Role of the mGRIP1 homologue DGrip in the *Drosophila* neuromuscular system

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LAURA ELIZABETH SWAN

born in

Launceston
Australia

2005

I hereby declare that this thesis has been written independently by me and with no other sources and aids than quoted,

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Appendix II: Published paper: Swan LE, Wichmann C, Prange U, Schmid A, Schmidt M, Schwarz T, Ponimaskin E, Madeo F, Vorbruggen G, Sigrist SJ. A glutamate receptor-interacting protein homolog organizes muscle guidance in Drosophila. Genes Dev. 2004 Jan 15;18(2):223-37.

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1. Introduction

1.1 *Drosophila melanogaster* as a model organism in neuroscience.

Drosophila, as one of the oldest genetic model organisms, has several particular advantages for the study of cellular processes, and has been the testing ground for very many works of cell autonomous and non-autonomous signalling and transcriptional cascades.
Several situations conspire to produce an organism that is convenient for many aspects of cell biology, and lately, neurobiology. The primary advantage is the short generation time. The *Drosophila* life cycle is approximately 10 days at 25°C, and dependent on temperature, such that animals raised at lower temperatures take longer to complete their life cycle. This provides an obvious advantage when compared to genetic approaches in higher animals, where transgenesis and breeding consume considerable amounts of time.

Figure A: life cycle of *Drosophila melanogaster* consists of 17 embryonic stages, 3 larval, a pre-pupal and pupal stage before adulthood. The entire cycle lasts 10 days.

Secondly, the first embryonic stages are accomplished extremely quickly. To aid in this process, the *Drosophila* female deposits so-called 'maternal' mRNAs and proteins in the unfertilised oocyte [1-11], allowing the oocytes' cells to divide and replicate without pausing for transcription of necessary genes, and effectively giving the early embryo the genotype of the mother, rather than its own (zygotic) genotype. This maternal contribution is then responsible for early embryogenesis, with the first zygotic transcripts appearing at stage 9 and the bulk of zygotic translation beginning during germ band

retraction [7, 12, 13]. The persistence of this maternal contribution is highly variable, depending on the gene, some even able to postpone functional defects for one more generation [10]. Since maternally contributed genes are usually those important for early cellular processes, embryos with a null mutation for a certain essential contribution can complete these processes, and researchers can focus on more subtle defects which arise in an established system in the absence of their gene of interest. This, in the case of neuroscientific studies, is a particular advantage, given that neurotransmission only begins in *Drosophila* neuromuscular synapses after neurons start to make contact with their target muscles after stage 13-14 of embryonic development [14].
A further advantage is that the use of P-element-based genetics has greatly simplified both targeted mutageneis and ectopic expression of genes. Using a technique developed by Brand and Perrimon [15], a minimal cassette of the yeast transcription factor Gal4 is inserted in a genetic locus, where it is expressed by surrounding promoters. This of itself should have no effect on gene expression. The second part of the expression system is a randomly inserted construct encoding the sequence to be expressed, fused downstream of the yeast upstream activating sequence (*UAS*), which is recognised by the Gal4 transcription factor. This leads to the desired gene only being expressed in those tissues and at those timepoints where the Gal4 cassette itself is expressed. Thus, using this system one can change expression pattern or the target gene to be expressed with relative ease, allowing the neuroscientist to differentiate between pre- and postsynaptic effects.
An advantage which has been exploited by our lab in particular is the ease of optical or even electrophysiological access to larval and even embryonic in particular using well-characterised model synapses, such as the embryonic and larval neuromuscular junction, treated below.

1.2 The *Drosophila* Neuromuscular Junction.

The *Drosophila* embryonic and larval neuromuscular system is a well-characterised and stereotypically arranged model synaptic system. An array of 70 motorneurons [16] form glutamtergic synapses [17], depending on their developmental program on multiple muscles, and others on a particular muscle only. While glutamate-immunoreactive motor endings innervate the entire larval bodywall musculature [18], specialised synapses utilise other transmitters including peptides [19] and aminergic cotransmitters [20-22].
In the early stages of myogenesis, from myoblast fusion through to the early stages of forming their mature morphology, myotubes are electrically inert and are electrically and dye coupled to adjacent myotubes [23]. This dye coupling is subsequently lost as motor neurons contact their target muscle [24] and a functional neuromuscular junction is formed.
A neuromuscular junction (NMJ) is typically arranged as a series of linked presynaptic specialisations (boutons, each housing about 10 to 20 individual synapses) which extend over a large fraction of the muscle. These NMJs are capable of growth, both due to developmental requirements as the larval muscles themselves grow -synaptic current per *Drosophila* NMJ increases by nearly two orders of magnitude to keep pace with the strongly growing postsynaptic muscle cell [25, 26] - and in response to elevated activity [26-28], thus exhibiting morphological and functional plasticity. Two morphological types of innervating nerve processes can be distinguished by their bouton size

distributions: (1) Type Ib processes, which have localised branching and a broad size distribution of relatively large boutons ranging up to 8 microns and (2) thinner Type Is processes, which have a narrower distribution of small boutons with a mean diameter of only 1.4 +/- 0.6 microns [18]. The most commonly used model synapse is the synapse of the ISNb neuron on muscles 6 and 7 (**Figure B**). Both Type Ib and Type Is synapses are present, but no peptidergic Type II synapses are formed by this junction.

The *Drosophila* neuromuscular synapse utilises an evolutionarily conserved molecular machinery for neurotransmission. Presynaptically, vesicle fusion and endocytosis are regulated by many of the same or homolgous proteins as in mammals [29-41], and neurotransmitter release is sensed by postsynaptic glutamate receptors [24, 42-45], which are expressed in muscle cells from stage 12 onwards [42, 45]. Glutamate receptor adaptor proteins, such as Discs-large, the homologue of mammalian PSD-95, have been extensively studied in this context, and much functional data has been derived from this system [46, 47]. Likewise a protein homologous to mammalian N-CAM, FasII [48, 49] has been found at this junction and its role in synaptic plasticity studied in detail. The original aim of this thesis was to use this well characterised system to understand the role of a putative glutamate receptor trafficking protein, DGrip, in glutamate receptor cycling at the postsynapse. Due to the discovery of a specific and striking defect in *dgrip* mutants earlier in the formation of the neuromuscular synapse, the focus of this work has instead turned to the process of muscle guidance, which was then used as a testing ground for many aspects of DGrip function *in vivo.*

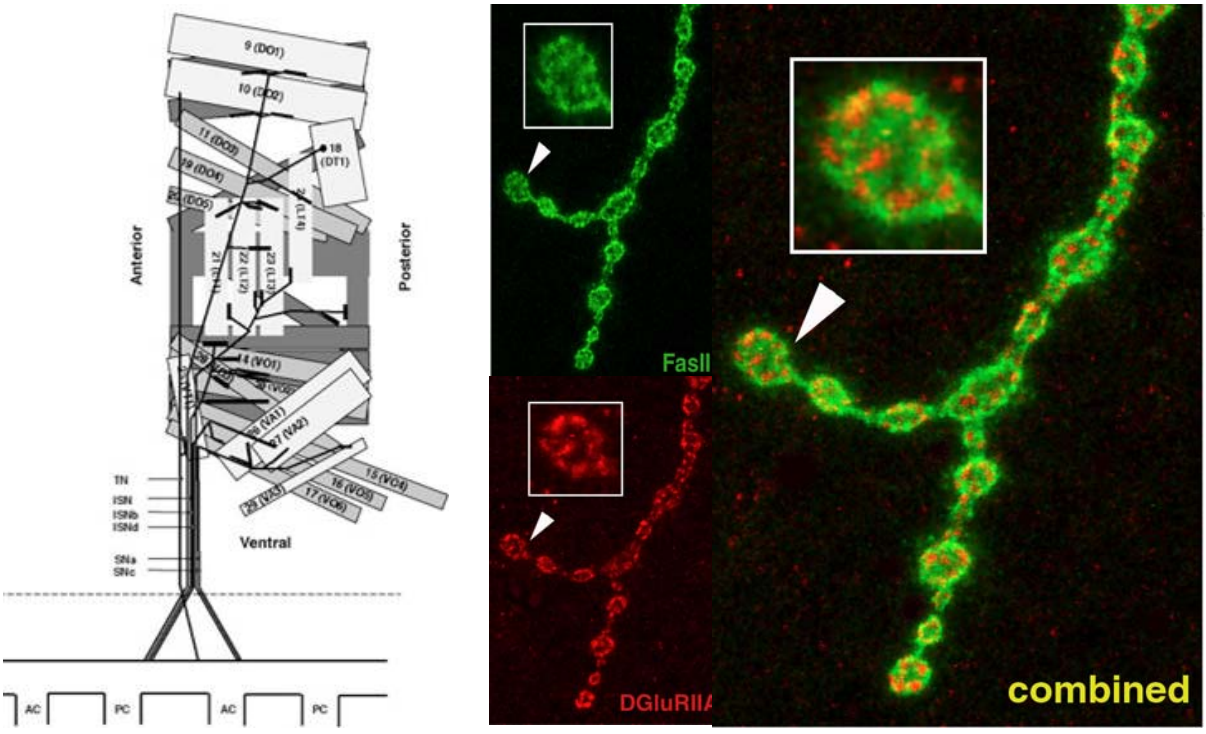


Figure B: The *Drosophila* Neuromuscular Junction. Presynaptic motorneurons pass out of the ventral nerve chord via the anterior and posterior commissures (AC and PC respectively). Motor neurons then synapse with specific groups of muscles, forming a series of postsynaptic varicosities called boutons (arrowhead). Each bouton contains a number of glutamatergic synapses, here labelled with an antibody to the postsynaptic receptor GluRIIA, surrounded by perisynaptic material, labelled by the cell adhesion molecule FasII. Schematic drawing from Hermann Aberle, confocal image from Stephan Sigrist.

1.3 Embryonic musculature in *Drosophila.*

The process of embryonic myogenesis is well characterised in its early stages, where tightly regulated signalling cascades are required to form a repeating pattern of 30 polynucleic embryonic muscles per hemisegment.
Due to the stereotyped and repetitive architecture of the embryonic muscle fields, this system has been used as a sensitive model system for the analysis of a variety of cell-fate specifying cascades and cell adhesion molecules [50-54]. *Drosophila* embryonic muscles are polynucleic cells, whose fate, and thus stereotypical positioning in the muscle field, are specified by a series of transcription factors and tightly regulated signalling cascades (The FGF and EGF receptor cascades among them [55]).
Muscle cell-type specification produces two kinds of cells: the so-called founder cells, whose fate and future morphology is already fixed, and a body of unspecified cells called the fusion competent myoblasts [56-59], which are programmed by founder cells when the founder myoblast fuses to them. The founder myoblast is in principle capable of forming a mature muscle cell whose volume is small, but with correct morphology and contractility- the undifferentiated myoblasts provide the necessary volume to the mature cell [58]. These muscles undergo a process where the founder muscle cell extends processes from its original position anchored to the epidermis, and stretches towards its second specific anchorage point (this process is henceforth referred to as 'muscle guidance'). At the same time whilst undergoing muscle guidance, the founder cell subsumes undifferentiated myoblast cells to increase the muscle volume.
While muscle guidance has not yet been investigated in great detail, some guidance factors have been identified. Mutants of the axonal guidance receptor Robo show misguided muscles specifically in the ventral longitudinal muscles (VLMs) [60, 61], showing that genes involved in axonal guidance can also have functions in guiding muscle morphology. *Drosophila* muscle guidance has so far not been subject to saturating genetic analysis and besides a few seminal studies [62-66] our understanding of the process is still rather poor. In several other models of cellular motility such as growth cone migration, cross-talk from several signalling complexes is required [67].
The later stage of muscle development, however, where muscles make contact and adhere to their epidermal target cells, and anchor to make a contraction-resistant junction, is better described.
Muscles in the *Drosophila* embryo can be classified in two simple groups, direct and indirect, with respect to the kinds of attachment they form. *Direct* muscle attachments are those formed by a single muscle attaching to a single apodeme and require little extracellular matrix (ECM) to maintain adhesion between cells. These muscles include the lateral transverse group of muscles (LTMs) (muscles 21-24). Muscles which span each segment of the animal, such as the ventral longitudinal group of muscles (muscles 6,7,12 and 13, see **Figure C**), form *indirect* connections, where several muscles converge on a point the centre of which is a single apodeme. The apodeme is a specialised epidermal cell which anchors muscle cells. The muscles at these junctions recruit a large amount of extracellular matrix, including the integrin ligand tiggrin [68], to the region, and adhesion is via Integrin-based [63, 69-73] adhesion of the muscle to the ECM, rather than to the apodeme itself. Thus muscle-ECM adhesion is as important as apodeme-ECM

adhesion in indirect junctions as opposed to direct junctions, and ectopic muscle-ECM-muscle junctions can be stably formed entirely in the absence of the apodeme [71].

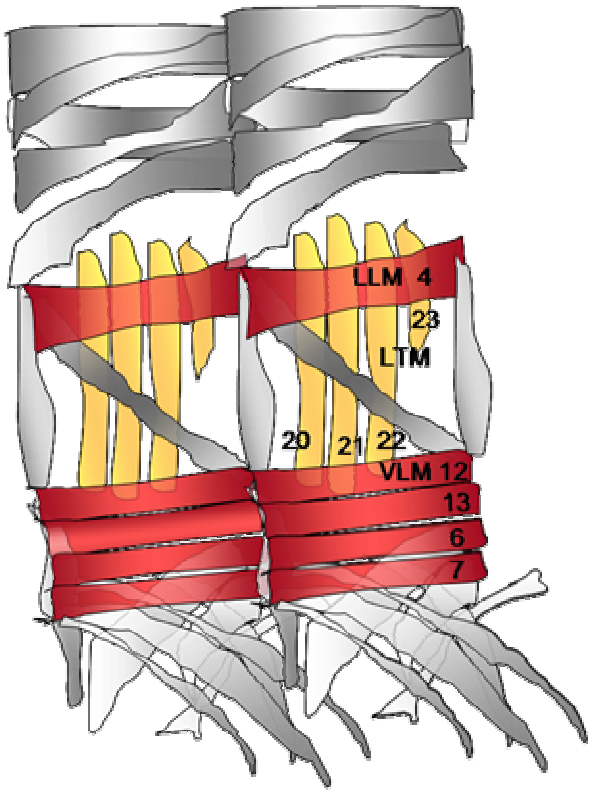


Figure C: Embryonic and larval musculature. Here, two hemisegments are shown as seen looking from the inside of a larva. Two segment-spanning groups of muscles, the ventral longitudinal and lateral longitudinal (VLM and LLM) muscles are marked in red. A group of non-segment spanning muscles (lateral transverse or LTMs), are coloured yellow. The numerical designation will be used when referring to specific muscles.

1.4 AMPA receptor trafficking

The topic of this study was to understand the role of the *Drosophila* homologue of the Glutamate Receptor Interacting Protein, GRIP, which was identified in this laboratory. The mammalian gene was first reported in 1997 [74], as a specific interactor of the GluR2 subunit.

Excitatory neurotransmission in mammalian central synapses is predominantly mediated by the neurotransmitter glutamate, which binds to both metabotropic and ionotropic transmembrane receptors. Ionotropc glutamate receptors fall into two classes which respond to the specific agonists N-methyl D-aspartate (NMDA) and either α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) or kainate. These last two are closely related, and in systems such as *Drosophila*, receptors responding one or the other agonist cannot be separated on a primary sequence level from one other [22, 45, 75, 76].

Plasticity, that is, the dynamic adjustment of signal transfer from one neuron to another, can be adjusted either by changing the amount of presynaptic release, or the postsynaptic response. One mechanism of changing postsynaptic response is to utilise neurotransmitter receptors which are functionally different from one another. This difference can take the form of receptors which allow different ion fluxes, or by receptors which are associated

with different signalling [77-79] or structural elements [80] in the postsynaptic cell. Then, the regulation of the functional behaviour at the synapse can be achieved by the presentation and modification of these different receptors. Here, a short summary of the vast body of research on this process is presented.

NMDA receptors are functionally different from AMPA receptors in that they require not only ligand binding, but also concomitant membrane depolarisation, for ions to pass through the channel, making them very likely to be the molecular mediators of Hebbian plasticity [81]. It is these receptors which are posited to occupy so-called silent synapses [82-85], where AMPA receptors are absent. Silent synapses are postulated to be electrically inactive during basal synaptic stimulation [86], but are necessary for the development of long-term potentiation (LTP). According to this model, upon activation by simulataneous ligand binding and membrane depolarisation, there is an influx of Ca^{2+} through NMDA channel. These silent synapses are then populated by complexes of AMPA receptors [85, 87-89], which are then responsible for increased levels of basal neurotransmission.

There are several subtypes of AMPA receptors, with different roles in synaptic processes. They can be easily separated on an interaction level by dividing them into receptors with short or long C-termini, which are the cytoplasmic interacting motifs for most GluR interacting proteins [90]. Long-tailed isoforms are GluR1, the rare splice variant GluR2L and the GluR4 subunit, which is mainly expressed during early development [91]. Three isoforms with short termini, all of which bind mGRIP [92], are GluR2, GluR3 and a short isoform of GluR4, GluR4c [93, 94]. These receptors constantly cycle between postsynaptic membrane and intracellular compartments [90], and the regulation of their membrane presentation is controlled by their interaction with scaffolding proteins, such as mGRIP, and by their association with other GluRs.

GluRs preferentially form either GluR1/2 or GluR2/3 complexes which are trafficked to the cell membrane and inserted as a functional unit into synapses [95-98]. At resting conditions, GluR1 is widely distributed in the dendritic arbor, but little is incorporated into synapses in the absence of activity [99, 100]. GluR1 has been described as the pioneer subunit, as the trafficking of GluR1, dependent on LTP or constitutively active CAMKII, can drive GluR1-GFP into NMDAR-only synapses, the previously described silent synapses. These GluR1/2 complexes are then steadily removed and replaced by complexes of GluR2/3 [101].

Thus, the mechanisms which guide the interaction and post-translational processing of GluRs have profound influence on the characteristics of synapses and their ability to be potentiated. Mechanisms such as phosphorylation of GluR2, which abolishes its interaction with mGRIP [102-104], can thus regulate the presentation of GluRs on the membrane surface, changing basal transmission mediated by GluR2/3 complexes. Removal of GluRs from the membrane surface can lead either to their being retained in endosomal compartments and recycled back to the postsynaptic membrane or to lysosomal degradation [105-107], a process determined by the GluR complexes' interacton partners. This leads to a complicated set of rules which govern the co-regulation of various synaptic complexes, without even considering the direct modulation of their ion channel properties, which can also be modulated by cytosolic factors, such as phosphorylation [90]. Interestingly, cell culture studies show that the intracellular accumulation of GluR2 and GluR3 but not GluR1 is enhanced by AMPA, NMDA, or

synaptic activity. After AMPA-induced internalisation, homomeric GluR2 enters the recycling pathway, but following NMDA, GluR2 is diverted to late endosomes or lysosomes. In contrast, GluR1, the pioneer subunit, remains in the recycling pathway, and GluR3 is targeted to lysosomes regardless of NMDA receptor activation. In these studies, GluR1/GluR2 heteromeric receptors behave like GluR2 homomers, and endogenous AMPA receptors show differential activity-dependent sorting similar to homomeric GluR2, indicating the central role of GluR2 trafficking in regulation of receptor complex presentation [105].

1.5 The Glutamate Receptor Interacting Protein, mGRIP

As mGRIP was originally identified as a specific GluR2-interacting factor (via interaction with mGRIPs 5th PDZ domain) that disturbs GluR clustering in the postsynapse [74, 108], it seemed possible that mGRIP, and that the complex that it builds, may be important for different aspects of plasticity. mGRIP, and another gene mGRIP2, or AMPA receptor binding protein (ABP) [108-111] have since been shown to be factors most likely involved in a subunit-specific and activity-dependent sorting of AMPA receptors in the postsynapse. There is an emerging consensus [102, 103, 112, 113] that mGRIP/ABP factors are primarily involved in stabilising intracellular pools of receptors, and protecting them from being sorted to degradation pathways.
However, the mechanics of this is still subject to debate, due to the lack of electrophysiological studies of the available mutants [114, 115]. Thus the interaction of the mGRIP/ABP trafficking pathways with other GluR trafficking signals, and particularly, their functional consequences, remains to be elucidated. However, one must mention within this context that it is well known that mGRIP is in fact more strongly expressed at GABA-ergic postsynapses than at glutamatergic terminals, suggesting it may well be a regulator of GABA-ergic transmission as well [116-119].
What then, is the mechanistic role of this molecule? mGRIP is composed of seven PSD-95/Discs-large/Zo-1 (PDZ) domains, and no other known functional domain. PDZ domains are structural motifs which preferentially recognise the C-termini of transmembrane proteins [120-123]. Proteins containing PDZ domains are typically scaffolding proteins [124-126] and can exhibit a broad array of ligand affinities [127], able to thus construct microdomains containing both transmembrane receptors and their downstream signalling components [128, 129]. It is not yet fully understood whether mGRIP is a postsynaptic scaffolding protein such as others like PSD-95, a NMDAR scaffolding protein, although biochemical evidence suggests likely not. The mGRIP protein is distributed in many cellular compartments, but unlike PSD-95, it is not detectable in those fractions most closely associated with the postsynaptic density [130].
Apart from another suggested interaction with kainate-type receptors [131], the mGRIP molecule appears to be involved in multiple protein complexes, with a role largely in membrane targeting of receptors, or segregation of these receptors in cellular subdomains. Many of these interactions may also affect the membrane presentation of AMPA receptors, although later work has indicated many roles for mGRIP apparently unrelated to GluR trafficking. Through interaction with Liprin-α via the 6th PDZ domain, a GluR2/3-mGRIP-Liprin-α-LAR-RPTP complex is formed which is required for

clustering AMPA receptors along dendrites [132, 133]. Likewise, interactions with ephrinB molecules over the 6th PDZ domain brings a mGRIP-ephrinB complex with kinase activity to sphingolipid-rich raft domains on the cell surface, which could be associated with retrograde ephrinB signalling [134-136]. EphrinB interaction with mGRIP has been implicated as a key component of mossy fibre LTP in the hippocampus, also dependent on retrograde signalling [135]. Segragation into lipid domains may also be accomplished by palmitoylatable isoforms of mGRIP, which then preferentially associate with postsynaptically located AMPA receptors, whereas unpalmitoylatable forms tend to associate with what appear to be intracellular pools of AMPA receptors [137, 138].

Further, non-receptor interactions include those with KIF5 [139], which steers kinesin and their cargoes (which include mRNAs and AMPA receptors) towards the somato-dendritic pole of neurons. mGRIP also associates with the ras-GEF factor GRASP1. Intriguingly, mGRIP has been proposed as a co-activator of DLX homeodomain transcriptional factors [140], an interaction which, in cell culture, is suppressed in the presence of GluR2. From this standpoint, one can consider mGRIP in the light of a more general function than its role in AMPA receptor trafficking, as one of a series of factors that establishes neuro-transmission competence, setting the conditions for AMPA receptor delivery, and co-ordination of responses to changed synaptic input.

Two studies producing mutants of the mammalian GRIP1 locus have been published [114, 115], both of which give strong support to the idea that mGRIP is in fact involved in several developmental pathways unrelated with neural transmission. The expression pattern of both mGRIPs 1 and 2 are wider than neuronal expression only [114, 115, 134], including a strong expression in the developing muscular system [115]. Phenotypic analysis of mGRIP1 mutant mice revealed several severe phenotypic defects, including fusion, or the production of supernumary digits, formation of eye blebs and lack of kidneys [114].

Both studies showed that mGRIP is required for adhesion between epidermal cells, but this effect is strongly dependent on the genetic background. In one case, loss of mGRIP1 leads to a severe, embryonic lethal defect, while in the other study animals could survive to adulthood. In both these cases, the defects could be linked to the mistrafficking of the extracellular matrix protein Fras1 [114]. Thus, the question of what is the general role of mGRIPs and of how the one molecule is able to co-ordinate such divergent functions are still largely unanswered. This thesis is an attempt to work on these questions in a simpler and more accessible system, so that general conclusions can be derived as to how this molecule works in terms of neurotransmission.

2.Materials and Methods:

2.1 Cloning:

Numbers in brackets indicate the position of PCR primers against the original cDNA sequence of the template. Full sequences of cDNAs can be obtained from the Berkley *Drosophila* Genome Project (http://weasel.lbl.gov/cgi-bin/EST/community_query/cloneReport.pl).

Construct Name: **pUAST-Grip**
Template: RE14068 cDNA
Forward Primer: ATAACAAGATCTCAAGATGAAACTGTGGAATCG (281-304)
Reverse Primer: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
Digest: XhoI/BglII
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip (no stop codon)**
Template: RE14068
Forward Primer: ATAACAAGATCTCAAGATGAAACTGTGGAATCG (281-304)
Reverse Primer: CATCCTCGAGAGAGCGCTGCATGATCATCTCG (3460-3441)
Digest: XhoI/BglII
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-PDZ1-3OE**
Template: RE14068
Forward Primer: ATACAAGATCTAAGATGAAACTGTGGAAATCGAAGAAGCC (218-312)
Reverse Primer: CATCCTCGAGCGGGCAGAGGCCCAGACCCATGCC (1675-1652)
Digest: XhoI/BglII
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-PDZ4-5OE**
Template: RE14068
Forward Primer: ATACAAGATCTAAGATGACGCAGATGCAGATCATGCCCGCTC (1420-1442)
Reverse Primer: CATCCTCGAGCGTGAAGATGATCTGGTGCAGACTGCTACC (2788-2759)
Digest: XhoI/BglII
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-PDZ6-7OE**
Template: RE14068
Forward Primer: ATACAAGATCTAAGATGATCGTCCTCCCGACTGCCTGCCC (2264-2287)
Reverse Primer: CATCCTCGAGAGAGCGCTGCATGATCATCTCG (3460-3439)
Digest: XhoI/BglII
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-Δ1-3**
Template: RE14068
Forward Primer1: ATAACAAGATCTCAAGATGAAACTGTGGAATCG (281-304)
Reverse Primer1: GGGTAGGTGGTACCCCTTTCTGG (529-506)
Forward Primer2::P*-CAGATGCAGATCATGCCCGCTCACGC (1421-1446)
Reverse Primer2: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
Digest: BglII (primer pair 1)/XhoI(primer pair2)
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-Δ4-5**
Template: RE14068
Forward Primer1: ATAACAAGATCTCAAGATGAAACTGTGGAATCG (281-304)
Reverse Primer1: GCAGAGGCCCAGACCCATGC (1672-1653)
Forward Primer2: P*-ATCGTCCTGCCCACTGCCTGCCC (2264-2287)
Reverse Primer2: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
Digest: BglII (primer pair 1)/XhoI(primer pair2)
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-Δ4-5L**
Template: RE14068
Forward Primer1: ATAACAAGATCTCAAGATGAAACTGTGGAATCG (281-304)
Reverse Primer1: CTGCGTGTAGCCGCGACCCG (1423-1404)
Forward Primer2: P*-ATCGTCCTGCCCACTGCCTGCCC (2264-2287)
Reverse Primer2: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
Digest: XhoI (primer pair 2)/BglII(primer pair1)
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-Δ4-5R**
Template: RE14068
Forward Primer1: ATAACAAGATCTCAAGATGAAACTGTGGAATCG (281-304)
Reverse Primer1: GCAGAGGCCCAGACCCATGC (1672-1653)
Forward Primer2: ACGGTGCGCTTGGAGCCC (2786-2803)
Reverse Primer2: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
Digest: XhoI (primer pair 2)/BglII(primer pair1)
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-Δ6-7L**
Template: RE14068
Forward Primer: ATAACAAGATCTCAAGATGAAACTGTGGAATCG (281-304)
Reverse Primer: ATATCTCGAGTTACCGCTTGATCGTCAGCGTGG (2263-2244)
Digest: XhoI /BglII
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-Δ6-7**
Template: RE14068
Forward Primer1: ATAACAAGATCTCAAGATGAAACTGTGGAATCG (281-304)
Reverse Primer1:CGTGAAGATGATCTGGTGCAGAC (2788-2766)
Forward Primer2: P*-GAGTGATGCTTTTGCCCGAGATCC (3458-3481)
Reverse Primer2: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
Digest: XhoI (primer pair 2)/BglII(primer pair1)
Vector: pUAST digested XhoI/BglII

Construct Name: **pUAST-Grip-Δint**
Template: RE14068
Forward Primer1: GGCATTATCTTGTCTGAAACGG
Reverse Primer1: P*-TGCGTATTGGGTGGGGCCAC (2599-2579)
Forward Primer2: ATACAAGATCCTCAAGATGAAACTGTGGAAATCG (2317-2340)
Reverse Primer2: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
Digest: XhoI (primer pair 2)/AspI(primer pair1)
Vector: pUAST-Grip digested XhoI/AspI

Construct Name: **pUAST-Grip-Δkhc**
Template: RE14068
Forward Primer1: GGCATTATCTTGTCTGAAACGG
Reverse Primer1: P*-TGCGTATTGGGTGGGGCCAC (2599-2579)
Forward Primer2: TAGCAGTCTGCACCAGATCATC (2761-2782)
Reverse Primer2: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
Digest: XhoI (primer pair 2)/AspI(primer pair1)
Vector: pUAST-Grip digested XhoI/AspI

Construct Name: **pUAST-Grip-C13S** (KPI GGCVPGKSAA - KPI GG**S**VPGKSAA)
Template: RE14068
Forward Primer: GGTGGCTCTGTGCCCGGAAAATCG (317-340)
Reverse Primer: CAGAGCATGTTCATCACCTTCTGGTTG (729-703)
To produce P1
Template: pFastbac-Grip
Forward Primer: P1
Reverse Primer: TAAATATTCCGGATTATTCA
Digest: BglII/SgrA1
Vector: pUAST-Grip digested BglII/SgrA1

Construct Name: **pUAST-Grip-x1** (LPRNALHLAIT- LP**A**NALH**A**AIT)
Template: RE14068
Forward Primer: ATACAAGATCTCAAGATGAACTGTGGAAATCG
Reverse Primer: TGTGATGGCCGCATGCAGGGCATTCGCTGGCAG
To produce P1
Forward Primer: P1
Reverse Primer: GCCCTCCTCCTTGAGCAGTGCGTCC
Digest: BglII/SgrA1
Vector: pUAST-Grip digested BglII/SgrA1

Construct Name: **pUAST-Grip-x2** (VERESGCLGL-VE**A**ESGC**A**GL)
Template: RE14068
Forward Primer: ATACAAGATCTCAAGATGAACTGTGGAAATCG
Reverse Primer: CAGGCCCGCGCATCCGCTCTCCGCCTCCAC
To produce P1
Template: RE14068
Forward Primer: P1
Reverse Primer: GCCCTCCTCCTTGAGCAGTGCGTCC
Digest: BglII/SgrA1
Vector: pUAST-Grip digested BglII/SgrA1

Construct Name: **pUAST-Grip-x3** (EIERPMN- EI**AA**PMN)
Template: RE14068
Forward Primer: ATACAAGATCTCAAGATGAACTGTGGAAATCG
Reverse Primer: CAACTTGTCGTTCATCGGTCGCGCGATCCTC
To produce P1
Template: RE14068
Forward Primer: P1
Reverse Primer: GCCCTCCTCCTTGAGCAGTGCGTCC
Digest: BglII/SgrA1
Vector: pUAST-Grip digested BglII/SgrA1

