated in horseradish peroxidase labeled secondary antibody (1:200) in PBT at room temperature for two hours. After the incubation period, the adult heads and nerve cords were washed of excess secondary with PBT overnight at 4°C. The next morning, the colourimetric reaction was performed. The adult fly parts were incubated for about five minutes in a 0.33 mg/ml solution of DAB in PBT. Following the brief incubation, 3 µl of 0.03% hydrogen peroxide was added and the color reaction was observed until the desired brown color was achieved. Once the color was developed to the suitable level, the reaction was terminated with several PBT washes and the adult tissues were stored in 70% glycerol/PBS.

2.13 Western Blot Analysis

Expression of dVELI through all stages of development was studied using Western blot. Embryonic, larval, adult head and adult body tissue was collected and then homogenized in cold RIPA lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 8.0], protease inhibitor cocktail tablets 1/10 ml (Roche, cat.# 1836170)). (1g tissue per 2 ml of RIPA). Once homogenized, the tissues were incubated on ice for 10 minutes and then centrifuged for five minutes at 3500 rpm to remove excess fly parts. The supernatant was collected, the concentration was determined and the samples were stored at -80°C.

Crude protein samples were defrosted and approximately 30 µg of protein (~20 µl) was mixed with 5 µl of 4X sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris [pH 8.0], 0.01% Bromophenol Blue) and boiled for five minutes. Protein samples were then loaded on a 12.5% Tris-HCl gel and run at 200V for 30 minutes. After the gel was run, the protein was transferred to a PVDF membrane. Before

assembly of the MiniProtean II (Bio-Rad), the sponges, 3MM whatman filter papers, PVDF membrane and the gel were soaked in transfer buffer for five minutes. The western sandwich was then assembled and the protein was transferred for one hour at 100V with an ice pack at 4°C. After transfer, the PVDF membrane was washed for five minutes each in two baths of post blot buffer (20 mM Tris-HCl, pH 7.2-7.4 , 150 mM NaCl, 01% (v/v) Tween 20) and then blocked for 60 minutes at room temperature on a shaker in 5% skim milk powder. After blocking, the membrane was incubated overnight at 4°C in a 1:5000 dilution of polyclonal antibody in blocking solution.

The next day, excess primary antibody was removed by washing 3X for ten minutes each in post blot buffer containing NP-40. Following the washes, the blot was incubated in 1:15000 dilution of horseradish peroxidase labeled secondary antibody for 30 minutes at room temperature. Secondary antibody was then removed, excess washed off with post blot buffer and then 10 mM Tris. The blot was then prepared for autoradiographic developing using Kodak X-ray film.

Equal volume of reagent A was mixed with reagent B from the Western Blot detection Kit (Amershan Pharmacia). 5 ml of this solution was spread evenly over the blot and incubated for 3 minutes. Excess liquid was then blotted off with a kimwipe, the blot was placed in saran wrap and exposed to Kodak X-OMAT AR (XAR) film in the dark for various amounts of time and then developed to determine the optimal exposure time.

2.14 Generation of UAS-*dveli* construct

A UAS-*dveli* construct was generated for future use by subcloning the *dveli* cDNA into pUASt (obtained from Gabrielle Boulianne). The cDNA was digested from pBSKSII using the *Kpn* and *NotI* sites. The orientation of the cDNA placed the 5' end at

the *Kpn* site and the 3' end near the *Not*I site. After digesting pUASt with the same restriction enzymes, the *dveli* cDNA was then ligated to pUASt with T4 ligase overnight at 14°C. The next day, the pUAS-*dveli* ligation product was transformed into DH5alpha competent cells (Gibco). After purifying the plasmid, potential transformants were screened by restriction digest using the *Kpn* and *Not*I restriction enzymes, as well as sequencing to confirm the insert was full length *dveli* cDNA.

2.15 Light Level Microscopy

Embryos and larval dissections were prepared for light level microscopy in slightly different ways. Embryos were mounted in methyl salicylate in permount on frosted glass slides (corning) using 18 mm^2 coverslips. Larval imaginal discs and nerve cords were mounted on glass slides directly in 70% glycerol/PBS using DPX mounting medium (Sigma), which were then covered with coverslips. All slides were viewed using a Zeiss Axiophot microscope and photographed using Kodak Ektachrome 64T professional color film. Processed film was scanned using a slide scanner (Minolta) and prepared as figures using Adobe Photoshop® 4.0.

Chapter 3
Results

The *dveli* cDNA was isolated by the McGlade laboratory at the University of Toronto and sent to Roger Jacobs laboratory for characterization. Based on the result from this laboratory that dVELI binds phosphorylated DER, experiments were undertaken that further characterized the expression patterns and structure of dVELI as well as the identification of other known binding partners from the *Drosophila* genome.

3.1 Identification and Sequence Analysis of the *Drosophila* ortholog of *C. elegans lin-7.*

Domains of *Drosophila* VELI

Figure 2 shows the cDNA sequence as well as the corresponding amino acid sequence of *dveli*. *dveli* coding region is 585 base pairs in length, corresponding to 195 amino acids. There are two domains recognized in the protein, the first being an L27 domain, a region recently identified as a conserved binding site in both LIN-2 and LIN-7 proteins responsible for the interaction of these two proteins (Doerks *et al*, 2000). This site consists of approximately 50 amino acid residues containing conserved negatively charged residues. The second domain identified is a PDZ domain. The PDZ domain is a protein motif found to function as a protein binding domain (reviewed in Kim, 1997). The dVELI PDZ domain contains 83 amino acid residues, containing the relatively conserved sites suspected of being responsible for it binding to the C-terminal end of other proteins.

Figure 2. Amino acid and nucleotide sequence of *Drosophila veli* (*dveli*). *dveli* is 585 base pairs long predicting 195 amino acids. Domains are colour coded. Pink = L27 domain, Blue = PDZ domain. Sequence accession number = AE003750.

```
ATG GCC GAT AAC GCA GAA CCA CTG ACT TTG TCC AGA GAT GTC AAA AGA
 M   A   D   N   A   E   P   L   T   L   S   R   D   V   K   R
TCG ATA GAG CTC TTG GAA AAG CTG CAA GCG AGT GGC GAC TTC CCC ACG
 S   I   E   L   L   E   K   L   Q   A   S   G   D   F   P   T
ACA AAA CTG GCC GCC CTG CAG AAG GTG CTC AAC TCG GAC TTC ATG ACC
 T   K   L   A   A   L   Q   K   V   L   N   S   D   F   M   T
TCC GTG CGG GAG GTG TAC GAG CAC GTC TAC GAG ACG GTG GAC ATC CAG
 S   V   R   E   V   Y   E   H   V   Y   E   T   V   D   I   Q
GGC TCC CAC GAC GTG AGG GCA TCC GCC ACT GCC AAG GCC ACT GTG GCA
 G   S   H   D   V   R   A   S   A   T   A   K   A   T   V   A
GCC TTC GCA GCC AGC GAG GGG CAC GCT CAT CCC AGA GTC GTC GAG CTC
 A   F   A   A   S   E   G   H   A   H   P   R   V   V   E   L
CCG AAA ACG GAG GAG GGC TTG GGT TTC AAT GTA ATG GGG GGC AAG GAG
 P   K   T   E   E   G   L   G   F   N   V   M   G   G   K   E
CAA AAC TCG CCC ATC TAT ATA TCC CGA ATA ATA CCT GGA GGC GTG GCT
 Q   N   S   P   I   Y   I   S   R   I   I   P   G   G   V   A
GAC AGG CAT GGT GGT CTG AAA AGG GGA GAT CAA CTT TTG TCT GTG AAT
 D   R   H   G   G   L   K   R   G   D   Q   L   L   S   V   N
GGT GTG TCT GTC GAA GGA GAA AAC CAC GAG AAG GCC GTA GAG CTA TTA
 G   V   S   V   E   G   E   N   H   E   K   A   V   E   L   L
AAG CAA GCT GTC GGA TCT GTA AAG CTG GTG GTA CGC TAC ACA CCA AAG
 K   Q   A   V   G   S   V   K   L   V   V   R   Y   T   P   K
GTT CTC GAG GAA ATG GAA ATG CGA TTT GAT AAG CAA CGC AAC ACA CGT
 V   L   E   E   M   E   M   R   F   D   K   Q   R   N   T   R
CGT CGC CAA TAA
 R   R   Q
```

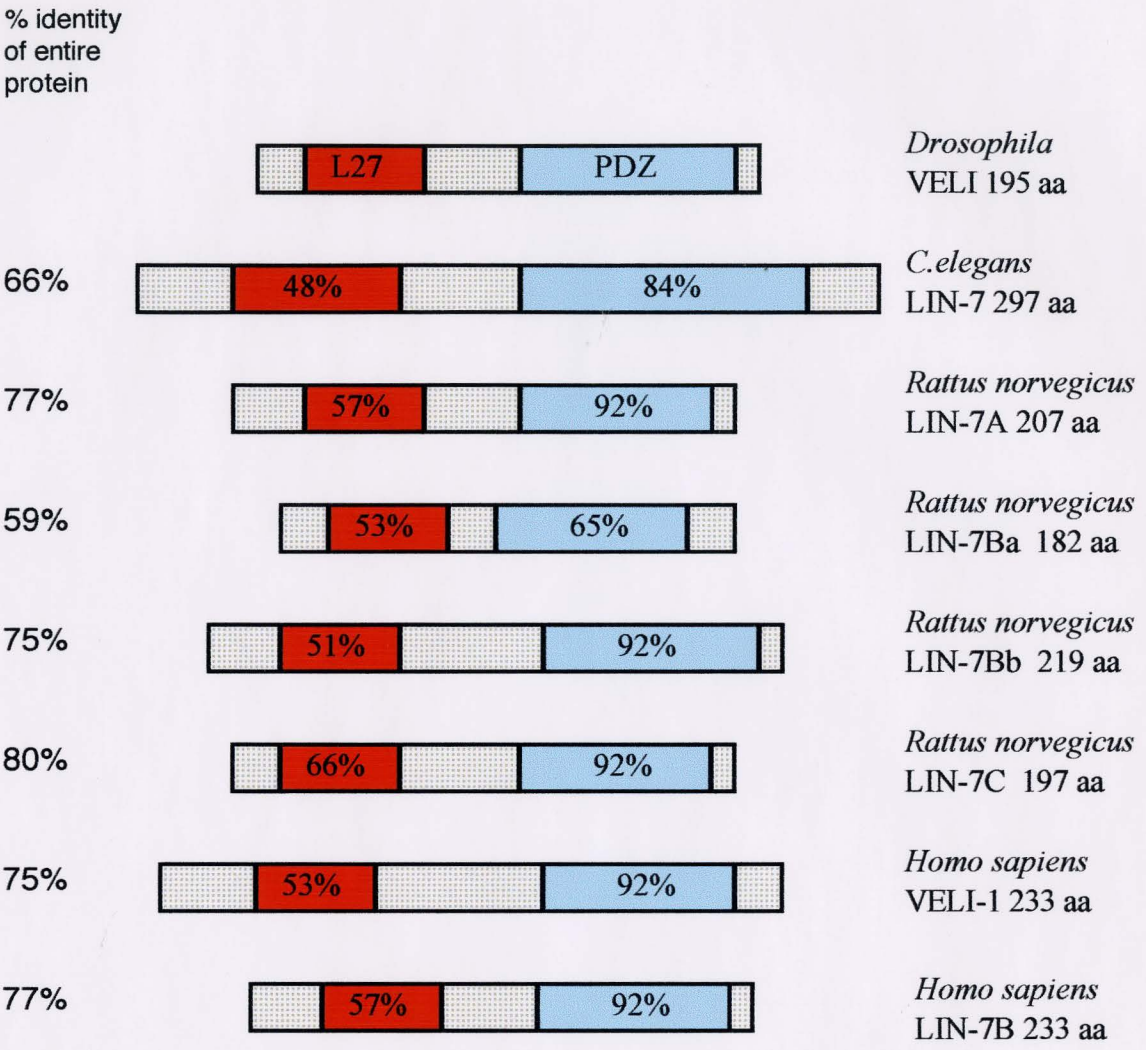
Amino Acid Sequence Comparisons Among VELIs/LIN-7 Orthologs

dVELI shows high percent identity to know VELIs/LIN-7 proteins. dVELI was compared both in its entirety as well as within recognized protein domains to known VELIs/LIN-7 proteins of *C. elegans, Rattus norvegicus* and *Homo sapiens* (Figure 3). *Drosophila* VELI shows the highest percent identity overall as well as within both the L27 domain and the PDZ domain with the LIN-7C protein (*Rattus norvegicus).* When comparing to all proteins, the PDZ domains are highly conserved, with identity anywhere from 84% to 92%, with the exception of LIN-7Ba, which is the lowest at 65%. The L27 domain is also relatively conserved among the proteins, ranging in identity to the *Drosophila* protein from 48% to 66%. The *Drosophila* protein appears to have a higher identity when comparing amino acids to the *Rattus norvegicus* and *Homo sapiens* proteins rather than the *C. elegans* ortholog. Amino acid alignment of the VELI/LIN-7 proteins can be seen in Appendix 3.

Genomic Structure

Several unordered BAC vectors were identified containing large genomic regions corresponding to parts of the *dveli* gene including upstream and downstream regions several base pairs from the start and stop codons, respectively. BACR03L02 (accession number AC007853) and BACR03I15 (accession number AC008206) were used to design primers just upstream and downstream of the gene in order subclone a genomic fragment containing *dveli* (see Appendix 2). The genomic *dveli* sequence was compared to that of the cDNA. The genomic sequence is 868 base pairs. It was determined that the genomic structure contained three introns dispersed through the gene. The introns range in size from 62 base pairs to 152 base pairs. Following the publication

Figure 3. *Drosophila* VELI and its orthologs showing percent identities at the amino acid level. Percent identity is shown for the entire protein as well as the domains. Numbers within domains indicate the identity of the domain of that species as compared with the similar domain of the *Drosophila* protein. Comparisons are made between *Drosophila* and *C.elegans* LIN-7, all the LIN-7 isoforms of *Rattus norvegicus* and the known *Homo sapiens* isoforms. L27 = LIN-2/LIN-7 biding domain, PDZ = PDZ domain.


% identity of entire protein
L27
PDZ
Drosophila VELI 195 aa
66%
48%
84%
C.elegans LIN-7 297 aa
77%
57%
92%
Rattus norvegicus LIN-7A 207 aa
59%
53%
65%
Rattus norvegicus LIN-7Ba 182 aa
75%
51%
92%
Rattus norvegicus LIN-7Bb 219 aa
80%
66%
92%
Rattus norvegicus LIN-7C 197 aa
75%
53%
92%
Homo sapiens VELI-1 233 aa
77%
57%
92%
Homo sapiens LIN-7B 233 aa

of the *Drosophila* genome (Adams *et al,* 2000), the genomic sequence of *dveli* was also obtained through a BLAST search of the *Drosophila* genome using the cDNA sequence. Gene CG7662 corresponds to *dveli*. The genomic structure confirms the presence of three introns dispersed throughout the gene. CG7662 also confirms the genomic size as well as the transcript size. These results are summarized in Figure 4.

Location of the *dveli* Gene by Polytene Chromosome *in situ* Hybridization

Polytene chromosome *in situ* hybridization was used to determine the location of the *dveli* gene before this data was available from the BDGP. Salivary glands of *Drosophila* contain large polytene chromosomes, which have distinct banding patterns. The chromosomal banding patterns are distinct enough that they allow the observer to map chromosome number and position based solely upon the bands. There are approximately 5000 of these bands which can be used to identify the location of a labeled probe. A biotin labeled probe was generated from the *dveli* sequence and was used to hybridize to third instar larval salivary gland chromosomes. The hybridized location was determined to be on the right arm of the third chromosome (3R) at region 96B. The location of the gene on 3R was confirmed using a probe to a P-element line known to be located at 3R97D. Figure 5 shows the 3R positive control band as well as the *dveli* band hybridized to the squashed chromosome.

The location of *dveli* was confirmed following the publication of the *Drosophila* genome. CG7662, identified above as *dveli*, is located at 3R96B16, supporting the result obtained through *in situ*.

Figure 4. *dveli* genomic structure. *dveli* is located at 3R96B16. The genomic interval contains three introns of varying sizes. Introns are indicated by black lines. Exons are color coded according to the domain which they code for. Pink = L27 domain, Blue = PDZ domain. dVELI protein is depicted at the bottom. All sizes are in base pairs.

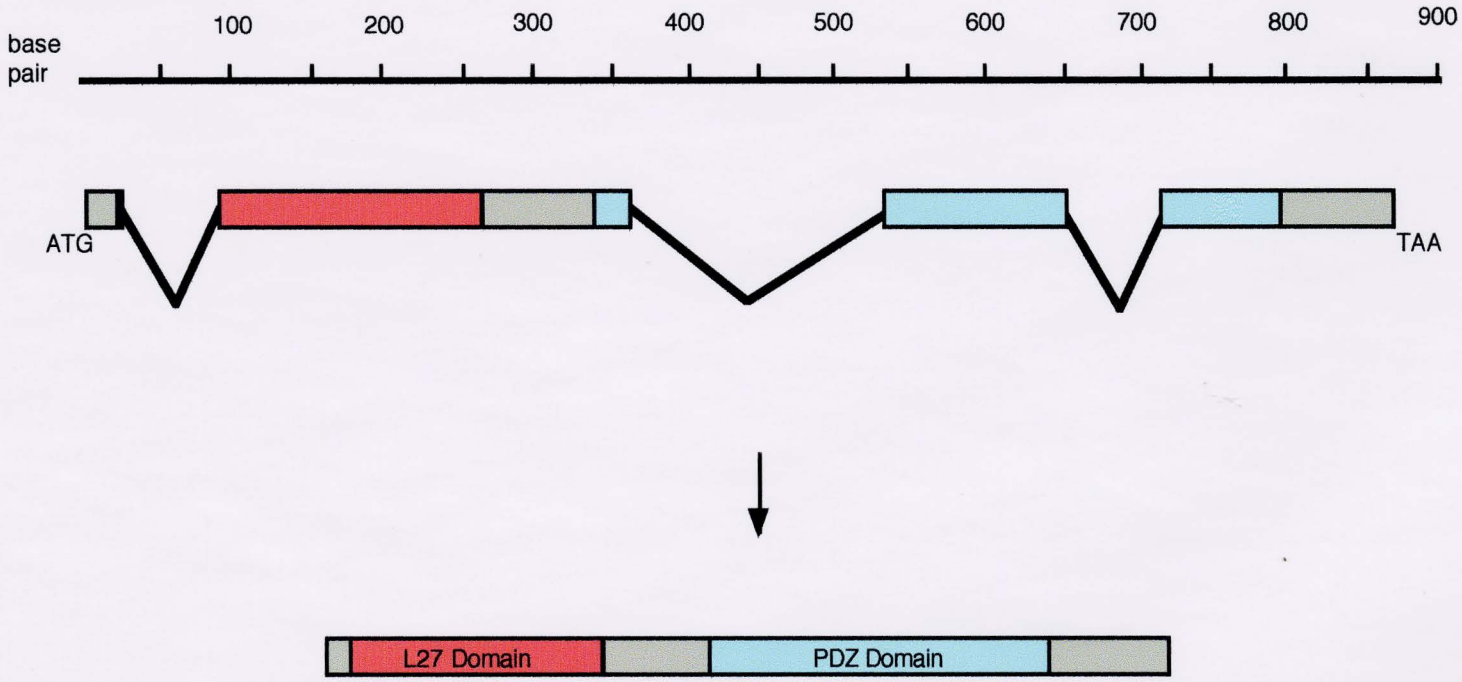
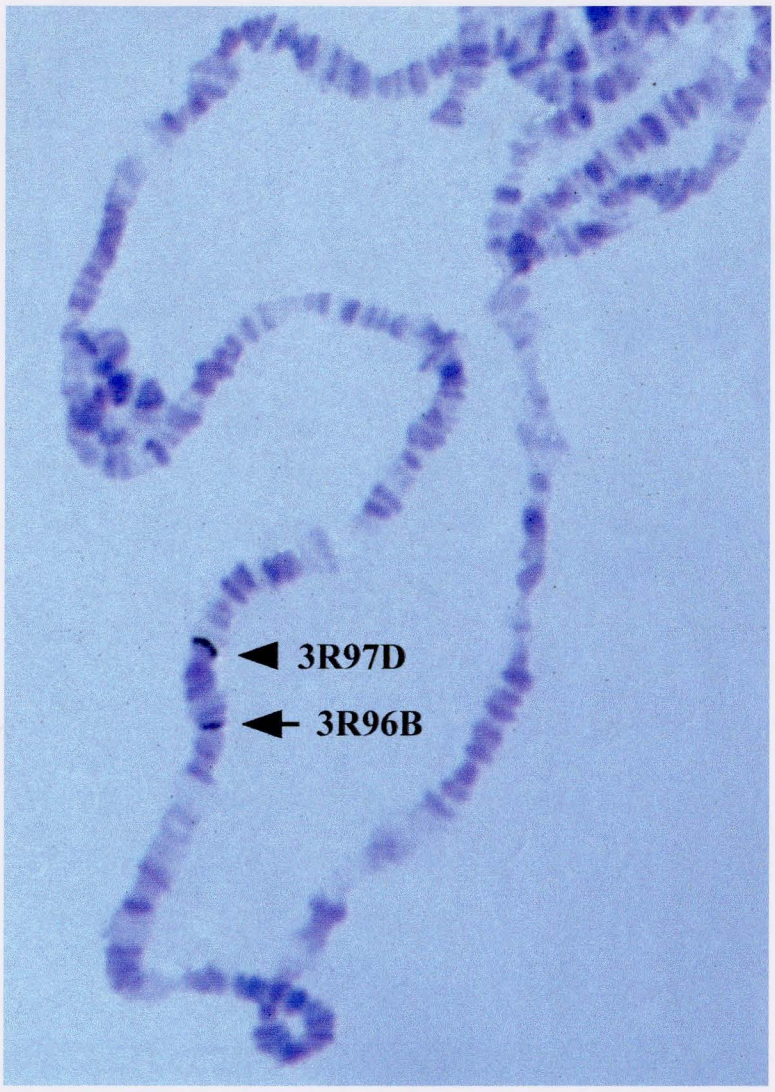

base
pair
100
200
300
400
500
600
700
800
900
ATG
TAA
L27 Domain
PDZ Domain

Figure 5. Polytene chromosome *in situ* hybridization to squashed salivary gland chromosomes using a Biotin-labeled DNA probe. The arrow indicates the location of *dveli* on the right arm of the third chromosome. Banding patterns further reveal this location as 96B (3R96B). The arrowhead represents a positive control for the right arm of the third chromosome. This is P-element F336 that is located at 97D (3R97D).


3R97D
3R96B

3.2 Identification and Sequence Analysis of the *Drosophila* ortholog of *C.elegans lin-10*

Identification of *Drosophila Mint/X11*

Following publication of the *Drosophila* genome, a search was undertaken to locate the binding partners of dVELI. One protein known to exist in a tripartite complex with LIN-7 and LIN-2 is LIN-10 (Kaech *et al,* 1998). The *Drosophila* ortholog of *C. elegans lin-10* was identified by comparing the amino acid sequence of LIN-10 against the *Drosophila* genome. The closest match pulled up the gene CG5678. Homologies noted among this gene included *Mint/X11alpha (Homo sapiens), lin-10 (C.elegans)* and *Mint/X11alpha (Rattus norvegicus).* CG5678 (herein referred to as *dMint*) is genomically 2064 base pairs, translating into 473 amino acids (1419 base pair cDNA length). It is located on the X chromosome at 16B8-16B9.

Domains of *Drosophila* MINT

Figure 6 shows the cDNA sequence as well as the amino acid sequence of *dMint*. dMINT consists of three protein domains, a PTB/PID domain and two PDZ domains. The first domain is a phosphotyrosine binding domain or phosphotyrosine interacting domain (PTB/PID). PTB domains are generally 100-150 amino acid residue motifs that commonly bind Asn-Pro-X-Tyr motifs (Bork and Margolis, 1995). The dMINT PTB domain is 165 amino acids long. The last two domains of dMINT are both PDZ domains. As noted already, most PDZ domains are approximately 90 amino acids long. The first PDZ domain is 87 amino acids while the second PDZ domain of dMINT is 76 amino acids in length.

Figure 6. Amino acid and nucleotide sequence of *Drosophila Mint/X11 (dMint).* *dMint* is 1419 base pairs long predicting 473 amino acids. *dMint* consists of 3 domains, which are color coded. Red = PTD domain, Blue = 1st PDZ domain, Green = 2nd PDZ domain. Sequence accession number = AE003506.

```
ATG AAT AAT AGC AAG CCG CAA ACC TAC TCG ACG GCC ACC ATT CGA CAA GGC ATC GGC ACC TCG
 M   N   N   S   K   P   Q   T   Y   S   T   A   T   I   R   Q   G   I   G   T   S
TTG ACA CCC AAT TCG CCG GAC ATC TGT CAG ATT GTG GGC ACG ACC GAC ATC TCC ATA AGC TCG
 L   T   P   N   S   P   D   I   C   Q   I   V   G   T   T   D   I   S   I   S   S
CCG GAA AAA CTG CAG TTC ACC AAA AGT CCC ACG GGC TCT ATT AAA TCC CTG AAG GAC TCT GCC
 P   E   K   L   Q   F   T   K   S   P   T   G   S   I   K   S   L   K   D   S   A
AAC TCG GAC AAG AAG GCC AAA TCG CGG AAC AAA GAG GGT CTC TTG GAA CCC AAA GTT CTC ATT
 N   S   D   K   K   A   K   S   R   N   K   E   G   L   L   E   P   K   V   L   I
GAG GGC GTG TTG TTT CGG GCC AGA TAC CTT GGA TCC ACT CAA CTT GTC TGC GAG GGT CAG CCG
 E   G   V   L   F   R   A   R   Y   L   G   S   T   Q   L   V   C   E   G   Q   P
ACC AAG TCG ACC AGA ATG ATG CAG GCC GAG GAG GCC GTT TCC AGG ATC AAG GCT CCC GAG GGC
 T   K   S   T   R   M   M   Q   A   E   E   A   V   S   R   I   K   A   P   E   G
GAA AGT CAG CCG AGC ACT GAA GTG GAC CTG TTC ATA TCA ACC GAA AAG ATA ATG GTG CTT AAC
 E   S   Q   P   S   T   E   V   D   L   F   I   S   T   E   K   I   M   V   L   N
ACG GAT CTC AAG GAG ATC ATG ATG GAC CAT GCG CTG CGT ACT ATA TCC TAT ATA GCT GAC ATT
 T   D   L   K   E   I   M   M   D   H   A   L   R   T   I   S   Y   I   A   D   I
GGC GAT CTG GTT GTG CTG ATG GCT CGT CGT CGA TTC GTA CCG AAT AGT GTT GTG GAT CCA TCG
 G   D   L   V   V   L   M   A   R   R   R   F   V   P   N   S   V   V   D   P   S
ATC ACC AGT CCA TTA GGT GAT GTT CCC ACT CCG GGC ATA GGC GAG GAG GAG TCG CCC CCC AAA
 I   T   S   P   L   G   D   V   P   T   P   G   I   G   E   E   E   S   P   P   K
GAG CCA CTC AGC AAG CAC AAT CGC ACG CCC AAA ATG ATC TGC CAC GTG TTC GAA AGC GAT GAG
 E   P   L   S   K   H   N   R   T   P   K   M   I   C   H   V   F   E   S   D   E
GCG CAG TTC ATA GCT CAG TCC ATT GGA CAG GCC TTC CAG GTG GCC TAC ATG GAG TTC CTG AAG
 A   Q   F   I   A   Q   S   I   G   Q   A   F   Q   V   A   Y   M   E   F   L   K
GCG AAC GGC ATC GAA AAC GAG AGT CTG GCC AAA GAG ATG GAC TAC CAG GAG GTG CTC AAC AGT
 A   N   G   I   E   N   E   S   L   A   K   E   M   D   Y   Q   E   V   L   N   S
CAG GAG ATT TTC GGT GAT GAG CTG GAG ATC TTT GCC AAA AAG GAG CTG CAA AAG GAG GTA GTT
 Q   E   I   F   G   D   E   L   E   I   F   A   K   K   E   L   Q   K   E   V   V
GTG CCG AAG GCG AAA GGC GAG ATC CTA GGC GTG GTG ATC GTA GAG AGT GGC TGG GGA TCC ATG
 V   P   K   A   K   G   E   I   L   G   V   V   I   V   E   S   G   W   G   S   M
CTG CCC ACC GTG GTG ATT GCC AAC CTG ATG AGT TCA GGA GCA GCT GCC CGC TGC GGC CAG CTG
 L   P   T   V   V   I   A   N   L   M   S   S   G   A   A   A   R   C   G   Q   L
AAC ATC GGT GAC CAG CTG ATC GCC ATT AAC GGC ATG AGC CTG GTG GGA CTG CCG CTG TCC ACC
 N   I   G   D   Q   L   I   A   I   N   G   M   S   L   V   G   L   P   L   S   T
TGC CAG AGC TAC ATA CGC AAT GCC AAG AAC CAA ACT GCC GTC AAG TTC ACC GTT GTG CCC TGT
 C   Q   S   Y   I   R   N   A   K   N   Q   T   A   V   K   F   T   V   V   P   C
CCG CCT GTC GTC GAG GTT AAG ATC CTG CGT CCC AAG GCG CTG TTC CAG TTG GGT TTT AGT GTT
 P   P   V   V   E   V   K   I   L   R   P   K   A   L   F   Q   L   G   F   S   V
CAA AAT GGC GTG ATC TGC AGT CTT TTG CGT GGA GGA ATC GCT GAG CGG GGT GGA GTA CGC GTA
 Q   N   G   V   I   C   S   L   L   R   G   G   I   A   E   R   G   G   V   R   V
GGC CAC CGC ATC ATT GAG ATT AAC AAC CAG AGC GTT GTG GCC GTG CCA CAC GAT ACC ATT GTC
 G   H   R   I   I   E   I   N   N   Q   S   V   V   A   V   P   H   D   T   I   V
AAG CTG TTG TCA TCC TCA GTG GGC GAG ATC CTG ATG AAG ACA ATG CCC ACG TCC ATG TTT CGT
 K   L   L   S   S   S   V   G   E   I   L   M   K   T   M   P   T   S   M   F   R
TTG CTC ACC GGT CAA GAG ACG CCA ATC TAT ATA TAA
 L   L   T   G   Q   E   T   P   I   Y   I
```

Amino Acid Sequence Comparisons Among MINT/LIN-10 Orthologs

dMINT was compared to the known MINT/X11 orthologs of *C. elegans*, *Rattus norvegicus* and *Homo sapiens* to determine percent identity based upon on identical amino acid residues of the entire protein as well as just among the domains. The results of this comparison are depicted in Figure 7. The *Drosophila* protein is most similar to *C. elegans* LIN-10 protein, with an overall identity of 58%. The PTB comparisons show the highest identity with *C. elegans* as well (64%). The first and second PDZ domains were compared between *Drosophila* and the above mentioned species as well. The second PDZ domain was more conserved from *Drosophila* to each respective species, ranging from 75% to 77%, than the first PDZ domain which had a slightly lower identity, ranging from 64% to 66%. Complete amino acid alignments of the MINT/LIN-10 orthologs can be seen in Appendix 4.

Genomic Structure of *dMint*

dMint was noted on FlyBase as having five introns dispersed throughout the genomic sequence, ranging in size from 54 base pairs to 223 base pairs. A summary of the location and size of the introns of *dMint* can be seen in Figure 8.

3.3 mRNA Transcript Distribution and Expression Analysis

***dveli* and *dMint* Transcripts are Present in all Developmental Stages**

Once both *dveli* and *dMint* were identified and characterized, the expression of mRNA transcripts during different developmental stages of *Drosophila* were sought. Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) was performed on total RNA preparations from embryo, larva and adult stages of development. The preparations were incubated with two sets of primers. The first primer added was a poly T primer

Figure 7. *Drosophila* MINT/X11 and its orthologs showing percent identities between the *Drosophila* protein and *C. elegans, Drosophila* and *Rattus norvegicus* , and finally *Drosophila* compared to *Homo sapiens*. Percent identity is shown at the amino acid level of the entire protein, as well as the indicated domains. Numbers within domains indicate percent identity of that domain compared to *Drosophila.* PTB = phosphotyrosine binding domain, PDZ1 = 1st PDZ domain, PDZ2 = 2nd PDZ domain.

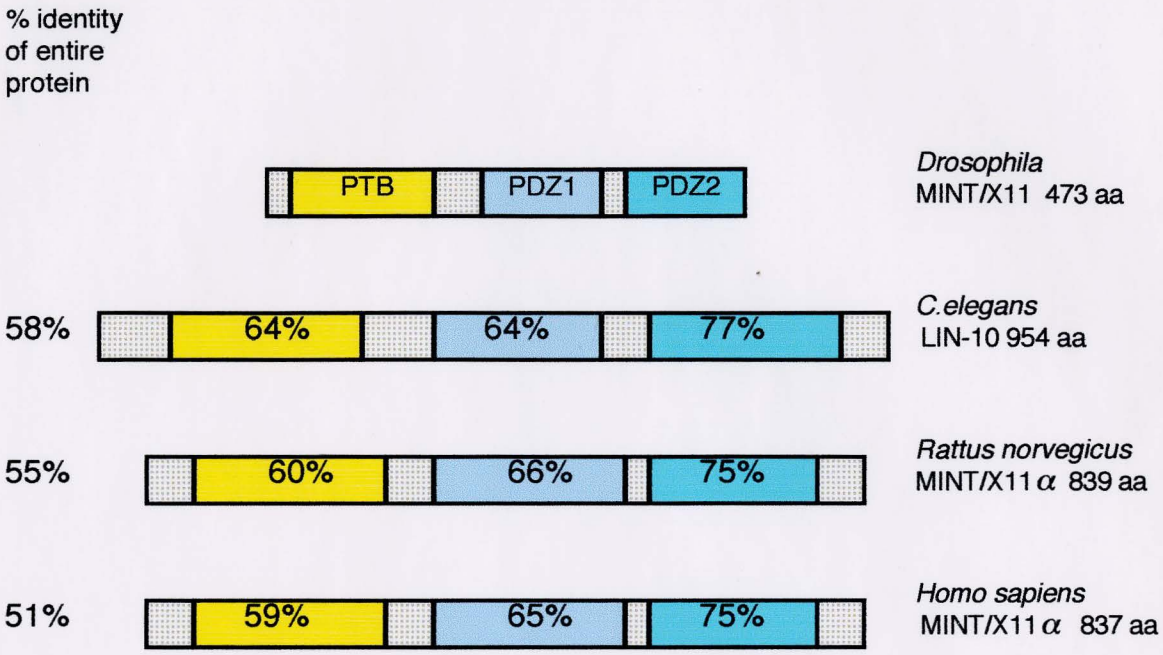
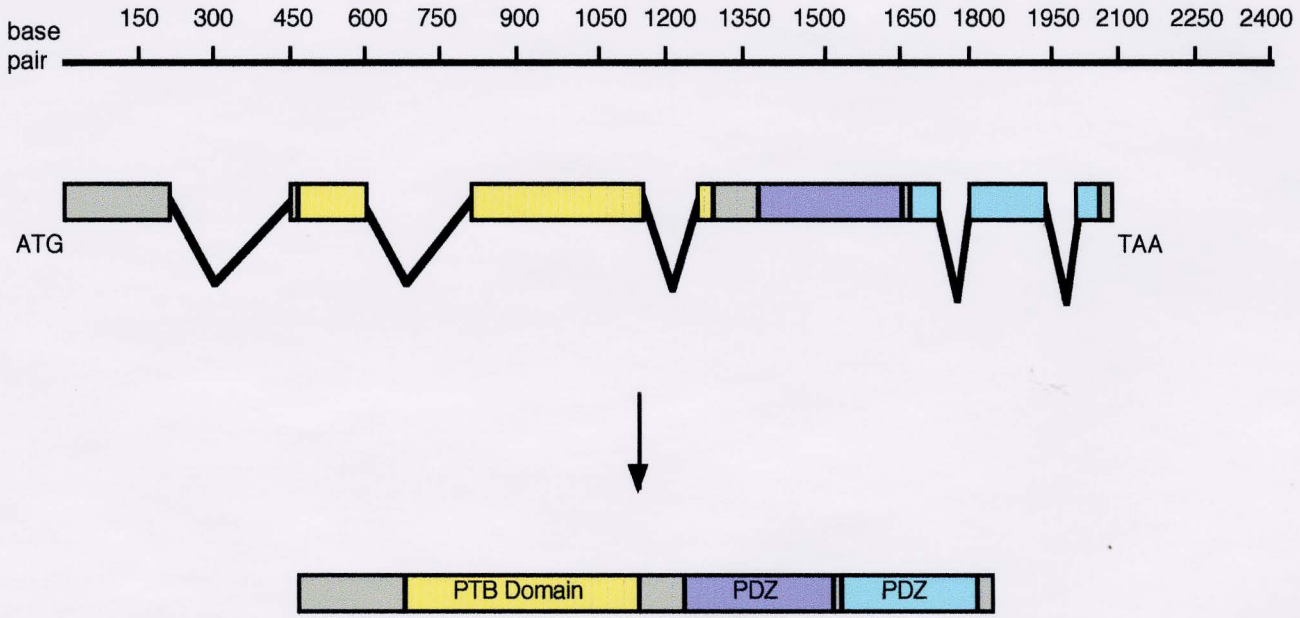

% identity
of entire
protein
PTB
PDZ1
PDZ2
Drosophila
MINT/X11 473 aa
58%
64%
64%
77%
C.elegans
LIN-10 954 aa
55%
60%
66%
75%
Rattus norvegicus
MINT/X11 α 839 aa
51%
59%
65%
75%
Homo sapiens
MINT/X11 α 837 aa

Figure 8. *dMint* genomic structure. *dMint* consists of 5 introns, indicated by black lines. The domain encoding nucleotides of *dMint* are color coded. Yellow = PTB domain, Purple = 1st PDZ domain, Blue = 2nd PDZ domain. dMINT protein is depicted at the bottom. All sizes are in base pairs.


base
pair
150
300
450
600
750
900
1050
1200
1350
1500
1650
1800
1950
2100
2250
2400
ATG
TAA
PTB Domain
PDZ
PDZ

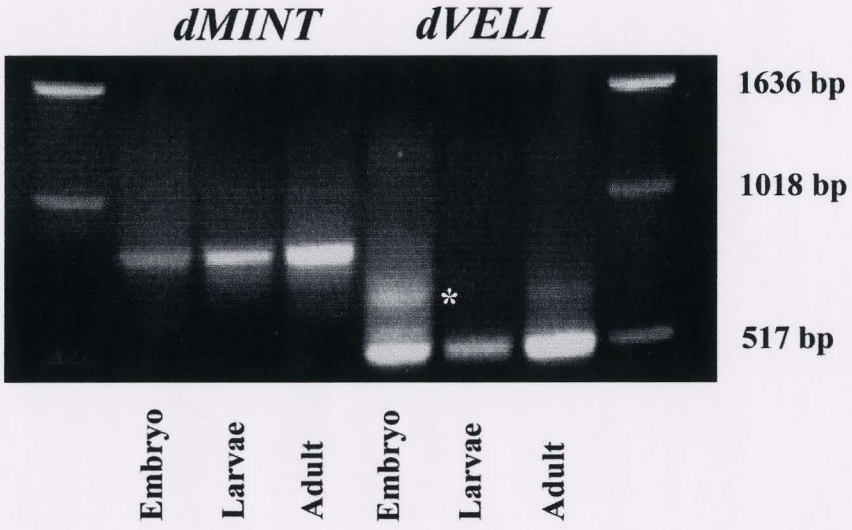
(pd(T)12-18) used to amplify cDNA from mRNA based on binding to the poly A tail of mRNA transcripts. The second set of primers were primers designed to *dveli* and *dMint* sequences that were capable of distinguishing cDNA transcripts from genomic DNA contamination (see Appendix 2).