Construct Name: **pUAST-Grip-x1,2**
Template: pUAST-Grip-x1
Forward Primer: ATACAAGATCTCAAGATGAACTGTGGAAATCG
Reverse Primer: CAGGCCCGCGCATCCGCTCTCCGCCTCCAC
To produce P1
Forward Primer: P1
Template: pUAST-Grip-x1
Reverse Primer: GCCCTCCTCCTTGAGCAGTGCGTCC
Digest: BglII/SgrA1
Vector: pUAST-Grip digested BglII/SgrA1

Construct Name: **pUAST-Grip-x1,2,3**
Template: pUAST-Grip-x1,2
Digest: BglII/SgrA1
Vector: pUAST-Grip-x3 digested BglII/SgrA1

Construct Name: **pUAST-Grip-x6** (EPKGGLLGITL-EP**A**GGL**A**GITL)
Template: RE14068
Forward Primer: GAGCCCGCAGGAGGATTGGCCGGCATCACTTTG (2938-2970)
Reverse Primer: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
To produce P1
Forward Primer: GGCATTATCTTGTCTGAAACGG
Reverse Primer: P1
Digest: XhoI /AspI
Vector: pUAST-Grip digested XhoI/AspI

Construct Name: **pUAST-Grip-x7** (FDCCLT- FD**GG**LTV)
Template: RE14068
Forward Primer: GGACTTCGATGGCGGTCTTACCGTTCCGC (3382-3410)
Reverse Primer: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
To produce P1
Forward Primer: GGCATTATCTTGTCTGAAACGG
Reverse Primer: P1
Digest: XhoI /AspI
Vector: pUAST-Grip digested XhoI/AspI

Construct Name: **pUAST-Grip-nd** (IIFTV-II**D**TV)
Template: RE14068
Forward Primer: CACCAGATCATCGACACGGTGCGCTTGG (2771-2798)
Reverse Primer: AGTACTCGAGGCTCGGTAAACAATACAGGA (3574-3554)
To produce P1
Forward Primer: GGCATTATCTTGTCTGAAACGG
Reverse Primer: P1
Digest: XhoI /AspI
Vector: pUAST-Grip digested XhoI/AspI

Construct Name: **pUAST-Grip-Δ1-3x6**
Template: pUAST-GripΔ1-3
Digest: AspI/BglII
Vector: pUAST-Grip-x6, digested AspI/BglII

Construct Name: **pUAST-Grip-Δ1-3x7**
Template: pUAST-GripΔ1-3
Digest: AspI/BglII
Vector: pUAST-Grip-x7, digested AspI/BglII

Construct Name: **pUAST-Grip-GFP**
Template: pEGFP (Clontech)
Digest: XhoI/NotI
Vector: pUAST-Grip no stop digested XhoI/NotI

Construct Name: **pUAST-Grip-C13S-GFP**
Template: pUAST-Grip-C13S
Digest: BglII/SgrA1
Vector: pUAST-Grip-GFP digested BglII/SgrA1

Construct Name: **pUAST-Grip-tdimer2**
Template: pSL tdimer2 (obtained from Tobias Rasse)
Forward Primer: GCCGCTCGAGATGGTGGCTTCGTCGGAGGACGTCATC
Reverse Primer: ATGATCTAGACTAGAGAAAGAGATGGTGGCGGCCCAC
Digest: XhoI/XbaI
Vector: pUAST-Grip-GFP digested XhoI/XbaI

Construct Name: **pFastbac-Grip**
Template: RE14068
Forward Primer: ATACAAGATCTCAAGATGAAACTGTGGAAATCG (281-304)
Reverse Primer: CATCCTCGAGAGAGCGCTGCATGATCATCTCG (3460-3439)
Digest: BglII/XhoI
Vector: pFastbac, digested BglII/XhoI

Construct Name: **pFastbac-Grip-myc**
Template: pFastbac-Grip
Digest: XhoI/HindIII
Add annealed oligos: TCGAGATGGAACAAAAACTTATTTGTGAAGAAGATCTGTAA
AGCTTTTACAGATCTTCTTCAGAAATAAGTTTTTGTTCCATC

Construct Name: **pFastbac-Grip-C13S**
Template: RE14068
Forward Primer: GGTGGCTCTGTGCCCGGAAAATCG (317-340)
Reverse Primer: CAGAGCATGTTCATCACCTTCTGGTTG (729-703)
To produce P1
Template: pFastbac-Grip
Forward Primer: P1
Reverse Primer: TAAATATTCCGGATTATTCA
Digest: XbaI/SgrAI
Vector: pFastbac-Grip,digested XbaI/SgrA1

Construct Name: **pGex4T3-PDZ1-3**
Template: pDNR-1-PDZ1-3
Digest: XhoI/NotI
Vector: pGex4T3-PDZ, digested XhoI/NotI

Construct Name: **pGex4T3-PDZ6-7**
Template: pDNR-1-PDZ6-7
Digest: XhoI/NotI
Vector: pGex4T3-PDZ, digested XhoI/NotI

Construct Name: **pQE-32-PDZ1-3**
Template: pDNR-1-PDZ1-3
Digest: XhoI/XbaI
Vector: pQE32, digested SalI/HindIII, ligated with the digestion product, blunted with Klenow and religated.

Construct Name: **pQE-32-PDZ6-7**
Template: pDNR-1-PDZ6-7
Digest: XhoI/XbaI
Vector: pQE32, digested SalI/HindIII, ligated with the digestion product, blunted with Klenow and religated.

Construct Name: **pRSETA-Antigen2**
Template: RE14068
Forward Primer: AGCGGGATCCTCAGACGTCAAAATGGGCAGTCCTG (2081-2105)
Reverse Primer: AGTCCCATGGCCTGCGGCAACGTGGAACCGTATT (3100-3076)
Digest: BamHI/KpnI
Vector: pRSETA, digested BamHI/KpnI

Construct Name: **pRSETA-Mint M1**
Template: LD29081 cDNA
Forward Primer: TATACTCGAGCAGCAGCAGCAATCGCATCAG (258-278)
Reverse Primer: ACACGGTACCGTCACTGGGTGGCTTTTTAGTAGGC (1059-1035)
Digest: XhoI/KpnI
Vector: pRSETA, digested XhoI/KpnI

Construct Name: **pRSETA-Mint M2**
Template: LD29081 cDNA
Forward Primer: TATACTCGAGCGGTCGCCTACTAAAAAGCCACC (1103-1125)
Reverse Primer: ACACGGTACCGAAGAAATCCTGCTCATCGTGCC (2256-2134)
Digest: XhoI/KpnI
Vector:pRSETA, digested XhoI/KpnI

Construct Name: **pDNR-1-PDZ1-3**
Template: RE14068
Forward Primer: GCGACTCGAGGCAGCAACAACAACAGCAACAGG (364-386)
Reverse Primer: GAGTCTAGATCTCCAACGGTAGGGAACTTTC (1577-1555)
Digest: XhoI/XbaI
Vector: pDNR-1,digested XhoI/XbaI

Construct Name: **pDNR-1-PDZ4-5**
Template: RE14068
Forward Primer: GAGCCTCGAGTCGCGGTCATACAACCTTGGGTAGTC (1456-1481)
Reverse Primer: GACTCTAGAGCTGGGCACTCGGAAGTAATC (2470-2450)
Digest: XhoI/XbaI
Vector: pDNR-1, digested XhoI/XbaI

Construct Name: **pDNR-1-PDZ6-7**
Template: RE14068
Forward Primer: GCGCCTCGAGCATGGGTGTCTCCACAAGCACAG (2344-2366)
Reverse Primer: GACTCTAGACGGGCAAAAGCATCACTCAG (3475-3456)
Digest: XhoI/XbaI
Vector: pDNR-1, digested XhoI/XbaI

Construct Name: **pGBK-PDZ1-3(1)**
Template: pDNR-1-PDZ1-3
Cre-recombinase driven recombination into pLP-GBK-T7

Construct Name: **pGBK-PDZ4-5(1)**
Template: pDNR-1-PDZ4-5
Cre-recombinase driven recombination into pLP-GBK-T7

Construct Name: **pGBK-PDZ6-7(1)**
Template: pDNR-1-PDZ6-7
Cre-recombinase driven recombination into pLP-GBK-T7

Construct Name: **pGBK-PDZ1-3(2)**
Template: RE14068
Forward Primer: GGAATCCCATATGCAGCAACAACAACAGCAACAGGAG (364-388)
Reverse Primer: GTCTCTCGAGTCTCCAACGGTAGGGAACTTTTC (1577-1555)
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ1-3x123(2)**
Template: pUAST-Grip-x1,2,3
Forward Primer: GGAATCCCATATGCAGCAACAACAACAGCAACAGGAG (364-388)
Reverse Primer: GTCTCTCGAGTCTCCAACGGTAGGGAACTTTTC (1577-1555)
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ1-3x1(2)**
Template: pUAST-Grip-x1
Forward Primer: GGAATCCCATATGCAGCAACAACAACAGCAACAGGAG (364-388)
Reverse Primer: GTCTCTCGAGTCTCCAACGGTAGGGAACTTTTC (1577-1555)
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ1-3x2(2)**
Template: pUAST-Grip-x2
Forward Primer: GGAATCCCATATGCAGCAACAACAACAGCAACAGGAG (364-388)
Reverse Primer: GTCTCTCGAGTCTCCAACGGTAGGGAACTTTTC (1577-1555)
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ1-3x3(2)**
Template: pUAST-Grip-x3
Forward Primer: GGAATCCCATATGCAGCAACAACAACAGCAACAGGAG (364-388)
Reverse Primer: GTCTCTCGAGTCTCCAACGGTAGGGAACTTTTC (1577-1555)
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ4-6(2)**
Template: RE14068
Forward Primer: CGAATTCCATATGCGCGGTCATACAACCTTGGGTAGTC (1457-1481)
Reverse Primer: CGCACTCGAGGGTTAGACTCTGCGTATTGGG (2608-2588)
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ7(2)**
Template: RE14068
Forward Primer: GGAATTGCATATGACCAAGTCCATTACGATTAGTGGC (2840-2872)
Reverse Primer: GACCTCGAGCGGGCAAAAGCATCACTCAG (3475-3456)
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ7x7(2)**
Template: pUAST-Gripx7
Forward Primer: GGAATTGCATATGACCAAGTCCATTACGATTAGTGGC (2840-2872)
Reverse Primer: GACCTCGAGCGGGCAAAAGCATCACTCAG (3475-3456)
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ6-7(2)**
Template: RE14068
Forward Primer: GGAATTCCATATGATGGGTGTCTCCACAAGCACAG
Reverse Primer: GACCTCGAGCGGGCAAAAGCATCACTCAG
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ6-7x6(2)**
Template: pUAST-Grip-x6
Forward Primer: GGAATTCCATATGATGGGTGTCTCCACAAGCACAG
Reverse Primer: GACCTCGAGCGGGCAAAAGCATCACTCAG
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGBK-PDZ6-7x7(2)**
Template: pUAST-Grip-x7
Forward Primer: GGAATTCCATATGATGGGTGTCTCCACAAGCACAG
Reverse Primer: GACCTCGAGCGGGCAAAAGCATCACTCAG
Digest: NdeI/XhoI
Vector: pGBK-T7, digested NdeI/SalI

Construct Name: **pGAD-PDZ1-3**
Template: RE14068
Forward Primer: GGAATCCCATATGCAGCAACAACAACAGCAACAGGAG
Reverse Primer: GTCTCTCGAGTCTCCAACGGTAGGGAACTTTTC (1577-1555)
Digest: NdeI/XhoI
Vector:pGAD-T7, digested NdeI/XhoI

Construct Name: **pGAD-PDZ4-6**
Template: RE14068
Forward Primer: CGAATTCCATATGCGCGGTCATACAACCTTGGGTAGTC (1457-1481)
Reverse Primer: CGCACTCGAGGGTTAGACTCTGCGTATTGGG (2608-2588)
Digest: NdeI/XhoI
Vector:pGAD-T7, digested NdeI/XhoI

Construct Name: **pGAD-PDZ7**
Template: RE14068
Forward Primer: GGAATTGCATATGACCAAGTCCATTACGATTAGTGGC (2840-2872)
Reverse Primer: GACCTCGAGCGGGCAAAAGCATCACTCAG (3475-3456)
Digest: NdeI/XhoI
Vector:pGAD-T7, digested NdeI/XhoI

Construct Name: **pGAD-khc**
Template: pEG202-khc (gift of Joseph Gindhardt, see [141] for details)
Digest: EcoRI
Vector:pGAD-T7 , digested EcoRI

Construct Name: **pGAD-klc**
Template: pEG202-klc-deltaH3 (gift of Joseph Gindhardt, see [142] for details)
Digest: EcoRI
Vector:pGAD-T7 , digested EcoRI

Construct Name: **pGAD-Robo1**
Template: HA-Robo1 (Gift of Barry Dickson)
Forward Primer: CTGAGAATTCGCGGCTATTTCGATGGTCTAC
Reverse Primer: CATTGAATTCTTAGGTGTTCTTAGCAGTCATTTGACGAGC
Digest: EcoRI
Vector:pGAD-T7, digested EcoRI

Construct Name: **pGAD-Robo2**
Template: HA-Robo2 (Gift of Barry Dickson[143-145])
Forward Primer: ATCGGAATTCGCAATGGTCTTTGTGAAGCG
Reverse Primer: TATCGAATCTCAGACATTGTGGCCAGCTGG
Digest: EcoRI
Vector:pGAD-T7, digested EcoRI

Construct Name: **pGAD-EGFR**
Template: LP05058 cDNA
Forward Primer: ATAACTCGAGCCAAGGCAGCACCTGGTCC (3436-3454)
Reverse Primer: TAATCTCGAGCCTACACCCTCGTCTCCGTGTTGCG (3972-3948)
Digest: XhoI
Vector:pGAD-T7, digested XhoI and CIP-treated

2.2 Production of mutants of the DGrip locus

P-element based mutagenesis is accomplished in the following way: P-elements are a naturally occurring phenomenon in *Drosophila*, relatively small sequences flanked by inverted terminal repeats. The sequence between these two repeats contains an enzyme (transposase) which recognises the flanking sequences and transposes the entire sequence to another part of the genome. Using modified P-elements, where the transposase gene is removed, and eye or body colour markers are incorporated to indicate the presence of the element, libraries are created of fly lines containing a stable insertion of P-elements in different genetic regions, stable because there is no transposase to remobilise the P-element. Using these modified P-element insertion points, a locus can be mutated. On crossing in a transposase-expressing chromosome, the P-element has the probability to remobilise, and in doing so, remove a random part of the surrounding chromosome. Animals exposed to transposase and who have lost the P-element marker are identified. As DGrip is on the X chromosome, hemizygous males with the candidate chromosome were identified then checked via the amplification of small (300-800bp) sections of genomic DNA, which parts of the X chromosome have been deleted.
DGrip deficient chromosomes were recovered from either P(KG)028662 (P-disruption project Baylor college) or P(GT1)BG01736 (generous gift of Ulrich Schäfer and Herbert Jäckle) after P-element mobilisation by crossing to Δ2-3-Transposase. Deficiencies *dgrip*^{ex36} and *dgrip*^{ex122} were identified and mapped with genomic PCR from mutant larvae or adults, respectively. The following primer pairs were used to identify the size of the genomic deletions:
CGAGAAGAAGGGGCAGTTTCCG, TTGTTGCTGTTGTTGTTGTTTTCGC;
GGAGATTTCTTCCACGCCACCC, GGAGGTTTACCAGTTGCCCAAGG;
GCCATTCAACCCATTGCGACAG, CGGAGAAAGCAGGACGGAGAGAC;
GGGGCATCCGTTGGGAACAC, TTGGGGGAGGGGTGACTTGG;
CCCTCCCCCAACACACTAAAC, GCCAAAACTCCCCAAAAAGC;
AGCAGCAACAACAACAGCAACAGG, AGCACCTTGGGCGGAAATGC;
CCCCCAACCCACTTCCTTCCAC, CCACAAGCACACACTGAGCGAAAAC;
AAGAGTATCAGTTTCGCCCACCTCC, CAGCCGCCTTTTTCCTCGC;
CGGAAGCGACGACGGAAGAAG, CCCTGGTTGGTGTGCCTCCATC;
TCCCCCCGCCGCTTTCTAAC, CGAACACGAGATGCCCTGGAAG;
GACCGATGAACGACAAGTTGGG, AATGGGAGACGAAGATGTGGGG;
TTCCCTACCGTTGGAGAATCCTGC, TTGACACTAAAGACACCACTGGCG;
CCAGCAAGGAGTCGGGTAGCG, GCGGAACGGTAAGACAGCAATCG;
AACCAGACATTGACACCACCACCTG, AACCCATTCGGCACGGCGAG;
GCTTGTTAGGTTAGTTGGCGG, GCTCATTGGTCAGTGTTGGC;
GGCGGCAGTCAACACCCTGG, GCGAGCAGCACTCAGCATCTTTG;
CGGCTGCTCCTCTCTTCACGGAC, CGGCTCCTCGCAACTGGTGC;
TCGTCCCACTCAAAGTCCCGC, CAAGAACAACAATGCCGCCAGC;
Primer pairs in bold span the P(KG02862) and P(GTi)BG01736 P-element insertion sites, respectively. These primers were used in conjunction with the P-element primer CGACGGGACCACCTTATGTTATTTCATCATG, to determine the presence of P-element ends.

2.3 Protein Expression and Purification

For the expression of recombinant proteins different *E.coli* strains were used, depending on the plasmid used: For pGex constructs BL21(DE3), for pRSET, BL21(DE3) pLysS (pLysS is a repressor, allowing the reduction of uninduced transcription of fusion proteins) and for pQE constructs XL1Blue .
To optimise the expression of fusion proteins pre-tests of expression were performed as follows:
A single clone was grown in 50ml LB medium in a 500ml flask, shaking at 37°C, until reaching OD_{600} 0.6. A 1ml sample of the culture was taken. Fusion protein expression was then induced by the addition of IPTG to a final concentration of 10µM. The culture was split in two, one half incubated at 25°C and the other at 37°C, and 1ml samples taken after 1 hour, 3 hours and the next morning after induction. These samples were pelleted and a) boiled in 200µl Laemmli buffer for 5 minutes to extract the total protein or b) treated with 160µl BugBuster (Novagen) plus Benzonase (Merck), then repelleted. The supernatant (soluble proteins) is separated from the pellet (insoluable) and analysed on a Coomassie-stained 8% PAA gel. These were then compared to obtain the best conditions for expression and solubility.
Three different protocols were followed then for protein purification, depending on the fusion protein, and solubility of the fusion protein. 1-2L cultures were grown and induced with IPTG before being split (500ml culture in a 2L flask) and cultured for maximal protein expression. Cultures were then pelleted and the pellet frozen before treatment.
For all His-tagged proteins (pRSET- and pQE- constructs) the standard protocol for TALON-resin purification was used (Clontech).
For GST-tagged proteins (pGex4T3) which were soluble were lysed in the appropriate amount of BugBuster lysis buffer (with Benzonase), subjected to repeated freeze-thaw cycles (stepping from liquid nitrogen to a 65°C waterbath), and spun down using a Sorvall SS34 rotor at 10,000 rpm for 10 minutes. The supernatant was incubated with approx 1ml per 250ml culture volume of glutathione-agarose resin at 4°C overnight, the pelleted resin washed three times with ice cold PBT, then PBS. Fusion proteins were then eluted in 1ml fractions with Elution Buffer (75mM HEPES, 150mM NaCl, 5mM DTT, 4.6g/L reduced glutathione, pH 7.8), and each fraction analysed for protein content.
For insoluble GST-proteins the protocol above was modified by extracting the protein in 6M Urea, before dialysis of the extract back to PBS. The protein was then purified over GST-agarose as above.
All fusion proteins were then dialysed into standard PBS before being sent for injection into rabbits or guinea pigs. Companies used were Cocalico Biologicals Inc, Reamstown, PA, USA; BioGenes, Berlin, Germany; BioScience PepScience, Goettingen, Germany.

2.4 Yeast-Two-Hybrid Retesting

For these experiments, co-transfection of both prey and bait constructs into yeast (strain AH109), using the following protocol:
Per transformation, the yeast strain AH109 was incubated in 5ml YPDA medium at 30°C until the OD_{600} reaches 0.5-0.6. The cells were pelleted, washed in SD-medium without amino acids or glucose, repelleted, suspended in 40µl 100mM LiAc pH 8.4 and incubated for 5 minutes. Cells were pelleted again and resuspended in 33µl H_20. The transformation mix was composed as follows, 100ng of each of the two plasmids, 2.4µl 50%PEG 3350, 36µl 1M LiAc pH 8.4, 50µl Herring Testes carrier DNA (Clontech), plus resuspended yeast cells. The mix was vortexed, left to incubate at 30°C for 30 minutes in a shaker and heat shocked for 25 minutes at 42°C. After heat shocking, the cells were carefully sedimented, the pellet suspended in 200µl YPDA medium and the transformation plated on nutrient-selective plates, to ensure the presence of both plasmids. As a quality control, clones for interaction experiments were only taken from plates with more than 20 transformants. These clones were then streaked out on –LT plates, selective for the presence of both plasmids, allowed to grow for 1-2 days so that clones are well grown, and then stamped across to both –HALT plates and –LT plates streaked with X-α-gal (2mg/ml in DMF, Clontech) to test interaction. Growth on selective media and 'blueness' were monitored separately, and recorded each day for five days after stamping the clones across, to control for the specificity of growth.

2.5 Palmitoylation Assay

Palmitoylation was assayed in insect cells (Sf9), infected with a baculovirus construct expressing DGrip. Constructs to be transfected into insect cells were cloned into the vector pFastbac (Gibco-BRL). The sequence of these clones was confirmed by sequencing. Constructs were transformed into the *E.coli* strain DH10BAC (Gibco-BRL), and grown on LB-agar plates containing 50µg/ml kanamycin, 7µg/ml gentamycin, 10µg/ml tetracycline, 100µg/ml Bluo-gal and 40µg/ml IPTG.
Plates with 100-200 transformants were grown for two days, and at least six white colonies (indicating the successful recombination of the donor cassette from the Fastbac vector into the bacmid genome) were picked and grown in LB with kanamycin, gentamycin and tetracycline. Bacmid DNA extraction followed a protocol for large (>100kb) plasmids[146]. The presence of the desired insert was then confirmed by PCR from the bacmid DNA.
The different isolates of the baculovirus DNA were then transfected into Sf9 cells at $9x10^5$ cells per 35mm well in serum-free medium using CellFECTIN reagent, incubated for 5 hours and then placed in serum-free cell culture medium with antibiotics. These cultures were allowed to grow for five to seven days, then the supernatant was harvested, clarified by centrifugation and stored at 4°C protected from the light. These supernatants were used to infect Sf9 cells, grown to approximately $2x10^6$ cells/ml, to determine the best viral stock. The cultures were allowed to grow for 24h hours before being harvested, lysed and analysed by Western blot (either anti-Grip or anti-myc (Santa Cruz)). The stock

which produced the highest amount of protein was amplified by infecting a large culture dish and harvesting viral particles as above.
The palmitoylation assay was performed in Sf9 insect tumour cells, transfected with either Baculo-Grip, Baculo-Grip-myc, an empty baculovirus or mock-transfected (transfected with no virus) as controls.
2ml culture dishes of cells were infected with the desired virus, grown for two days (the time point of maximal viral expression) and then placed in 600µl culture medium containing 3µl [S35]-Met (Hartmann Analytic) to label total protein production or 3µl of [9,10-H^3(N)]-palmitic acid (Hartmann Analytic) to label palmitoylated proteins. Cells are returned to the incubator to grow for 3hours at 29°C (instead of the normal culture temperature of 27°C). Cells were collected and lysed in 600µl NTEP buffer. A specific antibody was added at dilution 1:60 and incubated at 4°C shaking overnight. Then 30µl of Protein-A Sepharose (Santa Cruz) is added for 2h, shaking. The sepharose was pelleted, washed and then incubated with Laemmli buffer (without β-mercaptoethanol) for 30mins at 37°C. The supernatant from this sample was then run on an 8% PAA gel, the gel was fixed for 15 minutes with 10% acetic acid and 10% methanol, washed, treated for 30 minutes in 1M sodium salicylate and dried. The dried gel was then exposed to film (1-3 weeks) to analyse for the presence of labelled precipitates. Total lysates (without precipitation steps) were also analysed on gels in the same manner.

2.6 Forster Resonance Energy Transfer (FRET) assay in larvae

For analysis of FRET interactions, larvae from the following strains were used:
24B-gal4::UAS-Grip-GFP (negative control), *24B-gal4::UAS-Grip-GFP; UAS-Grip-tdimer2* (experiment) and *24B-gal4::UAS-Grip-tdimer2* (control for spectral contamination from the red fluorescent protein).
Larvae were fixed for thirty seconds in 4%PFA/PBS, washed and mounted in Slow Fade® Light antifade medium (Molecular Probes). The preparations were then imaged via confocal imaging (AOBS, Leica TCS NT). To take lifetime images of the samples, the samples were excited by a Mira two-photon laser with a femtosecond pulse, tuned to 900nm. Photons were detected by a SPC730 time-correlated single photon counting module (Becker-Hickl), for an average of five minutes. Only samples where more than 1000 photons per pixel were collected were used for analysis.
Analysis of the data proceeded as follows. Data was primarily analysed using the software package provided (Becker-Hickl). The threshold count of photons for each analysis was maintained at the same value for all comparable experiments, and pixel binning set to 3x3 pixels. The program then fit a single exponential decay to the time-gated photon counts (the lifetime). This program then provided three data outputs, the calculated lifetime, the χ^2 value for the fit in each pixel, and the number of photons collected in that pixel. Using a thresholding program (written by Massimiliano Stagi) written in Matlab 6, the lifetime data was 'cleaned', by multiplying the lifetime matrix by two thresholded matrices, one setting the tolerable error level ($\chi^2 < 2.5$) and the other the number of photons collected (thresholded so as to obtain an image comparable to the confocal images taken before). These settings for thresholds were then applied to all comparable experiments.

The ‘cleaned’ lifetime produced in this manner was then used as the basis for FRET analysis.

2.7 Fly Strains

Transgenic flies were by injecting pUAST constructs described above were into the w^1 fly line following standard protocols, and homozygous transgenic stocks created.
Stocks used for the suppression of lethality screen (**Section 3.7.5**) are described in **Appendix I**. Where no source for this stock is noted, stocks came from our own stock collection. Bloomington refers to the *Drosophila* stock collection hosted by the University of Indiana at Bloomington (http://flystocks.bio.indiana.edu/).
The wild-type fly line is w^1, unless otherwise described.
The recombined chromosome, *dgrip*^{ex36}, *twist-gal4* was produced as described[147].
The stocks *UAS-KZ* and *UAS-NZ* [148, 149] were a kind gift of Ira Clark. Alleles of *vein* *vn*$^{\Delta p25}$/TM3,lacz (kind gift of Gerd Vorbrueggen), *vein*^{dddL6} and *vein*^{p1749} (kind gifts of Talilah Volk) are described by Yarnitzky et al. [150, 151].

2.8 Statistical analysis of muscle phenotypes

This analysis was performed by looking at the non-GFP progeny of a cross of males homozygous for each candidate transgene to *dgrip*^{ex36}, *twist-gal4*/FM7, *act::GFP* virgins. Embryos from this cross were raised at 18°C (low transgenic expression), 25°C (standard raising condition) or 27°C for high levels of transgenic expression.
For each condition, at least three male and three female larvae were dissected and the muscles of each larval hemisegment scored individually. A so-called ‘clinical score’ was used, ranking clearly distinguishable morphologies with an arbitrary score.
We identified five categories of muscle defects, ranging from a score of 0.2 for wild-type morphology, to 1.0 for the most severe defects.
In the LTM group of muscles the score was as follows:
0.2: normal morphology.
0.4: slight segmentation: LTM muscles split into two processes.
0.6: ramification: LTM forms multiple processes, which make contact with oneanother.
0.8: loss of orientation: LTM group of muscles form multiple, ramified processes, and the main axis of the muscles is no longer perpendicular to the VLM muscles.
1.0: complete loss of orientation: LTM muscles form fan-like processes and are no longer individually identifiable.

The VLM group of muscles were also rated using this system, where the ‘clinical scores’ were as follows:
0.2: normal morphology.
0.4: slight: VLM muscles do not meet in register at the segment border.
0.6: mild: VLM muscles attach at the segment border, but are striated.
0.8: strong: VLMs from processes, which do not extend fully to the segment border, but ectopically fuse in mid-segment.

1.0: severe: VLMs form ‘ball’ shapes, do not extend to the segment border at all.

The average score for these animals were then plotted, and comparisons made between these conditions.

2.9 Coimmunoprecipitations

HA-Robo/Grip-GFP coprecipitation
pCNDA3.1-Grip-GFP and either HA-Robo1 or HA-Robo2 were singly or co-transfected in Cos-7 cells , and immunoprecipitations were made exactly following the protocol used to isolate a Robo-commissureless interaction, as previously described [143].

Grip-Kinesin heavy chain pulldown
$12x10^6$ Sf9 cells were transfected by baculovirus-Grip-myc, grown for two days, collected and lysed in 500µl NTEP buffer. This lysate is then the bait for the pulldown assay. 5g of adult flies were ground to powder with a mortar and pestle in liquid nitrogen and resupsended in 40ml of NTEP or Buffer A (30mM Tris pH 8.0, 4mM EGTA, 1mM ATP, 1mM $MgCl_2$, 1mM DTT, 10% Glycerol plus protease inhibitors). The lysate was incubated for 15 minutes on ice, and clarified repeatedly by centrifugation. 30ml of extract was separated into three samples where either 100µl of baculovirus-Grip-myc bait, 2mls of a hybridoma extract of an anti-myc antibody, or both, were added to 300µl of Protein A beads (Affi-prep Protein A support, Biorad). This was incubated for 4h at 4°C, washed in buffer three times, and then samples were boiled 10’ in Laemmli buffer and analysed by Western Blot (rabbit anti-*Drosophila* kinesin heavy chain AKIN01, (Cytoskeleton) 1:500).

2.10 Immunostaining

Immunocytochemistry on embryos and larvae was essentially performed as described [152]. Antibodies were usually preadsorbed to 0-4 h *Drosophila* embryos. Primary antibodies: FMM5A muscle myosin monoclonal (Christoph Schuster, 1:100), rabbit anti-muscle myosin (Dan Kiehardt, 1:100), mouse anti-βPS-integrin (Nick Brown, 1:100), rat anti-Delilah and guinea pig anti-Stripe (Talilah Volk [63], 1:500), anti-Sex Lethal (DSHB, 1:500), rabbit anti-β-Gal (Cappel, 1:500), monoclonal anti-β-Gal (Promega, 1:500), rabbit anti-pMad (Peter ten Dijke, 1:150), rabbit anti-Echinoid (Jui-Chu Hsu, 1:150), rat anti-EGFR (Pernille Rorth [153], 1:100), rabbit anti-*Drosophila* kinesin AKIN01 and mouse SUK4 anti-kinesin monoclonal (Cytoskeleton).
Embryos stained with mouse anti-dpERK (Sigma 1:100), were treated slightly differently, in that they were fixed in 8% PFA and that all fixation steps in methanol were performed at minus 20°C. Where two antibodies were required for co-staining, secondary antibodies were applied sequentially, with the mouse secondary applied first.
Secondary antibodies: Cy3- and FITC- conjugated goat anti-mouse, anti-rabbit or anti-rat FAB (Dianova), used at 1:200.