Figure 9 reveals the presence of cDNA transcripts in all stages of development for both *dveli* and *dMint*. Because the RT-PCR was not carried out quantitatively, no conclusions can be reached as to the level of expression in each developmental stage. RT-PCR is capable of detecting RNA transcripts that are present in as little as a few hundred copies per sample. Embryos, larvae and adults all express *dVELI* and *dMINT* mRNA transcripts, however further tests were used to reveal the location and levels of expression. *C. elegans* LIN-7 is known to bind LET-23, the ortholog of the *Drosophila* EGF receptor (DER), an area where much research has been carried out in Dr. Roger Jacobs laboratory. With this research focus, interest fell almost exclusively on determining expression patterns of *dVELI*.

Tissue RNA *in situ* Hybridization Detects First Expression of *dVELI* in the Central Nervous System of Larval Stages

pBS *dveli* cDNA construct (Appendix 1) was used to generate sense and anti-sense RNA probes for use in tissue *in situ* hybridization to detect RNA expression patterns. Probes were transcribed from linearized template as described in the methods section. Embryonic *in situ* hybridization using the generated probes was repeated several times with no expression detected. Positive controls were done along side the *dveli* probes to control for procedural errors. *Slit* sense and anti-sense probes were generated from linearized cDNA and labeled with digoxigenin UTP. All positive controls of genes known to be expressed in the embryo reacted as expected, while the *dveli* probed

Figure 9. RT-PCR of *dveli* and *dMint* through various stages of development indicates the presence of mRNA transcripts in all stages. cDNA and genomic DNA were differentiated by the use of primers designed to yield bands of significant size differences (see Appendix figure2). *dVELI* and *dMINT* are present in embryos, larvae and adults. *dveli* cDNA band length is 516 bp, while *dMint* product length is 809 bp. Asterisk (*) indicates genomic DNA contamination.


dMINT
dVELI
1636 bp
1018 bp
517 bp
*
Embryo
Larvae
Adult
Embryo
Larvae
Adult

embryos consistently detected no pattern. Because of the detection of mRNA by RT-PCR in the embryonic stage, it was expected that *dVELI* transcripts were present in the embryonic stage of development. The possibility of the *dveli* RNA probe degrading was explored by using a DNA probe in the tissue *in situ* as well. This also failed to produce an expression pattern. It is possible *dVELI* is expressed at such low levels in the embryo or at very late stages (just before 1st instar) *in situ* hybridization fails to detect the presence of mRNA while the extremely sensitive RT-PCR is able to amplify the product to detection quite possibly due to larval contamination.

Following failure to detect expression of *dVELI in situ* in embryos, larval stage *in situ* hybridization was used to show the distribution of *dVELI* transcripts in 1st, 2nd and 3rd instar larvae tissues. Larvae were dissected, fixed and probed as described in the methods section. As seen in figure 10, *dVELI* transcripts were detected in the central nervous system. The nerve cord is composed of a central neuropil area, the site of synaptic connections, and an outer ventral ganglion, composed mainly of cell bodies. *dVELI* appears to be produced in the bodies of cells located within the ventral ganglion of the nerve cord as well as those neurons present in the midline of the nerve cord. The central neurpile are lacking any *dVELI* expression, consistent with this being the site of synaptic connections rather than cell bodies. The larval brain also expresses *dVELI* transcripts. When comparing to the sense control, the brain appears to express transcripts throughout, with higher levels being produced in the inner portions of the brain, site of the optic lobes and the mushroom bodies. Although exact location of *dVELI* in the brain can not be determined with complete accuracy, the presence of the transcripts within the optic lobes and mushroom bodies suggests neuronal expression, as these are sites of very organized neuronal connections.

Figure 10. RNA transcript distribution in 3rd instar larval tissues. 3rd instar larvae were dissected, fixed and probed with DIG labeled sense and anti-sense probes to determine the distribution of *dveli* transcripts in larval development. A-D illustrates the sense control tissues. E-H depicts tissues probed with the anti-sense DIG labeled probe. CNS = central nervous system, L = leg imaginal discs, E-A = eye-antennal imaginal disc, W = wing imaginal disc. *dveli* transcripts are detected in the ventral ganglion cortical rind of the CNS (arrow), as well as some portions of the brain lobes (arrowheads). *dVELI* also appears to be present at low levels uniformly throughout the imaginal discs, particularly the leg imaginal discs (panel F). When comparing the sense controls, the imaginal discs appear more granular-like in staining, implicating low level expression throughout the discs.

CNS
L
E-A
W
sense
Anti-sense
A
B
C
D
E
F
G
H

The expression pattern of *dVELI* within the central nervous system of larval developmental stages suggests a function in neurons similar to that of the mammalian homologues (Butz *et al,* 1998; Jo *et al,* 1999). VELIs are thought to function in the synaptic connections of neurons, possibly aiding in the localization of receptors to the synaptic membranes. The localization of *dVELI* within cells (possibly neurons, neuroblasts, ganglion mother cells) of the CNS lends support to dVELI being more similar in function to its mammalian homologues rather than *C. elegans* LIN-7, which has no known neuronal role (Simske *et al,* 1996). It is hoped antibody production and subsequent testing to determine the protein location will help to elucidate the precise location and function of dVELI within these neurons and synaptic connections.

Possible Low Levels of RNA Expression in Some Imaginal Discs Suggests a Non-Neuronal Role for *dveli* as well

During the dissection and subsequent *in situ* hybridization on 3rd instar larval nerve cords, the imaginal discs of the developing larvae were also tested for *dveli* RNA expression patterns. The imaginal discs of *Drosophila* are clusters of primordial cells that are maintained separately from the developing larva, leading to the development of adult structure such as the legs, wings, eyes and antennae. When comparing to the sense control, *dVELI* appears to be uniformly expressed in low levels throughout the imaginal discs, particularly the leg imaginal discs (Figure 10). Expression in such non-neuronal structures implicates an epithelial cell role for *dveli* as well.

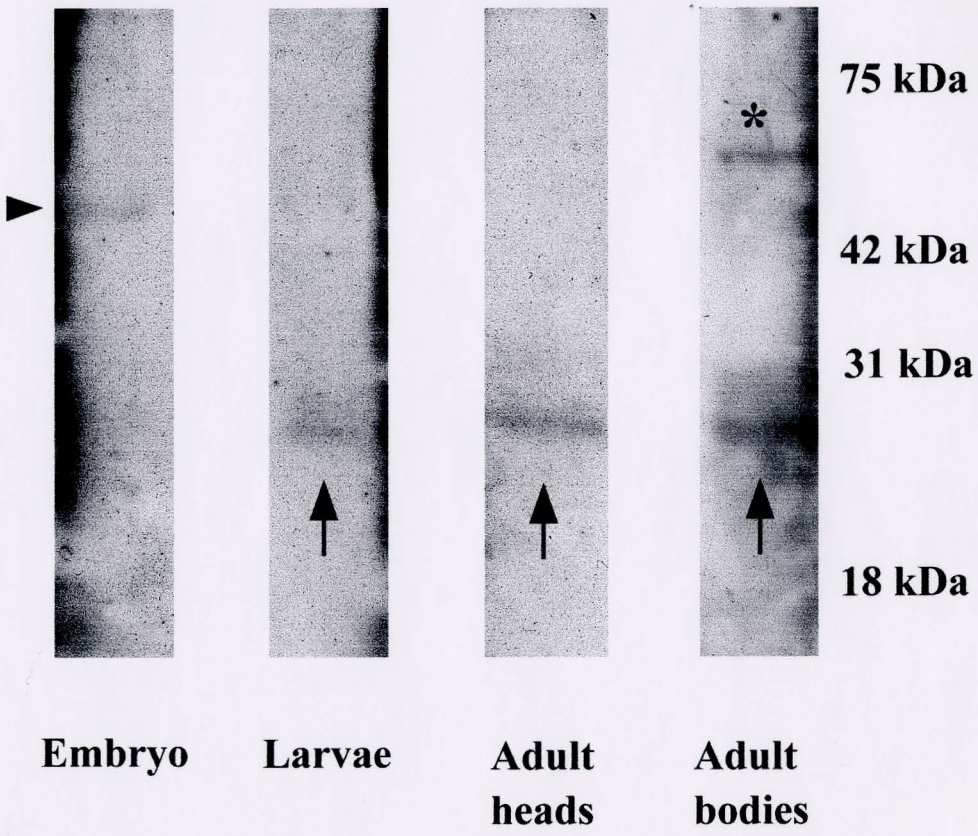
3.4 dVELI Protein Distribution and Developmental Stage Analysis

Western Blot Analysis of dVELI Supports Protein Expression Beginning in the Larval Stage and Continuing Through Adult Development

Whole protein lysates from embryos, larvae, adult heads and adult bodies were analyzed for the presence of dVELI protein expression through Western blot analysis. Blots were probed with varying concentrations (ranging from 1/1000 to 1/10000) of dVELI purified antibody to find the optimum concentration of the antibody (1/5000). dVELI antibody was generated in rats using a GST fusion protein and purified using CN-Br affinity purification (see Methods). ECL detection was used to develop the autoradiograph. As seen in figure 11, dVELI protein was detected in larvae, adult heads and adult bodies. No detectable level of embryonic protein expression was observed, even after prolonged exposure of the autoradiograph film to the blot (data not shown). dVELI appears as a single band located at approximately 24 kDa on the blot. This corresponds well with the expected mass of 22 kDa revealed through amino acid analysis. As noted on figure 11, two other bands not corresponding to the expected size dVELI were also observed - one in embryos and one in adult bodies. Interestingly these bands also appear in the pre-immunization control blot (data not shown). It is suspected these bands are background, resulting from the cross-reactivity of a rat antibody to some *Drosophila* proteins. This antibody possibly was purified along with the dVELI antibody when the antibody purification was performed (see Methods).

The absence of dVELI protein in embryonic protein lysates supports the above *in situ* data showing a lack of detection of transcripts in embryos. However, further support for this could come from timed embryo extracts. If dVELI expression begins very late in embryogenesis, pushing collections to later stages may reveal dVELI expression just before 1st instar. The spatial distribution of dVELI protein within these developmental

Figure 11. Western Blot Analysis of dVELI protein distribution in various developmental stages of *Drosophila*. Protein lysates of *Drosophila* embryos, larvae, adult heads and adult bodies were run on an SDS-PAGE gel, transferred to PVDF membrane, incubated with dVELI purified antibody and detected using ECL to elucidate the development stage of expression of dVELI protein. dVELI was not detected in embryonic protein lysates. Arrows indicate expression of a 24 kDa band corresponding to dVELI in larvae, adult heads and adult bodies. This corresponds well to the expected size of 22 kDa based on amino acid content. Arrowhead and asterisk indicate bands present in embryo and adult body lysates respectively that do not correspond to the size of dVELI. These bands are suspected to be background due to cross-reactivity as these are also present in the pre-immunization blot (data not shown).


75 kDa
42 kDa
31 kDa
18 kDa
Embryo
Larvae
Adult heads
Adult bodies

stages can not be determined from the Western analysis and instead will be determined through immunohistochemistry.

Drosophila Embryos Show No Distinct Pattern of dVELI Protein Expression

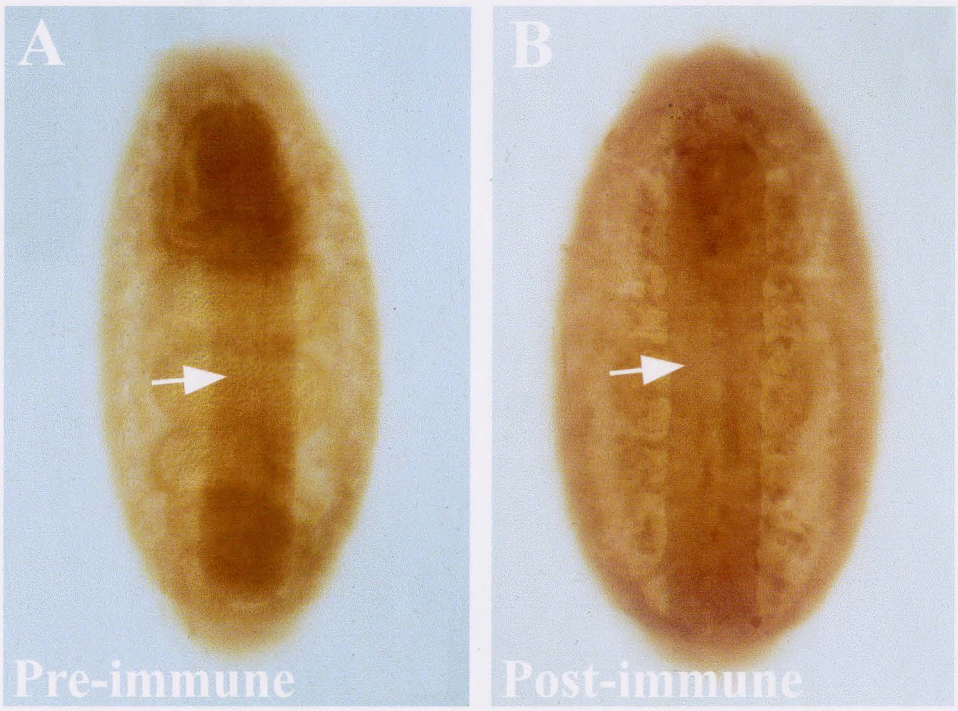
Following the finding that embryos lack any detectable dVELI levels within the Western, confirmation of this was sought through immunohistochemical testing ofwhole embryos with the dVELI generated antibody. Whole embryos were collected, fixed and stained as described in the methods. Stained embryos were mounted and were viewed under a microscope in order to detect any distribution of dVELI.

Figure 12 depicts a typical dVELI stained embryo. When comparing to the pre-immunization control, most if not all staining is attributed to background staining. No specific spatial distribution of dVELI protein is observed in embryos. This stain was repeated several times at varying concentrations with the same results each time. To further verify these results, crude pre and post-immune serum was tested at varying concentrations (from low to high) with the same outcome (data not shown). Embryonic collections contained a range of aged embryos to encompass all stages of development. Referring once again to figure12, even late stage 16-17 embryos display no antibody staining.

dVELI Localization in Larvae Supports a Role in the Central Nervous System

It was revealed earlier that *dveli* transcript expression begins in the larval stage, focused almost exclusively within the central nervous system. In order to confirm the protein functions within the nervous system, the temporal distribution of dVELI within larvae was tested immunohistochemically. 1st, 2nd and 3rd instar larvae were dissected,

Figure 12. *Drosophila* embryos show no distinct pattern of dVELI protein expression. *Drosophila* embryos were collected, fixed and prepared for immunohistochemistry to test the expression pattern of dVELI protein. Embryos were incubated with various dilutions of dVELI antibody and HRP labeled secondary was visualized using DAB. Embryos consistently showed no distinct protein expression (B) when compared to the purified pre-immunization serum (A). Arrows indicate general background staining of the nervous system present in both the pre- and post-immune embryos. Embryos shown are stage 16-17. Various stages of embryos were tested, even late stage 17 with no expression detected (not shown).


A
Pre-immune
B
Post-immune

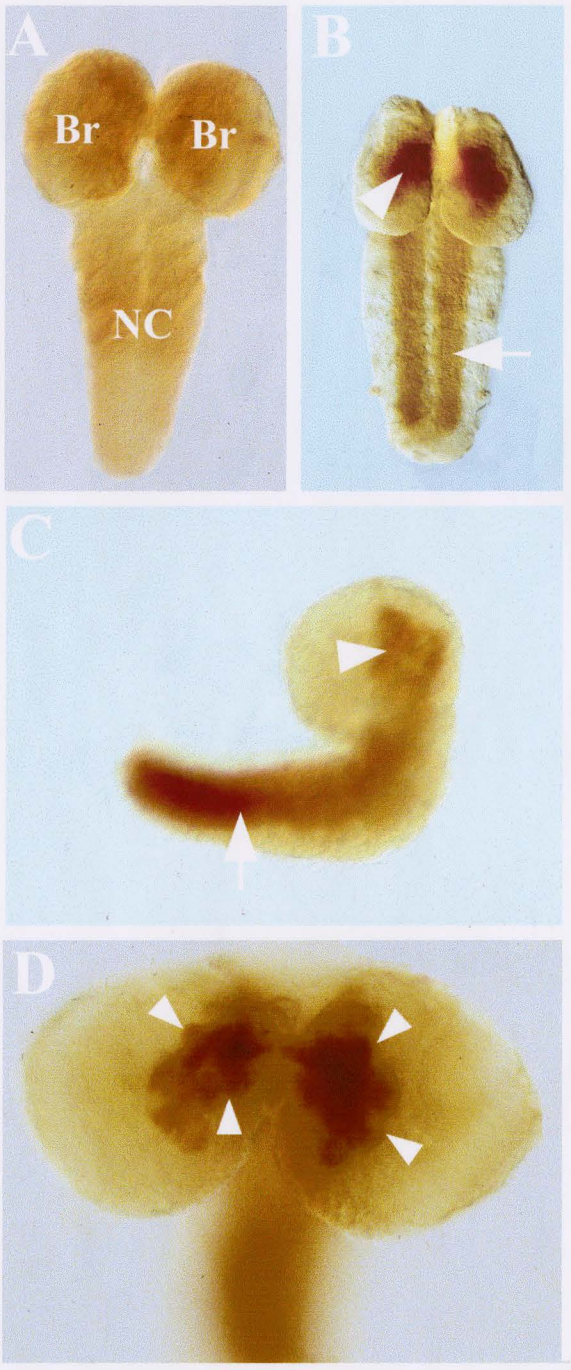
fixed and stained with dVELI generated antibody. The resulting distribution was similar in all larval stages. Figure 13 shows the location of dVELI within the central nervous system of larvae. dVELI is abundant within the neuropil of the larval ventral ganglion and also within what appear to be the mushroom bodies of the brain. Expression begins within early 1st instar larvae, continues through 2nd and 3rd instar larval stages, with the pattern being essentially identical in all stages of larval development.

The basic organization of the larval central nervous system consists of the brain connected to the nerve cord, which consists of segments of ventral ganglia. Neurons are arranged within the ventral ganglia such that the cell body is found in the outer parts, or the cortical rind and the axonal processes of these neurons extend centrally into what is known as the neuropil. The neuropil is the site of synaptic connections. The localization of dVELI within the neuropil of the nerve cord suggests a role in the synapse. dVELI distribution in the mushroom bodies of the brain also lends support to this role in synapses. The *Drosophila* mushroom bodies are paired structures consisting mainly of neuropil, with synaptic connections throughout the entire structure. The mushroom bodies consist of the cell bodies of neurons found at the dorsal-posterior surface of the brain, with their dendrites forming the calyx structure just below. The axons extend outward toward the anterior surface of the brain, resulting in the formation of five lobes - alpha, alpha', ß, ß' and gamma, where synaptic connections are formed. dVELI appears to be expressed throughout these lobe structures (see Figure 13).

Some Larval Imaginal Discs Express Low Levels of dVELI Suggesting a Possible Epithelial Cell Role

When dissecting and staining larvae, the imaginal discs were also stained. The cells that ultimately lead to the epidermal structures of the adult fly arise from separate

Figure 13. dVELI localization in larvae supports a role in the central nervous system. 1st, 2nd and 3rd instar larvae were dissected, fixed, incubated with dVELI antibody and HRP labeled secondary was visualized using DAB to determine the spatial distribution of dVELI within the larval stages of *Drosophila* development. Panel A is the pre-immunization control. Br are the brain lobes, while NC is the nerve cord. Panel B is a typical 1st instar larva when expression of dVELI begins. The white arrow shows the neuropil area of the nerve cord, where dVELI is prevalent. The white arrowhead indicates extensive staining in the brain of the central nervous system, most likely the mushroom bodies of the brain. Panel C is a sagittal view of a 3rd instar larval brain and nerve cord, revealing further the expression of dVELI in the nerve cord and brain (arrow and arrowhead, respectively). Finally, panel D takes a closer look at expression of dVELI within the brain. Several distinct lobular looking structures are stained (black arrowheads), similar to what the mushroom bodies of insect brains appear like (see Results, section 3.4). Little if no staining is observed in any other parts of the brain.


A
Br
Br
NC
B
C
D

cell populations referred to as imaginal discs. Such imaginal discs lead to the formation in the adult to such structures as the eye, antennae, the legs and the wings. Figure 14 shows the results of dVELI antibody staining on the eye-antennal imaginal disc, the leg imaginal discs and the wing imaginal disc. Both pre- and post-immunization imaginal discs from shown for 3rd instar larvae. dVELI appears to be expressed in low-levels uniformly throughout the leg imaginal discs. It is also present in the eye-antennal imaginal disc. The antennal portion of the disc is organized into a series of folds or segments. dVELI appears to be expressed within the second antennal segment. Within the eye imaginal disc, dVELI is found along the anterior rim within the morphogenic fold. Although the wing imaginal disc was stained as well, no distinct staining different from that of the pre-immunization control can be seen. The presence of dVELI within these non-neuronal structures implicates a possible role for the protein in epithelial cells.

dVELI is Present in Neuromuscular Junctions and can be Further Localized to the Post-Synaptic Membrane

The localization of dVELI within the neuropil of the larval nerve cord suggests a role in synaptic function, similar to the mammalian VELIs. In collaboration with Dr.Bryan Stewart, University of Toronto, dVELI expression within neuromuscular junctions was tested to determine the location of the protein within the synaptic junction. dVELI antibody was sent to the Boulianne lab where it was tested on larval neuromuscular junctions (NMJ). Figure 15 is a confocal image of the larval NMJ labeled for both dVELI and synaptotagmin antibodies. dVELI is in red, while synaptotagmin is green. Synaptotagmin is a synaptic vesicle protein with proposed exo- and endocytotic functions in the pre-synaptic membrane (Brose *et al,* 1992), making synaptotagmin an excellent marker for the pre-synaptic membrane. Co-localization of dVELI with

Figure 14. Some larval imaginal discs express low levels of dVELI implicating a possible epithelial cell role. Imaginal discs from 3rd instar larvae were dissected, fixed, incubated with dVELI antibody and HRP labeled secondary was visualized with DAB to characterize protein expression patterns of dVELI within these structures. W = wing imaginal disc, E-A = eye-antennal imaginal disc, L = leg imaginal discs. Both pre-immunization and post-immunization results are shown for all three imaginal disc types. Panel A and B depict typical results for the wing imaginal disc. No staining differing from that of the pre-immunization is observed. However, in Panel D, the eye antennal disc shows dVELI protein expression when comparing to its control, panel C. a = antennal portion, e = eye portion. Arrowheads show dVELI in the segments of the antennal disc as well as within the folds of eye disc. Finally, the leg imaginal discs were stained. When compared to the pre-immunization panel E, the post-immunization (F) shows uniform low levels of expression throughout the entire disc (arrow).

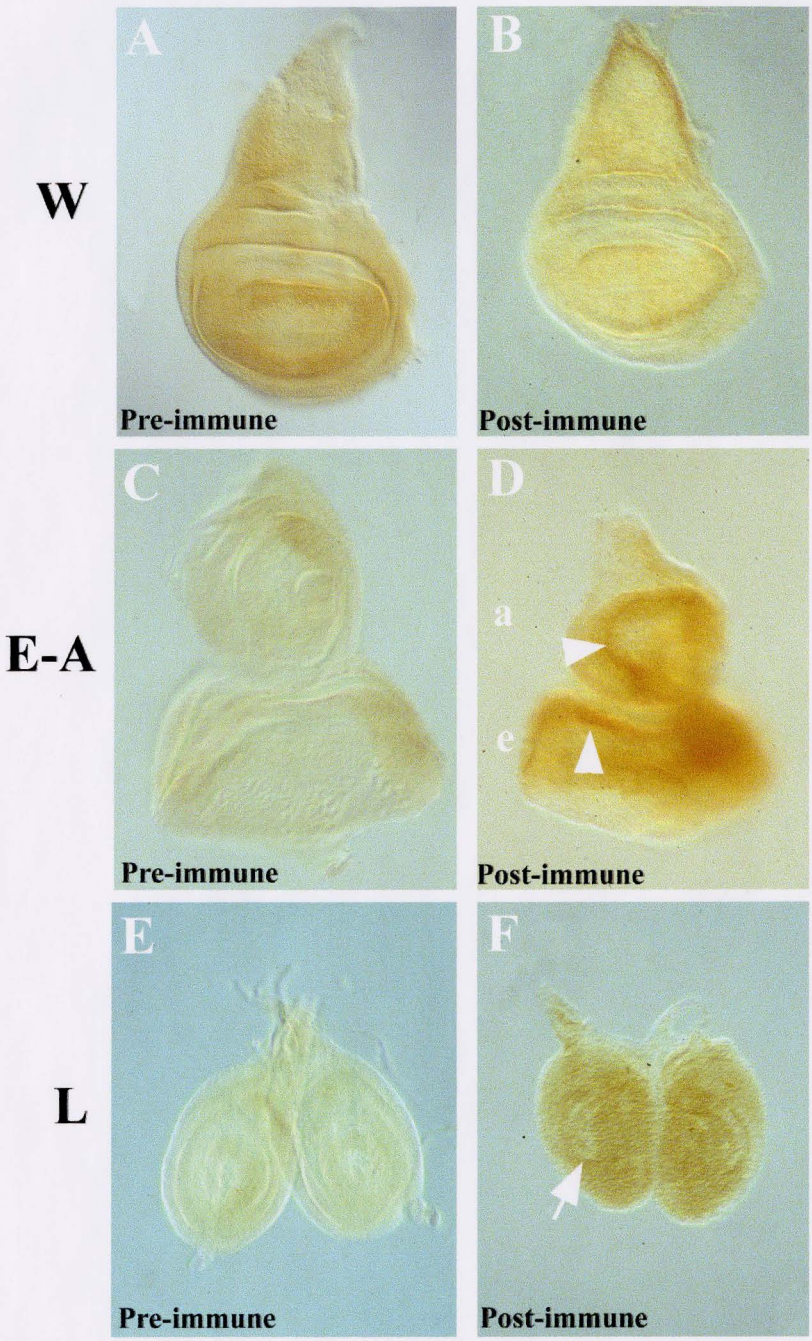
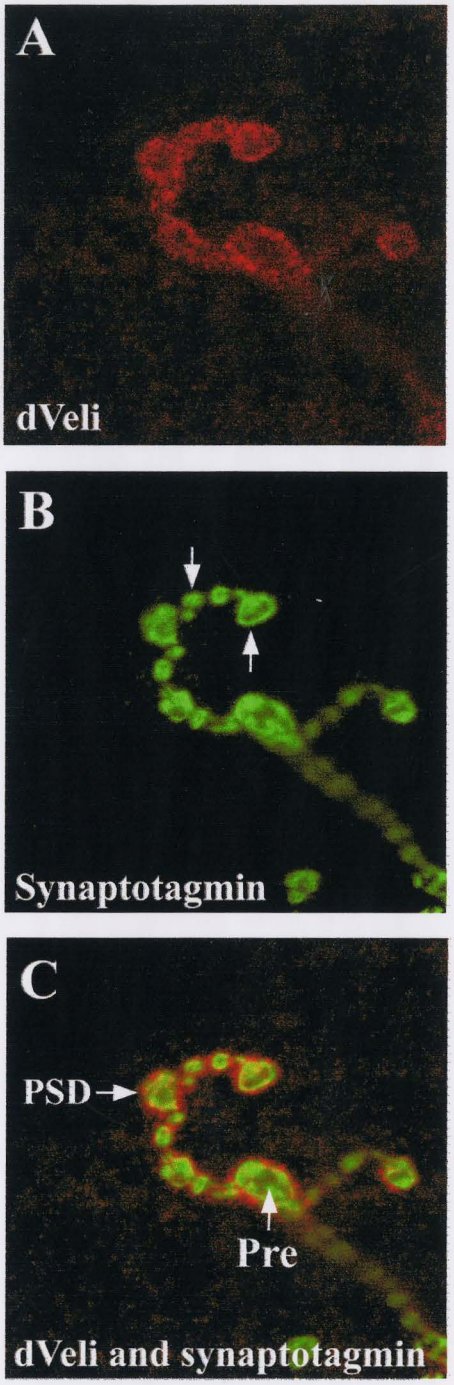

W
E-A
L
A
B
Pre-immune
Post-immune
C
D
a
e
Pre-immune
Post-immune
E
F
Pre-immune
Post-immune

Figure 15. dVELI is localized to the post-synaptic membrane of larval neuromuscular junctions (NMJ). In collaboration with Dr. Bryan Stewart, University of Toronto, dVELI antibody was tested on 3rd instar larval neuromuscular junctions to determine its location within the synapse using synaptotagmin as a pre-synaptic membrane control. Confocal images were obtained for dVELI (red) and synaptotagmin (green) alone (A and B respectively) as well as a merged image of both antibodies (yellow) to highlight areas of co-localization. The NMJ boutons appear as circular structures (panel B, arrows). The merged image, C, shows dVELI concentrated mostly at the post-synaptic density (PSD), while synaptotagmin is at the pre-synaptic membrane (Pre). Co-localization of both proteins does occur in some areas of the pre-synaptic membrane.


A
dVeli
B
Synaptotagmin
C
PSD
Pre
dVeli and synaptotagmin

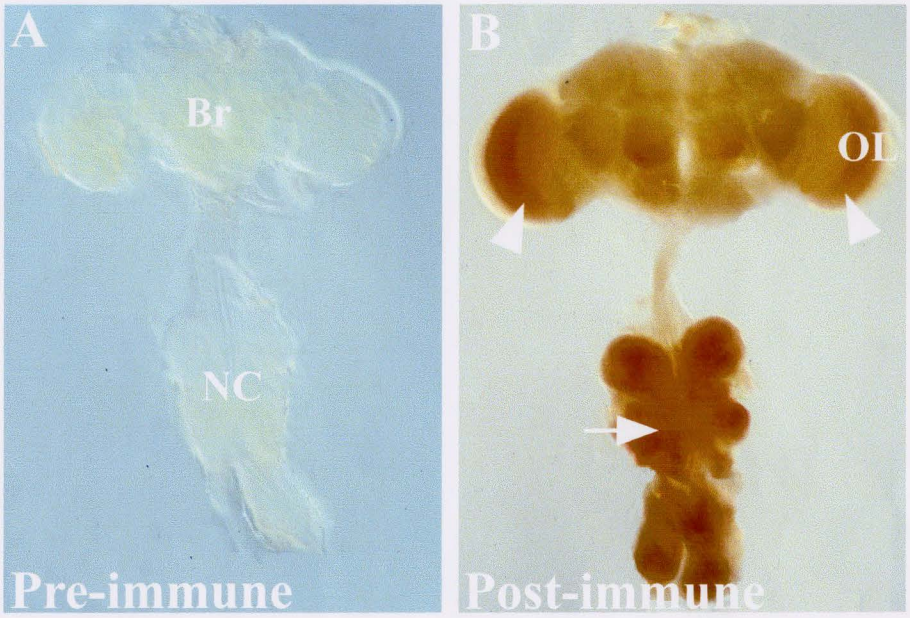
synaptotagmin in the pre-synaptic membrane would result in a yellow colour in the merged image. However, results show some yellow colour, indicating very little co-localization of dVELI with synaptotagmin. dVELI is almost exclusively located at the post-synaptic membrane, corresponding to the location of mammalian VELIs (Jo *et al,* 1999). The presence of dVELI in the post-synaptic membrane supports a role in the organization of the post-synaptic membrane, possibly localization of neurotransmitter receptors, similar to that of its mammalian counterparts (Jo *et al,* 1999).

dVELI Expression in the Adult CNS Includes the Nerve Cord and Several Brain Structures

dVELI was further studied in the adult fly to determine if expression in the CNS continued through adult development. Adult heads and nerve cords were dissected, fixed and immunostained to determine the spatial distribution of dVELI within the adult fly. Pre-immunization and post-immunization results are shown in Figure 16. Expression of dVELI can be observed in the motor pools of the thorax as well as within the optic lobes of the brain. No dVELI is found in the brain-thorax connective.

The adult nervous system contains many synaptic connections. The motor pools of the thorax are neuropile sites. The optic lobe of *Drosophila* is known to be the site of the most orderly of all the neuropile in the central nervous system , with carefully arranged synaptic connections (Meinertzhagen and Hanson, 1993), consistent with the orderly staining pattern of dVELI within these areas.

Figure 16. dVELI protein expression in the adult CNS includes the nerve cord and the optic lobe. CS-P adult heads and nerve cords were dissected, fixed and incubated with dVELI antibody. HRP labeled secondary was visualized with DAB to determine the spatial location of dVELI within the adult CNS. Panel A shows the pre-immunization control, while B illustrates a head and nerve cord after incubating with purified dVELI antibody. NC = nerve cord, Br = brain and head structures. As seen in B, the adult nerve cord is composed of neuropil that uniformly expresses dVELI when comparing to the control (arrow). Within the brain and head structures, dVELI is concentrated mostly to the optic lobes (OL, arrowheads), the sites of very organized neuropile. There also appears to be staining throughout the central brain regions as well.


A
Br
NC
Pre-immune
B
OL
Post-immune

3.5 The UAS-Gal4 System Will be Used in the Future to Help Determine the Normal Function of *Drosophila veli*

Using the UAS-Gal4 system in *Drosophila* it is possible to rapidly generate strains of flies in which ectopic expression of the candidate genes can be directed to a specific set of cells (Brand and Perrimon, 1993). The binary Gal4 system requires two different different fly lines. One line, UAS gene X, has the target sequence under the control of a yeast Gal4 upstream activation sequence (UAS) promotor, but no activator present. The other line, enhancer Gal4, encodes the UAS activator but no target sequence. When the lines are crossed, the progeny are able to activate the target sequence and express it in the pattern of the enhancer. It is hoped that overexpression and/or misexpression of *dveli* will help in the determination of its normal function. UAS-*dveli* was generated as described in the methods. However due to time constraint, injecting of the construct and testing of the subsequent transformed flies was not accomplished. Future work will include the completion of this task.

3.6 PALS (Proteins Associated With LIN-7) Appear to be Relatively Conserved From *mus musculus* to *Drosophila*

As mentioned previously, recent work has suggested that the amino terminus of the mammalian homologs of LIN-7 may have binding partners other than LIN-2. Kamberov *et al.* (2000) have supported this notion through the identification of proteins in *mus musculus* that contain the necessary LIN-7 binding domain, recently identified as an L27 domain (Doerks *et al*, 2000). These proteins were named PALS (**p**roteins **a**ssociated with **L**IN-**7**). Two novel members of this family were identified as PALS1 and PALS2. Both of these proteins are members of the MAGUK family of proteins. Because of the conservation of mammalian LIN-2, and as reported above, mammalian

LIN-7 and LIN-10 to their *Drosophila* orthologs, the task was undertaken to identify if the *Drosophila* genome also contains these novel MAGUK members.

PALS1 (accession number AF199008) amino acid sequence was aligned against the *Drosophila* genome to find a potential match. CG1617 (fly base accession number FBgn0030024) displays a 49% amino acid identity and a 63% similarity to PALS1. As with PALS-1, CG1617 contains the MAGUK domains of a PDZ domain, an SH3 domain and a guanylate kinase domain, along with the L27 binding domain. CG1617 is found at 7D18 on the X chromosome. It has a transcription length of 3041 base pairs and is 794 amino acids long when translated. Best fit amino acid alignment of PALS1 and CG1617 can be seen in Appendix 5.

As with PALS1, PALS2 (accession number alpha splice variant AF199009 and beta splice variant AF199010) splice variant amino acid sequences were subjected to BLAST searches against the *Drosophila* genome. The resulting matches were the same for both the alpha and the beta splice variant, therefore results are shown only for the alpha variant. Interestingly, two close matches were pulled out of the genome. CG9326 shows a 39% amino acid identity and a 58% similarity to PALS1. CG9326 contains all of the main MAGUK domains (PDZ, SH3 and guanylate kinase) as well as the L27 binding domain. CG9326 (fly base accession number FBgn0032885) is located on the second chromosome at 38E6. It is 3223 base pairs in length and when it is translated, 576 amino acids long. Best fit amino acid alignment of PALS2 alpha and CG9326 can be seen in Appendix 6. Also matching PALS2 closely was CG13219 (fly base accession number FBgn0033585), showing a 39% amino acid identity and a 57% similarity. As with CG9326 and PALS2, CG13219 contains all the same domains (SH3, guanylate kinase and L27), except it lacks a PDZ domain. CG13219 is located on the second chromosome at 47D7. It is 1826 base pairs long and when translated 416 amino acids in

length. Best fit amino acid alignment of CG13219 and PALS 2 can be seen in Appendix 7.

Kamberov *et al.* (2000) suggest that PALS compete for binding to mammalian LIN-7 proteins and that binding of PALS rather than LIN-2 to LIN-7 may target LIN-7 differently than when it is bound to LIN-2. These multiple binding interactions have yet to be studied further, however it does suggest multiple targeting roles for LIN-7. The presence of similar PALS proteins in *Drosophila* may lead to the discovery of a versatile role for dVELI similar to that of its mammalian ortholog.