Phalloidin-TRITC (Sigma) was used to visualise F-actin in larvae. Previously immunostained or freshly fixed larvae were incubated for 30 minutes in PBS/0.5%Triton-TX-100+5% Normal Goat Serum, protected from the light. Larvae were then rinsed and mounted as normal. Confocal images were taken on a Leica TCS NT system with a 63 fold objective (1.3 NA), 4x frame averaging, a ΔZ of 600 nm and image stacks projected in maximal intensity mode.
Wide field microscopy was performed with a Ziess Axioskop2 microscope, and acquired with Axiovision software.

3.Results

3.1 DGrip is the *Drosophila* homologue of mGRIP

The DGrip molecule was originally identified in our lab as a subunit-specific interactor in a yeast two-hybrid screen with the C-terminal EARV motif of the GluRIIC subunit, one of three glutamatergic subunits then known to be expressed at the *Drosophila* neuromuscular synapse. A cDNA fragment from DGrip, encoding PDZs 5-7 of the protein, specifically interacted with the GluR IIC/III subunit, and not with the other two tested C-termini from GluRs IIA and IIC. As the subunit IIC/III is described as the obligate member of GluR complexes at neuromuscular synapses [43, 44, 154], further examination of the function of this gene was warranted. DGrip, like its mammalian homologue, GRIP, has seven PDZ domains. When compared to one another, mGRIP and DGrip PDZs map in the same sequence (DGrip-PDZ1 is most similar to mGRIP-PDZ1 and so on) along the length of the protein (**Figure 1**), indicating a similar organisation of functional domains.

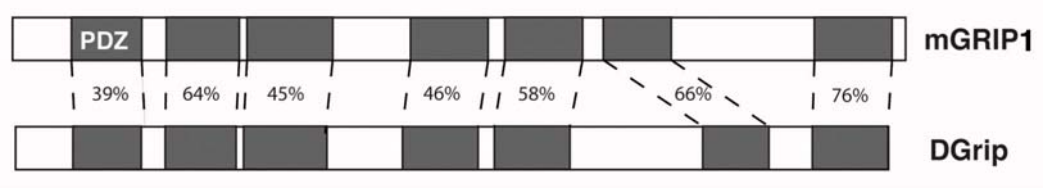


Figure 1: Percentage similarity between amino acids over the length of entire PDZ domains.

The PDZ-ligand specificity of individual PDZ domains is most strongly influenced by two positions in the 90aa motif: the last amino acid in the so-called carboxylate binding loop (the GLGF motif) and an amino acid at the beginning of the second α-helix (αB1) (**Figure 2**). These two positions are reasonable, but not infallible [155], predictors of the binding affinity of PDZ domains with various C-terminal ligands. A set of 25 possible combinations of carboxylate binding loop and α-helix motifs [120] have been used to predict theoretical binding affinities for PDZ domains, some of which have not been experimentally confirmed. However, these and other authors note that the vast majority of PDZ domains fall within two or three categories of affinities. The so-called Type I PDZ domains represent the bulk of PDZ domains (approximately 70% of all characterised PDZ domains [155]) - where residues of the βB strand and of the αB helix (in particular a histidine, highly conserved at position αB1) contact the C-terminal peptide and recognise a S/T-X-ϕ motif (ϕ is a hydrophobic residue). Type II (ϕ/ψ-X-ϕ, where ψ is an aromatic residue) and Type III (D/E-X-V) ligands are each recognised by several of the 25 combinations of structural motifs under this classification [120], while one particular group is associated with dual ligand specificity, such as that found for the Mint-1-PDZ1. It is also important to note, however, that the ligand binding preferences of PDZ domains

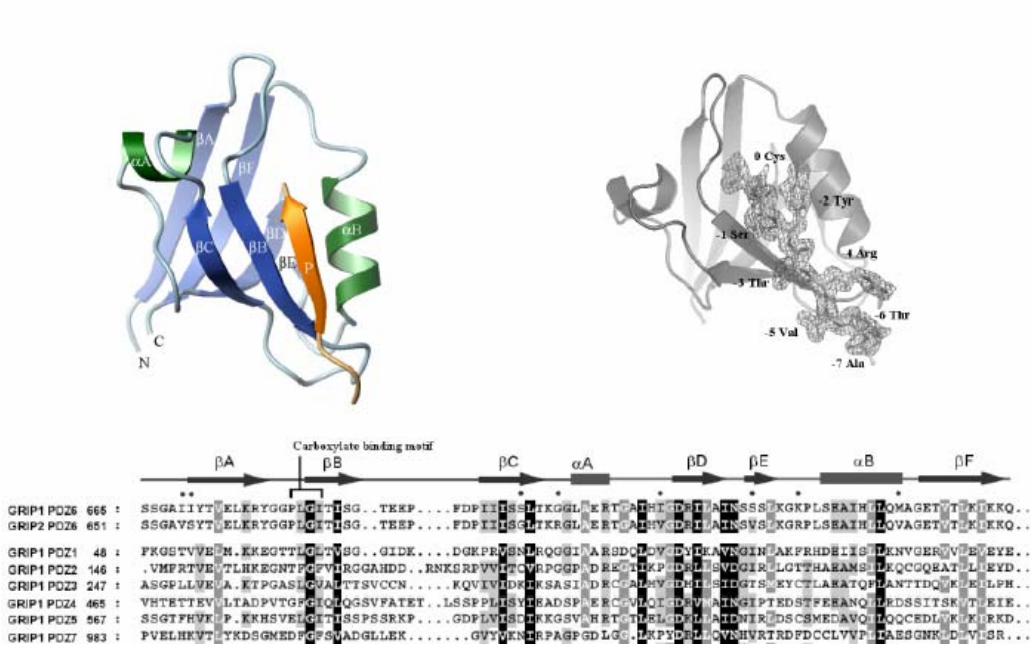


Figure 2: Structure of PDZ domains, modelled on mGRIP1 PDZ 6. Taken from Im. *et al.[156]*

differ between individual PDZs, even within the same class. This allows a PDZ domain protein to exhibit a vast array of ligand affinities.

The mGRIPs 1 and 2 are classified via this scheme as containing a Type I domain, two Type II domains, three poorly characterised domains and a seventh domain which has a dual ligand specificity for C-terminal sequences being either DXWC or EYYV [120]. This order and conservation is largely preserved in DGrip (**Table 1**).

The least conserved domain in terms of predicted binding affinities is PDZ 4, a fact which neatly converges with results obtained in studies of the mGRIP1 PDZ 4. This study suggested that the PDZ 4 had no ligand binding capability of itself, but was essential for maintaining the structure of PDZ 5 [157], relegating this PDZ domain to an accessory, chaperoning role.

Interestingly, the 7th PDZ domain is the most highly conserved of the domains and is the most unusual, in that this domain's binding modality, and thus affinity, has been shown to be different to that of most PDZ domains [158], also arguing a conservation of function over evolution for the *Drosophila* and mammalian proteins. Due to this homology of structure, study of DGrip was liable to shed some light on conserved functions of this molecule in a more genetically approachable system.

	I	II	III	IV	V	VI	VII
mGRIP1	Type I	Type II	Type II	G,a	Sp,h*	G,h*	Bimodal
mGRIP2	Type I	Type II	Type II	G,a	Sp,h*	G,h*	Bimodal
*D.mel*Grip	Type II	Type II	Type II	Bimodal	G,h*	G,h*	Bimodal
D.ps Grip	Type II	Type II	Type II	Bimodal	G,h*	G,h*	Bimodal
*A.gam*Grip	Type II	Type II	Sp, h*	Sp, h*	G,h*	Sp,h*	Bimodal

Table 1: Predicted classes of binding affinities of PDZ domains following the scheme of Bezprozvany and Maximov [120]. Where the binding affinity has been experimentally tested, the ligand binding Type is given. Otherwise, the predicted ligand binding type is given using Bezvprozany and Maximov's two-co-ordinate system. Those with asterices are predicted to be Type II binding motifs based on recently reported interactions [134, 135, 156, 159]. *D.mel*: *Drosophila melanogaster*, *D.ps*: *Drosophila psuedobscura*, *A. gam*: *Anopheles gambiae*.

3.2 DGrip expression pattern

To study whether the interaction of DGrip was in fact expressed in the same tissues as the GluRIIC subunit, the localisation of the DGrip transcript was determined via *in situ* hybridisation and immunolocalisation with antibodies specific to DGrip. The GluRIIC subunit is specifically expressed in the postsynaptic muscle cell from before the beginning of embryonic neuro-muscular transmission (stage 13-14), like other postsynaptic GluRs, at stage 12 [42] and then throughout larval development [44, 160]. It would thus be reasonable to see the DGrip transcript expressed in the same tissues and at a time point at or before that of the GluRIIC transcript.

3.2.1 *in situ* hybridisation (Ulrike Prange)

In situ hybridisation showed that the *dgrip* mRNA is indeed expressed in the postsynaptic muscle cells from very early stages. There is little or no signal present in early embryos, suggesting no maternal contribution of *dgrip* mRNA. At stage 9, transcripts appear in the mesoderm, first associated with the gut, and then at stage 10, in a segmental pattern in the somatic musculature. This pattern persists and intensifies in the somatic musculature throughout embryonic development, while not being present in the epidermis. Intriguingly, at stage 17, DGrip transcripts are visible in the embryonic heart, while mGRIP is also expressed in the murine heart. The expression of DGrip mRNA in muscles is well correlated with the timecourse of muscle development. After the process of germ band retraction, the specification of muscle precursor cells takes place from stage 10 until embryonic stage 12, after which point muscle guidance persists until the proper final morphology is reached at stage 16 [56, 150, 161, 162]. The *in situ* results indicate that DGrip mRNA is present during the entirety of the muscle guidance process.

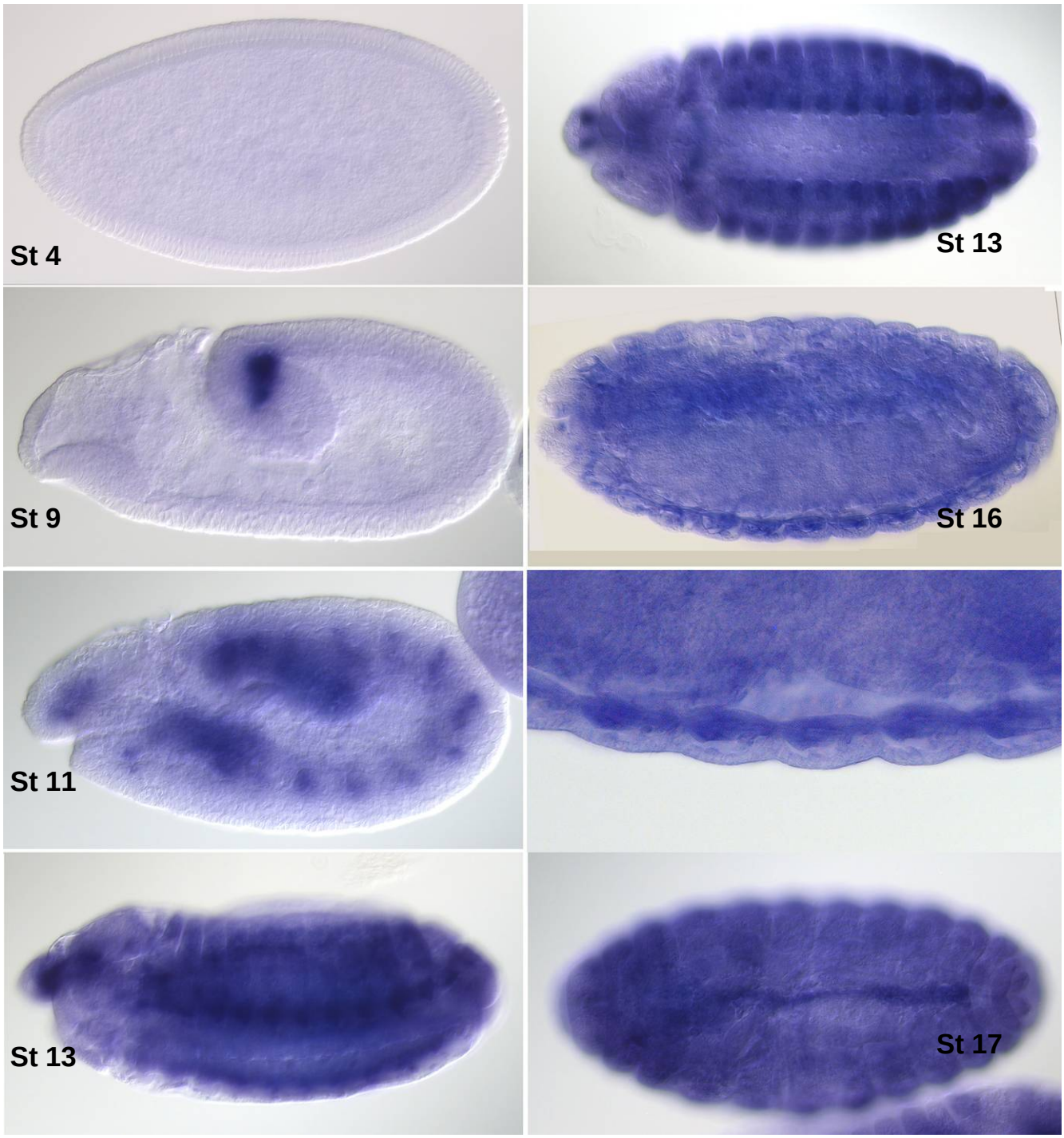


Figure 3: *In situ* hybridisation against DGrip during embryonic development (images from Stephan Sigrist).

3.2.2 Antibody generation

To localise the DGrip protein in *Drosophila* embryos and larvae, three series of antibodies were produced in both rabbits and guinea pigs. Both GST- and 6xHis-tagged fusion proteins, were expressed in bacteria, purified over Glutathione-coupled agarose (GST fusion proteins) or Talon resin (6xHis fusion proteins) and sent for injection (for more details, see **Materials and Methods**).

Antigen	**Company**	**Animal**	**Staining**
pRSET-Antigen2	Cocalico Biologicals	Rabbit 1	
		Rabbit 2	Yes
pGex PDZ1-3		Rabbit 3	
		Rabbit 4	
pGex-PDZ6-7		Rabbit 5	
		Rabbit 6	Yes
pRSET-Antigen2		Guinea pig 1	
		Guinea pig 2	
pGex PDZ1-3		Guinea pig 3	
		Guinea pig 4	weak
pGex-PDZ6-7		Guinea pig 5	weak
		Guinea pig 6	
pGex-PDZ1-3	BioGenes	Rabbit 5007	
		Rabbit 5008	
pGex-PDZ6-7		Rabbit 5015	Yes
		Rabbit 5016	
pQE-PDZ6-7		Rabbit 5009	
		Rabbit 5010	
pGex-PDZ1-3	BioScience	Rabbit 2098	Yes
		Rabbit 2099	
pGex-PDZ1-3		Guinea Pig 2713	Yes
		Guinea Pig 2714	
		Guinea Pig 2715	

Table 2: antibodies produced against DGrip. A map of relevant antigens is shown in **Figure 4** below. The staining pattern given by these antibodies is represented by the stain in **Figure 5**.

In total, 23 sera were produced, seven of which (stemming from different fusion proteins) produced a specific staining pattern. These antibodies were then affinity purified against His-tagged fusion proteins. All antibodies produced the same embryonic staining pattern, shown in **Figure 5** below.

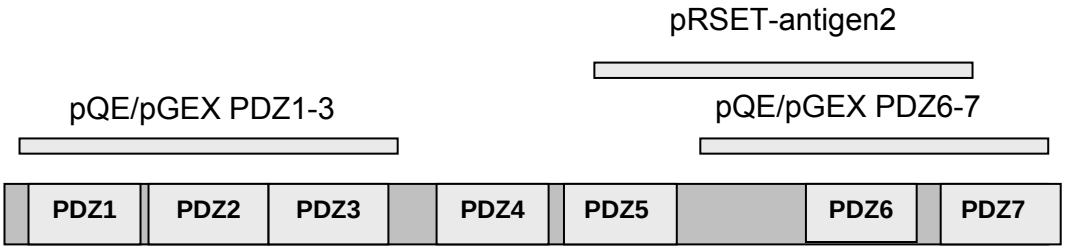


Figure 4: Map of epitopes. Antibodies against DGrip were generated against several fusion proteins (See **Materials and Methods**). Those fusion proteins that produced a specific stain are shown above.

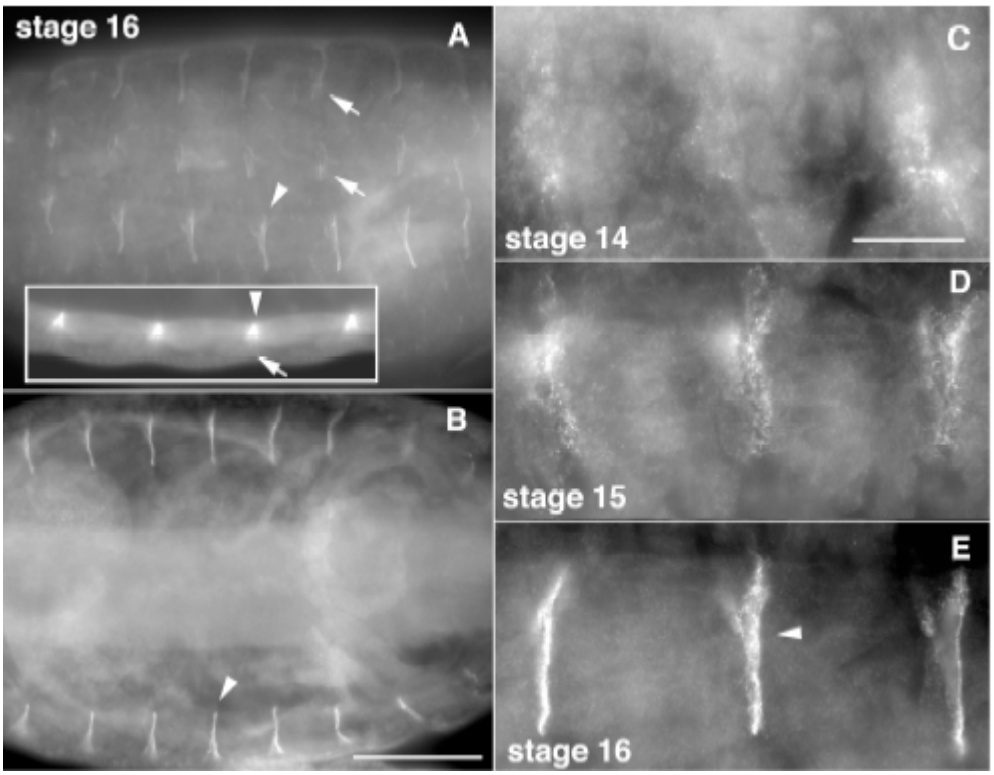


Figure 5: Staining with anti-DGrip antibody. **A,** Ventral view of a stage 16 embryo showing staining in specific groups of segment-spanning muscles. Inset: longitudinal view of the ventral longitudinal muscles (VLMs) showing that staining is present at the ends of muscles, but not in the epidermis. **B.** Dorsal view of a stage 16 embryo. **C-E:** Anti-DGrip staining in muscles condenses from a diffuse, punctuate pattern in muscles to a strong pattern at muscle ends as muscles form their final morphology.

3.3 Mutagenesis of DGrip

To produce mutants of the *dgrip* locus, two P-elements (P{y[+mDint2] w[BR.E.BR]=SUPor-P}KG02862a and P{w[+mGT]=GT1}BG01736, w[1118]) inserted 5' and 3' respectively of the locus that produces the DGrip cDNA, RE14068 (Bloomington) were utilised. More details of how P-element based mutagenesis is accomplished are given in **Section 2.2**.
Several deletions affecting the *dgrip* locus were isolated, stemming from the two independent P-element insertions, as well as so-called 'precise' excisions, where only the P-element itself is removed without deleting any of the surrounding chromosome.
The two mutant chromosomes shown below present the same phenotype, even though they arise from deletions of the *dgrip* locus derived from independent P-element insertions. Some deletions isolated in the two screens are shown below (**Figure 6**).

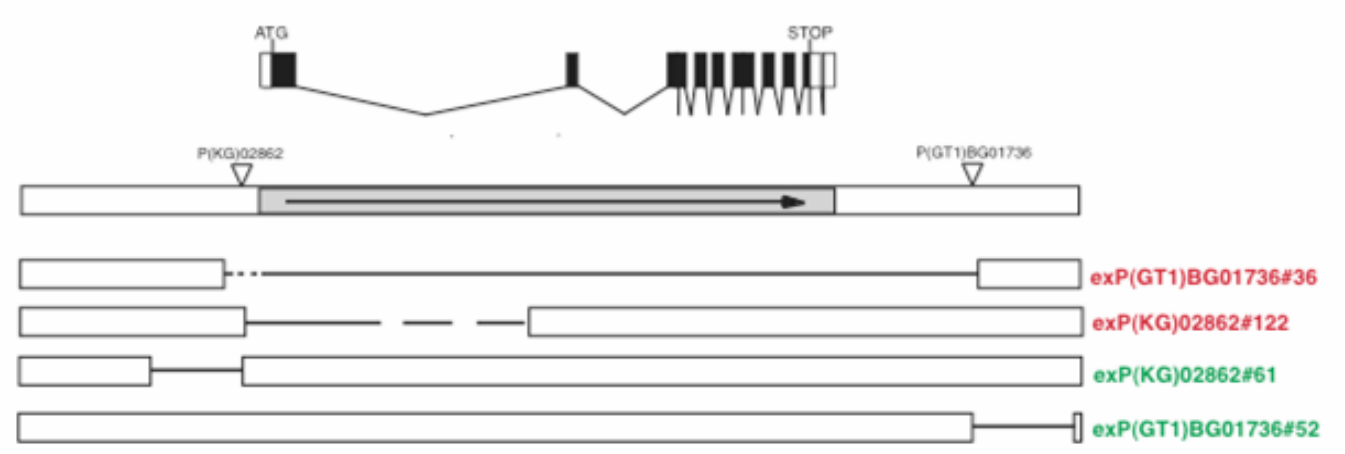


Figure 6: Mutants of the DGrip locus. All deletions of the *dgrip* locus (red text) present the same muscle phenotype, whereas precise excisions of the P-elements and excisions running away from the *dgrip* locus (green text) do not.

3.4 DGrip mutant phenotype

The phenotypes arising from the loss of the *dgrip* genomic locus were then characterised. The mutant chromosomes were tested by immunostaining and real-time PCR to identify if they still produced DGrip protein or mRNA (not shown). From this data, it was concluded that the $dgrip^{ex36}$ chromosome was a null mutant of DGrip and that $dgrip^{ex122}$ was a strong hypomorph. As DGrip is an X chromosomal gene, males are full mutants of the gene, whilst females carry one wild-type X chromosome. The following studies were carried out in mutant males, unless otherwise indicated. Several phenotypes arising from loss of the zygotic DGrip transcript were identified, two of which we studied in further detail. The two most dramatic defects were a specific inability of one group of embryonic muscles to form their proper morphology (treated in **Section 3.4.1**) and a very strong potentiation of Ca^{2+}-dependent neurotransmission in motor neurons of the larval neuromuscular synapse (**Section 3.10**).

3.4.1 DGrip mutant muscles show muscle-type specific guidance defects

The loss of the zygotic *dgrip* gene in *Drosophila* embryonic muscles has been the subject of a recent paper (see attached manuscript): Herewith, a brief treatment of the data presented in this paper, and a more detailed treatment of those data not shown in that article.

As demonstrated in our paper, the loss of DGrip has no effect on cell fate specification, and also no significant effect on the number of cells incorporated in the polynucleic muscle cells. Both *dgrip* alleles, $dgrip^{ex36}$ and $dgrip^{ex122}$, produce the same phenotype. Loss of DGrip leads to a dramatic and specific loss of the ability of a particular group of muscles to form their proper morphology (**Figure 7**). This group of muscles, the ventral longitudinal muscles (VLMs), are muscles which normally span each body segment, making indirect contact with an epidermal anchoring cell (the apodeme, or tendon cell) via aggregations of extracellular matrix, where several segment-spanning muscles connect together (For a schematic drawing, see **Figure C**).

Other mutants [163, 164] have been reported where muscle cells make initial contact with the apodeme, but are too weakly adhesive to remain attached, and then collapse when put under strain. This in principle could form the same collapsed muscle shape as shown in the $dgrip^{ex36}$ mutant. This in fact not the case, as not only is this aberrant muscle morphology retained throughout larval life, where these muscles are subjected to considerable strain from locomotion, but these misshaped muscles are able to ectopically recruit extracellular matrix to adhesion points in the middle of muscle segments, allowing the anchoring of the mutant muscle via integrins (**Figure 8**).

As muscle fate was not altered in *dgrip* mutants, and muscle cells are able to adhere to the epidermis, the intervening process of muscle guidance seemed to be compromised. We therefore conclude that DGrip VLMs are unable to respond properly to a guidance cue, which directs muscles to their proper target, but are otherwise capable of forming an adhesive and functional muscle. This interpretation was strengthened by imaging a specific VLM, muscle 12, over muscle development. It was clear that whereas wild-type muscle precursors extended one process across the segment to reach its future contact site,

dgrip^{ex36} muscles extend one or more processes in essentially random directions, and are considerably slower than wild-type muscles to grow in the proper direction (this experiment performed by Andreas Schmid).

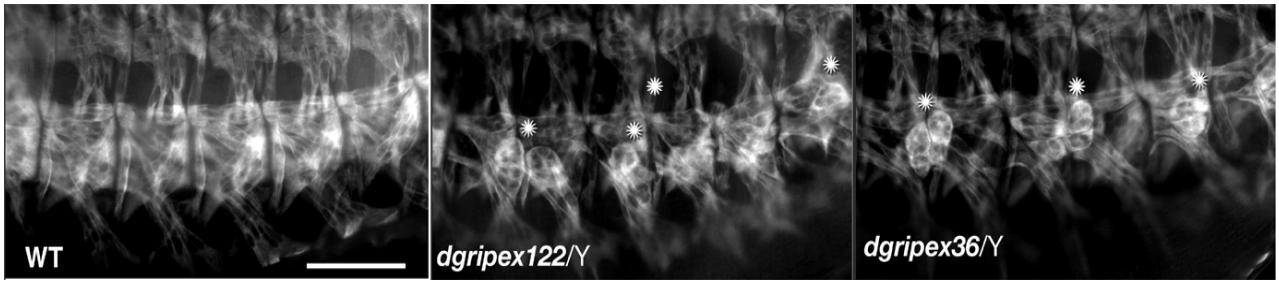


Figure 7: Muscle morphology of the ventral longitudinal muscles (VLMs) is disturbed (asterisk) in both a hypomorphic (*dgrip*^{ex122}) and a null (*dgrip*^{ex36}) allele of the DGrip locus.

Strong overexpression of a transgene encoding the DGrip cDNA was able to change the morphology of another group of muscles, the lateral transverse muscles (LTMs) (**Figure 9**). This class of muscles are not affected in the loss-of-function mutant of DGrip. These muscles normally have a bar-like shape, and make their contact with an individual apodeme cell within each segment, not at the segment border. Overexpression of DGrip caused the LTMs to form several long projections, which bend to make ectopic contact at the segment border (**Figure 9**), indicating that the LTMs had become ectopically sensitive to a guidance cue, further suggesting that DGrip was able to ectopically program a different mode of cell guidance (perhaps increased sensitivity to a certain ligand).

The muscle phenotype presented above could be explained by two separate phenomena, firstly, DGrip may be a factor which, like many PDZ domain proteins, acts as a polarity-determining molecules as do *Drosophila scribble* [165] and *bazooka* [166] in other tissues, and is involved in establishing the initial polarity of the muscle cell.

If this were the case, then receptors for guidance cues would be mislocalised due to a general absence of properly established polarity, thus causing cells to form random extensions depending on where functional receptors can be trafficked to. Alternatively, DGrip may act downstream of the establishment of cell polarity, as a receptor trafficking or signalling factor, and that the observed morphology is caused by mis-trafficking or mis-signalling from a receptor in an otherwise correctly polarised muscle.

To investigate if *dgrip*^{ex36} muscles show a general lack of muscle polarisation, a Nod:β-gal fusion protein was expressed in the *dgrip*^{ex36} mutant background, driven by the mesoderm-specific driver *twist-gal4* [161]. The Nod protein is a minus-directed kinesin-related motor protein [149], which when fused to β-gal, acts as a reporter of the minus-end of microtubulae. *dgrip*^{ex36}, *twist-gal4*::*UAS-Nod:β-gal* males and +/*dgrip*^{ex36},*twist-gal4*::*UAS-Nod:β-gal* females were identified and the distribution of Nod:β-gal staining compared. +/*dgrip*^{ex36},*twist-gal4*::*UAS-Nod:β-gal* females act as controls as they have no muscle phenotype due to the presence of the wild-type X chromosome.

If the muscle cell was unpolarised, the characteristic staining of Nod:β-gal would disappear, as no polar aggregates of microtubules would be present. If the defect was less severe and the microtubules could form microtubule organising centres, one might expect that the position of these would be shifted to one side of the cell, instead of in the centre of the cell. Examination of these animals showed that neither was the case. Comparison of

dgrip^{ex36} males with the control showed that there was no mislocalisation of the Nod:β-gal fusion protein (**Figure 10**), and that β-gal immunoreactivity concentrated in the centre of muscle cells. This lends weight to the hypothesis that general muscle polarity is normal in *dgrip*^{ex36} mutants, and that DGrip is involved in organising response to guidance cues.

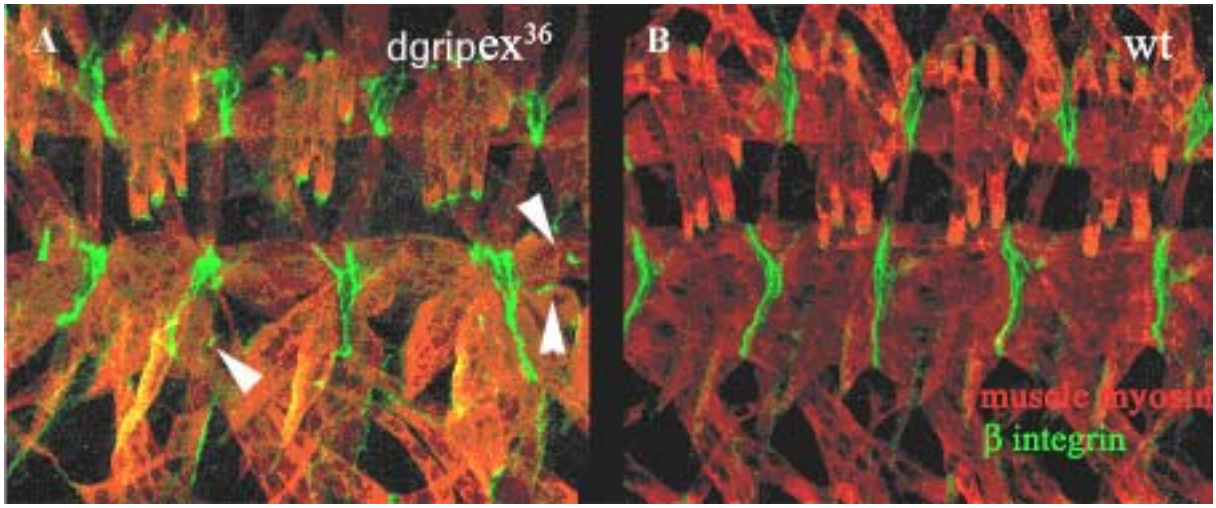


Figure 8: A. *dgrip*^{ex36} mutant muscles are capable of expressing the adhesion molecule integrin at ectopic attachment points (arrowheads), **B**. wild-type control.