3.7 The Discovery or Generation of a *dveli* Mutant is an Ongoing Task

Several Uncharacterized Lethals were Tested as Potential *dveli* Mutants

Following the confirmation of the location of *dveli*, the area around 3R96B was searched for existing mutants and deficiencies that could help in the isolation of a *dveli* mutant fly. This search led to the discovery of a set of uncharacterized lethals that were generated through an EMS mutagenesis screen by E.B Lewis (see Methods section for a further descriptive note). These lethals, named L(3)96Ba to L(3)96Bf, were characterized as best as possible genetically as well as phenotypically for any possible functional relationship to *dveli*. The results of this work are summarized in Appendix 8. After subsequent genetic, phenotypic and sequence analysis of these lethals, it is not certain that these are alleles of *dveli*. Some of the L(3)96B lethals were tested for viability, with those tested determined to be embryonic lethal. Furthermore, even though known information about these lethals indicate they are allelic, one of these line, L(3)96Bc, mapped outside of the 96B region based on complementation tests of a known large deficiency in the 96B region, Df(3R)96B.

An ensuing problem faced when working with these fly lines was the absence of balancer markers when the genotype stated all but one of the lines carried a stubble marker. After initially receiving the lines, all were phenotyped and it was found that most of the fly lines were not stably balanced. Several of the lines, L(3)96Bb, L(3)96Bd and L(3)96Bf, were very hard to work with as they seemed to lose their balancer markers. L(3)96Bf arrived from the Bloomington Stock Center without a stubble marker.

Sequence analysis was performed on three of the seven lethal lines - those lines that maintained their balancer. The L(3)96Ba, L(3)96Be and L(3)96Bg lines were first crossed to CS-P wild type flies to cross out the balancer. L(3)96B/+ was selected based on absence of the balancer phenotype. These flies were then crossed to each other (see Appendix 8 for genetics). Progeny were sequenced by first preparing single fly DNA preparations then using primers to PCR amplify the *dveli* gene (see Appendix 2 for primers). Because the flies would potentially be carrying a mutant copy of *dveli* as well as a wild type copy, base pair changes in the sequence indicating a potential mutation would appear as an N. Sequence data (not shown), showed several N's as a clean sequence product could not be achieved. Repeat sequencing yielded the same result. Therefore, sequencing was inconclusive about base pair changes in any of the three tested lethal lines.

Phenotypic analysis of embryos stained with fasciclin II, a protein expressed on the longitudinal axons of the nerve cord showed no significant phenotype in any of the lethals. Furthermore, expression of dVELI in these embryos could not be assessed as the lines are embryonic lethal. Therefore, based on all the results of all the tests on these potentially mutant in *dveli* lines, no direct conclusions can be reached. The lines can not for certain be named as alleles of *dveli*, nor can they be disregarded all together. Future work on these lines maybe will reveal further their genetic identity.

Expression of dVELI in a Synaptic Mutant

Expression analysis of dVELI in a *dveli* mutant was not possible since a mutant line has not been isolated. However, since dVELI appears to function at synaptic connections, expression of dVELI in mutants where these synaptic connections may be disrupted may lead to information about its functionality in the synapse. *paralytic* encodes for a class of sodium channels found in the nervous system of *Drosophila* (Loughney *et al,* 1989). *para*^{ID30} is an inversion within the transcribed portion of the gene, resulting in a non-functional product. Budnick *et al* (1990) studied the plasticity of synapses at the neuromuscular junction in *Drosophila* hyperexcitable mutants and mutants with reduced excitability. Hyperexcitability mutants showed an increase in the number of axonal branches and subsequent synaptic sites, while a mutation in a regulator the *para* locus, *no action potential* (*nap*^{ts}), showed a decrease in branching of axon terminals when compared to wild type. *nap*^{ts} is thought to act to decrease the number of sodium channels (Loughney *et. al,* 1989). Therefore, it is hoped that dVELI expression within *para*^{ID30} mutants will show a decrease in the amount of dVELI staining due to a subsequent lack of synaptic connections resulting from less branching in the axonal terminals, similar to that of *nap*^{ts}.

para^{ID30} is larval lethal such that the embryo develops to the 1st instar stage, but can not hatch due to paralysis. These 1st instar larvae were removed from their cases, dissected, fixed and stained for dVELI expression using DAB. The resulting pattern of dVELI protein expression was compared to that of wild type larvae to assess for changes. No observable changes were seen (data not shown). The expression patterns appeared to be similar for both *para*^{ID30} larvae and wild type larvae. It is possible that the potential decrease in synaptic connections and possibly dVELI is below the sensitivity and detection level of the method of choice for staining. Therefore, no conclusions can be

reached as to the expression of dVELI within a synaptic mutant. It is possible a more sensitive method for detecting the loss of synaptic sites and the potential corresponding decrease in dVELI expression would reveal more supportive results. NMJ preparations may be more useful.

P-element Mutagenesis Hopefully Will Generate a *dveli* Mutant Fly Line

The precise function of *dveli* can not be determined, only speculated upon, without the existence of a mutant for the gene. From mutational effects one can usually deduce wild type function of a gene. Therefore, the generation of a mutation within *dveli* is an important task. The *Drosophila* genome contains transposable genetic elements, portions of DNA, which are capable of jumping from one place to another within the genome. These transposable elements, or P-elements (for paternal elements), can be moved from their normal repressive condition and hopped to another location when a "hopping" source, or transposase, is supplied (reviewed in Robertson *et al*, 1988). P-element mutagenesis involves hopping a nonautonomous P-element from a nearby location using an immobile copy of the transposase gene into the gene of interest. The resulting P-element insertional mutations can be screened by PCR using primers designed to a portion of the P-element and the gene of interest to see if the P-element has inserted itself within the gene, resulting in a mutation.

In the case of *dveli,* a P-element insertion, P{lacW}L(3)J2D9, is present interrupting the gene *OstStt3* at approximately 3(R)96B15-17, 2 kb away from *dveli*. *OstStt3*, or *Oligosaccharyl transferase 3,* codes for an enzymatic portion of the plasma membrane (BDGP, 1994-1999). It is hoped P{w^+=lacW}L(3)J2D9 can be mobilized within *dveli* using an immobile copy of the transposase gene. Dr/TMSP{$ry^+\Delta 2$-3}(99B) is a fly stock available at Bloomington stock center containing one of the most widely

used sources of transposase, {Δ2-3}(99B). This transposase gene is lacking in the 2-3 intron and can not transpose itself (Robertson *et al,* 1988). Crossing the P{w^+=lacW}L(3)J2D9 fly line to Dr/TMSP{ry^+Δ2-3}(99B) results in progeny where mutagenesis may occur in the germ cells. Potential mutants are then screened against the original P-element line to determine if it is the same one or if the P-element has hopped. Once this is determined, any potentially new lethal lines are stabilized over a balancer and sequenced to determine where the P-element has inserted. This entire procedure can be monitored for P-element activity based on the presence of the mini white insert, w^+, which results in orange eyes in flies containing this P-element in a w^- background. See appendix 9 for complete mutagenesis scheme.

This P-element mutagenesis is an ongoing task. To date, only two hopped lethal lines have been isolated (not alleles of *OstStt*). However, testing on whether or not these lines are in fact the P-element inserted within *dveli* has yet to be determined. It is hoped that by continually trying to generate new fly lines in which the P-element has been mobilized differently will result in the eventual production of a *dveli* mutant strain.

Chapter 4
Discussion

4.0 The *Drosophila melanogaster* Orthologs of *lin-7/veli* and *lin-10/Mint1/X11alpha*

The LIN-2/LIN-7/LIN-10 complex of proteins has been previously been shown to be conserved between *C. elegans* and mammalian species (Butz *et al,* 1998; Borg *et al,* 1998a). The identification of potential orthologs of this complex in *Drosophila* could lead to a further understanding of its function because of the accessibility of molecular and genetic manipulation of gene function. The fully sequenced genome of *Drosophila* also facilitates rapid identification of other potential scaffolding proteins associated with this complex.

The *Drosophila* ortholog of *lin-2* has been previously identified (Martin and Ollo, 1996; Dimitratos *et al,* 1997). We have identified the *Drosophila* orthologs of *lin-7* and *lin-10*, which we have termed *dveli* and *dMint*, respectively. *dveli* chromosomal location was determined by polytene chromosome *in situ* hybridization to be 3R(96B)16, which was confirmed following the sequencing of the genome. The area around this genomic location was studied for deficiencies and other fly lines potentially useful in a genetic analysis of *dveli* function. Interestingly, a set of previously uncharacterized lethals reported to be allelic were discovered in the area of *dveli*. These fly lines were assayed for viability and phenotypic characteristics as well as sequenced in the *dveli* region to assess any base pair changes or deletions in the gene to determine if these lines were indeed *dveli* mutants. Unfortunately, no conclusive data can be reached about these

lethal lines. Sequencing data was inconclusive and no phenotypic changes from wild type were observed. P-element mutagenesis is currently underway and will hopefully generate a *dveli* mutant fly line.

Without a mutant fly line, research on *dveli* fell exclusively on characterizing homology of the protein to its known orthologs and the location of those proteins that potentially interact with dVELI, as well as characterizing RNA expression patterns and protein expression patterns.

4.1 LIN-7/VELIs and LIN-10/Mint1/X11alpha Proteins are Conserved in *Drosophila melanogaster.*

dVELI has a 66% amino acid identity to *C. elegans* LIN-7. However, interestingly it appears to be more similar to the mammalian VELIs, particularly VELI-3 (or LIN-7C) with which it shares a 80% amino acid identity. dVELI protein structure is characterized by the same domain recognitions as the other known LIN-7/VELIs, possessing a single PDZ domain and an L27 domain. These domains are highly conserved from *Drosophila* to the LIN-7/VELIs proteins of other species, with the PDZ domain showing a 92% amino acid identity in some of the known proteins. In a similar manner, dMINT has a 58% identity to *C. elegans* LIN-10 and a 50-55% identity with the mammalian Mint1/X11alpha orthologs. The conservation of the recognized domains of LIN-10 in dMINT is evident in the PTB domain as well as both of the PDZ domains, with amino acid identity ranging from 59-64% and 64-77%, respectively. The observed similarity of the *Drosophila* proteins to the *C. elegans* and mammalian proteins suggests conserved function for the LIN-2/CASK, LIN-7/VELI, LIN-10/Mint1/X11alpha complex in *Drosophila*.

4.2 *Drosophila* VELI Functions in Synaptic Connections Similar to the Mammalian VELIs

Developmental transcript analysis of both *dveli* and *dMint* reveals the presence of both transcripts in all stages of development - embryo, larva and adult. With the possible association of dVELI with DER, focus fell mainly on the characterization of dVELI. RNA *in situ* hybridization shows the spatial distribution of *dVELI* in neurons of the ventral ganglion cortical rind as well as neurons within the brain beginning at 1st instar larval stage. The *Drosophila* nerve cord is composed of a central neuropil area, the site of synaptic connections, and an outer ventral ganglion, composed mainly of the cell bodies of neurons. *dVELI* appears to be produced in the cell bodies of neurons located within the ventral ganglion as well as those in the midline of the nerve cord. The neuropil areas lack any *dVELI* expression. The neuropil areas are the sites of synaptic connections. This expression pattern of *dVELI* suggests a function in neurons similar to that of the mammalian orthologs (Butz *et al,* 1998; Jo *et al*, 1999).

dVELI protein distribution in the CNS further supports the function of dVELI in neurons. Western blot analysis detects a 24kDa band corresponding to dVELI in larval and adult stages of development. No protein was detected in the embryonic stage, even after prolonged exposure. Within larvae and adults, immunocytochemistry reveals dVELI is mainly concentrated in neuropile of the nerve cord and brain. Within the nerve cord, the neuropile areas show distinct dVELI expression, suggesting a function in synaptic connections. The expression of dVELI in the mushroom bodies of the brain as well as the optic lobe areas also supports the notion of dVELI function in synaptic connections. The *Drosophila* mushroom bodies are paired structures consisting mainly of neuropil, with synaptic connections throughout the entire structure. The mushroom bodies consist of the cell bodies of neurons found at the dorsal-posterior surface of the

brain, with their dendrites forming the calyx structure just below. The axons extend outward toward the anterior surface of the brain, resulting in the formation of five lobes - α, α'. ß, ß' and γ, where synaptic connections are formed (Itoh *et al,* 1997). Similarly, the optic lobe areas are the sites of the most orderly of all neuropil areas (Meinertzhagen and Hanson, 1993), with synaptic connections prevalent in these areas.

Mammalian VELIs are believed to have a role in the synapse (Butz *et al,* 1998; Jo *et al,* 1999), however *C. elegans* LIN-7 is not (Simske *et al,* 1996). Mammalian VELIs are thought to function in both the pre-synaptic and post-synaptic membrane (Butz *et al,* 1998; Jo *et al,* 1999). Within the presynaptic membrane, VELI is associated with CASK and Mint1/X11alpha complex (Butz *et al,* 1998). At the post-synaptic membrane, VELI functions in NMDA glutamate receptor sorting (Jo *et al,* 1999). Direct evidence of CASK and Mint1/X11alpha within the VELI complex with the NMDA receptor remains to be demonstrated.

NMJ staining with antibodies to dVELI localizes the protein mainly to the post-synaptic membrane. However, there may also be expression at the pre-synaptic membrane. The presence of dVELI in the post-synaptic membrane supports a role in the organization of this specialization, possibly localization of neurotransmitter receptors, similar to that of its mammalian counterparts (Jo *et al,* 1999). Its presence in low levels in the pre-synaptic membrane, however, may reveal a multi-functional role for dVELI in the synapse.

The synaptic localization of dVELI in neurons of the CNS compared to the basolateral localization of LIN-7 in *C. elegans* is consistent with the idea that protein complexes and machinery involved in polarization of receptors and ion channels are shared between cell types (Dotti and Simons, 1990). Proteins that are basolaterally located in epithelial cells are found at the post-synaptic membrane of neurons. The

localization of dVELI within synapses suggests that dVELI functions in neurons like the mammalian orthologs, localizing neurotransmitter receptors and/or ion channels the way in which *C. elegans* LIN-7 functions to localize LET-23 to the basolateral membrane of epithelial cells.

4.3 A *Drosophila* Model for the CAMGUK/dVELI/dMINT Complex in Neurons

A Pre-synaptic Membrane Complex in *Drosophila* may Function in Linking Cell Adhesion to Signal Transduction and Synaptic Vesicle Release

CAMGUK is localized primarily in the neuropile of the CNS - the ventral ganglion and the optic lobe areas (Dimitratos, 1999; Martin and Ollo, 1996), with expression patterns in the larva and adult almost identical to that of dVELI. The similar spatial locations of both CMG and dVELI suggests they function together as a complex similar to the mammalian complex. The similarity of dMINT amino acid sequence to LIN-10 and the mammalian Mint1/X11alpha suggests that dMINT also functions as part of this *Drosophila* complex.

The CASK/VELI/Mint1/X11alpha complex has been implicated to function at the pre-synaptic membrane in neurons (Butz *et al,* 1998). CASK is anchored to the membrane through its interaction with the cell adhesion protein neurexin (Hata *et al,* 1996). Neurexin is associated with the post-synaptic adhesion molecule neuroligin, which is bound by PSD-95 at the PSD, acting as a scaffolding molecule to recruit receptors and other molecules to the neurexin-neuroligin junction (Irie *et al,* 1997). CASK/VELI/Mint1/X11alpha bind each other via various domains, however the potential for other binding partners exist in the complex as each of the proteins has other protein domain motifs available for binding. At the pre-synaptic membrane, several binding partners for the complex have been identified by different research groups (Hsueh *et al,*

1998; Maximov *et al,* 1999; Perego *et al,* 2000). Interestingly, multiple binding partners have been found for some of the recognized domains of the complex proteins, particularly the PDZ domains. This is consistent with the idea that PDZ domains are capable of binding several different carboxy protein motifs (Songyang *et al,* 1997).

CASK has been shown to associate via its PDZ domain not only with the cell adhesion molecule neurexin in neuronal synapses, but also with the syndecans, a family of transmembrane heparan sulfate proteoglycans important in cell matrix interactions as well as cell-cell interactions (Hsueh *et al,* 1998). Mint1/X11alpha associates with Munc 18-1, a synaptic vesicle trafficking protein in the pre-synaptic membrane (Okamato and Sudhof, 1997). Recently, a binding partner for the first PDZ domain of Mint1/X11alpha has been identified that links the tripartite complex to ion influx that mediates synaptic vesicle fusion to the presynaptic membrane and subsequent neurotransmitter release. The C-terminal amino acids of the N-type Ca^{2+} channel α_{1B} subunit define a novel type III PDZ binding motif that interacts with the first PDZ domain of Mint1/X11alpha (Maximov *et al,* 1999).

This interaction with the Ca^{2+} channels mediates a link between synaptic vesicle fusion (via Munc 18-1) and neurotransmitter release. The potential for a link to signal transduction exists via an interaction with VELI. However, what VELI binds at the pre-synaptic membrane is still poorly understood. Presynaptically, VELI is believed to contribute to adhesion. Mammalian VELI associates with the β-catenin-cadherin complex at neuronal synapses through its single PDZ domain (Perego *et al,* 2000). Cadherins are transmembrane Ca^{2+} dependent adhesion molecules that are linked to the cytoplasm by catenins (Gumbiner, 1996). Catenin-cadherin complexes are a major component of cellular junctions which organize signaling and machinery complexes. VELI association with the catenin-cadherin complex may function in localizing CASK

and Mint1/X11alpha to the pre-synaptic membrane and in turn linking adhesion to synaptic vesicle fusion and neurotransmitter release. It is possible VELI functions at the pre-synaptic membrane to localize proteins involved in ion channel mechanics through its role in adhesion, indirectly involved in proper localization of a Ca^{2+} ion channel and subsequent synaptic vesicle release into the synaptic cleft.

Drosophila 3rd instar larval NMJ antibody staining suggests some pre-synaptic localization of dVELI. The similar structure and conservation of dVELI to the mammalian VELIs implies dVELI may function in a similar manner as the mammalian proteins. Therefore, the possibility of dVELI localizing synaptic vesicle fusion and neurotransmitter release machinery to the pre-synaptic membrane via adhesion mechanisms is entertained. The *Drosophila* α_{1B} Ca^{2+} subunit is known as *cacophony* (*cac*) or *night blind A* (*nbA*) (Kawasaki *et al,* 2000). nbA carboxy terminal terminates in the amino acid sequence E-D-W-C, consistent with the type III PDZ binding motif identified in the mammalian α_{1B} subunit, with the consensus sequence of E/D-X-W-C/S-COOH (Maximov *et al,* 1999). Similar to the way in which the mammalian subunit binds Mint1/X11alpha, it is possible that dMINT binds this PDZ motif of nbA via its first PDZ domain, linking the potential tripartite complex of CMG/VELI/dMINT to ion channel influx and subsequently synaptic vesicle release.

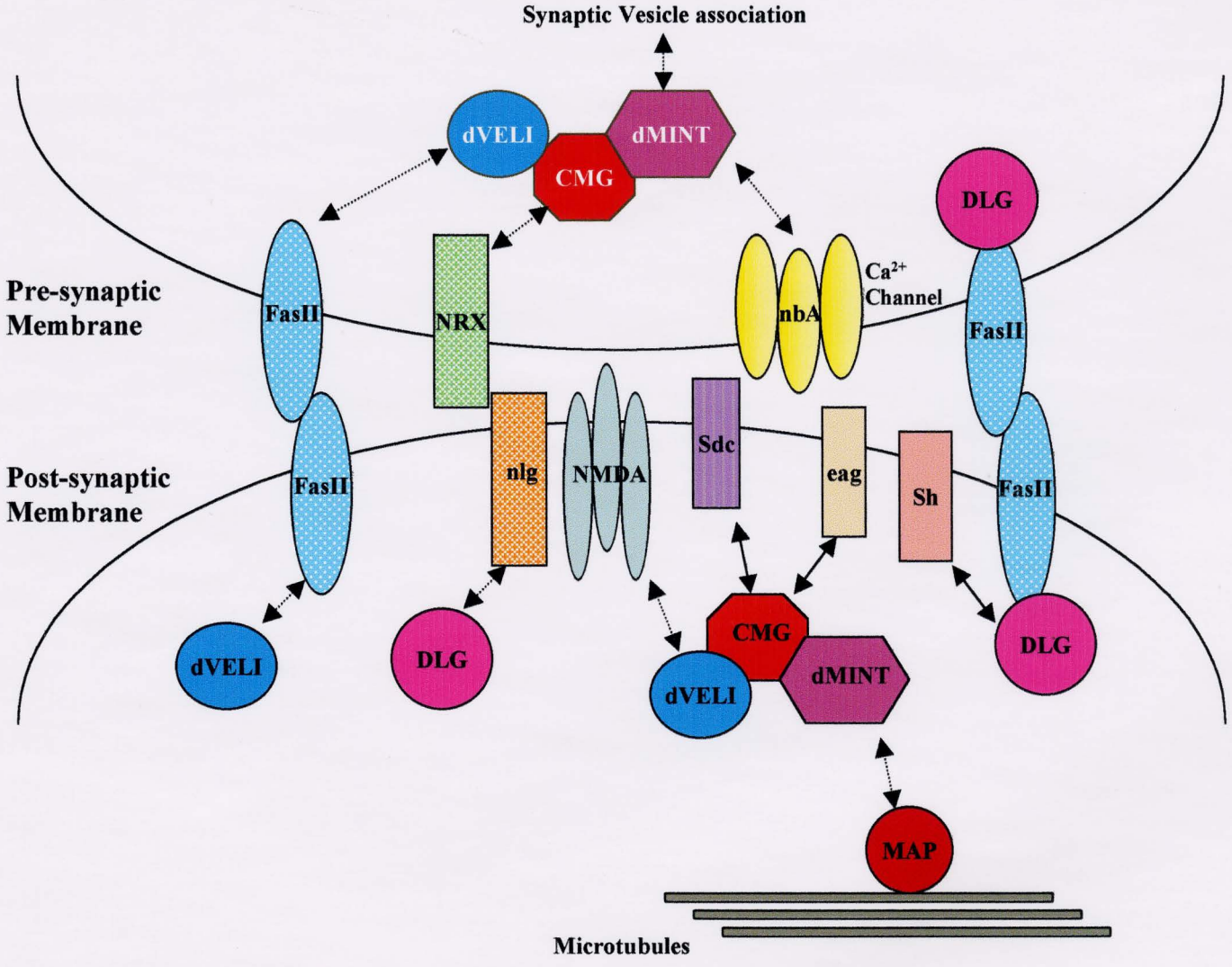
CMG binds to syndecan with its PDZ domain, analogous to the manner in which mammalian CASK binds syndecans (Dimitratos, 1997). However, until recently the *Drosophila* ortholog of *neurexin 1* has not been known. Therefore, the binding of CMG to this NRX has not been shown. The recent identification of NRX1, known as dNRX-1 suggests CMG may bind to this cell adhesion protein the way in which CASK binds neurexin in mammals. dNRX-1 is expressed in neurons of the CNS starting in embryonic

stages of development (Salinas *et al,* 2001). dNRX-1 ends in a type I PDZ binding motif, consistent with the idea that CMG may bind to this newly identified neurexin.

Mammalian VELI at the pre-synaptic membrane binds cell adhesion molecules (Perego *et al,* 1999). dVELI may function in adhesion as well. One identified cell adhesion molecule present in neurons and ending in a type I PDZ binding motif (S-A-V-COOH) is fasciclin II (FasII). FasII is localized at synaptic boutons of the NMJ, both pre-synaptically and post-synaptically (Schuster *et al,* 1996). It has been shown that DLG, the *Drosophila* MAGUK protein similar to PSD-95, acts to cluster FasII to the pre- and post-synaptic membranes of type I boutons (Thomas *et al,* 1997). Type I and type II synaptic boutons have been shown to differ at the ultrastructural level. DLG is localized only in type I boutons, while FasII is present in both type I and type II boutons (Thomas *et al,* 1997). It has been suggested that signaling complexes similar to DLG may act to localize FasII in the areas where no DLG exists. PSD-95, the mammalian MAGUK similar to DLG, has been shown to bind the NR2 subunit of NMDA glutamate receptor (Kornau *et al,* 1995). Similarly, VELI has been shown to associate with the NR2 subunit (Jo *et al,* 1999). Therefore, the idea of DLG and dVELI competing for binding to the cell adhesion molecule FasII is consistent with previous results that show their mammalian orthologs compete for binding to other proteins. Finally, the lack of expression of DLG in type II boutons where FasII is expressed leaves available the possibility that dVELI clusters FasII to the membrane in these boutons where DLG is not expressed.

This *Drosophila* model (figure 17) of a tripartite complex working to link cell adhesion to synaptic vesicle fusion and neurotransmitter release will have to be studied further. However, with the similarity of the various proteins to the mammalian proteins, the idea of this complex functioning in a manner similar to the way in which the mammalian complex functions in neurons provides a sound model for investigation.

Figure 17. A *Drosophila* model for the function of the CMG/dVELI/dMINT tripartite complex. Presynaptically, the complex is hypothesized to link cell adhesion to synaptic vesicle fusion and subsequent neurotransmitter release. dVELI may bind the cell adhesion molecule FasII, competing for binding with DLG in type I boutons but potentially binding exclusively in type II boutons, where DLG is absent. CMG may interact with the newly identified neurexin, NRX-1, analogous to the manner in which CASK binds neurexin presynaptically in mammals. Finally, a dMINT interaction with nbA, the Ca^{2+} subunit, links ion channel influx and subsequent synaptic vesicle release via an association with a *Drosophila* synapse associated protein, such as an ortholog of Munc18-1. At the post-synaptic density, the complex may aid in glutamate receptor localization and sorting. Mammalian Mint1/X11alpha binds KIF17, a MAP motor, which moves cargo along microtubules. dMINT may bind a similar MAP, aiding in sorting of the NMDA glutamate receptor, which is subsequently bound by dVELI and localized to the post-synaptic membrane. This process is aided in turn by CMG association with syndecan (Sdc), acting in a cell adhesion manner. CMG has also been shown to bind eag (ether-a-go-go), an ion channel (Dimitratos, 1999). Therefore, CMG may also function in ion channel mechanics. It is interesting to note that many other protein interactions occur at the post-synaptic density, which may be revealed to be important in the CMG/dVELI/dMINT scaffolding complex. DLG is bound to FasII, however it has also been shown to associate with Shaker (Sh) K^+ channels (Tejedor *et al*, 1997) which associates with eag channels, thereby indirectly linking DLG with the scaffolding complex. Furthermore, DLG may associate with a neuroligin (nlg) ortholog, helping in the formation of a cellular junction that helps to anchor the presynaptic complex. Legend: white text - denotes possible presynaptic expression and function, broken arrows: suspected protein interactions, solid arrows: known protein interactions


Synaptic Vesicle association
dVELI
dMINT
CMG
DLG
Pre-synaptic Membrane
FasII
NRX
nbA
Ca2+ Channel
FasII
Post-synaptic Membrane
FasII
nlg
NMDA
Sdc
eag
Sh
FasII
dVELI
DLG
CMG
dMINT
dVELI
DLG
MAP
Microtubules

A Post-synaptic Membrane Model for the Functioning of dVELI

Mammalian VELIs are present at the post-synaptic membrane in association with the NR2 subunit of the NMDA type glutamate receptor (Jo *et al,* 1999). Direct evidence for a tripartite complex of CASK/VELI/Mint1/X11alpha at the post-synaptic side has not been shown, however models that suggest this have been proposed (Jo *et al,* 1999; Setou *et al,* 2000).

Mint1/X11alpha functions in the vesicular transport of the NMDA receptor NR2 subunit via an interaction with the kinesin motor protein KIF17 (Setou *et al,* 2000). KIF17 is a neuronal motor protein that is a member of the kinesin superfamily of proteins which function to move cargo along microtubules (Nakagawa *et al,* 1997). KIF17 terminates in a PDZ binding motif and directly interacts with the first PDZ domain of Mint1/X11alpha (Setou *et al,* 2000). Immunoprecipitation experiments using KIF17 showed the presence of Mint1/X11alpha, CASK, VELI and NR2B in the complex (Setou *et al,* 2000). Although it has been shown that PSD-95 also binds NR2B, PSD-95 was not detected with immunoprecipitation experiments with KIF17 (Setou *et al,* 2000). Therefore, it seems plausible the tripartite complex of CASK/VELI/Mint1/X11alpha functions in the localization of the NMDA receptor NR2 subunit, moving the cargo along the micrtotubules through Mint1/X11alpha interaction with the MAP motor, finally localizing the glutamate receptor to the PSD. This function is similar to the manner in which LIN-7 localizes LET-23 to the basolateral membrane surface in *C. elegans* (Kaech *et al,* 1998).

dVELI is highly concentrated at the PSD in larval NMJs. Therefore, the possibility of dVELI functioning in glutamate receptor localization and sorting, the way in which VELI functions in mammals, was examined. The NR2B subunit of the NMDA glutamate receptor in *Drosophila* , CG14793, has been identified from the *Drosophila*

genome as part of an analysis of the genome for ion channels and synapse proteins (Littleton and Ganetzky, 2000). The NR2B subunit ends in a type II PDZ binding motif, which has been shown to consist of X-Ø - X - Ø - COOH, where Ø is a hydrophobic amino acid residue (Songyang *et al,* 1997). *Drosophila* NR2B carboxy terminus ends in V-P-R-V-COOH, consistent with the type II, since P (proline) and V (valine) are hydrophobic residues. Subsequently, a model can be proposed in which dVELI acts to localize the NR2B subunit to the PSD via binding by its PDZ domain. One can further infer that dVELI exists in a complex with CMG and dMINT at the PSD, acting to sort and localize the glutamate receptor subunit (see figure 17).

dVELI may also have a multi-functional role in the PSD, with localizing the NR2B subunit being just one of its roles. Mammalian VELIs associate with the catenin-cadherin complex not only in the pre-synaptic membrane, but also in the post-synaptic membrane of neurons (Perego *et al,* 2000). In a similar manner, the hypothesized role of dVELI in cell adhesion, perhaps through an association with FasII, may also occur at the PSD, needs to be examined further.

4.4 *Drosophila* VELI Epithelial Function with DER may be Similar to that of *C. elegans* LIN-7

C. elegans LIN-7 associates in a complex with LIN-2 and LIN-10, which acts to localize the EGFR ortholog, LET-23 to the basolateral membrane surface of epithelial cells (Kaech *et al,* 1998). Initial identification of dVELI showed it pulled down the *Drosophila* EGFR, DER, in immunoassays. Given that DER ends in a type I PDZ binding motif (T-R-V-COOH), the possibility of the CMG/dVELI/dMINT complex functioning in epithelial cells as well as neurons was examined.

Tissue *in situ* hybridization of *dVELI* in 3rd instar imaginal disc epithelia detect low levels of expression in imaginal discs. Subsequent antibody staining with dVELI antibody also shows dVELI in imaginal disc epithelia. This supports the notion of both an epithelial and a neuronal function for dVELI. Mammalian VELIs have also been shown to have both a neuronal function and an epithelial function (Butz *et al,* 1998; Jo *et al,* 1999; Irie *et al,* 1999; Perego *et al,* 2000).

Although mammalian VELIs have been detected in epithelial tissues, an association with the mammalian EGFR has not been detected (Borg *et al,* 1998a; Irie *et al,* 1999; Jo *et al,* 1999). Very recent research on dVELI by another research group has focused on the presence of dVELI in imaginal disc epithelia. DER and dVELI (referred to as FLIN-7 by this other group) have been shown to co-localize in imaginal disc epithelia and dVELI binds directly to the C-terminal of DER (DeLorenzo *et al,* 2001). It has also been shown that dMINT and CMG co-localize in the same areas. Interestingly, DLG has also been shown to be co-localized in the same areas as DER, dVELI, dMINT and CMG (DeLorenzo *et al,* 2001). Co-immunoprecipitation studies show DLG is a member of a large complex that also consists of dVELI, dMINT, CMG and DER (DeLorenzo *et al,* 2001).

The association of dVELI with DER is suggestive of an epithelial role similar to that of LIN-7 localizing LET-23 to the basolateral membrane surface of vulval epithelial cells (Simske *et al,* 1996; Kaech *et al,* 1998). The presence of the complex members in imaginal disc epithelia suggests DER may be localized to the membrane surface by the tripartite complex of CMG/dVELI/dMINT. However, as in mammals, the potential for other players in the complex exists, the way in which DLG has been shown to associate (DeLorenzo *et al,* 2001). The identification of other members associated with this tripartite complex is the focus of recent research.

4.5 The *Drosophila* Genome Contains Proteins that may Interact with dVELI and Compete with Binding to dVELI with CMG/dMINT

Because of the multi-functional role for mammalian VELIs, it is suspected VELIs may also have a function outside of binding to CASK. Recent studies have suggested that the L27 domain in VELIs may have binding partners other than CASK (Butz *et al,* 1998). Newly identified proteins, known as PALS (**p**roteins **a**ssociated with **L**IN-7), have been found to associate with VELI via the L27 domain (Kamberov *et al,* 2000). PALS1 and PALS2 are MAGUK family members found in both neurons and epithelia (Kamberov *et al,* 2000).

Multiple binding partners for VELI may assist in the different targeting of VELI for its different functions. It has been suggested that VELI bound to CASK may be targeted differently than VELI bound by PALS proteins (Kamberov *et al,* 2000). Regardless of the function, the existence of multiple binding partners for VELI has been shown. Research into the exact function behind differing binding partners and its affects on targeting of VELI should further reveal the multi-functional role of VELI.

The conserved amino acid identity of CMG, dVELI and dMINT to that of the mammalian orthologs implies other potential scaffolding proteins within this complex may be conserved from mammals to *Drosophila*. The *Drosophila* genome was assessed for the presence of potential PALS1 and PALS2 proteins. dPALS1 (CG1617) and dPALS2 (CG9326) were identified from the *Drosophila* genome and show a 49% amino acid identity and a 39% amino acid identity, respectively. This conserved identity of the PALS proteins suggests they may function in a similar manner in *Drosophila*, competing for binding to dVELI and assisting in the multiple roles of dVELI.

4.6 Direction of Future Research

The initial identification and characterization of dVELI leaves open a number of questions as to its function in the *Drosophila* CNS and epithelia. One ongoing objective of this project is the generation of a *dveli* mutant. It is hoped that with a mutant, the requirement for dVELI in synaptic differentiation and signaling will become more evident. If dVELI indeed acts to localize the NR2B subunit of the NMDA glutamate receptor, loss of *dveli* function should result in a loss of or delay in the formation of the glutamate receptor clusters. Such structural defects can be detected by electron microscopy. Furthermore, the role of dVELI in cell adhesion can be examined in greater detail. It is suspected that in a *dveli* mutant, FasII will fail to cluster properly if dVELI indeed play a role this process. Therefore, *dveli* mutants labeled with FasII antibody would display a decreased level of FasII staining, evident under microscopic examination.

Further dVELI function can be observed by mis-expression or over-expression of dVELI using a UAS-dVELI construct (described in Results). Recent transformants for a UAS-dVELI transgenic fly line will be useful in determining function of dVELI in the pre-synaptic membrane (through use of neuronal GAL4 lines) and the post-synaptic membrane (through muscle GAL4 lines), giving an overall view of its effects on the NMJ.

The scaffolding proteins associated with dVELI need to be examined further for direct relationships to dVELI. In particular, with CMG expression patterns already known, focus on dMINT is essential to establish a tripartite complex in *Drosophila*. RNA expression patterns must be examined further, as well as antibody distribution following generation of a dMINT polyclonal antibody. Ideally, PALS proteins will also need to be examined further in their relationship to dVELI, including both transcript and

protein distribution. In vitro binding assays can be employed to show direct interactions between dVELI and the above mentioned proteins.

It is hoped that with further research on dVELI, this protein will emerge as one of the main components linking cell adhesion to signal transduction and synaptic transmission. This conserved scaffolding complex is essential for proper targeting and function of receptors and ion channels in mammals and worms and it is suspected it serves a similar purpose in *Drosophila*. It is hoped the foundation has been laid to continue research in this area and to provide further insight into the function of this complex and subsequently the organization and functional assembly of synapses.

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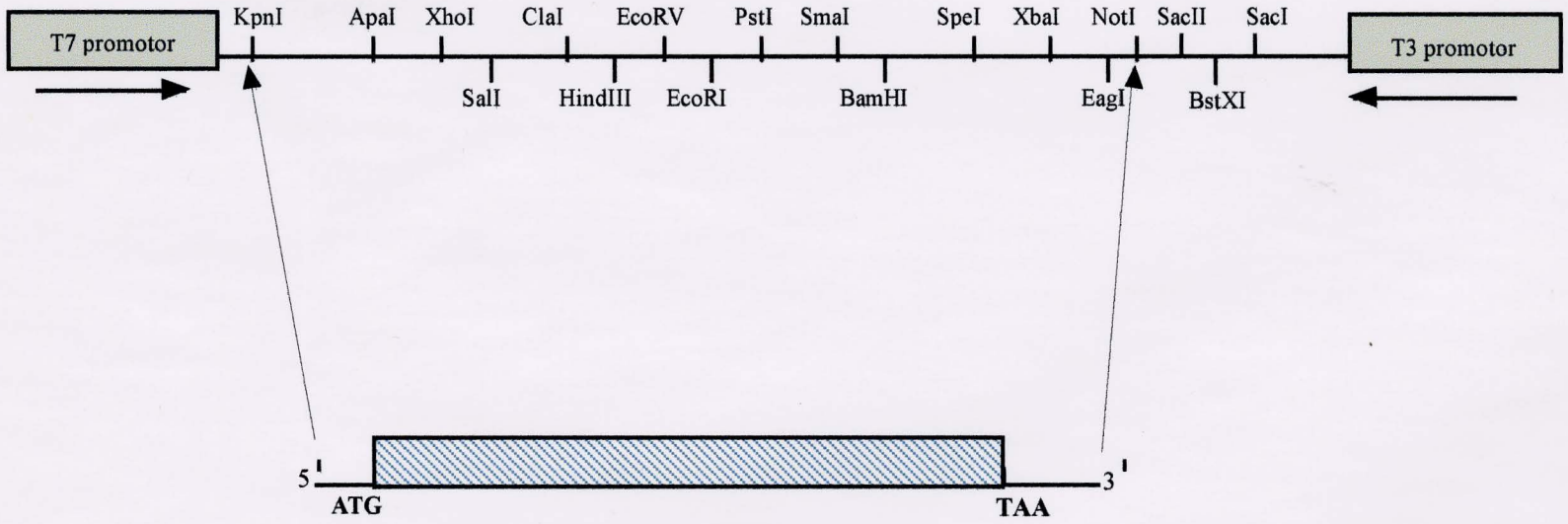
Appendix 1. pBluescript KSII multiple cloning site showing the location of *dveli* cDNA insert. *dveli* cDNA was cloned into the KpnI and NotI sites of pBSKSII. The presence of the T7 and T3 promotors allow for sequencing of the insert as well as generation of sense and anti-sense RNA probes for the purposes of *in situ* hybridization. The total insert length including upstream and downstream sequences is 853 base pairs. Construct was generated by Christian Smith, Hospital for Sick Kids, Toronto, Ontario.

pBluescript KSII (+/-) Multiple Cloning Site



dveli cDNA insert is 853 base pairs

Appendix 2. Olignonucleotide primers used in the PCR analysis of *dveli.* Table lists all sets of primers used, the sequence of the oligonucleotides, amplification region within or near the gene and the length of the amplified product. Shown in bold within the sequences are restrictions sites that were either designed into the sequence or were present originally. All primers were generated through MOBIX at McMaster University.