This second possibility, that DGrip is a factor involved in a specific receptor signalling or trafficking pathway, requires a closer understanding of what pathway DGrip may be operating in. A screening approach to look for candidate genes was used (**Section 3.7**), and candidate genes were identified based on known phenotypes. It is known that VLMs are known to be guided by the Slit-Robo ligand-receptor system, better known for its role in axonal guidance, but an interaction between the DGrip and Robo-dependent guidance pathways has not conclusively been demonstrated. For a more detailed account of experiments to uncover a role of DGrip in Robo-Slit signalling see **Section 3.7.1** below.

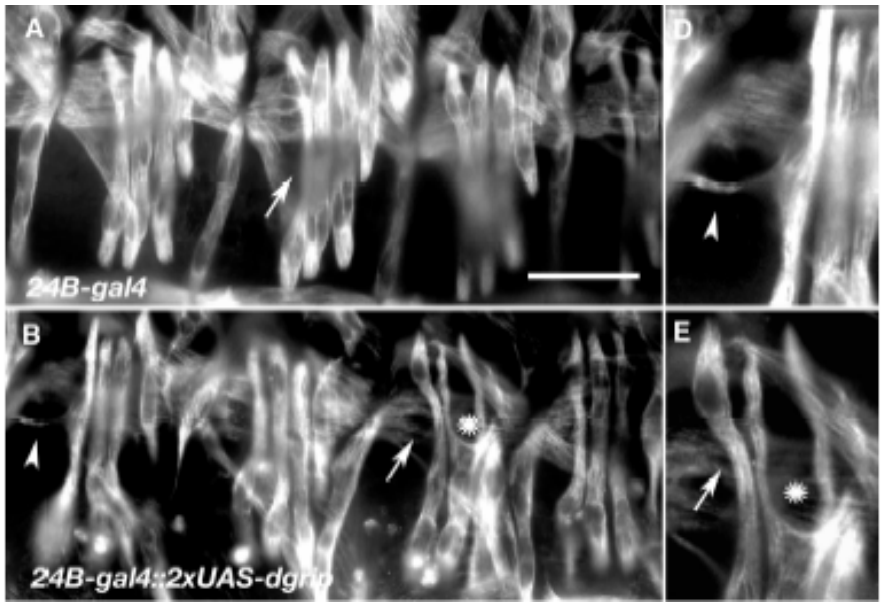


Figure 9: Overexpression of two copies of a *UAS-dgrip* transgene with the muscle-expressed driver *24B-gal4* causes the LTM group of muscles (arrows, control, **A**) to form extra processes (**B-E**, asterisks), which extend towards the segment border, rather than to their normal attachment sites which are within the same hemisegment

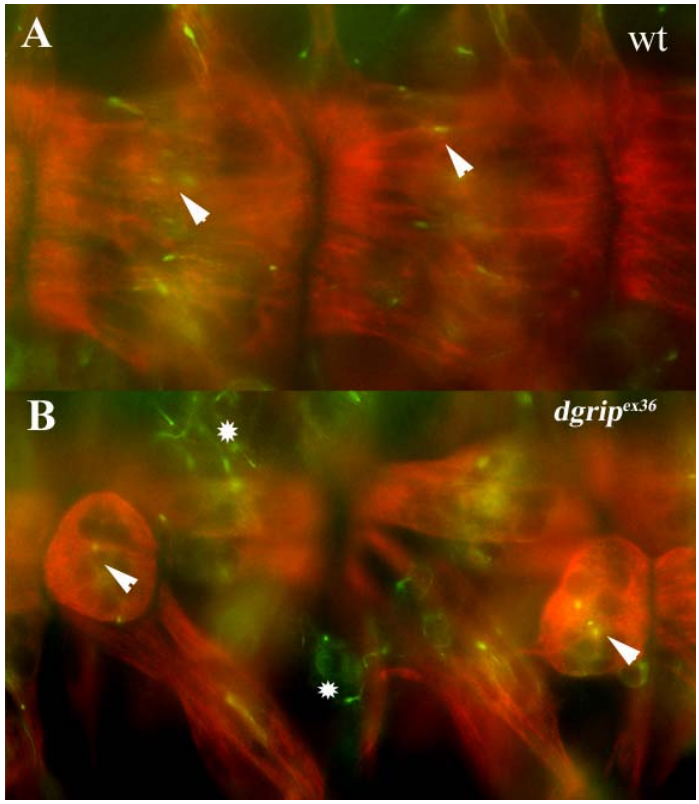


Figure 10: The position of microtubule minus ends is not changed for *dgrip*^{ex36} mutants. Expression of the polarity marker Nod:β-gal (green) expressed in wild-type (**A**) and *dgrip*^{ex36} (**B**) backgrounds with the driver *twist-gal4* reveals that in both situations, microtubule minus ends (arrows) are oriented correctly in muscles (stained by anti-muscle myosin (red)). Green staining in the lower panel, not colocalised with the red muscles (asterices), are other Nod:β-gal expressing cells.

3.5 A structure-function characterisation of DGrip function

What are DGrip's seven PDZ domains used for, and how do they function together? Given the vast array of possible combinations of ligand-binding partners, it seemed possible that loss of DGrip may affect many different signalling pathways. In the DGrip muscle guidance phenotype, a fully penetrant (100% of hemizygous males are affected), and easily scorable defect was identified, which lends itself to genetic analysis. Using the established advantages that *Drosophila* displays as a genetic model system, one can then efficiently screen for functional domains of the protein and their interactors. The rescue of the muscle phenotype requires the expression of one isoform of DGrip [147] (it is not yet clear how many isoforms of DGrip there may be). To understand whether DGrip has its own molecular logic in dealing with interactions over various domains, a rescue assay was designed to study the function of each domain of the protein.
Virgins carrying one $dgrip^{ex36}$ chromosome and the muscle-specific driver *twist-gal4* were crossed to animals homozygous for transgenes expressing mutated forms of DGrip. The rescue function of the transgene in muscles was assayed in males which are $dgrip^{ex36}$/Y, (indicated as $dgrip^{ex36}$) hemizygous for the mutant chromosome. Females $dgrip^{ex36}$/+ were used to analyse dominant defects in muscle guidance.
Plentiful *in vitro*, structural and cell-culture studies strongly suggest some functions for mGRIP, although none of these have been demonstrated *in vivo*. Thus, understanding how these motifs interact with one another represents a necessary step to uncover how DGrip can mediate multiple functions, and in what way several ligands converging on pathways in the same synapse (such as the Ephrin [127, 134-136] and Liprin [132, 133] pathways) can be integrated. Based on structural studies on mammalian GRIP domains [113, 137, 138, 140, 156-158, 167], and on conservation in DGrip, several putative motifs apart from the seven known PDZ domains were identified. These were namely palmitoylation, dimerisation and potential kinesin binding motifs.

3.5.1 Palmitoylation

Previous studies have investigated the role of palmitoylation of mGRIPs[137, 138]. The mGRIP2/ABP isoform has a splice variant, ABP-L, which associates with synaptic clusters of GluR2 in cell culture, whereas the non-palmitoylated form of ABP is associated instead with intracellular GluR2 clusters, suggesting a likely functional difference between the two isoforms. In comparing DGrip with the palmitoylated and non-palmitoylated forms of mGRIP2/ABP, a putative N-terminal motif was identified in the first exon of DGrip (**Table 3**). Palmitoylation is a post-translational modification that consists in the addition of a 16 carbons fatty acid, palmitate, to a cysteine residue through the creation of a thioester link. Unlike other lipid modifications such as myristoylation and prenylation, which are irreversible, palmitoylation is a dynamic modification involving palmitoylation/depalmitoylation cycles suggesting a regulatory role. We attempted to show a functional relevance for this motif in three ways: by demonstrating

DGrip palmitoylation biochemically in cell culture, by demonstrating a change in DGrip distribution in *Drosophila* muscle on point mutation of the putative motif and by demonstrating a lack of rescue of the muscle defect when expressing a point mutated version in the *dgrip*^{ex36} background.

Non-palmitoylated forms	
mGRIP-1a	MIAVSFKCRCQILRRLTK
mGRIP2/ABP-L	MLAVSLKWRLGVVRRRPK
Palmitoylated forms	
mGRIP1b	MPGWKKNIPI**C**LQAEEQER
pABP-L	MRGWRRNLAL**C**LQRLPDEDD
DGrip	MKLWKSKKPIGG**C**VPGKSAA

Table 3: Sequence comparisons between the N-termini of palmitoylated and non-palmiltoylated forms of mGRIP. The putitatively palmitoylated N-terminal sequence for DGrip is included for comparison. The cysteine to which palmitate is anchored is indicated in bold.

For the biochemical analysis of DGrip palmitoylation, two baculoviruses, Baculo-DGrip and Baculo-DGrip-myc, with a c-terminal myc tag (see **Materials and Methods**), were created. These viruses were used to transfect Sf9 cells which were incubated for 24 hours before being labelled with either [S35]-Met (to label the total amount of protein manufactured) or [^{3}H] palmitic acid (to label palmitoylated proteins). These cell cultures were labelled for three hours before being lysed and the lysates immunoprecipitated with antibodies against DGrip or myc. These were compared against radiolabelled immunopreciptates from control cultures not infected with a virus, or infected with an empty baculovirus as controls. The anti-DGrip antibody was not able to efficiently precipitate DGrip from the control [S35]-Met –labelled lysate and so the anti-myc precipitated Baculo-DGrip-myc lysates were analysed. Only a very weak, specific band of palmitoylated protein at approximately 120kDa in Baculo-DGrip lysates (**Figure 11**) was identified, but more robust labelling was not found despite several repetitions or with the Baculo-DGrip-myc construct, and precipitation with an anti-myc antibody. Thus a weak palmitoylation of DGrip is possible, but the assay was not robust enough to study DGrip palmitoylation in detail.

It was then examined if palmitoylation of DGrip has a functional consequence for muscle guidance. For this, three transgenes were constructed: *UAS-dgrip-C13S, UAS-dgrip-GFP* and *UAS-dgrip-C13S-GFP*, where the C13S is a point mutation to destroy the putative N-terminal palmitoylation motif (**Materials and Methods**, **Table 3**). Expressing DGrip-C13S in *dgrip*^{ex36}*,twist-gal4* animals was enough to rescue muscle guidance, even when the transgene was expressed at minimal levels (embryonic raising temperature 18°C), indicating that this motif is not necessary for muscle rescue function.

Curiously, when expressing DGrip-GFP and DGrip-C13S-GFP with the muscle driver *G14-gal4*, a slightly different distribution of the GFP positive signal in larval muscle was detected (**Figure 12**), despite the fact that both GFP transgenes rescue the embryonic muscle defect. This may indicate that the putative palmitoylation motif is in fact functional, but that it is not essential for DGrip's function in muscle guidance.

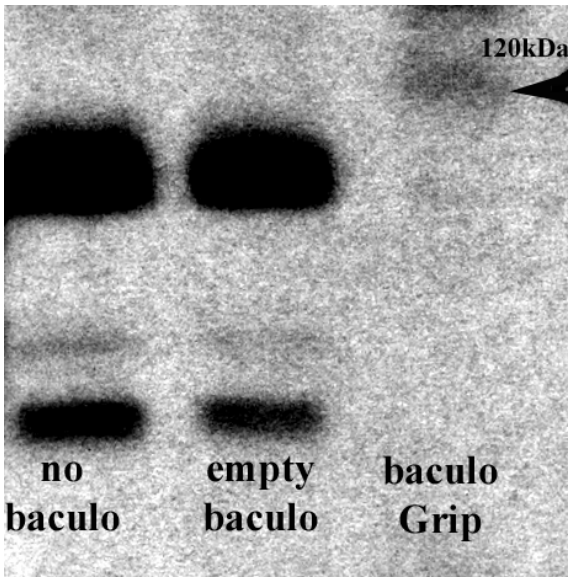


Figure 11: A weak band of palmitoylated protein at 120kDa is seen in anti-DGrip precipitates of Baculo-DGrip transfected Sf9 cells. Strong bands, which are non-specifically labelled material, are present in controls, and serve to show that the film exposure was sufficiently long to detect a signal.

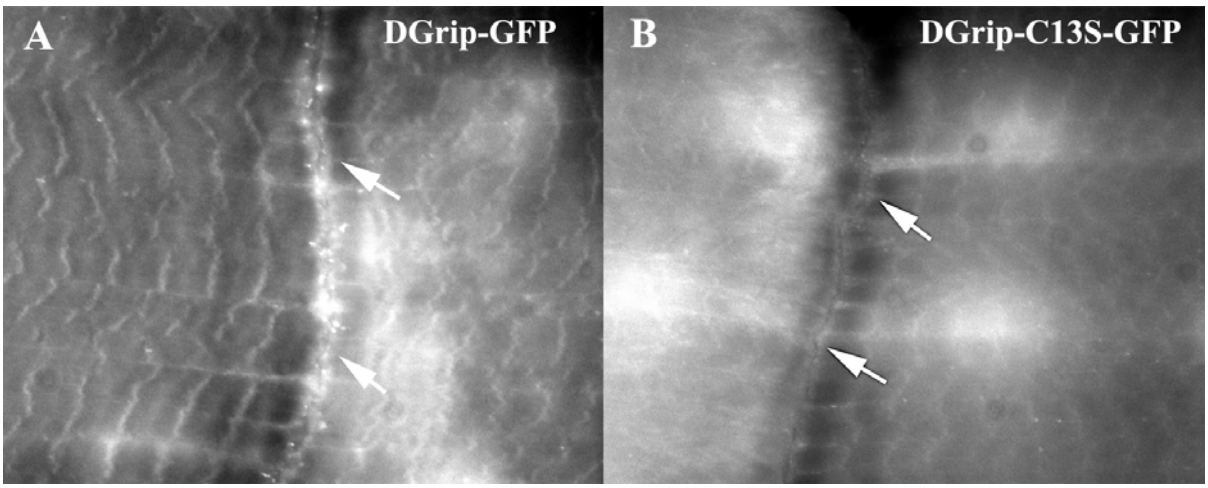


Figure 12: The distribution of DGrip-GFP and DGrip-C13S-GFP differs in larval muscles, when driven by the muscle driver *G14-gal4*. Arrows indicate the border between two VLM muscles, where DGrip-GFP, but not DGrip-C13S-GFP, aggregates. This pattern persists also when these transgenes are expressed at lower levels by raising animals at 18°C.

3.5.2 Dimerisation

Several investigators have remarked on the possibility of mGRIP homo or hetero dimerisation [110, 137, 156, 157] between mGRIP1 and mGRIP2, based on dimerisation of the PDZ456 tandem cluster of PDZs. However, little has been shown in terms of a functional consequence of this dimerisation. One crystallographic study [156] was able to show that the disruption of a single amino acid just N-terminal to PDZ 6 (Y671D) was able to disrupt dimers formed by PDZ6. This N-terminal sequence was also conserved in *Drosophila*, despite the fact that the relative positioning of PDZ6 relative to the PDZ 45 tandem is altered.

DGrip	II**F**TVRLEP
mGRIP1	II**Y**TVELKR

Table 4: Comparison of sequences N-terminal to PDZ6 for mammalian and *Drosophila* Grips. In bold is the residue point mutated to Asp in a previous study[156], and in this study.

Two approaches were taken to study the possibility of DGrip dimerisation: the first was using the FRET/FLIM technique to look for an interaction between wild-type transgenes labelled with GFP and with tdimer2 (a self-dimerising RFP variant). Both fluorophore-tagged constructs were found to be fully functional in terms of their ability to rescue the *dgrip*^{ex36} muscle defect (not shown). A genetic approach was also taken to see if the motif identified was necessary for muscle rescue.

Fluoresecent Lifetime Imaging (FLIM) was used to determine the characteristic lifetime of GFP-tagged constructs. If the GFP and tdimer2-tagged proteins were in close enough proximity, then Forster Resonance Energy Transfer (FRET) could occur, reducing the amount of time GFP remained in the excited state. It is this phenomenon which is measured by lifetime measurements.

A limiting problem for all uses of GFP variants in *Drosophila* is that all GFPs are bleached by fixation methods, meaning that fixation of tissue can only be minimal (10 to 30 seconds allows almost full preservation of the signal) before the endogenous GFP signal is lost. In the setup used for these experiments, the FLIM module is coupled to a two-photon laser. This means that excitation generates a large amount of heat, forcing a compromise between the length of data acquisition and physical destruction of the sample, particularly in the case of weakly fixed samples, which contain a large amount of water. Modification of the strength of the two-photon excitation did not improve the number of photons collected before the sample was destroyed. By modifying the mounting media used, data could maximally be acquired for five minutes (approximately 1,000 - 4,000 photons per pixel) from dissected larvae. The population of photons collected, when combined with strong auto-fluorescence from larval muscle preparations, only allowed the identification FRETting populations if the FRET efficiency was above 20%. FRET efficiencies of this magnitude between DGrip-GFP and DGrip-tdimer2 (**Figure 13**) were not identified. This naturally does not preclude the existence of dimerisation between DGrips.

As to the genetic approach, a point mutation (F->D) was introduced in the putative DGrip dimerisation motif to create DGrip-ND (see **Table 4**). In a previous crystallographic study [156], this had the effect of abolishing dimers between PDZs 6, although this has not been proven to be the case *in vivo*. Transgenic flies bearing the *UAS-dgrip-ND* construct were examined for their ability to rescue the *dgrip*^{ex36} muscle defect. This transgene was fully able to rescue the *dgrip*^{ex36} muscle defect, indicating that this conserved motif is not necessary for DGrip function in myotube guidance.

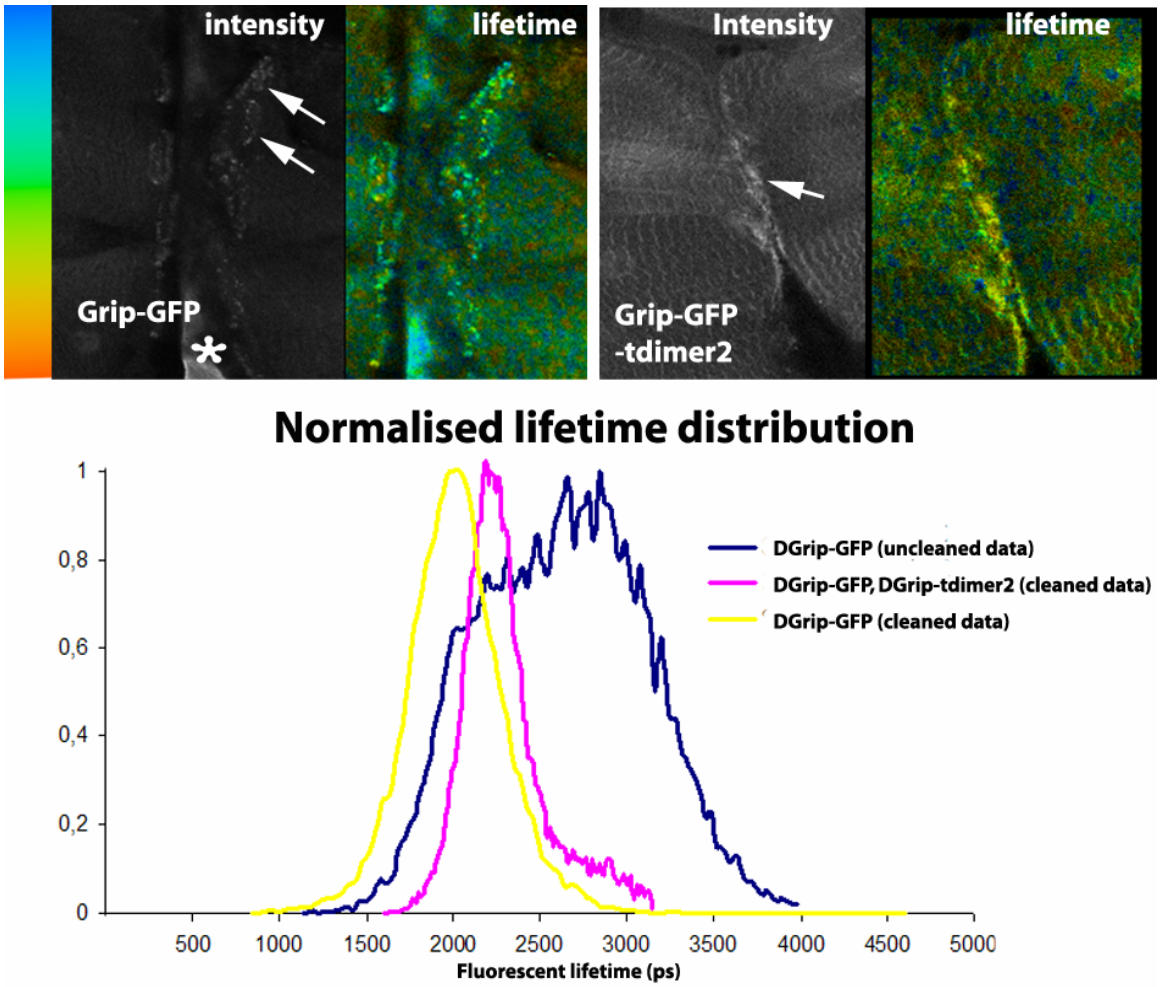


Figure 13: FLIM-based FRET experiments provide no conclusive data on DGrip dimerisation. Upper panels show two-photon intensity images of the ends of larval muscles expressing DGrip-GFP or co-expressing DGrip-GFP and DGrip-tdimer2. No photons were collected from animals expressing DGrip-tdimer2 only, indicating that there was no spectral contamination from the tdimer2 fluorophore. Arrows indicate aggregates of DGrip-GFP. The asterisk indicates strong autofluorescence from tissue not expressing DGrip-GFP. Lifetime images are presented with a colour scale running from 1000ps (red) to 4000ps (blue). The normalised lifetime distribution is strongly contaminated by membrane autofluorescence (blue line). The data was 'cleaned' by accepting only pixels where the lifetime was fitted with $\chi^2 < 2.5$, and where the intensity of the two-photon signal was comparable with images obtained with the AOBS confocal image made of the DGrip-GFP signal, to exclude as much membrane autofluorescence as possible (yellow and pink lines).

3.5.3 PDZ binding

The seven PDZ domains of DGrip were the most probable interaction motifs capable of organising the DGip muscle guidance process. The approach of deleting large parts of the *UAS-dgrip* transgene was taken to coarsely orient as to which domains are necessary for function. These same domains were also reintroduced fused to the DGrip Kozack sequence and start codon. The rescue ability of these constructs was assessed in *dgrip*^{ex36} males by muscle specific expression with *twist-gal4*. Simultaneously, in females carrying one copy of the wild-type DGrip, dominant defects in embryonic or larval musculature were assayed. The results of this first, orientation screen are presented below in **Figures 14 and 15.**

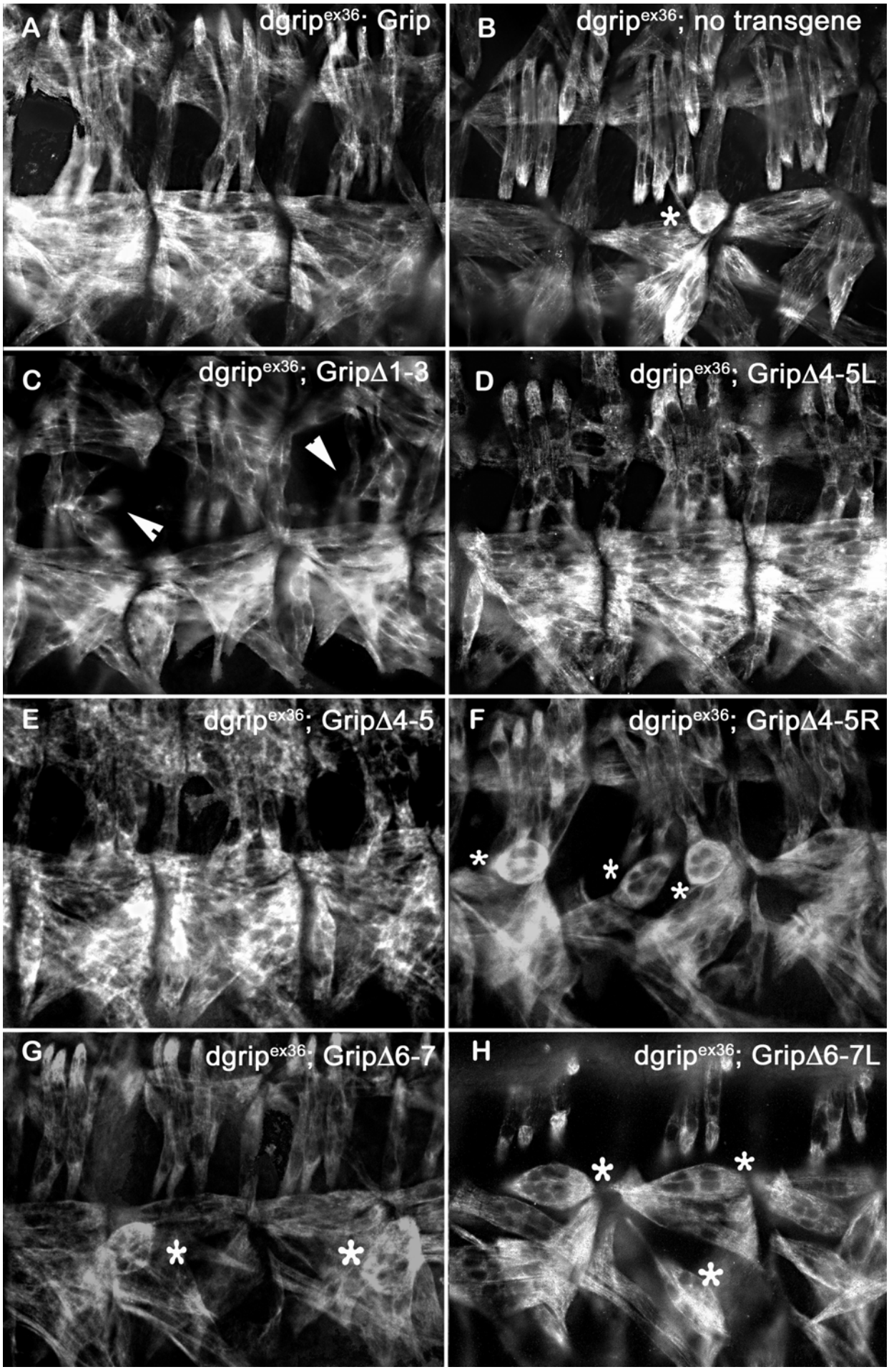
The results of this screen provided several interesting structural motifs in the DGrip protein. First, the muscle function of DGrip is regulated by PDZ domains 1-3 of the protein, which is treated in **Section 3.5.4**.

Second, PDZ domains 4 and 5 appear to have no function in rescuing the *dgrip*^{ex36} muscle defect, as transgenes missing these domains behave in the same way as the complete DGrip cDNA in terms of muscle rescue. Furthermore, when strongly overexpressed (using the stronger muscle driver *24B-gal4* at 29°C) the DGripΔ4-5 protein elicits overexpresion defects in the same manner as overexpression of DGrip, suggesting DGripΔ4-5 is a fully functional protein in terms of muscle function (not shown).

Thirdly, it could be demonstrated that the rescue of the *dgrip*^{ex36} muscle defect is dependent on the function of no single PDZ. Removal of PDZs 6 and 7 produces a transgene which can only partially rescue the *dgrip*^{ex36} muscle phenotype (**Figures 14 and 15**), indicating PDZs 6 or 7 are responsible for at least part of the rescue function, but constructs containing PDZs 6 and 7 only do not improve the *dgrip*^{ex36} phenotype (see **Figure 15**). Nor do any transgenes deleting any single tandem group of PDZs show a complete loss-of-function phenotype, suggesting that multiple domains of the protein are involved in muscle guidance and that they can to some extent compensate for one another.

Following Page:

Figure 14: An analysis of rescue function using mutant DGrip transgenes. *dgrip*^{ex36}, *twist-gal4*/FM7*ftz*::*lacZ* virgins crossed to **A.** *UAS-dgrip* **B**. no transgene **C**. *UAS-dgripΔ1-3* **D**.*UAS-dgripΔ4-5L* **E.** *UAS-dgripΔ4-5* **F.***UAS-dgripΔ4-5R* **G**.*UAS-dgripΔ6-7* **H**.*UAS-dgripΔ67L*. Embryos were stained with an anti-muscle myosin antibody to visualise somatic musculature. DGripΔ1-3 produced abnormal morphologies in the LTM group of muscles (**C**), while several constructs gave an incomplete rescue of VLM defects (Compare **B** with **F,G,H**). These results consistently identified in at least two independent transgenic lines per construct.


A
dgrip^{ex36}; Grip
B
dgrip^{ex36}; no transgene
C
dgrip^{ex36}; GripΔ1-3
D
dgrip^{ex36}; GripΔ4-5L
E
dgrip^{ex36}; GripΔ4-5
F
dgrip^{ex36}; GripΔ4-5R
G
dgrip^{ex36}; GripΔ6-7
H
dgrip^{ex36}; GripΔ6-7L

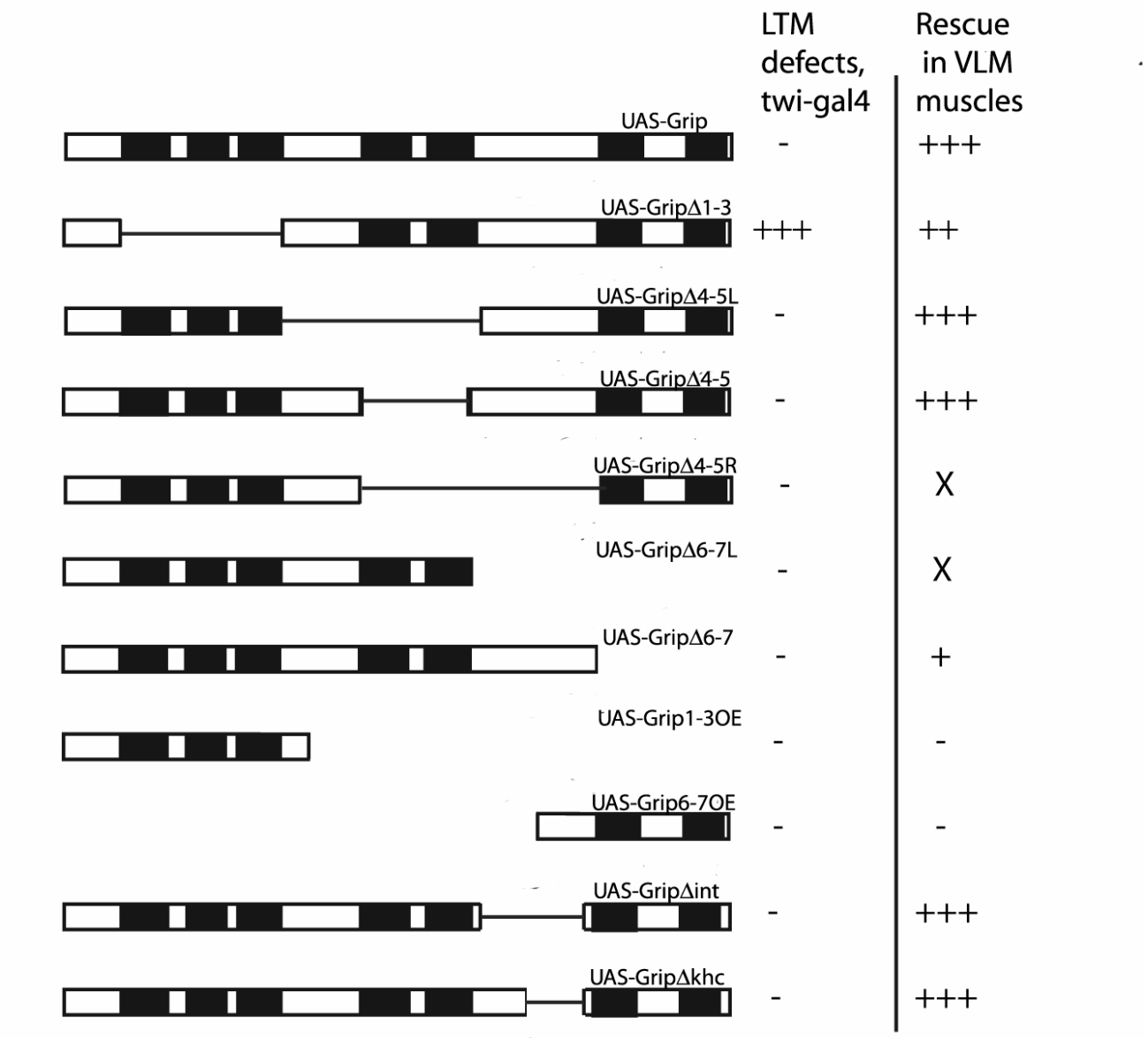


Figure 15: Summary of mutant phenotypes, examined in both embryos and larvae, performed with the driver *twist-gal4* at 25°C. The *UAS-dgripΔ1-3* transgene was the only transgene to elicit muscle morphologies such as those seen with strong DGrip overexpression (see below for a more detailed treatment). No single domain is essential for the rescue function of the DGrip transgene. Key: +++ complete rescue, +/++ partial rescue, - no rescue, X, more severe muscle disturbance than *dgrip*^{ex36}.