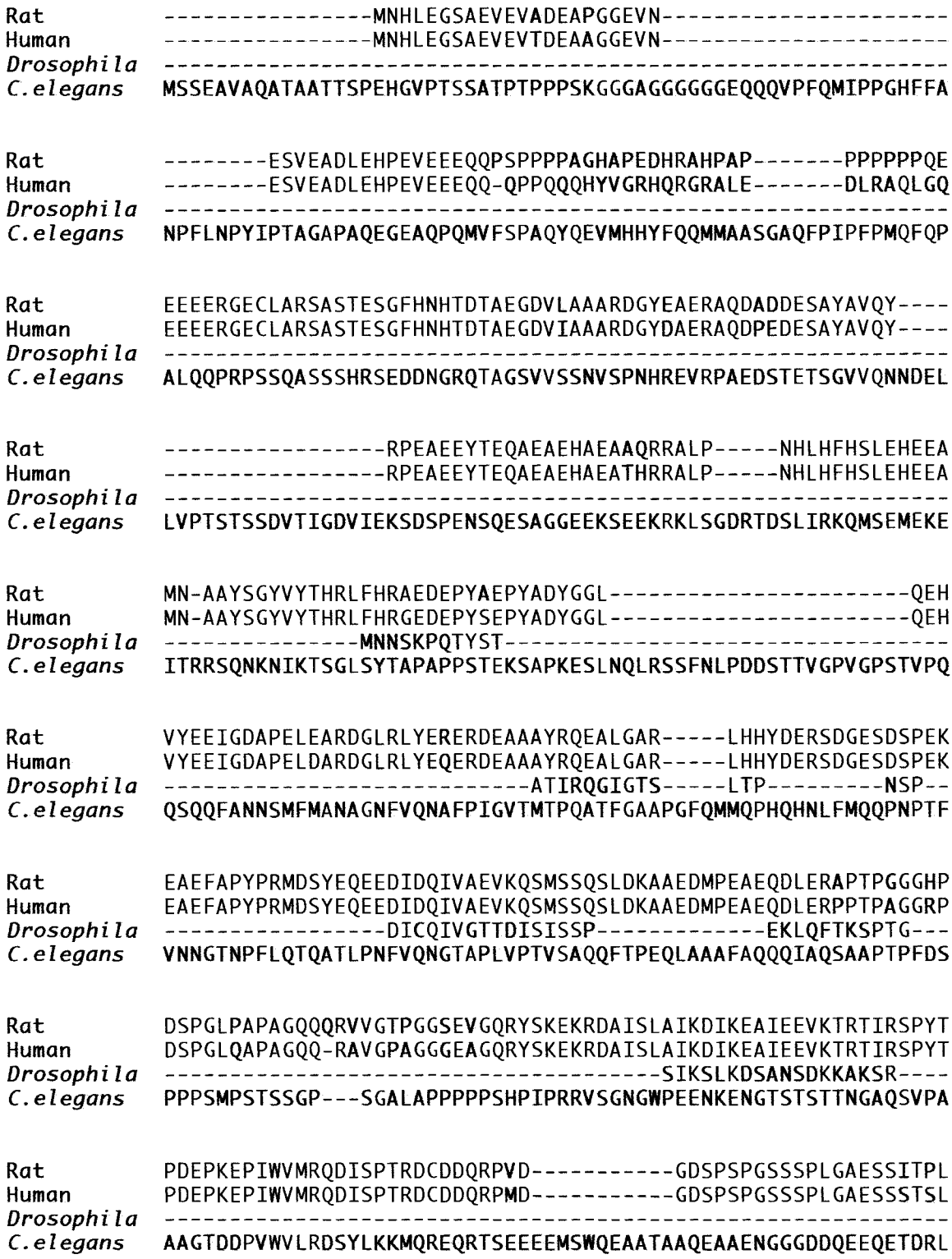
Primer Name (Id#)	Primer Sequence	Amplification Region	Length of Product
EcoRI F (AB19576)	5' CAG CTG CTC **GAA TTC** TAT TTC AGG 3' (EcoRI site)	Forward primer begins 65 bp upstream of the start codon.	Primers were used to amplify full length genomic *dveli*. Product length is 957 bp.
NotI R (AB19575)	5' GGC ACA AGT T**GC GGC CGC** TGT GC 3' (NotI site)	Reverse primer begins 24 bp downstream of the stop codon.	
EcoRI F (AB19576)	5' CAG CTG CTC **GAA TTC** TAT TTC AGG 3' (EcoRI site)	Forward primer begins 65 bp upstream of the start codon.	Primers were used for RT-PCR. cDNA product length is 516 bp. Genomic DNA product is 796 bp.
Lin-7R-RTPCR (AB23852)	5' CTC CTT CAA CAG ACA CAC CAT TCA CAG 3'	Reverse primer begins at bp 451 of *dveli* cDNA.	
EcoRI F (AB19576)	5' CAG CTG CTC **GAA TTC** TAT TTC AGG 3' (EcoRI site)	Forward primer begins 65 bp upstream of the start codon.	Primers were used to sequence potential mutants. Genomic DNA product length is 996 bp.
L7mut R (AB23740)	5' GGT GCA AAA TCA GCA TGGTCT AAT GG 3'	Reverse primer begins 63 bp downstream of the stop codon.	
geLin-7F (AB20289)	5' GAG GGC ATC CGC CAC TGC CAA GGC 3'	Forward primer begins amplification at bp 157 of *dveli* cDNA	Primers were used to confirm sequence of UAS-*dveli* construct. *dveli* complete coding region was amplified along with bits of the UAS construct itself.
geLin-7R (AB20290)	5' GCG TGC CCC TCG CTG GCT GCG 3'	Reverse primer begins amplification at bp 266 of *dveli* cDNA	
Lin10 F (AB23272)	5' CTG **TCT AGA** TTG TGG GCA CGA CCG AC 3' (XbaI site)	Forward primer begins amplification at bp 87 of *dMint* cDNA.	Primers were used for RT-PCR. cDNA product length is 809 bp. Genomic DNA product is 1326 bp.
Lin10 R (AB23273)	5' GGT **GAA TTC** GCC TTC GGC ACA ACT AC 3' (EcoRI site)	Reverse primer begins at bp 896 of *dMint* cDNA.	

Appendix 3. Alignment of LIN-7/VELIs proteins of various species. Sequences are shown in single letter amino acid codes. L27 domain is underlined in a blue line while the PDZ domain is underlined in a red line. Amino acid residues identical for at least four of the eight aligned sequences are coloured either blue or purple - blue if the aligned sequences includes *Drosophila* and purple if the aligned sequences do not include the *Drosophila* protein. Accession numbers = *Drosophila* (AE003750), *C.elegans* (U78092), *Rattus norvegicus* LIN-7A (AC78072), *Rattus norvegicus* LIN-7Ba (AC78073), *Rattus norvegicus* LIN-7Bb (AC78074), *Rattus norvegicus* LIN-7C (AC78075), *Homo sapiens* VELI-1 (AD48500) and *Homo sapiens* LIN-7B (NP071448).

```
Drosophila  ------------------------------------------------------------
C.elegans   MGLKGFTGSFQQIRGLLRPPKNLPFRGIFRKDGEVVRKDDLLVNQFKMNYHPGLNVYYEN
RatLIN7A    ------------------------------------------------------------
HumanLIN7B  ------------------------------------------------------------
RatLIN7C    ------------------------------------------------------------
RatLIN7BB   ------------------------------------------------------------
HumanVELI1  ------------------------------------------------------------
RatLIN7Ba   ------------------------------------------------------------

Drosophila  ----------------------------------------------MADNAEPLTLSRD
C.elegans   DRGERLLRAHCDGIVRISQEKCDPDYEIEEMKGYEYRKDVDLYKMTFNMDNPDGPNLERD
RatLIN7A    ----------------------------------------------MAALVEPLGLERD
HumanLINB   ----------------------------------------------MAALVEPLGLERD
RatLIN7C    ----------------------------------------------MAALGEPVRLERD
RatLIN7BB   ----------------------------------------------MATLTVVQPLTLDRD
HumanVELI1  -------------------------------MLKPSVTSAPTADMATLTVVQPLTLDRD
RatLIN7Ba   ----------------------------------------------MATLTVVQPLTLDRD

Drosophila  VKRSIELLEKLQASGDFPTTKLAALQKVLNSDFMTSVREVYEHVYETVDIQGSHDVRASA
C.elegans   VQRILELMEHVQKTGEVNNAKLASLQQVLQSEFFGAVREVYETVYESIDADTTPEIKAAA
RatLIN7A    VSRAVELLERLQRSGELPPQKLQALQRVLQSRFCSAIREVYEQLYDTLDITGSAEVRAHA
HumanLIN7B  VSRAVELLERLQRSGELPPQKLQALQRVLQSRFCSAIREVYEQLYDTLDITGSAEIRAHA
RatLIN7C    ICRAIELLEKLQRSGEVPPQKLQALQRVLQSEFCNAVREVYEHVYETVDISSSPEVRANA
RatLIN7BB   VARAIELLEKPQESGEVPVHKLQSLKKVLQSEFCTAIREVYQYMHETITVNGCPEFRARA
HumanVELI1  VARAIELLEKLQESGEVPVHKLQSLKKVLQSEFCTAIREVYQYMHETITVNGCPEFRARA
RatLIN7Ba   VARAIELLEKLQESGEVPVHKLQSLKKVLQSEFCTAIREVYQYMHETITVNGCPEFRARA

Drosophila  TAKATVAAFAASEGHAHPRVVELPKTEEGLGFNVMGGKEQNSPIYISRIIPGGVADRHGG
C.elegans   TAKATVAAFAAAEGHAHPRIVELPKTDQGLGFNVMGGKEQNSPIYISRIIPGGVADRHGG
RatLIN7A    TAKATVAAFTASEGHAHPRVVELPKTDEGLGFNIMGGKEQNSPIYISRVIPGGVADRHGG
HumanLIN7B  TAKATVAAFTASEGHAHPRVVELPKTDEGLGFNIMGGKEQNSPIYISRVIPGGVADRHGG
RatLIN7C    TAKATVAAFAASEGHSHPRVVELPKTEEGLGFNIMGGKEQNSPIYISRIIPGGIADRHGG
RatLIN7BB   TAKATVAAFAASEGHSHPRVVELPKTDEGLGFNVMGGKEQNSPIYISRIIPGGVAERHGG
HumanVELI1  TAKATVAAFAASEGHSHPRVVELPKTDEGLGFNVMGGKEQNSPIYISRIIPGGVAERHGG
RatLIN7Ba   TAKATVAAFAASEGHSHPRVVELPKTDEGLGFNVMGGKEQNSPIYISRIIPGGVAERHGG

Drosophila  LKRGDQLLSVNGVSVEGENHEKAVELLKQAVGSVKLVVRYTPKVLEEMEMRFDKQRNTRR
C.elegans   LKRGDQLIAVNGN-VEAECHEKAVDLLKSAVGSVKLVIRYMPKLLDEMERRFERQRIP--
RatLIN7A    LKRGDQLLSVNGVSVEGEHHEKAVELLKAAQGSVKLVVRYTPRVLEEMEARFEKMRSARR
HumanLIN7B  LKRGDQLLSVNGVSVEGEQHEKAVELLKAAQGSVKLVVRYTPRVLEEMEARFEKMRSARR
RatLIN7C    LKRGDQLLSVNGVSVEGEHHEKAVELLKAAQGKVKLVVRYTPKVLEEMESRFEKMRSAKR
RatLIN7BB   LKRGDQLLSVNGVSVEGEHHEKAVELLKAAKDSVKLVVRYTPKVLEEMEARFEKLRTARR
HumanVELI1  LKRGDQLLSVNGVSVEGEHHEKAVELLKAAKDSVKLVVRYTPKVLEEMEARFEKLRTARR
RatLIN7Ba   LKRGDQLLSVNGVALEEKLAGQSSNSHKFGNPCSGIPAHRK----------------RK
```

```
Drosophila RQ---------------------- 195
C.elegans  ----------------------
RatLIN7A   RQQHHSYSSLESRG---------- 207
HumanLIN7B RQQHQSYSSLESRG---------- 207
RatLIN7C   RQQT-------------------- 197
RatLIN7BB  RQQQQLLIQQQQQQQQQ-PQQNHMS 219
HumanVELI1 RQQQQLLIQQQQQQQQQQTQQNHMS 233
RatLIN7Ba  RKYQ-------------------- 182
```

Appendix 4. Amino acid alignment of LIN-10/MINT proteins of various species. Sequences are shown in single letter amino acid codes. PTB domain is underlined in a red line, 1st PDZ domain is underlined in a dark blue line and the 2nd PDZ domain is underlined in a light blue line. Amino acid residues identical in 2 or 4 of the aligned sequences are coloured blue or purple - blue if the aligned sequence includes the *Drosophila* protein and purple if it does not. Sequence accession numbers are as follows: *Drosophila* (AE003506), *C.elegans* (AB40208), *Rattus norvegicus* (O35430) and *Homo sapiens* (Q02410).


Rat ----------MNHLEGSAEVEVADEAPGGEVN----------
Human ----------MNHLEGSAEVEVTDEAAGGEVN----------
Drosophila ----------
C.elegans MSSEAVAQATAATTSPEHGVPTSSATPTPPPSKGGGAGGGGGGEQQQVPFQMIPPGHFFA
Rat --------ESVEADLEHPEVEEEQQPSPPPPAGHAPEDHRAHPAP-------PPPPPPQE
Human --------ESVEADLEHPEVEEEQQ-QPPQQQHYVGRHQRGRALE-------DLRAQLGQ
Drosophila ----------
C.elegans NPFLNPYIPTAGAPAQEGEAQPQMVFSPAQYQEVMHHYFQQMMAASGAQFPIPFPMQFQP
Rat EEEERGECLARSASTESGFHNHTDTAEGDVLAAARDGYEAERAQDADDESAYAVQY----
Human EEEERGECLARSASTESGFHNHTDTAEGDVIAAARDGYDAERAQDPEDESAYAVQY----
Drosophila ----------
C.elegans ALQQPRPSSQASSSHRSEDDNGRQTAGSVVSSNVSPNHREVRPAEDSTETSGVVQNNDEL
Rat ----------RPEAEEYTEQAEAEHAEAAQRRALP-----NHLHFHSLEHEEA
Human ----------RPEAEEYTEQAEAEHAEATHRRALP-----NHLHFHSLEHEEA
Drosophila ----------
C.elegans LVPTSTSSDVTIGDVIEKSDSPENSQESAGGEEKSEEKRKLSGDRTDSLIRKQMSEMEKE
Rat MN-AAYSGYVYTHRLFHRAEDEPYAEPYADYGGL----------QEH
Human MN-AAYSGYVYTHRLFHRGEDEPYSEPYADYGGL----------QEH
Drosophila ----------MNNSKPQTYST----------
C.elegans ITRRSQNKNIKTSGLSYTAPAPPSTEKSAPKESLNQLRSSFNLPDDSTTVGPVGPSTVPQ
Rat VYEEIGDAPELEARDGLRLYERERDEAAAYRQEALGAR-----LHHYDERSDGESDSPEK
Human VYEEIGDAPELDARDGLRLYEQERDEAAAYRQEALGAR-----LHHYDERSDGESDSPEK
Drosophila ----------ATIRQGIGTS-----LTP---------NSP--
C.elegans QSQQFANNSMFMANAGNFVQNAFPIGVTMTPQATFGAAPGFQMMQPHQHNLFMQQPNPTF
Rat EAEFAPYPRMDSYEQEEDIDQIVAEVKQSMSSQSLDKAAEDMPEAEQDLERAPTPGGGHP
Human EAEFAPYPRMDSYEQEEDIDQIVAEVKQSMSSQSLDKAAEDMPEAEQDLERPPTPAGGRP
Drosophila ----------DICQIVGTTDISISSP----------EKLQFTKSPTG---
C.elegans VNNGTNPFLQTQATLPNFVQNGTAPLVPTVSAQQFTPEQLAAAFAQQQIAQSAAPTPFDS
Rat DSPGLPAPAGQQQRVVGTPGGSEVGQRYSKEKRDAISLAIKDIKEAIEEVKTRTIRSPYT
Human DSPGLQAPAGQQ-RAVGPAGGGEAGQRYSKEKRDAISLAIKDIKEAIEEVKTRTIRSPYT
Drosophila ----------SIKSLKDSANSDKKAKSR----
C.elegans PPPSMPSTSSGP---SGALAPPPPPSHPIPRRVSGNGWPEENKENGTSTSTTNGAQSVPA
Rat PDEPKEPIWVMRQDISPTRDCDDQRPVD----------GDSPSPGSSSPLGAESSITPL
Human PDEPKEPIWVMRQDISPTRDCDDQRPMD----------GDSPSPGSSSPLGAESSSTSL
Drosophila ----------
C.elegans AAGTDDPVWVLRDSYLKKMQREQRTSEEEEMSWQEAATAAQEAAENGGGDDQEEQETDRL

```
Rat          HPGDPTEASTNKESRKSLASFPTYVEVPGPCDPEDLIDGIIFAANYLGSTQLLSDKTPSK
Human        HPSDPVEVPINKESRKSLASFPTYVEVPGPCDPEDLIDGIIFAANYLGSTQLLSDKTPSK
Drosophila   ----------NKE--------------GLLEPKVLIEGVLFRARYLGSTQLVCEGQPTK
C.elegans    LNGGTTGASTKGAERRGSVDKKKNSKETMVHEPAVLIEGVLFRARYLGSTQMLCESRGSK

Rat          NVRMMQAQEAVSRIKTAQKLAKSRKKAPEGESQPMTEVDLFISTQRIKVLNADTQ-----
Human        NVRMMQAQEAVSRIKMAQKLAKSRKKAPEGESQPMTEVDLFILTQRIKVLNADTQ-----
Drosophila   STRMMQAEEAVSRIK----------APEGESQPSTEVDLFISTEKIMVLNTDLK-----
C.elegans    AARMAQAQEAVARVK----------APEGDVQPSTEIDLFISTEKIMVLNTDLQRISDT

Rat          ----EPMMDHPLRTISYIADIGNIVVLMARRRMPR----------------SNSQENV
Human        ----ETMMDHPLRTISYIADIGNIVVLMARRRIPR----------------SNSQENV
Drosophila   ----EIMMDHALRTISYIADIGDLVVLMARRRFVPNSVVDPSITSPLGDVPTPGIGEEES
C.elegans    DVRQDILMDHALRTISYIADIGDLVVLMARRMSTS----------------HSDESCS

Rat          EASHPSQDAKRQYKMICHVFESEDAQLIAQSIGQAFSVAYQEFLRANGINPEDLS-QKEY
Human        EASHPSQDGKRQYKMICHVFESEDAQLIAQSIGQAFSVAYQEFLRANGINPEDLS-QKEY
Drosophila   PPKEPLSKHNRTPKMICHVFESDEAQFIAQSIGQAFQVAYMEFLKANGIENESLAKEMDY
C.elegans    DGDSSGGGVRKTPKVICHVFESDEASFIAQSIGQAFQVAYVEFLRANGIDDPSYLRQIDY

Rat          SDLLNTQDMYNDDLIHFSKSENCKDVFIEKQKGEILGVVIVESGWGSILPTVIIANMMHG
Human        SDLLNTQDMYNDDLIHFSKSENCKDVFIEKQKGEILGVVIVESGWGSILPTVIIANMMHG
Drosophila   QEVLNSQEIFGDELEIFAKKELQKEVVVPKAKGEILGVVIVESGWGSMLPTVVIANLMSS
C.elegans    QEVLNSQELLGDELEMFAKKETQKEVVVPKKAGEPLGIVVVESGWGSMLPTVVLAHMNPV

Rat          GPAEKSGKLNIGDQIMSINGTSLVGLPLSTCQSIIKGLKNQSRVKLNIVRCPPVTTVLIR
Human        GPAEKSGKLNIGDQIMSINGTSLVGLPLSTCQSIIKGLENQSRVKLNIVRCPPVTTVLIR
Drosophila   GAAARCGQLNIGDQLIAINGMSLVGLPLSTCQSYIRNAKNQTAVKFTVVPCPPVVEVKIL
C.elegans    GPAAHSNKLNIGDQIININGISLVGLPLSAAQTQIKNMKTATAVRMTVVSTPPVVEVRIR

Rat          RPDLRYQLGFSVQNGIICSLMRGGIAERGGVRVGHRIIEINGQSVVATPHEKIVHILSNA
Human        RPDLRYQLGFSVQNGIICSLMRGGIAERGGVRVGHRIIEINGQSVVATPHEKIVHILSNA
Drosophila   RPKALFQLGFSVQNGVICSLLRGGIAERGGVRVGHRIIEINNQSVVAVPHDTIVKLLSSS
C.elegans    RPDTKYQLGFSVQNGVICSLLRGGIAERGGIRVGHRIIEINGTSVVAVAHDRIVNMLATA

Rat          VGEIHMKTMPAAMYRLLTAQEQPVYI 839
Human        VGEIHMKTMPAAMYRLLTAQEQPVYI 837
Drosophila   VGEILMKTMPTSMFRLLTGQETPIYI 473
C.elegans    VGEIHMKTMPTSMFRLLTGQEQPQYI 954
```