3.5.4 DGripΔ1-3 is a dominant active form of DGrip

As low levels of DGripΔ1-3 expression phenocopies very high levels of DGrip expression (**Figures 9, 14 and 16**), it was suspected that DGripΔ1-3 was a dominant active form of DGrip. This would naturally suggest that the PDZ domains 1-3, or some subset of these domains, exert a repressive effect on muscle guidance functions. It was necessary to study the function of PDZs 1-3 of the DGrip protein in more detail, first from a genetic standpoint, to then be able to move to a functional understanding of the repression mediated by these PDZ domains.
Firstly, the defects arising from expression of DGripΔ1-3 was characterised in more detail. As defects in the embryonic muscle system were harder to identify, the majority of experiments were carried out in larvae, stained for F-actin by phalloidin toxin to visualise the larval musculature. To confirm if the expression of the DGripΔ1-3 protein was dominant, both *dgrip*^{ex36}, *twist-gal4*::*UAS-dgripΔ1-3* and +/*dgrip*^{ex36},*twist-gal4*::*UAS-dgripΔ1-3* larvae were dissected to see if these larvae displayed the same phenotype. DGripΔ1-3 expressing animals showed the same muscle phenotype regardless of the presence of one intact copy of DGrip, indicating that the action of DGripΔ1-3 is dominant (**Figures 16 and 18**). The mild defects in VLM muscles (**Figure 18**), are also present in both genotypes. That suggests that DGripΔ1-3 is also dominant in VLMs, where there is positive evidence of endogenous DGrip expression (**Figure 5**).

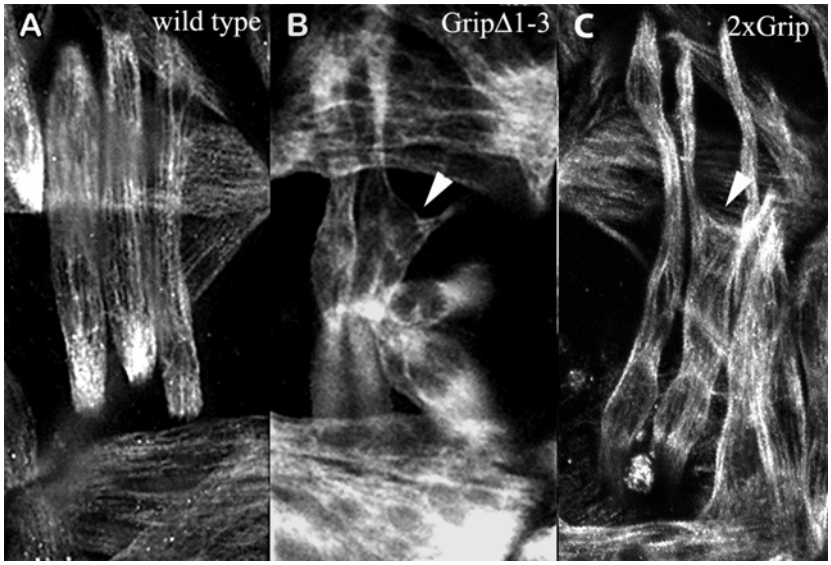


Figure 16: Low levels of a construct missing PDZ1-3 show the same phenotype as very high levels of ectopic DGrip when expressed in the LTM group of muscles. **A**. Wild-type **B**. *twist-gal4*::*UAS-dgripΔ1-3* driven at 18°C (minimal expression conditions). **C**. *24B-gal4*::*2xUAS-dgrip* driven at 29°C. Arrows indicate LTM muscles, which from multiple processes, bending towards the segment border. Muscles visualised with an anti-muscle myosin antibody.

DGripΔ1-3-expressing LTMs show defects which are highly indicative of DGrip having a role in muscle guidance. The LTMs form more than one long, filopod-like extension, which then attaches to ectopic targets, sometimes at the segment border. As the defect becomes more severe, more processes are formed by the muscle, forming multiple muscle subsegments, 'ramifications', which form contacts to one another and to ectopic targets.

To quantify the extent of muscle defects in these various mutants, a scoring system for the severity of muscle defects in either the VLM or LTM groups of muscles was devised. Five categories of muscle defects were identified, ranging from a score of 0.2 for normal wild-type morphology, to 1.0 for the most severe defects.

In the LTM group of muscles the score was as follows:
0.2: normal morphology
0.4: slight segmentation: LTM muscles split into two processes
0.6: ramification: LTM forms multiple processes, which make contact with oneanother
0.8: loss of orientation: LTM group of muscles form multiple, ramified processes, and the main axis of the muscles is no longer perpendicular to the VLM muscles.
1.0: Complete loss of orientation: LTM muscles form fan-like processes and are no longer individually identifiable.

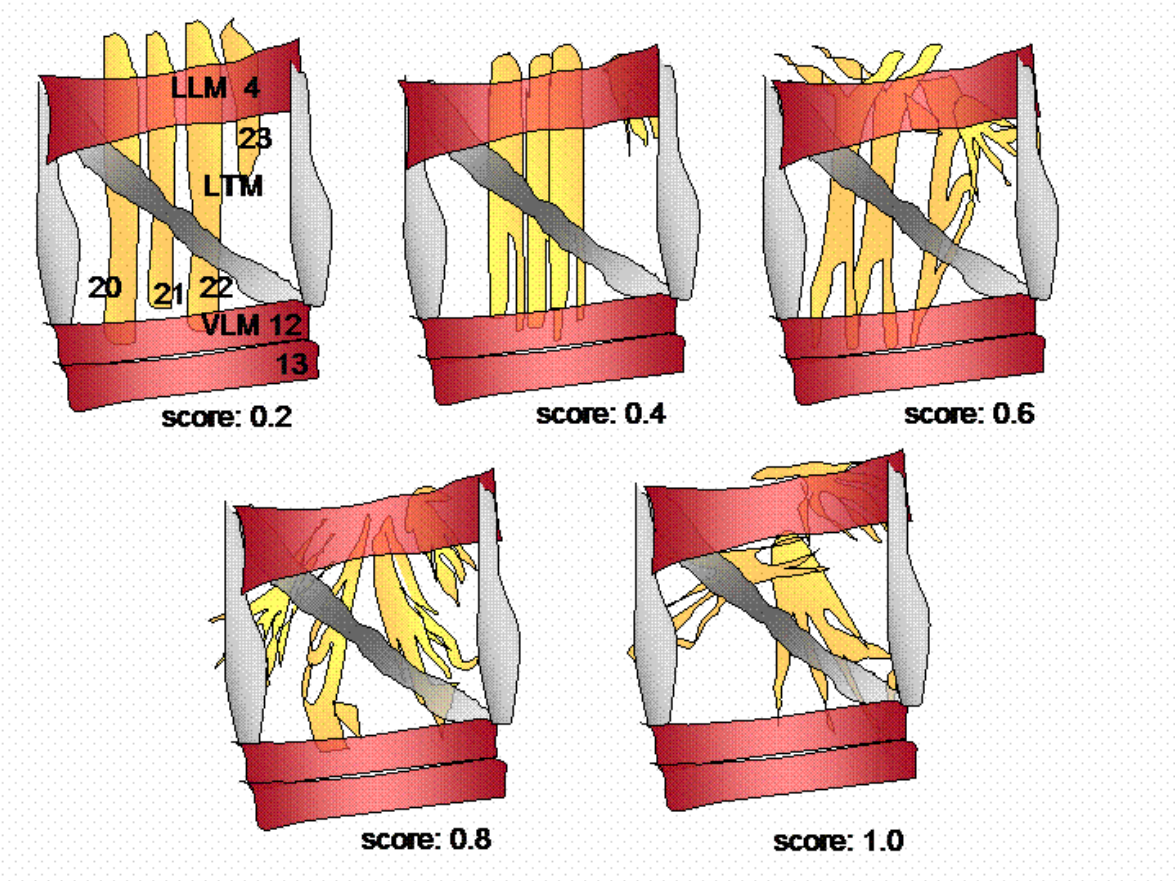


Figure 17: schematic representation of classes of LTM defects.

These phenotypes were scored in each hemisegment of larval muscles stained with phalloidin (which visualises F-actin), in at least three animals for every expression condition, and the average score plotted. It was confirmed that expression of DGrip does not produce a strong phenotype in the LTM group of muscles when expressed with *twist-gal4*; whereas DGripΔ1-3 can elicit strong LTM defects, such that DGripΔ1-3 animals are immediately identifiable, whether endogenous DGrip is present or not (**Figure 18**).

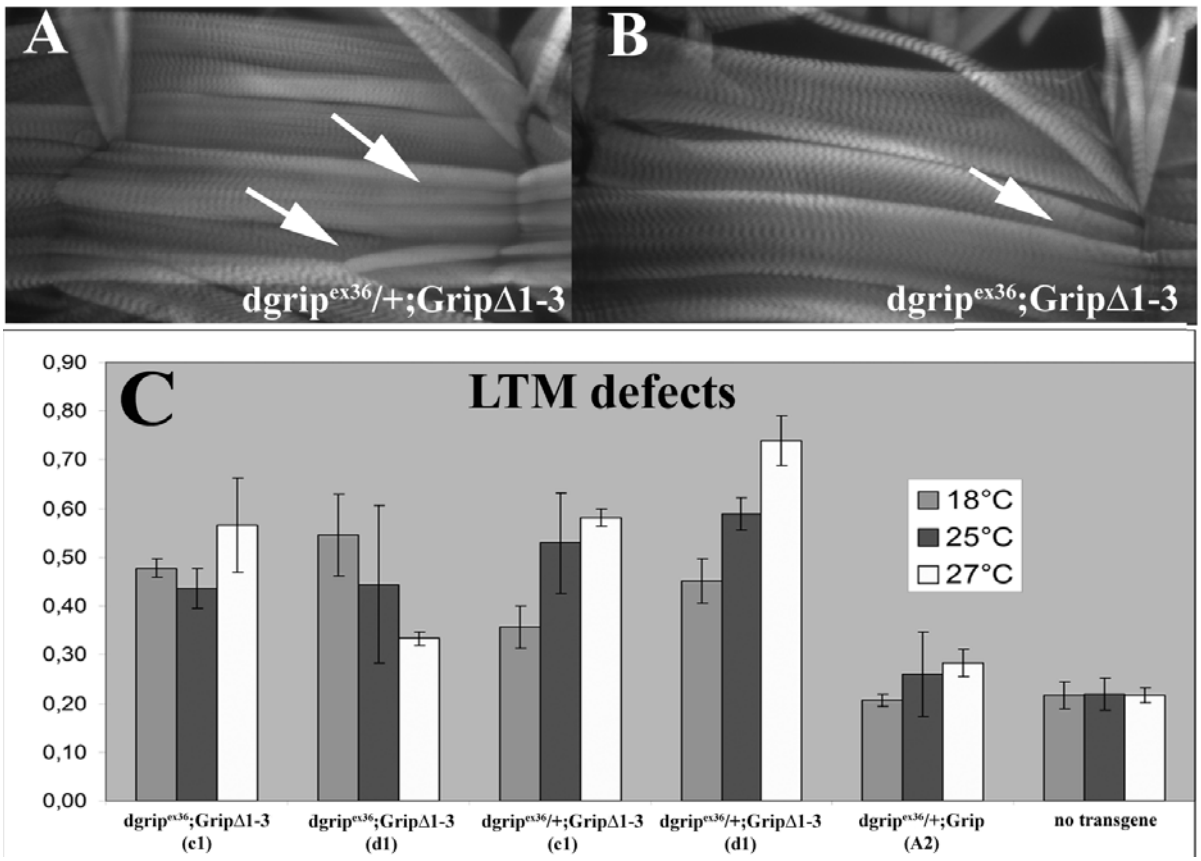


Figure 18: DGripΔ1-3 dominantly causes defects (arrows) in both VLM and LTM muscles. **A**. +/*dgrip*^{ex36},*twist-gal4*::*UAS-dgripΔ1-3* larva. **B.** *dgrip*^{ex36},*twist-gal4*::*UAS-dgripΔ1-3* larva **C.** Analysis of defects (score= 0.2 is wild-type, >0.2 defective) in the LTM group of muscles at different expression levels of the driver *twist-gal4*, controlled by raising temperature. Data presented as average score +/- StDev. No threshold for the onset of DGripΔ1-3-driven defects could be found.

One theory could be that PDZs 1-3 function as repressors by binding proteins which otherwise would be functional in organising muscle motility. In this case PDZs1-3 alone could deplete active components in the muscle and make them inactive for the muscle guidance process. To look at this possibility, DGrip-1-3OE, containing only the PDZs1-3 was examined (see **Figures 15 and 19**). If DGrip-1-3OE was able to cause muscle defects, either by making the *dgrip*^{ex36} muscle defect more severe, or by causing defects in wild-type muscles, then it could suggest that PDZs1-3 act as a kind of 'titrating' repressor. However, this transgene did not cause severe muscle defects caused when expressed (at either 25°C or 29°C) in the *dgrip*^{ex36} background with *twist-gal4*, or expressed in the wild-type background with *24B-gal4* at 29°C, suggesting that this transgene does not interfere in the muscle guidance process.

To then understand if PDZs 1-3 alone were able to repress the DGripΔ1-3 construct, larvae with the genotype *twist-gal4*::*UAS-dgripΔ1-3, UAS-dgrip1-3OE*, were dissected. In this way, it could be determined if PDZs 1-3 need to be part of the same protein to repress DGrip muscle function, or if PDZs1-3 could act in *trans*.

Co-expression of DGrip1-3OE could not repress the LTM phenotype obtained with *twist-gal*::*UAS-dgripΔ1-3*. In fact the defect appeared worse, indicating that isolated PDZs1-3 cannot function as repressors of DGrip function.

As PDZs 1-3 function only in the context of the entire DGrip protein, it is more probable that DGrip PDZs 1-3 are responsible for binding a complex which binds at a second position on DGrip. Thus the activity of this complex (repression or function) would be determined by the position of binding on the DGrip molecule. This would suggest that DGripΔ1-3 can be repressed by a point mutation of another PDZ domain in the protein (**Section 3.5.8**).

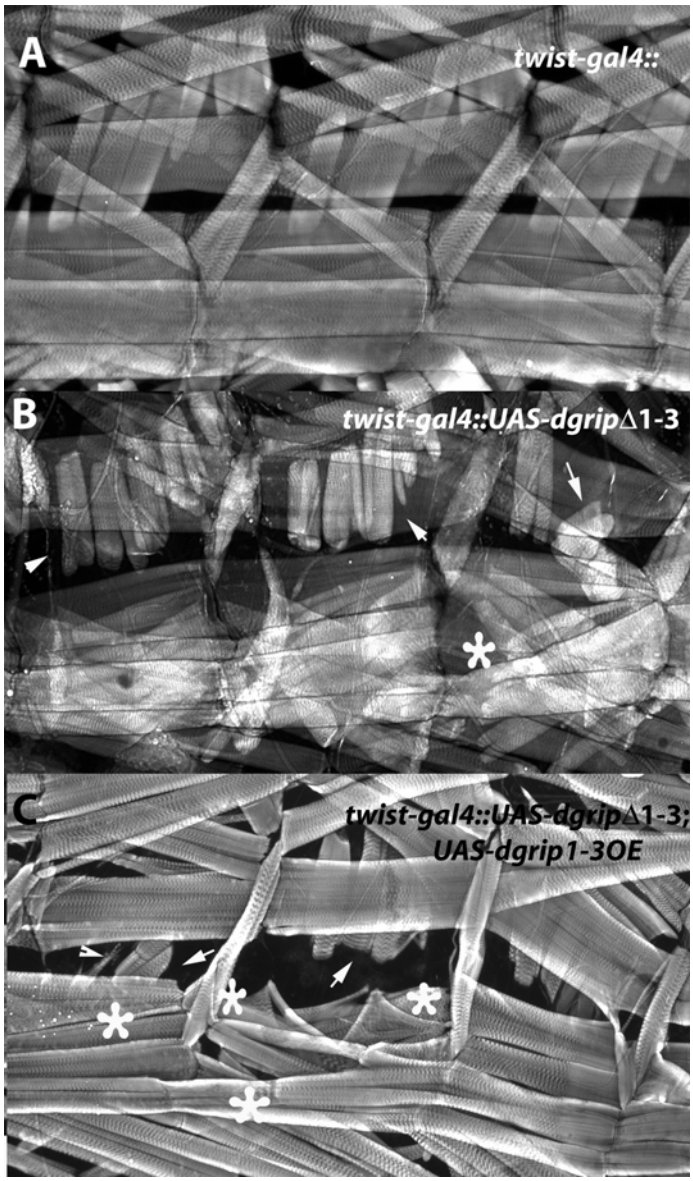


Figure 19: DGrip1-3OE does not repress DGripΔ1-3 in muscles. **A.** Control larva, expressing only *twist-gal4*. Both LTMs (arrows) and VLMs (asterices) are still affected by DGripΔ1-3, with (**C**) or without (**B**) the presence of the DGrip1-3OE protein.

The localisation of the DGripΔ1-3 protein, when compared with transgenically expressed full-length DGrip, was also examined. Using *dgrip*^{ex36} embryos, which are protein nulls for DGrip, and re-expressing either DGrip or DGripΔ1-3 with *twist-gal4*, the cellular distribution of DGripΔ1-3 could be visualised. Both DGrip and DGripΔ1-3 are readily detected in the VLM field, where the distribution is the same as the endogenous pattern of DGrip staining, showing that the DGripΔ1-3 protein is trafficked normally in VLMs.

However, in the LTM field of the same animals, clusters of DGripΔ1-3 protein can be detected, whereas transgenically expressed full-length DGrip could not be seen in LTMs, even though VLMs showed a robust DGrip staining in these embryos. This suggests that the DGripΔ1-3 protein more readily forms aggregates, although whether these are functional aggregations, i.e. trafficking or signalling aggregates, is as yet unknown.

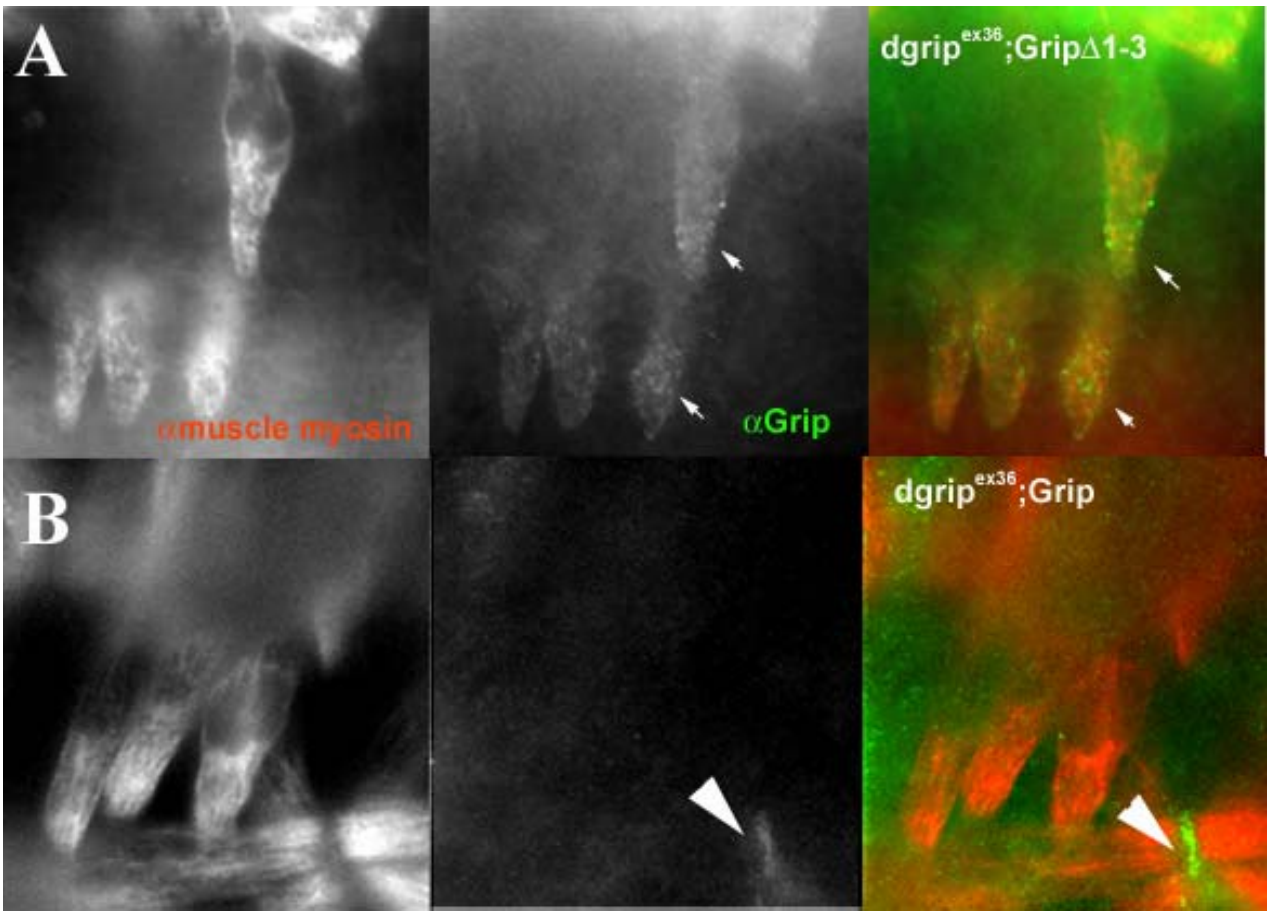


Figure 20: DGripΔ1-3 forms aggregates in the LTM group of muscles. **A:** Ectopic DGripΔ1-3 expression in the lateral transverse muscles (LTMs) in *dgrip*^{ex36} embryos raised at 25°C. Aggregates of DGrip immunoreactivity, using an antibody against PDZs 6-7, can be identified in the LTM group of muscles (arrowheads). **B:** No DGrip staining can be detected in LTMs using the same expression and staining conditions, whereas rexpressed DGrip is readily detected in the rescued VLM muscles (arrows).

3.5.5 DGrip binds repressors of muscle function over PDZs 1-3

The mechanism behind the repression mediated by PDZs 1-3 was examined in more detail. The function of the missing PDZs 1-3 could be explained by the ligand-binding capabilities of PDZs 1-3, but this group of PDZs could also act by other, structural, functions. Therefore, point mutations were made to specifically disturb PDZ binding. Several approaches have been used to do this [168 and Ingrid Ehrlich, personal communication]: by either mutating or deleting the GLGF motif, by mutating a conserved histidine in the αB motif, or by mutating charged residues just N-terminal of the GLGF motif. A combination of the first and third strategies was chosen, a substitution from R/K xxx GLGF PDZ domains to **A** xxx G**A**GL (see **Table 5**).

PDZ domain	**Sequence**
PDZ 1	ITLP**R**NALH**L**AIT
PDZ 2	ITVE**R**ESGC**L**GLT
PDZ 3	EI**ER**PMNDKLGLV

Table 5: Point mutations of PDZ domains 1-3 of DGrip. The underlined residues were mutated to alanine (see **Materials and Methods**).

Four transgenes were produced: *UAS-dgripx123*, where all three PDZ domains were mutated, and three more -*UAS-dgripx1*, *UAS-dgripx2* and *UAS-dgripx3*- where each PDZ was mutated separately. These constructs were then expressed in muscle using *twist-gal4*. These experiments show that dominant active DGripΔ1-3 phenotype stems largely from the loss of PDZ1 ligand binding capability. Likewise, mutations of PDZ2 alone, and even PDZ3 to a much lesser extent cause milder LTM defects (see **Figure 21 and**, **Discussion**). The data above suggests that the DGripΔ1-3 dominant phenotype is due to the loss of PDZ-ligand binding over PDZs 1-3, and predominantly PDZs 1 and 2. Given that mutation of PDZ1 cause the majority of the defect, why mutations in PDZ2 and an even lesser extent PDZ3, also cause dominant phenotypes in LTMs could have one of two explanations. All three PDZ domains could co-operate to produce repression by each binding their own ligands. A second possibility is that the disturbance of PDZs 2 or 3 by point mutation could cause PDZ 1 to loose its ligand binding capability to a greater or lesser extent, via allosteric modulations. Once the ligands of these three PDZs are known, these two possibilities can be examined further.

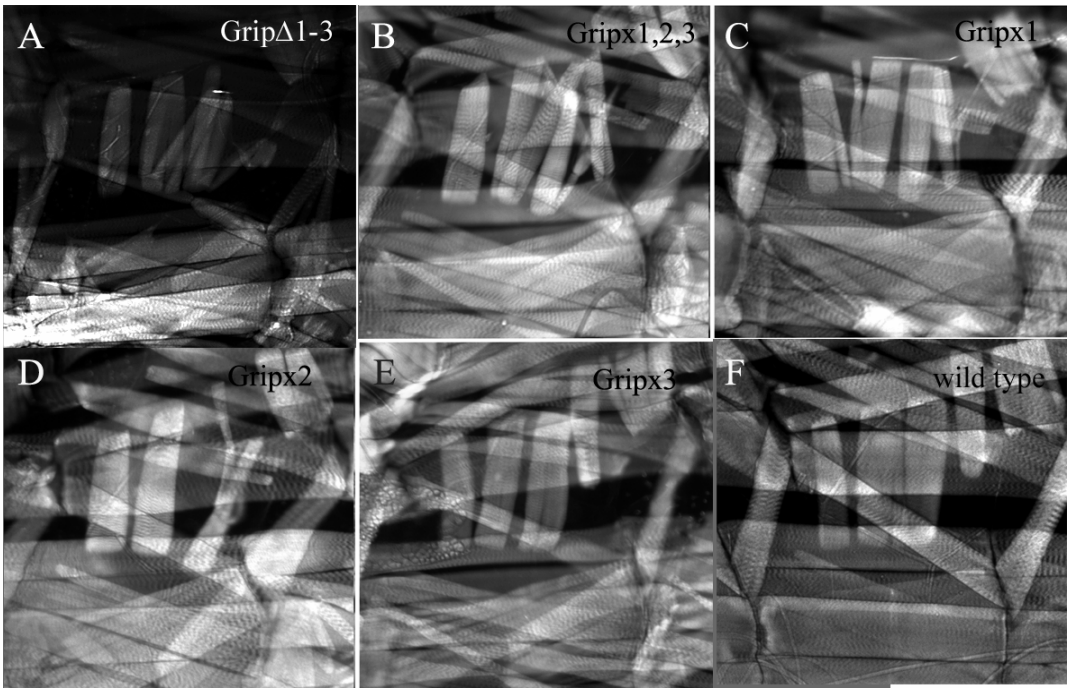


Figure 21: The dominant muscle phenotype in DGripΔ1-3 animals is mediated by the loss of PDZ-ligand binding over these domains. Here, the LTM phenotype is shown. The DGripΔ1-3 phenotype in LTMs can be recapitulated by mutating PDZ1, and to a lesser extent, PDZ2. **A** *dgrip$^{ex36;}$twist-gal4*/+;*UAS-GripΔ1-3* **B** *dgrip$^{ex36;}$twist-gal4*/+;*UAS-dgripx123*, **C** *dgrip$^{ex36;}$twist-gal4*/+;*UAS-dgripx1*, **D** *dgrip$^{ex36;}$twist-gal4*/+;*UAS-dgripx2*, **E** *dgrip$^{ex36;}$twist-gal4*/+;*UAS-dgripx3*, **F** wild-type (no transgene).

3.5.6 Mutations of PDZs 1-3 have defects in VLM formation

Mutations in PDZ1, PDZ2 or PDZs1,2 and 3 together gave the same mild, dominant effect on VLM morphology as the DGripΔ1-3 protein (**Figure 22**). The only exception was the point mutation of PDZ3, which was not able to completely rescue *dgrip^{ex36}* muscles (**Figure 22 E**), but in the wild-type background (**Figure 22 F**) still gives a dominant effect in VLM morphology. This suggests that some of the same domains necessary for muscle rescue function are also involved in the production of the overexpression phenotype.

Surprisingly, this means that rescue deficiencies caused by the loss of PDZ3 binding can be masked by overactivity caused by loss of PDZ interactions over PDZs 1 and 2. This explains why both DGripΔ1-3 and DGripx123, being dominantly active, are able to rescue the *dgrip^{ex36}* muscle defect even though PDZ3 binding is compromised, while point mutation of PDZ3 alone compromises the DGrip transgene's rescue function.

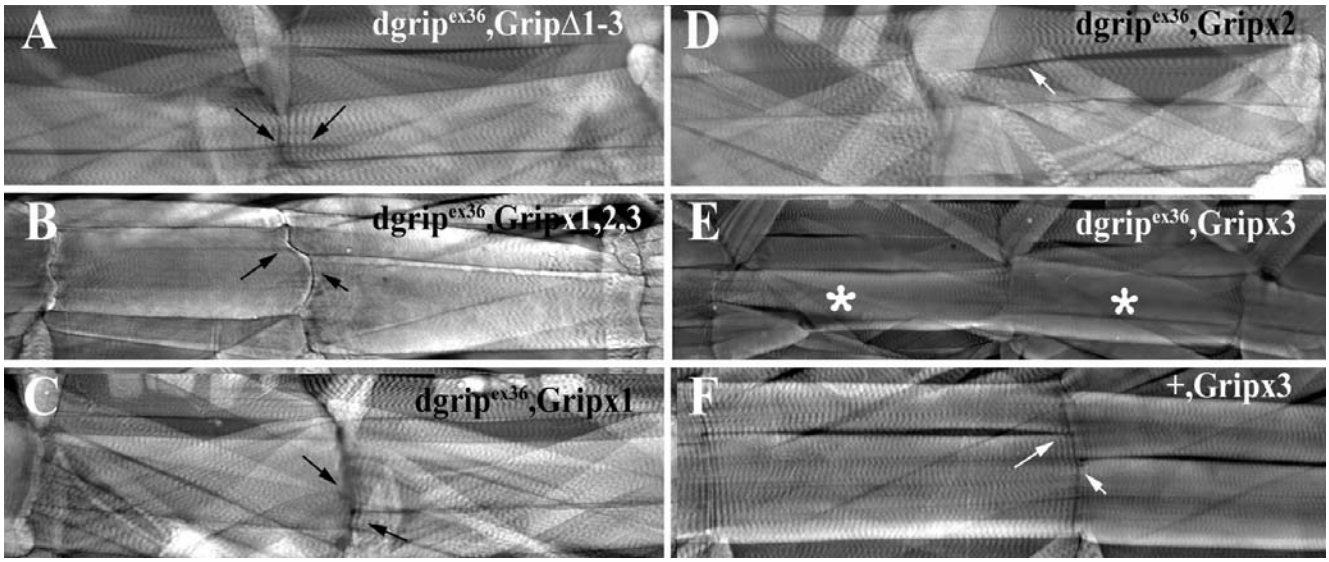


Figure 22: Mutations in PDZ1-3 also give mild VLM defects**.** Dominant phenotypes in the VLM field of muscles driven by *twist-gal4*. The defect consists of muscles meeting out of register at the segment border (arrowheads). **A** *UAS-dgripΔ1-3*, **B** *UAS-dgripx123* **C** *UAS-dgripx1* **D** *UAS-dgripx2* **E** *UAS-dgripx3*, all in the *dgrip*^{ex36} background. **F** *UAS-dgripx3* in wild-type background. DGripx3 cannot fully rescue *dgrip*^{ex36}(a strong example in **E,** asterices), but in wildtype animals, DGripx3 gives a mild dominant defect (**F**).

Protein	Rescue (VLM)	Dominant defects (VLM)	Dominant defects (LTM)
DGripΔ1-3	+++	+++	+++
DGripx123	+++	+++	+++
DGripx1	+++	+++	+++
DGripx2	+++	++	++
DGripx3	+	+	-/+
DGrip	+++	-	-

Table 6: summary of defects when expressing transgenes with the driver *twist-gal4*. Rescue of VLMs: +++ full rescue- - no rescue. Dominant defects: +++ strong defects- + weak defects – no defects.

3.5.7 PDZs 6 and 7 in muscle guidance.

Constructs missing both PDZs 6 and 7 could only partially rescue the *dgrip*^{ex36} muscle guidance defect (see **Figure 14** above, and **Figure 24** below for quantification). This suggested that one or both of these domains were involved in the positive function of DGrip in muscle guidance, although they could not be the only domains involved.

The VLM group of muscles were also rated using the 'clinical score' system where the different ratings were as follows:
0.2: normal morphology
0.4: slight: VLM muscles do not meet in register at the segment border.
0.6: mild: VLM muscles attach at the segment border, but are striated.
0.8: strong: VLMs from processes, which do not extend fully to the segment border, but ectopically fuse in mid-segment.
1.0: severe: VLMs form 'ball' shapes, and do not extend to the segment border at all.

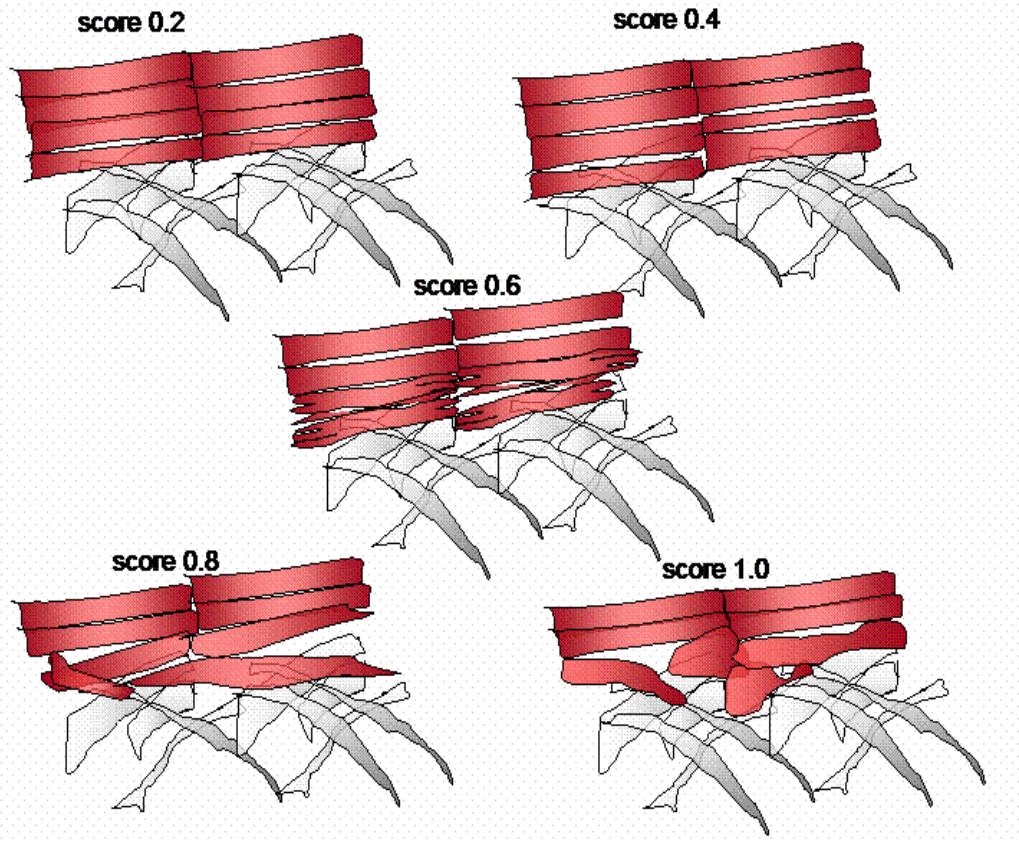


Figure 23: Schematic of defects in the VLM group of muscles.

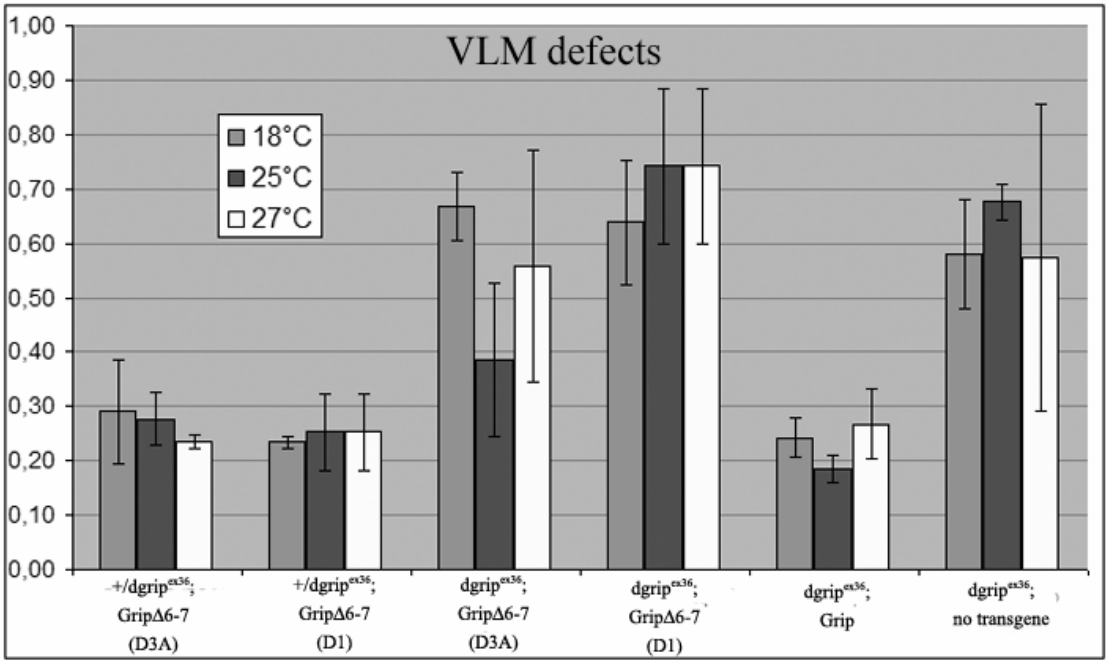


Figure 24: Quantification of defects in *dgrip*^{ex36} animals re-expressing DGripΔ6-7. This transgene is unable to completely rescue *dgrip*^{ex36}, and causes no defects when overexpressed with a wild-type copy of DGrip. The full-length DGrip fully rescues the *dgrip*^{ex36} defect. Results presented as average +/- StDev.

To understand the roles of PDZs 6 and 7 in muscle guidance, a similar strategy of point mutation to these PDZs was applied. However, as PDZ7 has an unusual, bimodal, form of ligand binding [158], highly conserved residues in the domain which are predicted be involved in both modes of binding were mutated (see **Materials and Methods**). Mutations in PDZ6 showed that PDZ6 is not responsible for rescue function of the DGrip transgene, as rescue was unaffected by this point mutation (**Figure 25**). The DGrip x7 protein showed an impaired ability to rescue the *dgrip*^{ex36} muscle defect in VLMs (**Figure 26 B**), which phenocopied the lack of rescue function with DGripΔ6-7. However, when *dgrip*^{ex36}/+, *twist-gal4*::*UAS-dgripx7* animals were examined for any dominant defects, a surprising result was found. Unlike the DGripΔ6-7 protein, point mutations in PDZ7 caused a mild dominant defect in both VLM morphology (**Figure 26 A**) and LTM morphology (**Figure 26 E**). A question for further study is then why it is that the simple absence of PDZs 6 and 7 (DGripΔ6-7) does not cause these dominant defects. One hypothesis is that PDZ7x7 can cause steric interference even though it cannot bind ligands.

3.5.8 DGripΔ1-3 can be repressed by mutating PDZ7

Simultaneously, two other transgenes incorporating the same point mutations, namely *UAS-dgripΔ1-3x6* and *UAS-dgripΔ1-3x7* were constructed. The purpose of this was twofold. As it was known that point mutations of simply PDZ6 or 7 alone is unlikely to give a strong loss-of-function phenotype, given that constructs missing both PDZs 6 and 7 are already capable of partial rescue, residual muscle rescue function could be associated with one of the PDZs 1-3. It also allowed the investigation of whether the dominant active activity of DGripΔ1-3 is caused by the de-repression of PDZs 6 and 7 (or their interaction

partners), or whether it is based purely on the binding capability of PDZs 1-3 alone (also treated in **Section 3.5.5**).

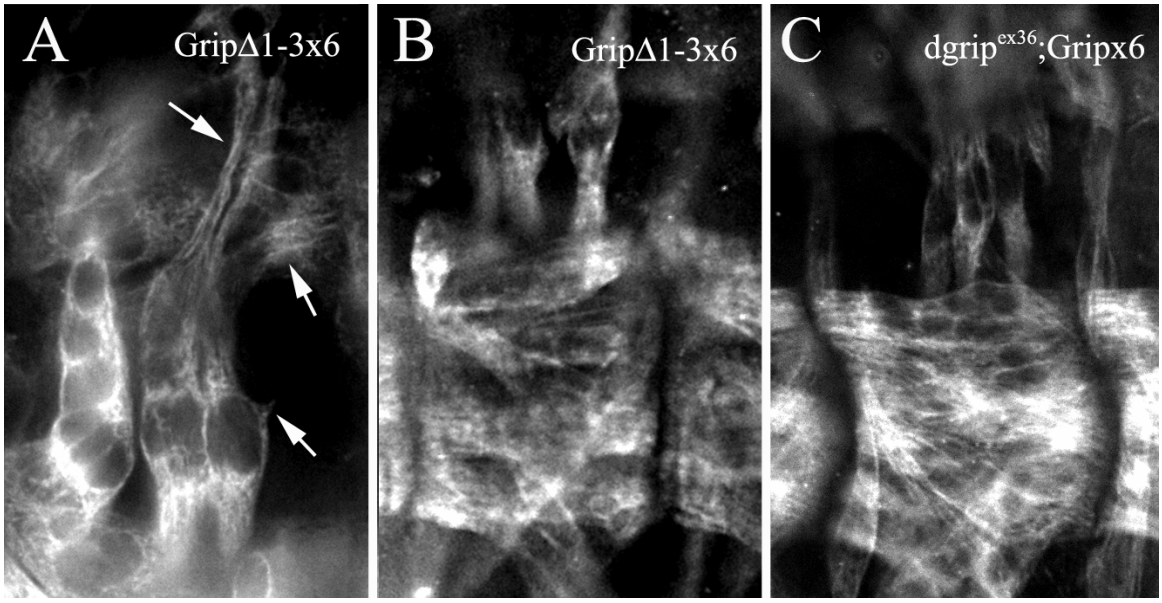


Figure 25: Mutations in PDZ6 do not suppress the DGripΔ1-3 phenotype. **A:** *twist-gal4*::*UAS-dgripΔ1-3x6*. Expression of DGripΔ1-3x6 tstill shows LTM defects (arrows) **B.** *twist-gal4*::*UAS-dgripΔ1-3x6* shows no more defects in VLMs than *UAS-dgripΔ1-3*. **C.** *dgrip^{ex36}*, *twist-gal4*::*UAS-dgripx6*. Mutations in PDZ6 cause no defects in the ability of the transgene to rescue *dgrip^{ex36}*. Embryos stained with an anti-muscle myosin antibody.

The PDZ6 point mutation could not suppress the dominant action of DGripΔ1-3, suggesting that the Δ1-3 mutation does not function by de-repressing ligand binding to PDZ6 (**Figure 25**).

Interestingly, DGripΔ1-3x7 showed a severely impaired ability to rescue *dgrip^{ex36}* (**Figure 26 D**), suggesting that PDZ7 co-operates with one or more of PDZs 1-3 for rescue function.

The severity of the LTM defect was decreased for DGripΔ1-3x7 animals when compared to DGripΔ1-3 animals (**Figure 26 F**), suggesting that at least part of the dominant action of the Δ1-3 mutation is mediated by de-repression of PDZ7 binding. The DGripx7 defects are comparable with mild dominant defects obtained with the DGripΔ1-3x7 transgene (**Figure 26 C, F**). As point mutations in PDZ7 caused mild dominant defects in LTMs, it was not clear if the mutation of PDZ7 fully represses DGripΔ1-3, the residual LTM phenotype is associated with the DGripx7 mutation alone.

This result would suggest that DGrip binds a complex in two different positions. If this complex binds to PDZs1-3, the activity of this complex is repressed, but if it binds to PDZ7, then the complex is available for function.

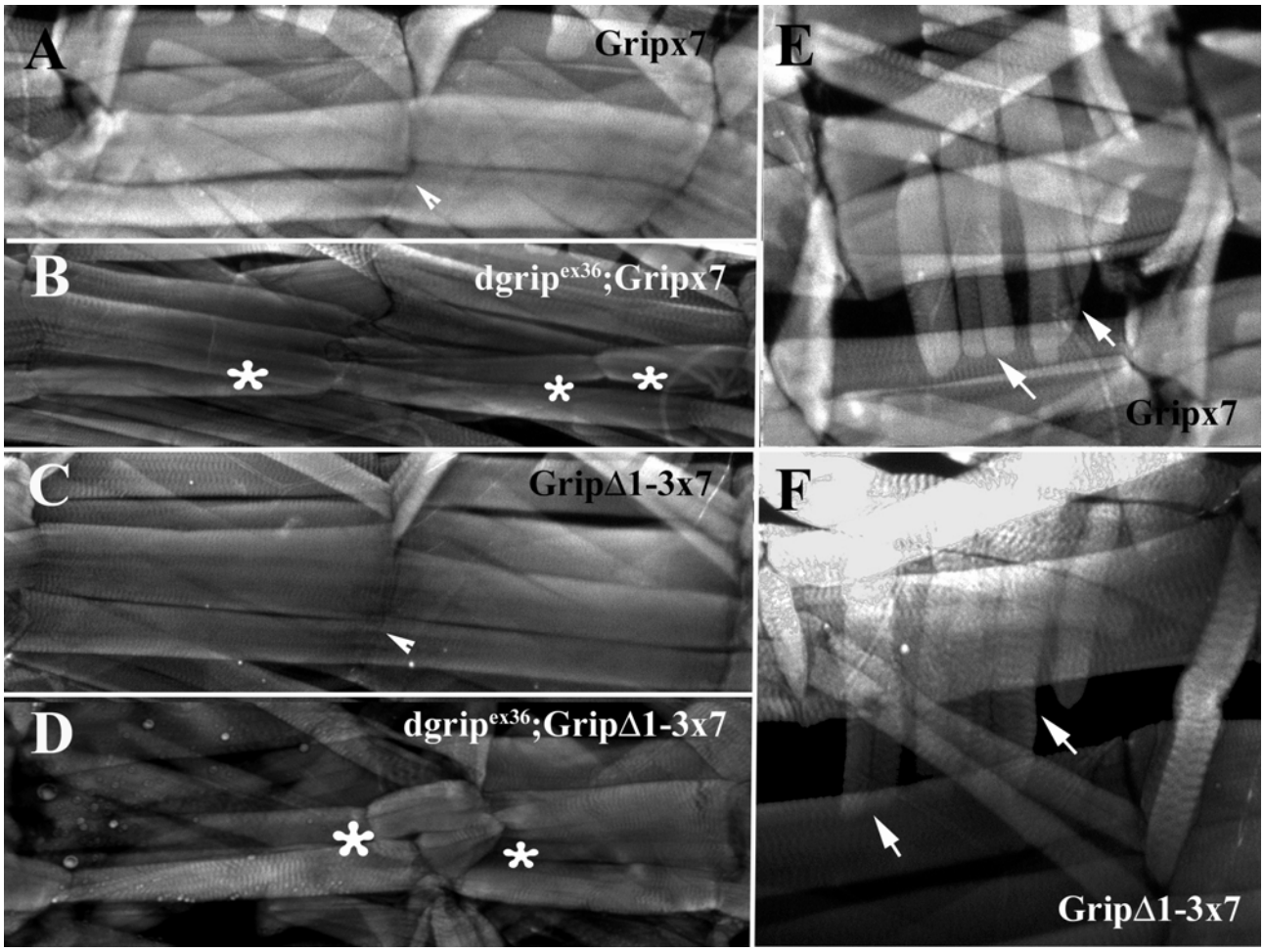


Figure 26: Mutation of PDZ7 represses DGripΔ1-3. **A.** *twist-gal4*::*UAS-dgripx7* VLMs **B**. *dgrip*^{ex36},*twist-gal4*::*UAS-dgripx7*VLMs **C.** *twist-gal4*::*UAS-dgripΔ1-3x7* VLMs, **D.** *dgrip*^{ex36},*twist-gal4*::*UAS-dgripΔ1-3x7*VLMs **E.** *twist-gal4*::*UAS-dgripx7* LTMS, **F.** DGripΔ1-3x7 LTMs. Neither DGripΔ1-3x7, nor DGrip x7 can fully rescue the *dgrip*^{ex36} VLM phenotype (**B,D** asterices), although both produce mild dominant VLM defects (**A,C** arrowheads). DGripΔ1-3x7 does not produce a strong LTM phenotype (compare **F**, arrows with **Figure 21** above), instead a mild defect which is comparable with mutation of PDZ7 alone (**E,** arrows).

3.5.9 The role of interdomain motifs in DGrip muscle guidance function.

The last result from the coarse orienting screen (**Figure 14**), showed that the loss of the second interdomain region (between PDZs 5 and 6) give rise to proteins (DGripΔ4-5R, and DGripΔ6-7L), which when expressed in the *dgrip*^{ex36} background show stronger loss-of-function phenotypes than could be obtained for the pure *dgrip*^{ex36} mutant.
These animals had not only the typical defects of the VLMs 6 and 7 associated with the *dgrip*^{ex36} mutant, but other muscles, including muscles 12 and 13 were affected at a much higher rate than in *dgrip*^{ex36} mutants.
This was particularly the case for the DGripΔ6-7L mutant, where the severity of the *dgrip* loss-of-function phenotype significantly increased. This did not occur if the DGripΔ6-7 protein was strongly expressed (**Figure 24**), even though it was not capable of fully rescuing *dgrip*^{ex36}. Expression of DGripΔ4-5R did not give as strong a phenotype as

DGripΔ6-7L, but it still showed an increased effect on *dgrip*^{ex36} muscles with higher levels of expression.
This, however, was not a dominant phenotype, as all animals expressing these two transgenes in the presence of endogenous DGrip showed normal muscle morphologies.

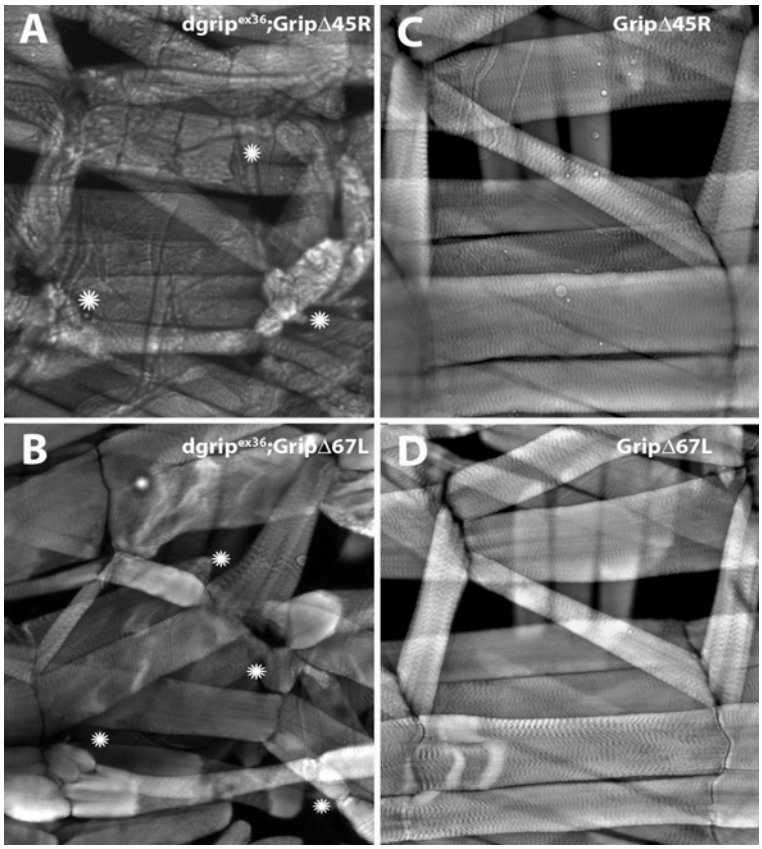


Figure 27: Constructs missing the second interdomain produce stronger defects, affecting more muscles than *dgrip*^{ex36} when expressed in the *dgrip*^{ex36} background. This effect is blocked by the presence of one wild-type copy of DGrip **A**.*dgrip*^{ex36}, *twist-gal4*::*UAS-dgripΔ4-5R* B. *dgrip*^{ex36}, *twist-gal4*::*UAS-dgripΔ6-7L* show strong defects in the larval musculature (asterices) whereas control animals: **C**.*dgrip*^{ex36}/+, *twist-gal4*::*UAS-dgripΔ4-5R* **D**. *dgrip*^{ex36}/+, *twist-gal4*::*UAS-dgripΔ6-7L* have normal muscle morphologies. All transgenes were expressed at 29°C.

Why is it, that DGripΔ6-7, which has a loss-of-function phenotype, does not become more severe with more transgenic expression, where DGripΔ6-7L does? There were two main possibilities- first that this interdomain contains a dimerising region, or second, that this domain could bind kinesins. Loss of the second interdomain region would then produce a transport inactive form of DGrip which could bind ligands, but not transport them to their sites of action. This would mean that muscles which express DGrip, but which are not strongly affected in the *dgrip*^{ex36} mutant, would become misguided when transgenes are expressed which bind components that DGrip uses for muscle guidance, and stops them being transported properly.

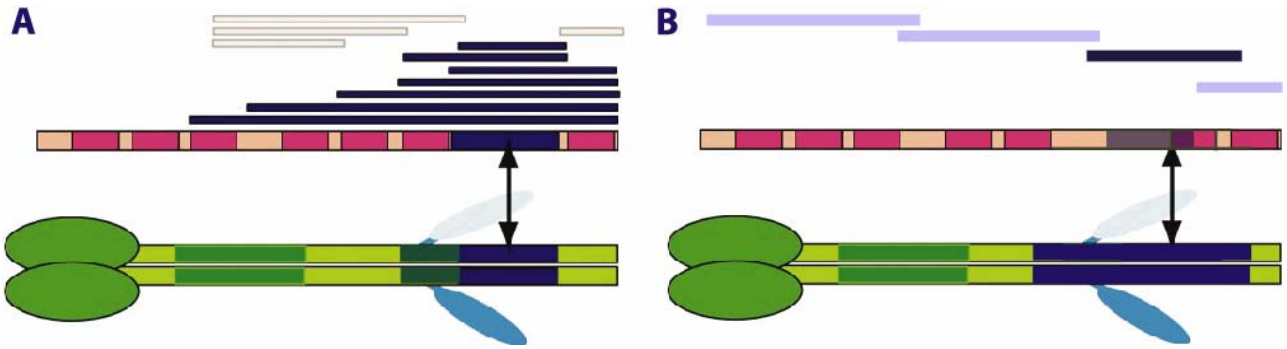


Figure 28: The yeast two-hybrid system suggested that the DGrip second interdomain interacted with the heavy chain of conventional kinesin, but not kinesin light chain (not shown). **A.** Previously published results showing a similar interaction between mGRIP and KIF5s, the conventional kinesins [169]. **B**. Yeast two-hybrid result using *Drosophila* Grip and conventional kinesin.

To further investigate the possibility of DGrip-kinesin interactions, DGrip constructs were tested against *Drosophila* kinesin heavy and light chains in yeast two-hybrid (**Figure 28**). One construct strongly reacted with the kinesin heavy chain but not with the light chain, suggesting a possible interaction over this interdomain segment.
This interaction was examined on the genetic level, by testing constructs missing the second interdomain for their rescue ability. Two constructs were made: *UAS-dgripΔint* which deletes the entire second interdomain, while leaving PDZs5 and 6 undisturbed, and *UAS-dgripΔkhc* which deletes the sequence N-terminal to PDZ6 which interacted with kinesin heavy chain in the yeast two-hybrid screen above.
Surprisingly, neither one of these deletions had an effect on the rescuing ability of the transgene (**Figure 15** above), suggesting, that on its own, the second interdomain region is not essential to DGrip function, and that it is only necessary when other PDZ domains are compromised.

3.6 Rescue of pupal lethality and adult abdominal phenotypes.

To begin to understand if the PDZ domain organisation uncovered in the study of the muscle phenotype is transferred to other functions of DGrip in *Drosophila*, a screen was made of these transgenes to see if two other *dgrip*^{ex36} phenotypes [147] responded in the same manner. The two phenotypes are recessive, so visible only in males carrying the mutant X chromosome over Y (hemizygotes). One phenotype is a pupal lethality and the other is that the rare animals that do not die as pupae (so-called 'escapers') show a markedly malformed abdomen.
These later phenotypes are not likely associated with defects in embryonic musculature. Expression of DGrip with *twist-gal4* completely rescues defects in muscle morphology, but cannot rescue pupal lethality associated with *dgrip*^{ex36} (not shown). Thus, these two phenotypes represent two new processes where DGrip is necessary, a functional understanding of DGrip domain structure in another context can be obtained, and a statistical result could be generated.
The experiment consisted of crossing males homozygous for various DGrip transgenes to virgins from a stock *dgrip*^{ex36}/FM7 *ftz::lacz;;24B-gal4*. This allows the simultaneously screening of the rescue of the pupal lethality and rescue of the abdominal phenotype.

Unexpectedly, *24B-gal4* driven expression of some DGrip transgenes, including DGripΔ1-3, was itself lethal at the pupal stage (see a detailed treatment in **Section 3.7.5**). This lethality was another dominant effect which allowed the screening of repression of DGripΔ1-3 in another system besides larval muscles. Percentage lethality was calculated as the number of dead pupae from divided by the total number of animals (both live adults and dead pupae) recovered from the cross. Rescue percentage was calculated as percentage of the expected mendelian ratio (here, 1:4) where all animals including dead pupae were counted.

Interestingly, several elements of the functional organisation of domains identified through the muscle guidance phenotype are preserved for these later phenotypes. DGripΔ1-3, the dominant active form of DGrip in the muscle guidance process is 100% lethal when expressed with the *24B-gal4* driver. A DGripx1,2,3 transgene seemed only to partially phenocopy the DGripΔ1-3 effect, producing only 100% lethality in males, whilst females survived to adulthood, giving overall 65% lethality. However, this may have only been a question of expression strength as a point mutation of PDZ2 only (DGripx2) was fully lethal, phenocopying the DGripΔ1-3 defect in this lethality assay, as it did in assays of muscle morphology. Significantly, point mutation of PDZ7 was able to suppress the DGripΔ1-3 phenotype, while DGripΔ1-3x6 was still lethal with 24B-gal, as was the case for the dominant active LTM phenotype. This would suggest that some elements of the signalling logic uncovered for muscle guidance is preserved in later developmental stages, perhaps involving even the same signalling complexes. Most interestingly, PDZs 1-3 alone (DGrip1-3OE) could provide a partial rescue of male lethality, indicating that that construct, and these domains, are functional in later DGrip dependent processes.

Transgene	Rescue male lethality	Rescue abdomen	Lethality with *24B-gal4*
None	No	-	No
UAS-dgrip	96%	Yes	No
UAS-dgripΔ1-3	-	-	100%
UAS-dgripx1,2,3	-	-	65% (male lethal)
UAS-dgripx1	120%	Yes	61% (female lethal)
UAS-dgripx2	-	-	100%
UAS-dgripx3	5%	No	No
UAS-dgripΔ45L	-	-	44% (male lethal)
UAS-dgripΔ45R	No	-	No
UAS-dgripΔ67L	No	-	No
UAS-dgripx6	120%	Yes	No
UAS-dgripx7	60%	Yes	No
UAS-dgripΔ1-3x6	-	-	100%
UAS-dgripΔ1-3x7	53%	No	No
UAS-dgripΔint	117%	Yes	No
UAS-dgrip-ND	69%	Yes	No
UAS-dgrip1-3OE	38%	No	No

Table 7: Some aspects of DGrip functional organisation found for the muscle phenotype are preserved in later functions. Mutations of PDZ7, but not PDZ6, can suppress lethality mediated by DGripΔ1-3 when driven by *24B-gal4*. Mutations of PDZ2 only are also lethal. Interestingly, a transgene missing PDZs 1-3 and 7 (DGripΔ1-3x7) can still partially rescue male lethality, but not the adult abdominal phenotype, indicating that these two phenotypes may rely on different PDZ domains.

3.7 Identifying interactors:

Given that the work above demonstrates that DGrip function is more complicated than that of a simple adaptor protein, and that DGrip itself may have regulatory functions converging on several different proteins, it is very interesting to understand how the PDZ binding logic uncovered in the study above correlates to actual binding partners. Based on the understanding of ligand–binding phenotypes for PDZ domains, interaction partners can be approached with an understanding of what the actual molecular role of DGrip may be in muscle guidance.