Appendix 5. Amino acid alignment of *mus musculus* PALS1 to its best match *Drosophila* protein, CG1617. PALS1 was compared to the *Drosophila* genome to find the best match in amino acid identity and similarity. CG1617 displays a 49% amino acid identity and a 63% similarity to PALS1. Both proteins contain a PDZ domain, an SH3 domain, guanylate kinase domain and the L27 binding domain (not indicated on the alignment). Alignment shown is the best fit identity and similarity wise and does not necessarily line up the domains. Amino acids identical between PALS1 and CG1617 are highlighted in blue. Accession number: PALS1 (AF199008), CG1617 (FBgn0030024).

```
PALS1    MTTSYMNGHVTEESDSGIKNLD---LASPEEYPKHREMAVDCPGDLGTRMMPVRRSAQLE 57
CG1617   MRIKCATQESTPREQLRRRREEEERIAQQNEFLRNSLRGSRKLKALQDTATPGKAVAQQQ 60

PALS1    R-------IRQQQEDMRRRREEEGKKQELD----------LNSSMRLKKLAQIPPKTGI 99
CG1617   QQATLATQVVGVENEAYLPDEDQPQAEQIDGYGELIAALTRLQNQLSKSGLSTLAGRVSA 120

PALS1    DNPIFDTEEGIVLESPHYAVNILDVEDLFSSLKH----IQHTLVDSQSQEDISLLLQL-- 153
CG1617   AHSVLASASVAHVLAARTAVLQRRRSRVSGPLHHSSLGLQKDIVELLTQSNTAAAIELGN 180

PALS1    -VQNRDFQNAFKIHNAVTVHMSKASPPFP----------LIANVQDLVQEVQTVLKP 199
CG1617   LLTSHEMEGLLLAHDRIANHTDGTPSPTPTPTPAIGAATGSTLSSPVAGPKRNLGMVVPP 240

PALS1    -VHQKEGQELTALLNAPHIQALLLAHDKVAEQEMQLEPITDERVYESIGHYGGETVKIVR 258
CG1617   PVVPPPLAQRGAMPLPRGESPPPVPMPPLATMPMSMPVNLPMSACFGTLNDQNDNIRIIQ 300

PALS1    IEKARDIPLGATVRNEMDSVIISRIVKGGAAEKSGLLHEGDEVLEINGIEIRGKDVNEVF 318
CG1617   IEKS-TEPLGATVRNEGEAVVIGRIVRGGAAEKSGLLHEGDEILEVNGQELRGKTVNEVC 359

PALS1    DLLSDMHGTLTFVLIPSQQ----------IKPPPAKETVIHVKAHFDYDPSDD 361
CG1617   ALLGAMQGTLTFLIVPAGSPPSVGVMGGTTGSQLAGLGGAHRDTAVLHVRAHFDYDPEDD 419

PALS1    PYVPCRELGLSFQKGDILHVISQEDPNWWQAYREGDEDNQPLAGLVPGKSFQQQREAMKQ 421
CG1617   LYIPCRELGISFQKGDVLHVISREDPNWWQAYREGEED-QTLAGLIPSQSFQHQRETMKL 478

PALS1    TIEEDKEPEKS-GK---------LWCAKKNKKKRKKV-------LYNANKNDDYDNEEIL 464
CG1617   AIAEEAGLARSRGKDGSGSKGATLLCARKGRKKKKKASSEAGYPLYATTAPDETDPEEIL 538

PALS1    TYEEMSLYHQPANRKRPIILIGPQNCGQNELRQRLMNKEKDRFASAVP---------- 512
CG1617   TYEEVALYYPRATHKRPIVLIGPPNIGRHELRQRLMADS-ERFSAAVPLFYLLEERLKPA 597

PALS1    -------HTTRNRRDHEVAGRDYHFVSRQAFEADIAAGKFIEHGEFEKNLYGTSIDSVRQ 565
CG1617   KIKAQVKDTSRARREGEVPGVDYHFITRQAFEADILARRFVEHGEYEKAYYGTSLEAIRT 657

PALS1    VINSGKICLLSLRTQSLKTLRNSDLKPYIIFIAPPSQERLRALLAKEGKNPKP------- 618
CG1617   VVASGKICVLNLHPQSLKLLRASDLKPYVVLVAPPSLDKLRQKKLRNGEPFKVRFQHSNL 717

PALS1    --------------EELREIIEKTREMEQNNGHYFDTAIVNSDLDKAYQELLRLINKLDT 664
CG1617   SNKTQSKSNLFKQEEELKDIIATARDMEARWGHLFDMIIINNDTERAYHQLLAEINSLER 777

PALS1    EPQWVPSTWLR------ 675
CG1617   EPQWVPAQWVHNNRDES 794
```

Appendix 6. Amino acid alignment of *mus musculus* PALS2 alpha to the first of its best fit matches in the *Drosophila* genome, CG9326. PALS2 alpha was compared to the *Drosophila* genome to search for the possible orthologs. Two subsequent matches were found. The first of these proteins, CG9326, is shown aligned to PALS2 to show the best fit based on amino acid identity and similarity. CG9326 shows a 39% identity and a 58% similarity to PALS2. As with PALS2, CG9326 contains a PDZ domain, an SH3 domain, a guanylate kinase domain and an L27 binding domain (not indicated on figure). Domains are not necessarily aligned. Amino acid residues identical between PALS2 and CG9326 are shown in blue. Accession numbers for PALS2 and CG9326 are AF199009 and FBgn0032885, respectively.

```
PALS2a  MQQVLENLTELPSSTGAEEIDLIFLKGIMENPIVKSLAKAHERLEDSKLEAVSDNNLELV 60
CG9326  MTYVHLNPTEP------QPVPLFLPAHLNNKPICDDIIRKFS--PSRRLESR-----ELA 47

PALS2a  NEILEDITPLISVDENVAELVGILKEPHFQSLLEAHDIVASKCYDSPPSSPEMNIPSLNN 120
CG9326  KLLAQ---PHFRALLRAHDEIGALYEQRLKAAGGSTSQLEIASQ-RQTGGYLFTEDVLNT 103

PALS2a  QLPVDAIRILGIHKKAGEPLGVTFRVEN-NDLVIARILHGGMIDRQGLLHVGDIIKEVNG 179
CG9326  KMPVETIKMVGLRRDPSKPLGLTVELDEFKQLVVARILAGGVIDKQSMLHVGDVILEVNG 163

PALS2a  HEVGNNPKELQELLKNISGSVTLKILP-------SYRDTITPQQV--------------- 217
CG9326  TPVR-TPDELQVEVSRAKENLTLKIGPNVDEEIKSGRYTVSGGQVKQNGIASLETGKKLT 222

PALS2a  -FVKCHFDYNPFNDNLIPCKEAGLKFSKGEILQIVNREDPNWWQASHVK-EGGSAGLIPS 275
CG9326  CYMRALFTYNPSEDSLLPCRDIGLPFKSGDILQIINVKDPNWWQAKNITAESDKIGLIPS 282

PALS2a  QFLEEKRKAFVR--RDWDNSGPFCGTISNKKKKKMMYLTTRNAEFDRHEIQIYEEVAKMP 333
CG9326  QELEERRKAFVAPEADYVHKIGICGTRISKRKRKTMYRSVANCEFDKAELLLYEEVTRMP 342

PALS2a  PFQRKTLVLIGAQGVGRRSLKNRFIVLNPARFGTTVPFTSRKPREDEKDGQAYKFVSRSE 393
CG9326  PFRRKTLVLIGVSGVGRRTLKNRLINSDVDKFGAVIPHTSRPKRALEENGSSYWFMDREE 402

PALS2a  MEADIKAGKYLEHGEYEGNLYGTKIDSILEVVQTGRTCILDVNPQALKVLRTS-EFMPYV 452
CG9326  MEEAVRNNEFLEYGEHNGNLYGTHLQSIKDVINSGRMCILDCAPNALKILHNSQELMPFV 462

PALS2a  VFIAAPELETLRAMHK----------------AVVDAGITTKLLT-----------DSDL 485
CG9326  IFVAAPGMEQLKTIYADRRATGSNRNLSATLVALLDLCLNTQTLLNTFAFYDFSEQDDDL 522

PALS2a  KKTVDESARIQRAYNHYFDLIIVNDNLDKAFEKLQTAIEKLRMEPQWVPISWVY 539
CG9326  VATVEESSFVQRKYEKYFDMVIVNEDFDETFRQVVETLDQMSHEEQWVPVNWIY 576
```

Appendix 7. PALS2 alpha aligned to the second of its best fit matches from the *Drosophila* genome, CG13219. PALS2 alpha was compared to the *Drosophila* genome to locate the orthologs to the PALS proteins. PALS2 revealed two potential matches. CG13219 is 39% identical and 57% similar to PALS2 based on amino acid sequence. However, PALS2 and CG13219 are slightly different in that CG13219 lacks a PDZ domain. Sequences are aligned such that the best fit match is achieved, and not by location on domains. Identical amino acids between PALS2 and CG13219 are shown in blue. Accession numbers are: PALS2 (AF199009), CG13219 (FBgn0033585).

```
PALS2a  MQQVLENLTELPSSTGAEEIDLIFLKGIMENPIVKSLAKAHERLEDSKLEAVSDNNLELV 60
CG13219 ------------------------------------------------------------

PALS2a  NEILEDITPLISVDENVAELVGILKEPHFQSLLEAHDIVASKCYDSPPSSPEMNIPSLNN 120
CG13219 ------------------------------------------------------------

PALS2a  QLPVDAIRILGIHKKAGEPLGVTFRVENNDLVIARILHGGMIDRQGLLHVGDIIKEVNGH 180
CG13219 ----------------------------------MHGGAADRSGLIHVGDEVIEVNNI 24

PALS2a  EVG-NNPKELQELLKNISGSVTLKILPS-YRDTITPQQVFVKCHFDYNPFNDNLIPCKEA 238
CG13219 NVEGKTPGDVLTILQNSEGTITFKLVPADNKGAQRESKVRVRAHFDYNPDVDPYIPCKEA 84

PALS2a  GLKFSKGEILQIVNREDPNWWQASHVKEGGS-AGLIPSQFLEEKRKAFVRR-----DWDN 292
CG13219 GLAFQRGDVLHIVAQDDAYWWQARKEHERSARAGLIPSRALQERRILHDRTQKNGTDLDS 144

PALS2a  SGPFCGTISNK-------------------KKKKMMYLTTRNAEFDRHEIQIYEEVAKMP 333
CG13219 KPGSCASLCTTPPGSPRLPASSSTSSCRQPKTKKIMYDLTENDDFDREQIATYEEVAKLY 204

PALS2a  PFQR--KTLVLIGAQGVGRRSLKNRFIVLNPARFGTTVPFTSRKPREDEKDGQAYKFVSR 391
CG13219 PRPGVFRPIVLIGAPGVGRNELRRRLIARDPEKFRSPVPYTTRPMRTGEVAGREYIFVAR 264

PALS2a  SEMEADIKAGKYLEHGEYEGNLYGTKIDSILEVVQTGRTCILDVNPQALKVLRTSEFMPY 451
CG13219 EKMDADIEAGKFVEHGEYKGHLYGTSAESVKSIVNAGCVCVLSPHYQAIKTLRTAQLKPF 324

PALS2a  VVFIAAPELETLRAMHKAVVDAG----ITTKLLTDSDLKKTVDESARIQRAYNHYFDLII 507
CG13219 LIHVKPPELDILKATRTEARAKSTFDEANARSFTDEEFEDMIKSAERIDFLYGHFFDVEL 384

PALS2a  VNDNLDKAFEKLQTAIEKLRMEPQWVPISWVY 539
CG13219 VNGELVNAFEQLVQNVQRLENEPVWAPSMWVQ 416
```

Appendix 8. Summary of phenotypic, genotypic and sequence analysis data for several uncharacterized lethal lines in the 3R96B region of the *Drosophila* genome. Seven uncharacterized lethal lines were tested through various ways to determine if they were possibly mutant in the *dveli* gene. The lethal lines, L(3)06Ba-g, were tested by complementation to a known deficiency in the area, Df(3R)96B/TM3, to see if they fell within the 96B region. Bloomington stock centre, where the flies were obtained from, indicate they all fall within the 96B area. However, L(3)96Bc mapped outside of the defiency based on complementation tests. Other factors looked at included viability, embryonic phenotype based on fasII staining and sequence analysis of *dveli* to determine if any base pair changes were present (refer to results section 3.7). Note: fly crosses performed before isolating embryos for the sequence analysis is also shown.

	L(3)96Ba	L(3)96Bb	L(3)96Bc	L(3)96Bd	L(3)96Be	L(3)96Bf	L(3)96Bg
Location (Bloomington)	3R96B	3R96B	3R96B	3R96B	3R96B	3R96B	3R96B
Within Df96B?	Yes	Yes	No	Yes	Yes	Yes	Yes
Balancer Lost?	No	Yes	No	Yes	No	Yes	No
Viability	Embryonic lethal	-	Embryonic lethal	-	Embryonic lethal	-	Embryonic lethal
Embryonic phenotype (fasII)	wild type	wild type	wild type	wild type	wild type	wild type	wild type
Sequence Analysis*	Sequencing inconclusive	-	-	-	Sequencing inconclusive	-	Sequencing inconclusive

*Genetics behind Sequence Analysis of Potential Mutants

Step 1: L(3)96B/TM3Sb X +/+ → **L(3)96B/+** , +/TM3Sb
50% 50%
Select for non-Sb flies

Step 2: L(3)96B/+ X L(3)96B/+ → L(3)96B/L(3)96B , **L(3)96B/+**
Embryonic Lethal Sequence these flies

NB: Because L(3)96Be is over Tubby balancer, there is no visible adult phenotype to distinguish Step 1 progeny, therefore an initial step was carried out for this lethal that first crossed in the Stubble phenotype using dichaete (D).

L(3)96Be/TM3Tb X D/TM3Sb → L(3)96Be/D , **L(3)96Be/TM3Sb** , D/L(3)96Be , TM3Tb/TM3Sb
Select for stubble flies to be used for Step 1
Embryonic lethal

Appendix 9. P-element mutagenesis genetics. In the first step, males containing the P-element are crossed to females containing a transposase source (2-3). From the progeny are chosen females that contain both the P-element and the transposase (chosen based on orange eye colour and stubble phenotypes). From here, in Step 2, the previously mentioned females are crossed to the TM3 balancer (present in the dichaete line, an airplane like phenotype of the wings) to select for flies lacking the transposase, which is no longer required, and also containing the P-element possibly mobilized in a differing location from the original. Only males from the resulting progeny are chosen to go on to Step 3, because no recombination occurs in males. Males are chosen based on orange eyes, either yellow or normal body and dichaete wings. The potentially new lethal line (represented as P{w$^+$=lacW}J2D9?) containing the dichaete wing phenotype is chosen over the balanced lethal line (see Step 2, progeny P{w$^+$=lacW}J2D9?/TM3Sb) because in Step 3 it will be impossible to tell apart the balanced line from the original lethal line. Finally Step 3 is the complementation test to determine if the P-element is allelic to the original J2D9. If the P-element is in the same location, the bold face genotype of Step 3 will not be present among the population of progeny flies, indicating the P-element has not hopped. However, if these flies are present, the P-element is in a new location and the balanced line should be collected and tested by PCR. In a similar manner, a complementation test will also be performed to determine if the P-element is now located within the 96B region through the use of Df(3R)96B/TM3 (step not shown).

P-element Mutagenesis Genetics

Step 1: w /w ; + ; Dr/TMS 2-3Sb X yw /Y ; + ; P{w $^{+}$=lacW}L(3)J2D9/TM3Sb

↓

w/Y or yw/w ; + ; P{w+=lacW}J2D9/Dr , w^{-}/Y or yw/w ; + ; TM3Sb/TMS 2-3Sb
Embryonic Lethal

w/Y or yw/w ; + ; Dr/TM3Sb , w/Y or **yw/w ; + ; P{w+=lacW}J2D9/TMS 2-3Sb**
select females based on orange eyes, stubble for Step 2

Step 2: yw/w ; + ; P{w+=lacW}J2D9/TMS 2-3Sb X yw/Y ; + ; D/TM3Sb
pairwise ↓

yw/yw or yw/w or **yw/Y or w/Y ; + ; P{w+=lacW}J2D9?/D**
choose males only based on orange eyes, either yellow or normal body, dichaete wings

yw/yw or yw/w or yw/Y or w/Y ; + ; P{w+=lacW}J2D9?/TM3Sb

yw/yw or yw/w or yw/Y or w/Y ; + ; D/TMS 2-3Sb

yw/yw or yw/w or yw/Y or w/Y ; + ; TM3Sb/TMS 2-3Sb

Step 3:
yw /yw ; + ; P{w^{+}=lacW}L(3)J2D9/TM3Sb X yw/Y or w/Y ; + ; P{w+=lacW}J2D9?/D
↓ potential new lethal line

without showing body colour, progeny are:

P{w+=lacW}J2D9?/P{w^{+}=lacW}L(3)J2D9 **non-Sb, no dichaete wings. If these are present, collect both males and females of the next indicated fly line**

P{w+=lacW}J2D9?/TM3Sb **Potentially balanced new lethal line within *dveli***

P{w^{+}=lacW}L(3)J2D9/D

D/TM3Sb