3.7.1 Robo-Slit pathway

Some candidate proteins were approached based on known phenotype, rather than on screening results. A prime candidate was the Robo receptors, which are one of the few other molecules known to a have a specific function during the muscle guidance part of muscle development.
The Robo-Slit signalling system is well known in both *Drosophila* and in mammals to be necessary for axonal guidance [170-179], particularly in the determination of midline crossing of spinal cord neurons (or ventral nerve cord neurons in *Drosophila*). Robo also has a role in the postsynaptic muscle cell during muscle guidance [60, 61]. Two Robo receptors, Robo1 and Robo2, are redundantly involved in the guidance of the VLM muscles 6 and 7. Loss of both Robo1 and Robo2 in the muscle, or alternatively, the loss of the Slit ligand (in this case, an attractive ligand) expressed in the apodeme, leads to a loss of muscle guidance in VLMs 6 and 7, strongly reminiscent of the *dgrip*^{ex36} defect. Furthermore, overexpression of either Robo1 or Robo2 in LTMs causes these muscles to respond to the Slit cue emanating from the apodemes at the segment border [60]. As both the loss-of-function and gain-of-function of Robo signalling and DGrip are very similar, it seemed possible that DGrip may be involved in Robo signalling, likely as a Robo trafficking or signalling factor.
To demonstrate that DGrip is involved in the Robo-Slit pathway, the genetic interaction of the Robo and DGrip pathways was examined. Expression of a Robo2 transgene in the *dgrip*^{ex36} background did not cause the same muscle defect in the LTM field, as overexpression of Robo2 causes in wild-type (compare **Figure 29 B** and **C**). Furthermore, expression of Robos2 and 1 (not shown) both made the muscle phenotype of *dgrip*^{ex36} mutants more severe, affecting all of the VLM group of muscles (6,7, 12 and 13), rather than predominantly muscles 6 and 7, as found in *dgrip*^{ex36} mutants.
Furthermore, muscle 4 (see **Figure C** for a schematic drawing), which shares the same mode of muscle guidance as the VLM group, and which expresses both Robo [60] and DGrip (**Figure 5**), also shows misguidance defects which do not occur in either *robo1,robo2* mutants, or *dgrip*^{ex36} alone.

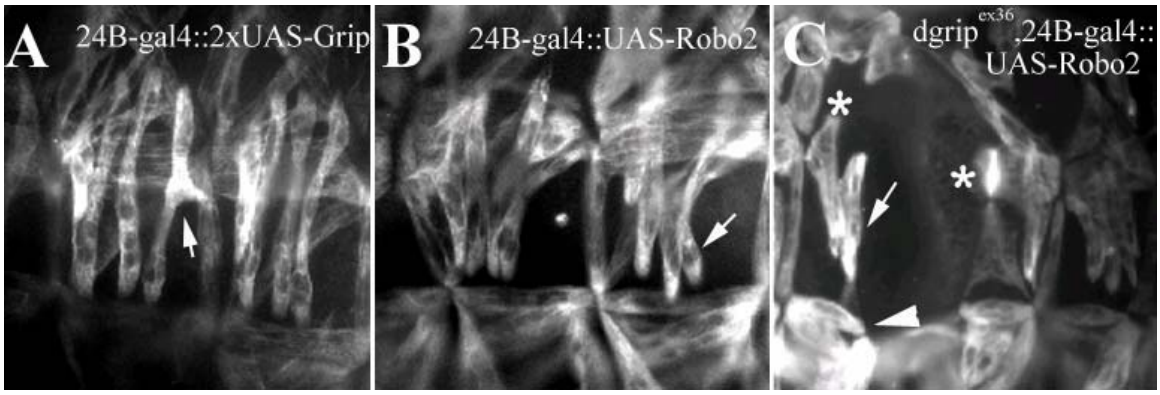


Figure 29: Robo2 overexpression exacerbates defects in *dgrip*^{ex36} animals **A**. *24B-gal4*::2x*UAS-dgrip* **B**. *24B-gal4*::*UAS-Robo2* **C**. *dgrip*^{ex36}; *24B-gal4*::*UAS-Robo2*. Overexpression of Robo2 in the *dgrip*^{ex36} background causes far more severe defects, the VLM muscles 6,7,12 and 13 are strongly affected (arrowhead) as well as muscle 4 (asterix). Furthermore, overexpression of Robo2 causes different morphologies in LTMs in the *dgrip*^{ex36} background, than in wild-type (compare arrows in **B** with **C**). Images from Carolin Wichmann.

These experiments suggest that the Robo-Slit system for muscle guidance is indeed involved in the same process as DGrip, and that the two proteins cannot compensate for one another. However, it is not explicit proof that DGrip acts in Robo signalling, as no combination of expression was able to rescue the muscle guidance defect produced.
This may be due to the nature of the two proteins: the Robo proteins as transmembrane proteins and DGrip as a cytosolic trafficking or signalling factor. If DGrip is indeed a trafficking protein that interacts with Robos, then in *dgrip*^{ex36}, Robo1 or Robo2 would likely be mistrafficked. Thus, overexpression of either Robo1 or Robo2 would only increase the *dgrip*^{ex36} defect, as the overexpressed protein would also be mislocalised in the muscle. Likewise, in the case of a Robo-DGrip interaction, *dgrip*^{ex36} should not be rescued by *robo1 robo2*. This, in fact, would most likely also cause a worsening of the *dgrip*^{ex36} defect, as the remaining mistrafficked Robo proteins would be removed, abolishing the remaining response to the Slit ligand in *dgrip*^{ex36} animals. As this experiment could not prove conclusive evidence either for or against a role for DGrip in Robo signalling, other approaches were tried.
To approach this interaction biochemically, N-terminally HA-tagged Robo1 and Robo2 (Kind gift of Frank Schnorrer, Barry Dickson) were expressed in both neuroblastoma and Cos-7 cells, both in the presence and absence of DGrip-GFP (constructed by Tobias Schwartz). The HA-tagged Robo constructs did not co-localise with DGrip-GFP in Cos-7 or neuroblastoma cell lines (**Figure 30**), their distributions on the membrane were not affected by the presence of DGrip-GFP, neither was co-immunoprecipitation of HA-Robo and DGrip-GFP possible from Cos-7 cells. Curiously, HA-Robo1 or HA-Robo2 and DGrip-GFP staining in both unpermeabilised and Tween-20 permeablised cells showed mutually exclusive distributions (**Figure 30**), showing that DGrip-GFP does not localise to the same compartments as the Robo receptors, at least in the two cell lines used.

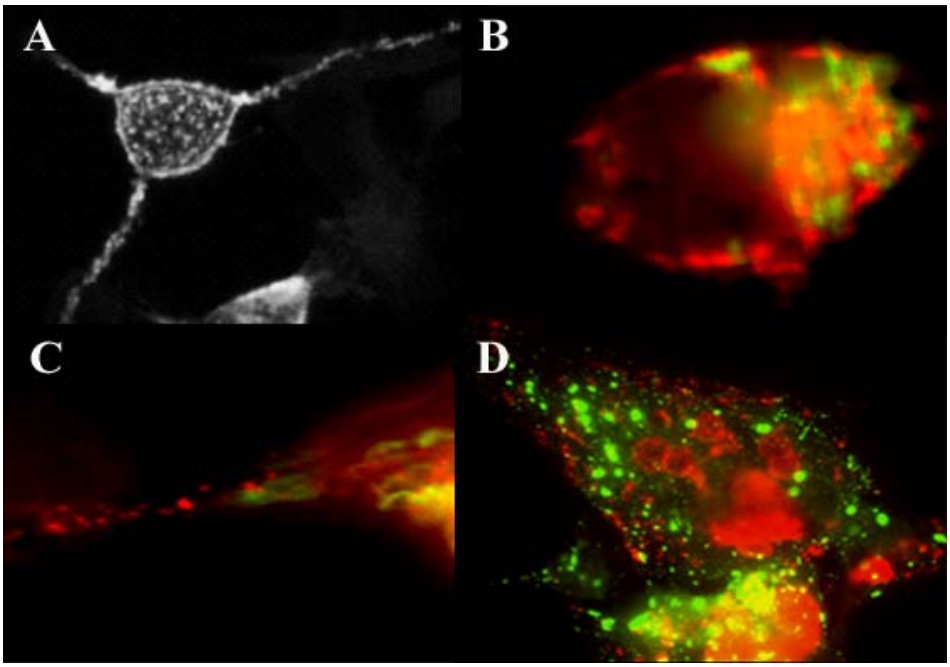


Figure 30: Co-expression of N-terminally HA-tagged Robos 1 and 2 in **A.**neuroblastoma, and **B-D** Cos-7 cells. **A.** Surface presentation of the HA-tagged Robo1 occurs without co-transfection of a DGrip plasmid (confocal projection of HA stained, unpermeabilised neuroblastoma cell). This distribution is also not changed by co-transfection with DGrip (not shown) **B-C** HA-tagged Robo1 (red) forms mutually exclusive domains with DGrip-GFP (green) (wide-field image of Cos-7 permeabilsed with 1% Tween-20 for 1 minute). (**D**) Neither does HA-Robo2 (red) co-localise with DGrip-GFP (projection of confocal z-stack of Cos-7 cell permeabilised as above)

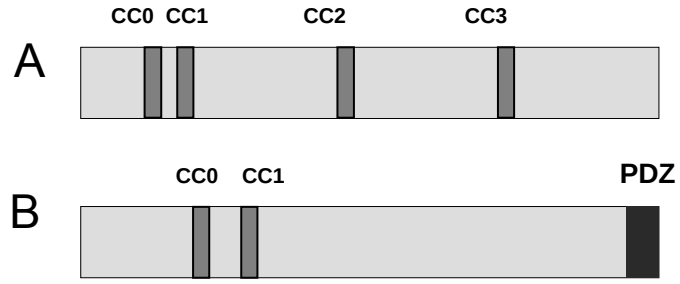


Figure 31: To test the direct interaction of Robo and Robo2 with DGrip in yeast two-hybrid, the entire C-termini were cloned into pGAD (**Materials and Methods**). **A**. Robo1 C-terminus, **B**. Robo2 C-terminus, with a potential PDZ ligand (GHNV) at the C-terminus.

To check the direct association of Robo1 or Robo2 C-termini with DGrip, two yeast-two-hybrid 'prey' clones containing the complete cytoplasmic domain of each protein were tested against the DGrip bait constructs. More details of this experiment are provided below (**Figure 31** and **Section 3.7.4**).

3.7.2 Yeast-two-hybrid analysis.

To identify possible interaction partners, two yeast two-hybrid screens were undertaken, using various tandem PDZs as baits **(Figure 32**). These screens were performed:

1. Via co-transfection (Ulrike Prange)
2. Via yeast mating (Tobias Boeckers, University of Ulm, Manuela Schmidt)

Some candidates from these screens (see below) were then further examined.

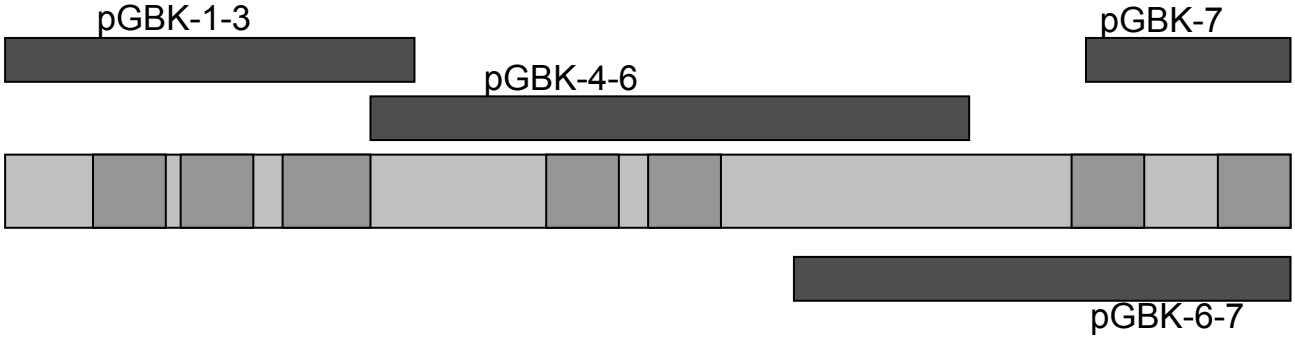


Figure 32: Yeast two-hybrid baits constructed for the DGrip protein.

3.7.3 Mint

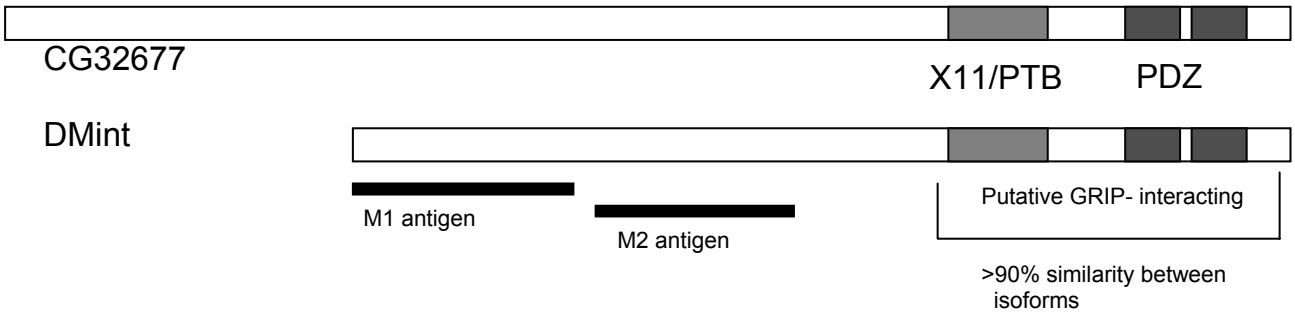


Figure 33: Comparison between the two *Drosophila* Mint isoforms, and constructs for generation of DMint antibodies.

A promising interactor on the yeast-two-hybrid level was the gene *CG32677*, a homologue of Mint, the Munc-18 interacting protein, also known as lin-10. There are two isoforms in *Drosophila*, *CG32677* and *DMint/X11*. Like isoforms of Mints in mammalians and *C. elegans* [180-183], only the C-terminals are conserved between isoforms, although to an astonishingly high degree (>90% amino acid conservation). The putative DGrip interacting domain is fully conserved, suggesting that the interaction may be valid for both DMint isoforms. The N-termini are however completely different, allowing for the generation of isoform-specific antibodies.

Mints have many known functions, which when considered in the light of the known phenotypes of DGrip, would make the Mints genes attractive candidates as DGrip interactors or effectors. Presynaptically, Mints are involved in N-type Ca^{2+} clustering and potassium channel trafficking [184-186], with a direct role in neurotransmitter release which affects many mammalian synapses [187-191]. As an interactor of the amyloid

precursor protein (APP) [180, 192-195], Mints regulate APP cleavage and APP-dependent transcription.
Postsynaptically, Mint is part of a highly conserved complex providing localisation of the EGF receptor [196, 197] and is also involved in both AMPA and NMDA receptor transport [139, 198]. As such, an interaction between DGrip and DMint isoforms could potentially explain both the presysnaptic (Ca^{2+} channel clustering, **Section 3.10**) and muscle guidance phenotypes (trafficking of EGFRs, or transcriptional regulation) in DGrip mutants.
As no full-length *CG32677* cDNA had been isolated by this time, antibodies against the *DMint/X11* isoform were made instead. Two plasmids, pRSET-Mint-M1 and pRSET-Mint-M2 were constructed, and 6xHis fusion protein expressed and purified (see **Materials and Methods**). The purified protein was sent for injection in both rabbits and guinea pigs (Cocalico Biologicals) to make a total of eight sera. To this date, one serum gives a promising staining pattern, which corresponds to the known presynaptic distribution of DMint [199].

3.7.4 Yeast-two-hybrid retest with point mutations

The results of the structure-function study suggested that PDZs 1-3 and 7 are necessary for DGrip function for muscle guidance. This raised the interesting question of if the PDZs 1- 3 and 7 bind the same ligands, or if DGrip requires the co-incidence of two different ligands binding for function. To test whether any potential interactors could interact with both PDZ domains, positive candidates from both yeast two-hybrid screens (see **Section 3.7.2** above) and the C-termini of other candidate proteins were retested against the original DGrip yeast two-hybrid baits. To control for ligand binding specificity, these constructs were also tested against the same baits with the same point mutations present in the transgenes used to assay the rescue function of DGrip. The results of the experiment are shown below in **Table 8**.

Prey:	**Robo1**	**Robo2**	**Ed** (no.42)	**Mad** (no. 73)	**Sax** (no.56)	**CG5053** (no.218)	**EGFR**	**Phyl.** (no.14)
pGBK-**laminin**	✗	✗	✗	✗	✗	✓	✗	✗
pGBK-**PDZ1-3**	✗	✓	✓	✓	✓	✓	✗	✓
pGBK-**PDZ1-3 x123**	✗	✗	✗	✗	✓	✗	✗	✓
pGBK-**PDZ1-3 x1**	nd	✗	(✓)	✗	nd	nd	✗	✓
pGBK-**PDZ1-3 x2**	nd	✗	✗	✗	nd	nd	✗	✓
pGBK-**PDZ1-3 x3**	nd	✗	✓	✗	nd	nd	✗	✓
pGBK-**PDZ6-7**	✓	✓	✓	✓	✓	✓	nd	nd
pGBK-**PDZ6-7 x6**	✓	✓	✓	✓	✓	✓	nd	nd
pGBK-**PDZ6-7 x7**	✓	✓	✓	✓	✓	✓	nd	nd
pGBK-**PDZ7**	nd	✓	✓	✓	nd	nd	✗	✗
pGBK-**PDZ7x7**	nd	✗	✗	✗	nd	nd	✗	✗

Table 8: Results of a yeast two-hybrid retest of interactors against point-mutated PDZ domains. X= no interaction √= positive interaction (√) =weak interaction nd= not determined

The results of the experiment are presented as follows: the PDZ6-7 construct is of itself active, meaning that no results produced in this assay could be used to determine the specificity of interactions with the point mutations x6 and x7.
Neither Robo1 nor the EGFR interacted with PDZs 1-3 or 7. However, Robo2 (C-terminal GHNV, a Type II PDZ ligand), Echinoid (C-terminal EIIV, a Type II ligand) and Mad (C-terminal ISVS, atypical ligand, possibly Type I) all specifically interact with the PDZ domains 1-3. This confirms the specificity of both the interactions of the yeast two-hybrid prey with the bait PDZ1-3, and the specificity of the point mutations abolishing PDZ function in PDZs 1-3. To correlate each ligand with an individual PDZ domain, candidates were specifically checked against point mutants of each of the first three PDZs and against PDZ7.
Most intriguingly, the pattern of Echinoid (Ed) binding to these yeast two-hybrid constructs follows the DGrip-dependent LTM phenotype. Ectopic DGrip missing PDZs 1,2 and 3 or PDZ1 only show strong dominant active phenotypes (**Section 3.5**). Mutation

of PDZ 2 only also causes a dominant phenotype in LTMs, and point mutation of PDZ7 suppresses the DGripΔ1-3 phenotype. Echinoid binding in this assay, using the same point mutations, is abolished by point mutations of 1,2 and 3 together, by mutation of PDZ 2 alone and greatly weakened by point mutation of PDZ1. Binding of Echinoid to DGrip's PDZ 7 is also abolished by point mutation.
The Mad C-terminal motif, which is an atypical PDZ ligand, is the characteristic C-terminal motif for all Smads (effector proteins in the TGFβ signalling pathway, of which Mad is one). During TGFβ signalling, this C-terminal SxS motif is phosphorylated by its respective the TGFβ Type-I receptor [200], adding a potential layer of regulation to any interaction of DGrip with smads such as Mad.
Mad binding to DGrip constructs is disturbed by destruction of PDZs 1, 2, 3 or 7 ligand binding motifs. This is also the case for Robo2, which has the same interaction pattern with DGrip baits as Mad. This does not mean that these proteins can bind to all of PDZ domains 1-3. It may also mean that binding of these C-termini to their target PDZ is sensitive to conformational changes in neighbouring PDZs caused by the introduction of point mutations.
Point mutation of PDZs 1-3 does not disturb Saxophone (Sax) or Phyllopod (Phyll) binding to PDZs1-3. As neither of these constructs interacted with the laminin negative control, it is possible that these proteins interact with DGrip via an unknown motif. However, it is not clear how this interaction could be correlated with any DGrip muscle phenotype.

3.7.5 DGripΔ1-3 lethality screen

To look for another approach to screen for interaction partners, heterozygous suppressors of the dominantly active DGripΔ1-3 were screened for. All DGripΔ1-3 animals die as pupae when the transgene is driven by either *24B-gal4* or *G14-gal4* (**Tables 7 and 9**). This offers a simple criterion to screen bulk numbers of flies for a genetic interaction. First, however, it is necessary to establish the screen conditions - whether expression of this transgene is lethal in all tissues or if this lethality is associated with a certain time point and tissue. If the construct is not lethal in all tissue, it much increases the likelihood that lethality is associated with the transgene's interference in a certain pathway or at a certain time point.
To check that the expression of DGripΔ1-3 is not itself intrinsically lethal, in a system where loss of zygotic DGrip has no effect, either *UAS-dgrip*, *UAS-dgripΔ1-3* or a wild-type line containing no transgene was crossed to the eye-specific driver *GMR-gal4*. The animals were raised at 29°C for maximal levels of transgenic expression.
If a transgene is cell lethal, one would expect to the so-called 'rough eye' phenotype, where individual cells in the compound eye are absent or misformed. A rough eye phenotype was not elicited (not shown), indicating that neither DGrip nor DGripΔ1-3 are cell lethal, but that DGripΔ1-3 is lethal due to its interaction in a tissue-specific pathway.

3.7.5.1 Pre-test

To better determine what the time point and tissues are that elicit lethality in this experiment, DGripΔ1-3 was expressed with various drivers and the level of expression was controlled by changing the temperature at which the experiment was performed. Due to the fact that the majority of driver lines are created by insertions of the Gal4 cassette in random genetic loci, the reported expression of the driver may not represent the full extent of the expression driven by each driver. Mortality was calculated as (number of dead pupae)/(total animals, including dead pupae)

Based on the data presented in **Table 9**, the driver *G14-gal4* was chosen to screen with at a raising temperature of 25°C, as at 25°C 100% mortality was elicited, making statistical analysis of the situation easier. Furthermore, since at 18°C mortality with *G14-gal4* was greatly reduced, it shows that the lethality of this line can be titrated out, unlike *24B-gal4*, which is still lethal with very low levels of expression at 18°C. The choice of the 90 lines to screen was based on members of pathways represented in the two yeast two-hybrid screens performed with DGrip, and on *Drosophila* homologues of published interactors with mGRIPs 1 and 2. The lines screened included mutations (deletions, ethane methyl sulfonate and x-ray mutagenised chromosomes), P-element insertions (including EP insertions which drive the expression of mRNAs from the neighbouring locus) as well as transgenes which are co-expressed in the same tissue as the DGripΔ1-3 construct. See **Appendix I** for the specifics of the lines screened.

Driver line	Published expression pattern	Lethality @ 18°C (%)	Lethality @ 25°C (%)	Lethality @ 29°C (%)	control: driver x w [1] @ 29°C Lethality (%)
24B	Muscle, motorneuron, trachea, and apodeme [15]	97	100	100	5
G14	muscle and motorneuron [201]	47	100	100	0
Twist	muscle [161]		0	75	0
OK319	Neuronal		0		
Sim	General [202]		0		
Stripe	General [147, 203]		0		
Elav	Panneuronal [147]		0	82	0
C57	muscle and ? [204]		0	80	
D42	muscle and motorneurons, interneurons [205, 206]		0	68	8
B185	muscle and motorneurons [207]		0	55	
OK6	Motorneuron [208]		0	26	
S59	muscle [147]		0	5	
Mhc	muscle [209]		0	2	
Actin	Ubiquitous		0	0	

Table 9: Pre-test to determine lethal combinations of drivers with *UAS-dgripΔ1-3*. The known expression patterns of these drivers are shown, and the level of transgenic expression is controlled by the temperature at which the experiment is performed. To control that the lethality is not associated with the driver line itself, drivers were crossed to wild-type and raised at maximum levels of expression (29°C). The data shows that lethality is elicited by both pre- and postsynaptic drivers in combination with *UAS-dgripΔ1-3*, allowing the screening of DGrip-related interactors in both the pre and post synapse.

3.7.5.2 Crossing Schemes

In order to bring candidate chromosomes into the *G14-gal4*::*UAS-dgripΔ1-3* background in a manner that ensures that these chromosomes can be reliably followed in the process of making the relevant stocks, the following schemes were designed. The candidate chromosomes are indicated with an asterisk *. The expected mendelian ratio if the mutant chromosome were to completely rescue the DGripΔ1-3 lethality (ie the experiment produces adults with the genotype *; *G14-gal4*:: *UAS-dgripΔ1-3*) is indicated below.

Mutations on X chromosome:

*/Balancer ♀ *x FM7i,act-GFP;+/+ ;+/MKRS* ♂

↓

**/FM7i,act-GFP;+/+ ; +/MKRS* ♀ x *FM7i,act-GFP; ;UAS-dgripΔ1-3* ♂

↓

**/ FM7i,act-GFP; +/+; UAS-dgripΔ1-3/MKRS* (establish stock as backup)

Experiment: Cross males (if transgene or mutation is non-lethal) or virgins to *G14-gal4*/*CyO,act-GFP*. Expected mendelian ratio: 1: 8.

Mutations on II chromosome:

*/Balancer ♂ x *7005-GFP/CyO,act-GFP;TM3,Ser/MKRS* ♀
↓
**/CyO,act-GFP;+/MKRS* ♂ x *+/CyO; UAS-dgripΔ1-3* ♀
↓
**/ CyO; UAS-dgripΔ1-3/MKRS* (establish stock as backup)

Experiment: Cross males from this stock to virgins from *G14-gal4*/*CyO,act-GFP*. Expected mendelian ratio: 1 :6. (CyO/CyO,act-GFP is embryonic lethal, therefore is not counted).

Mutations on III chromosome:

*/Balancer ♂ x *7005-GFP/CyO,act-GFP;TM3,Ser/MKRS* ♀
↓
+/CyO,act-GFP;/TM3,Ser* ♂ x *G14-gal4/CyO; TM3,Ser/MKRS* ♀
↓
*G14-gal4/ CyO,act-GFP; */MKRS* (establish stock as backup)

Experiment: Cross males from this stock to virgins from *UAS-dgripΔ1-3*. Expected mendelian ratio: 1 :4.

The mendelian ratio of positively interacting flies was calculated as follows: the number of animals with the genotype *G14-gal4*::*UAS-dgripΔ1-3*; * over the total of all (animals including dead pupae) divided by the expected mendelian ratio.
Each experiment was designed such that each individual experiment has its own control (ie animals which were *G14-gal4*::*UAS-dgripΔ1-3* and should not survive to adulthood) to compare against any potential positive interactor (adult flies with the genotype *, *G14-gal4*::*UAS-dgripΔ1-3*). In this screen, the number of escaper animals (*G14-gal4* ::*UAS-dgripΔ1-3* adults) was small, varying between 0% of the observed adults to, in extreme cases, 3% of the total number of adults in individual experiments.
Screening on the third chromosome produced the highest level of escapers (*G14-gal4*::*UAS-dgripΔ1-3*), on average representing one percent of the total population, or four percent of the expected mendelian ratio. Screening on the X and second chromosomes produced an average escaper rate of 0.29% of total flies. These escapers, which were predominantly male, had no discernable morphological or behavioural defect- they responded to CO_2 (the gas used to immobilise flies for sorting) in the same manner as their siblings, were able to fly and to respond to sudden movements, and had no gross defects in wing, eye or leg morphology. The fact that the screen of the third chromosome habitually produced higher escaper rates may be attributed to one of two factors: The

experiment is susceptible to a genetic background effect carried in one of the stocks used for the experiment, or incorporating the *G14-gal4* chromosome in the generation before crossing to *UAS-dgripΔ1-3* may have caused difficulties. Such difficulties may be, particularly in the case of screening with transgenic chromosomes, that the transgene to be screened is itself lethal (or at least sick) in the presence of *G14-gal4*, thus leading to the selection pressure on males for the experiment which do not express *G14-gal4* well. To reduce this problem, males for the experiment were taken where possible directly from the first cross that produced the correct genotype, and the stock was maintained as a backup, so as to reduce selection pressure against *G14-gal4* expression.
Several transgenes (*UAS-Baboon* and *UAS-Ras8D* among them) were pupal lethal with the driver *G14-gal4* and thus could not be screened. Due to the fact that they were lethal at the same stage as *UAS-dgripΔ1-3*, they were in fact good candidate chromosomes and were examined via alternate methods.

3.7.5.3 Screen – Result

The results for each individual line are presented in **Appendix I**. Selection criteria for a positively interacting stock were as follows: The number of escaper (*G14-gal4*::*UAS-dgripΔ1-3*) or positively interacting (*;*G14-gal4*::*UAS-dgripΔ1-3*) flies was significantly larger than that present in the negative control for that experiment, and was greater than 5% of the expected Mendelian ratio if the gene was completely able to rescue the DGripΔ1-3-induced lethality. Several lines were found, and were classified into either weak (5- 30%, coded orange) or strong (>30% Mendelian ratio, coded yellow). These results were then confirmed by performing the same experiment again from the same stock where possible, or by recreating the stock again and repeating the experiment. Positively interacting adults were collected and the presence of the mutated chromosome was confirmed by single-fly PCR.

Strong interactors:
Baboon (32), protein null..... **93%**
Echinoid (slH8), ems allele, possibly null..... **91%**

Weak interactors:
Echinoid (slA12), hypomorphic allele..... **20%**
Ras opposite (G27) ems allele, functional null [40]..... **10%**
RhoL (*UAS-RhoL* N25) dominant negative transgene **7%**
Phyllopod (2245),x-ray allele..... **5%**

Two of these interactors, Baboon and Echinoid, are studied in more detail below. In passing, it is interesting to note that one of the weak interactors in this screen was an allele of Ras Opposite (*rop*), which is the *Drosophila* homologue of Munc-18 [38, 40, 210-212]. As such, it is a good candidate as a weak direct, or possibly indirect, interactor which may go in some way to explain the presynaptic vesicle release phenotype obtained in DGrip mutants (**Section 3.10**).

3.8 Echinoid and the EGFR pathway

3.8.1 Echinoid and DGrip genetically interact

Thus far, Echinoid was identified as a DGrip interactor in several experiments, as a yeast-two-hybrid interactor which was specifically abolished in point mutations of PDZs 1-3 and 7 (**Section 3.7.4**), and as a strong repressor of DGripΔ1-3 mediated pupal lethality (**Section 3.7.5**). This interaction was studied in more detail, first to understand whether Echinoid is involved in the muscle guidance process at all, or whether its interactions with DGrip are only relevant in the other later processes that contribute to the DGripΔ1-3 mediated pupal lethal phase.
Echinoid has not yet been reported as having a phenotype related to muscle development, although it is described as an antagonist of EGFR signalling, particularly in photoreceptor development [213-220]. However, *in situ* hybridisation and antibody stainings for Echinoid in embryonic tissues show *echinoid* expression in embryonic mesoderm and CNS [213]. To look if Echinoid has a specific role in muscle guidance, the ed^{SlH8} allele which had interacted in the DGripΔ1-3-suppression screen was examined for muscle formation defects. This allele of *echinoid* was induced by ems mutagenesis [221] and is described as loss-of-function allele with recessive pupal lethality, although the exact molecular lesion is not reported [214]. The ed^{SlH8}/ed^{SlH8} larvae were retarded in their growth compared to their $ed^{SlH8}/+$ siblings, but were able to survive to at least early third larval instar, allowing dissection.
The dissected muscles clearly showed that ed^{SlH8} homozygotes have muscle defects (**Figure 34**), arguing that Echinoid is also involved in the muscle guidance process. The ed^{SlH8} muscle phenotype shows a mild disturbance of both the LTM and VLM muscles, where the VLM muscle defect is reminiscent of partial loss of DGrip function.

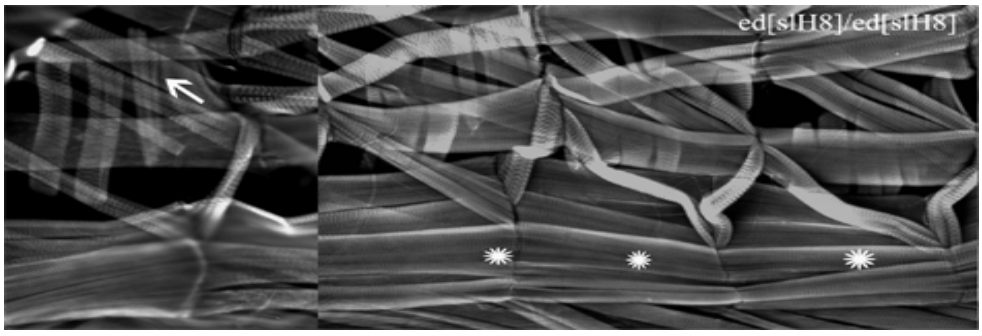


Figure 34: ed^{SlH8} homozygotes show several muscle defects in both VLM and LTMs. ed^{SlH8} LTMs split into multiple processes, instead of preserving their bar-like morphology (arrow), and the VLMs of these animals (particularly muscles 6 and 7 are most affected, similar to DGrip mutants) are also mildly misguided (asterices).

To understand whether Echinoid signalling interacts with DGrip-dependent muscle guidance, the phenotype of $dgrip^{ex36}$; ed^{SlH8} transheterozygotes was examined.
Naturally, as $dgrip^{ex36}$ is on the X chromosome, the males identified in this experiment are full mutants for DGrip, with one copy of the mutated *ed* chromosome, whereas females are classical transheterozygotes with each mutant chromosome over a wild-type copy. In

embryos, males with the *dgrip*^{ex36} chromosome present a more severe muscle phenotype when the mutated *echinoid* chromosome is present (**Figure 35**). Both the LTMs and VLMs are affected in *dgrip*^{ex36} animals with a half-dose of Echinoid, again suggesting that perhaps DGrip is also expressed at a low level in the LTM group of muscles, albeit at level which cannot be detected by antibody staining (see **Discussion**).

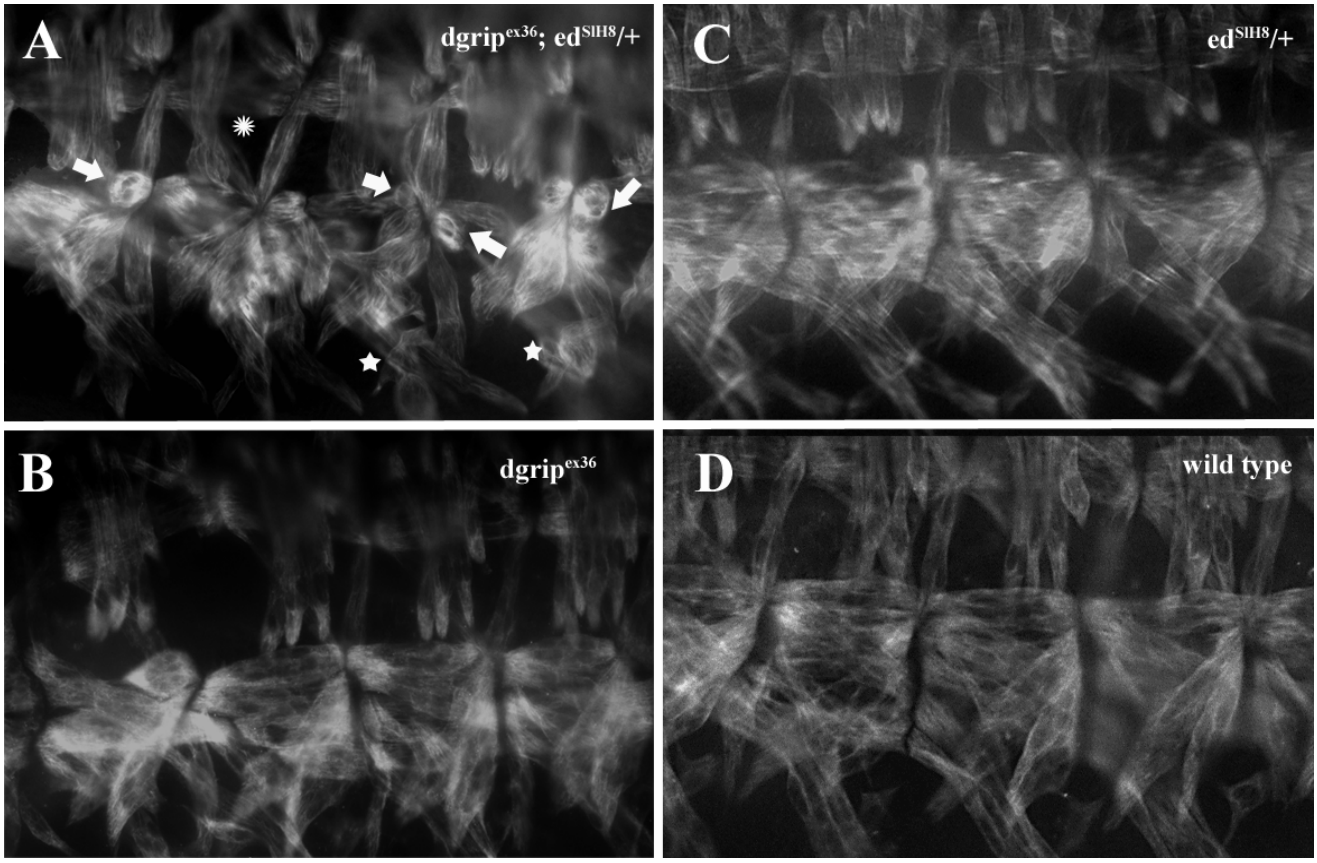


Figure 35: loss of one copy of *echinoid* enhances *dgrip*^{ex36} defects, affecting more muscle groups. **A.** a *dgrip*^{ex36};*ed*^{SlH8}/+ embryo, demonstrating a more severe muscle defect than *dgrip*^{ex36}. VLM muscles 6 and 7 are affected as in the *dgrip*^{ex36} mutant, but also the other two muscles of the VLM group, 12 and 13, show a greater number of guidance defects (arrows). The LTMs are occasionally missing (asterisk), a defect not observed in *dgrip*^{ex36} mutants, and the ventral oblique muscles (stars) are also mildly misguided. **B.** *dgrip*^{ex36} male animal. **C.** *ed*^{SlH8}/+ embryos do not show detectable defects. **D**. Wild-type embryo.

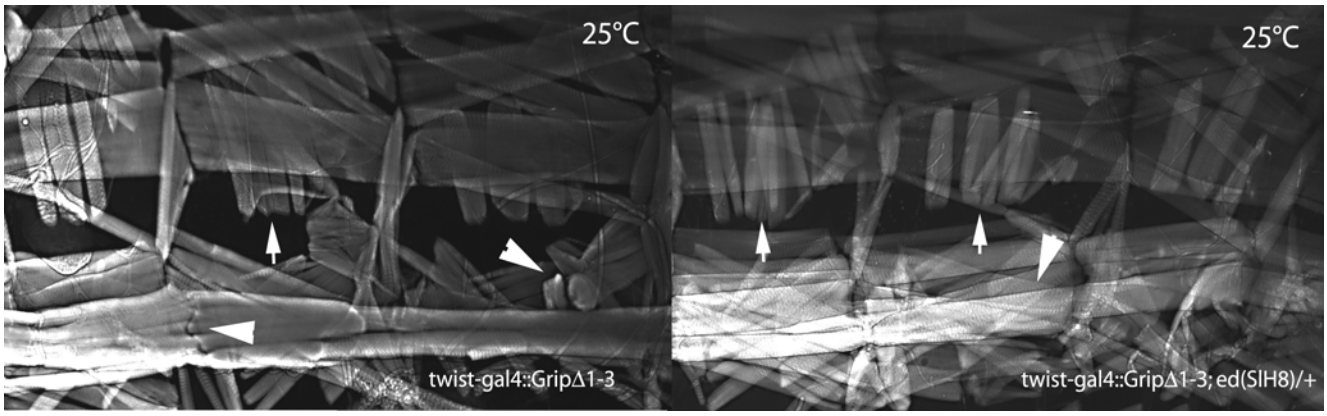


Figure 36: One copy of ed^{SlH8} does not transheterozygously repress the DGripΔ1-3 muscle defect. **A.***twist-gal4*::*UAS-dgripΔ1-3*; ed^{slH8}/+ larva. **B.** *twist-gal4*::*UAS-dgripΔ1-3* larva. This experiment was repeated at lower levels of transgenic expression (18°C), but gave similar results. Arrowheads indicate VLMs, arrows indicate LTMs.

It was tested if transheterozygosity for the ed^{SlH8} chromosome can suppress the DGripΔ1-3-mediated LTM phenotype, as this chromosome had been able to suppress DGripΔ1-3-mediated pupal lethality (**Section 3.7.5.3**). This was not the case (**Figure 36**). In the future, it would be most instructive to test if the DGripΔ1-3 LTM phenotype can be repressed by the complete absence of Echinoid protein.
However, there is some evidence that Echinoid in muscles is regulated by DGrip, as $dgrip^{ex36}$ larval muscles are sensitive to *twist-gal4* expressed Echinoid protein (**Figure 37**). When Echinoid is expressed in a wild-type background with *twist-gal4*, very minor defects are found, but in the absence of DGrip, these defects become considerably stronger.

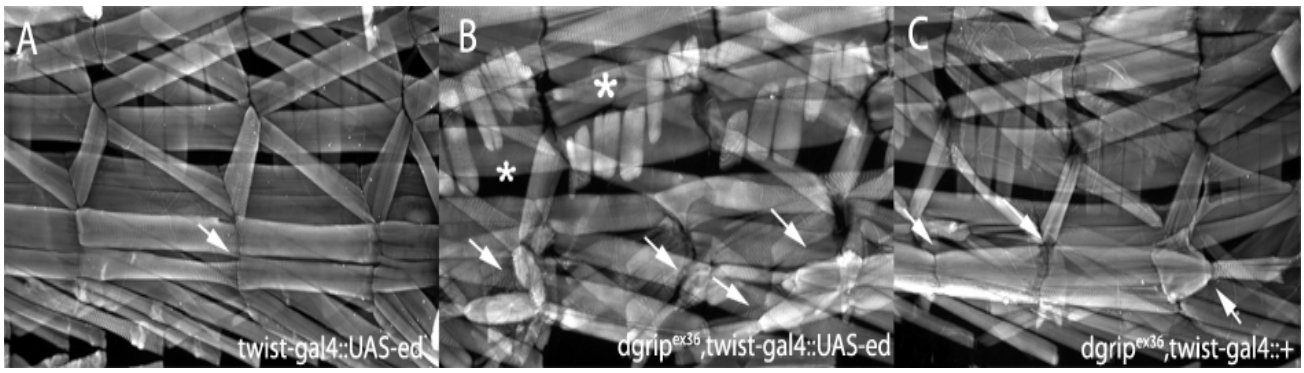


Figure 37: $dgrip^{ex36}$ animals are sensitive to Echinoid expression in muscles. Muscle-specific expression of Echinoid in the presence of wild-type DGrip (**A**) causes minor VLM defects (arrows), while in the $dgrip^{ex36}$ animal (**B**), *UAS-ed* evokes mild LTM defects (asterices) and strongly enhances the VLM phenotype of $dgrip^{ex36}$ mutants (**C**, arrows).

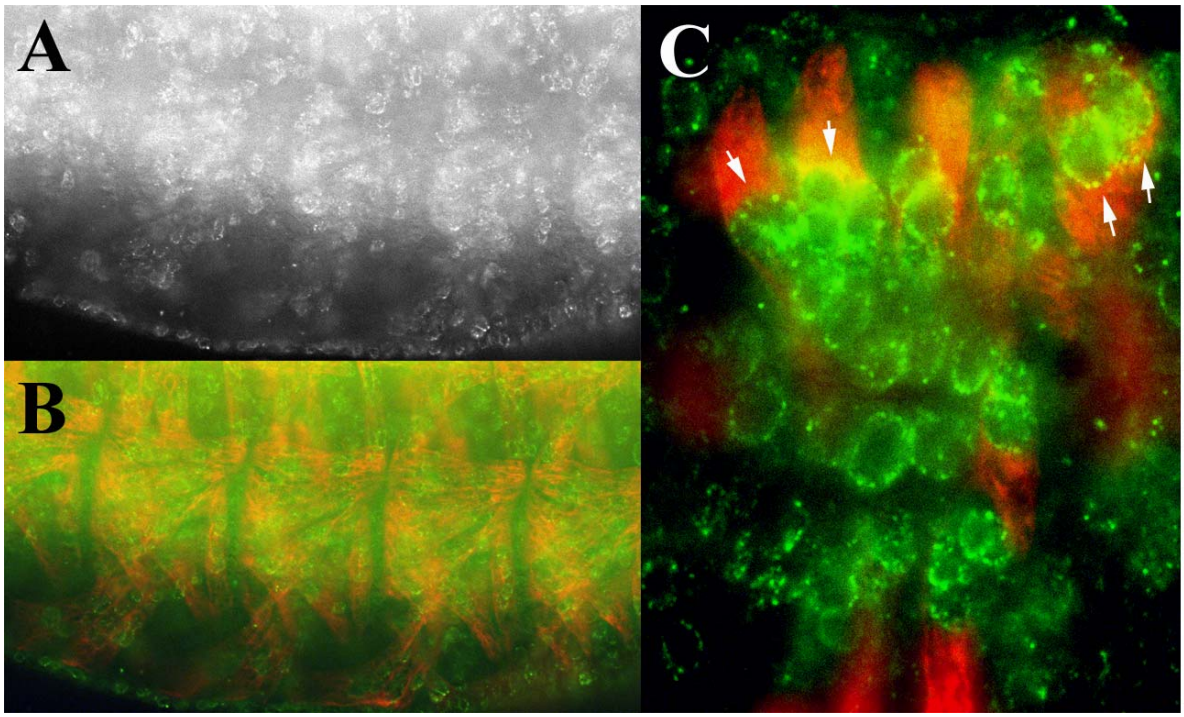


Figure 38: Echinoid staining in wild-type embryos. **A** anti-ed staining is stronger near, but not restricted to, muscle cells **B**. Colocalisation of Echinoid (green) with muscle myosin (red) in wild-type muscles. **C**. Echinoid appears in a perinuclear pattern, sometimes around muscle nuclei (arrows), both in VLM and LTM muscles (here, the LTM group of muscles are shown). However, Echinoid staining is also apparent around nuclei of other cells.

3.8.2 EGFR signalling in DGrip mutants

Echinoid is described as a negative regulator of Epidermal Growth Factor Receptor (EGFR) signalling, particularly in the process of photoreceptor development and specification in the *Drosophila* eye. To understand whether Echinoid also functions in the same manner in myotubes, EGFR-dependent signalling in the muscle guidance process was investigated. The regulation of EGFR activity was visualised in embryos by markers downstream of EGFR activation.

A long-established marker of EGFR activation [222-226] in *Drosophila* was used, a phospho-specific antibody against the activated, doubly phosphorylated form of Rolled, otherwise known as dpERK. This marker is downstream of Ras activation elicited by receptor tyrosine kinases [227, 228], not only EGFR, and as such must be controlled for pathway specificifty. While activation of dpERK was not revealed in myotubes, although it has been reported in detailed studies by Gabay and coworkers [227, 228], dpERK staining in late-stage embryos was identified in the target cells of the developing myotubes, the apodemes.

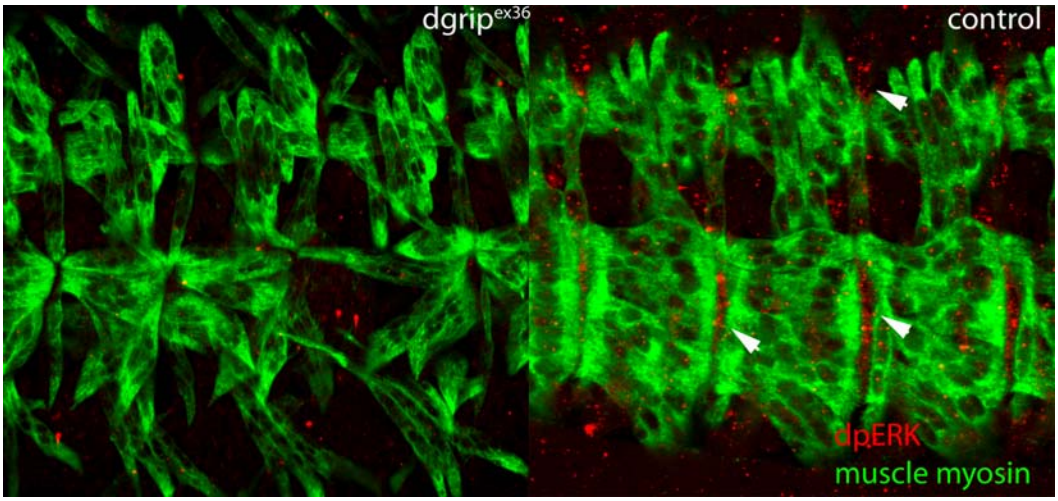


Figure 39: Z-projection of confocal scans in embryos stained for muscle myosin and dpERK in late-stage *dgrip*^{ex36} and control embryos. DpERK staining is found in the apodemes of control embryos, while it is strongly reduced in *dgrip*^{ex36}, suggesting dpERK signalling is reduced in the apodemes of *dgrip*^{ex36} muscles, even when muscles are able to span the segment and attach to their segment border.

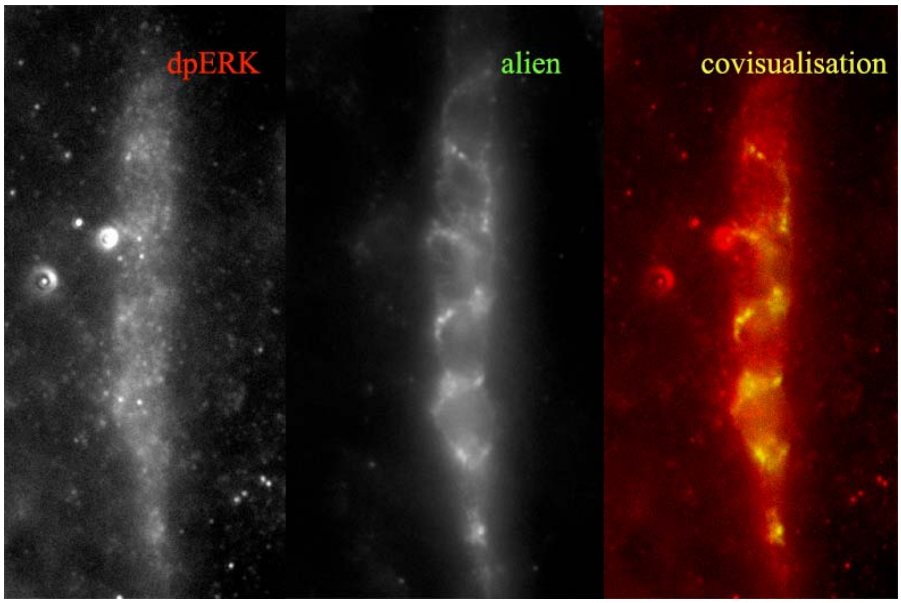


Figure 40: dpERK is expressed in apodemes, marked by Alien [229] in wild-type embryos.

Curiously, *dgrip*^{ex36} embryos show much weaker dpERK staining in these cells than in control embryos (**Figure 39**). Yarnitzky and co-workers [150] have studied this process in some detail: They found that somatic muscles produce a weak EGFR ligand, Vein, which signals to the apodeme, which, via a ras-dependent pathway induces the expression of terminal markers of apodeme differentiation, proteins like Delilah and β-tubulin. Thus, the last stage of apodeme maturation is dependent on signalling from the muscle. The observation had been made that Delilah staining was reduced in *dgrip*^{ex36} embryos (Stephan Sigrist, personal communication). It was now checked to see if Alien protein expression, which in *vein* mutants is not decreased [150], was also reduced in *dgrip*^{ex36} animals (**Figure 41**). Like published *vein* mutants, Alien expression is not strongly affected in *dgrip*^{ex36} animals (**Figure 41**).

These results strongly suggest that the process of EGFR-dependent apodeme maturation is not completed in *dgrip*^{ex36} mutants, even in those muscles which make complete contact to the apodeme. It is interesting to note that this maturation process seems not to be completely necessary for the integrity of the apodeme-muscle contact, as, as mentioned before, these muscles are fully resistant to the strain placed on them by larval locomotion.
Nevertheless, this process is a most interesting model system to understand how DGrip may interact in EGFR signalling. dpERK staining could be restored to apodemes by the muscle-specific expression of DGrip (**Figure 42**), indicating that DGrip acts in *trans* on the EGFR during this process. Further work must be done to understand if this is due to the action of Echinoid on EGFR or the Vein ligand.
To then return to the muscle guidance process with information provided by this model system may prove most instructive, in particular to see if EGFR signalling is regulated by DGrip in the same way in both tissues.

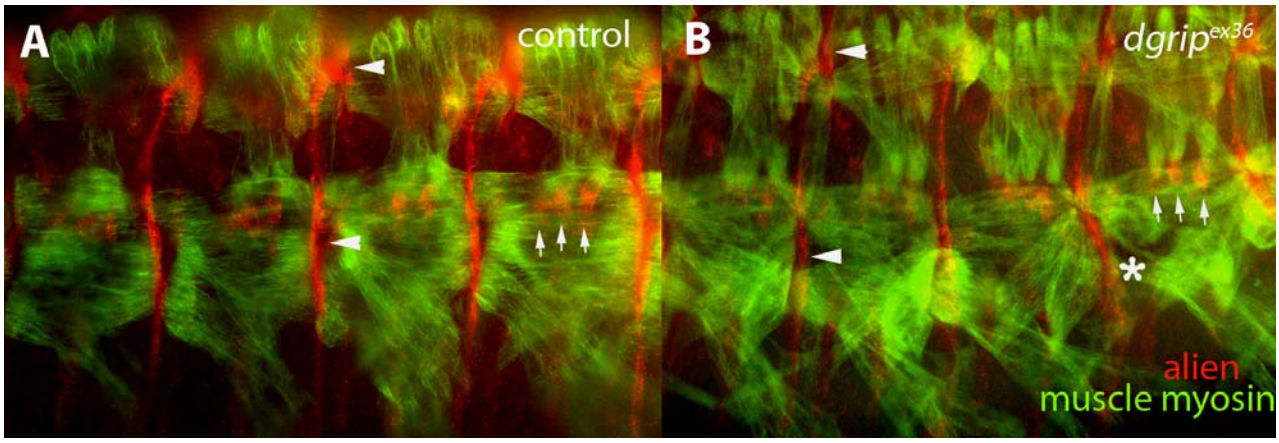


Figure 41: Alien staining is not affected in *dgrip*^{ex36} apodemes, indicating that apodemes are present and otherwise normal. Alien is expressed at apodemes for the LTM group (arrows), and at the segment border (arrowhead), even at positions where the *dgrip*^{ex36} muscle does not make full contact to the segment border (asterisk).

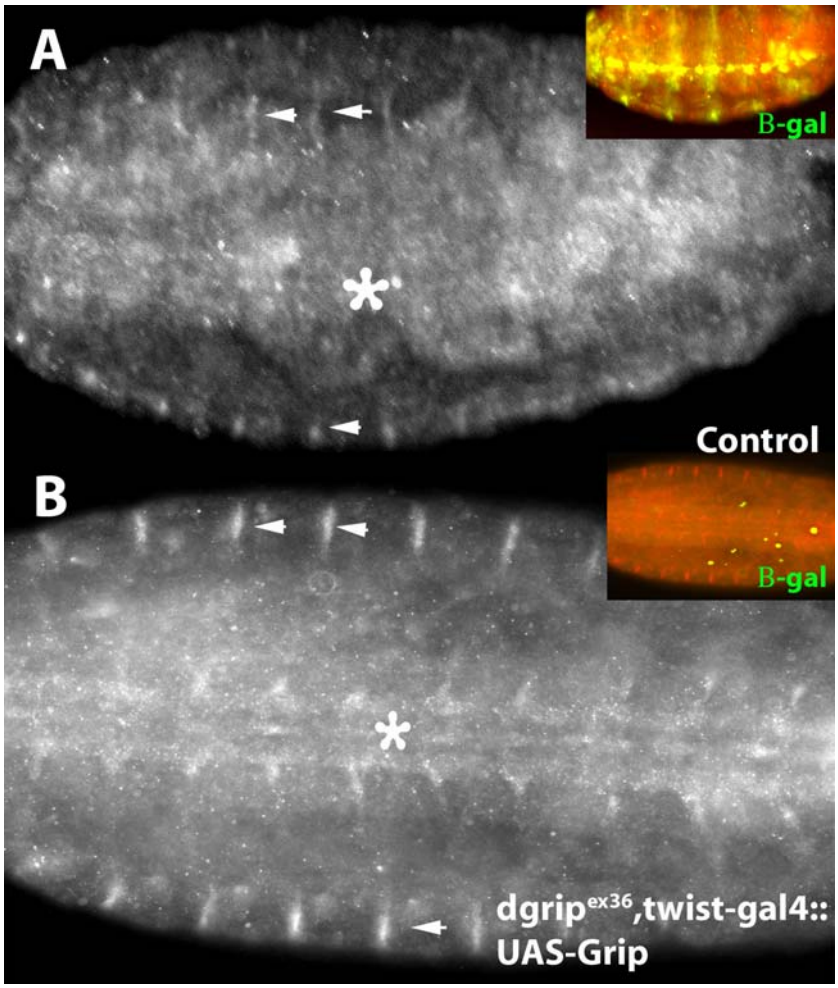


Figure 42: dpERK staining is restored by muscle-specific expression of DGrip in *dgrip*^{ex36} mutant embryos. *dgrip*^{ex36}, *twist-gal4*/FM7, *ftz::lacZ* virgins were crossed to *UAS-dgrip* males and the resulting embryos stained with anti-Sex Lethal to detect females and anti-β-gal to detect non-mutants. **A**. A β-gal stained control male embryo shows the same distribution of dpERK as the β-gal negative *dgrip*^{ex36}, *twist-gal4*::*UAS-dgrip* male (**B**). Apodemes (arrowheads) both show strong dpERK staining. The asterisk indicates dpERK in the ventral nerve chord. Insets: β-gal staining.

Looking at muscle guidance, it was established that EGFR signalling could exert an influence on muscle morphologies (**Figure 44**). While EGFR expressed with the pan muscular driver *twist-gal4* in a wild-type background has very little effect on muscle morphology, *dgrip*^{ex36} muscles are sensitive to EGFR expression. *dgrip*^{ex36}, *twist-gal4*:: *UAS-EGFR* segment spanning muscles, such as muscles 4, 6,7,11 and 12 show greater defects than *dgrip*^{ex36} muscle alone. The fact that EGFR expression in the wild-type background produces no visible effect, while it is able to produce relatively severe effects in *dgrip*^{ex36}, suggests that DGrip is involved in EGFR regulation, and that loss of DGrip allows EGFR to become ectopically active.

Vein is involved in the EGFR-dependent specification of muscle precursors, and in muscle-dependent maturation of apodemes [150], but has not before been demonstrated as necessary for the intervening process of muscle guidance. Preliminary results (**Figure 43**) suggest that the Vein ligand may in fact also act during muscle guidance, as mutants of *dgrip*^{ex36} show stronger muscle defects when one copy of the Vein ligand is missing. As Vein is a ligand produced by the muscles themselves, it will be most interesting to

understand how Vein acts on the muscles during muscle guidance, and what is the role of EGFR signalling in this process.

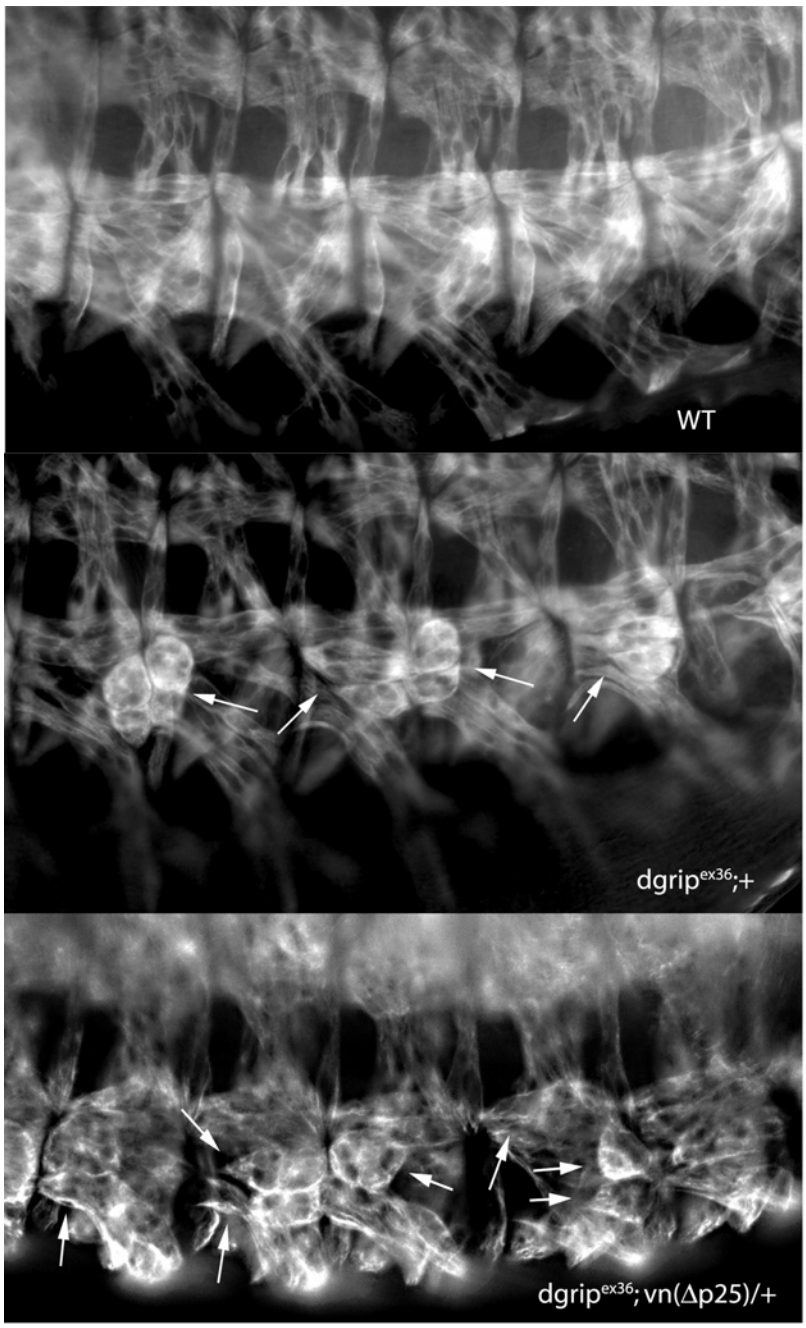


Figure 43: *dgrip^{ex36}* embryonic muscles are sensitive to mutations in the EGFR ligand Vein. *dgrip^{ex36}* males, carrying one copy of *vn$^{\Delta p25}$*, a strong mutant of the Vein ligand, show increased defects in the VLM group of muscles, (arrowheads), including more severe defects in muscles 12 and 13. This is a first indication that the Vein ligand may in fact signal to the muscles in which it is produced.

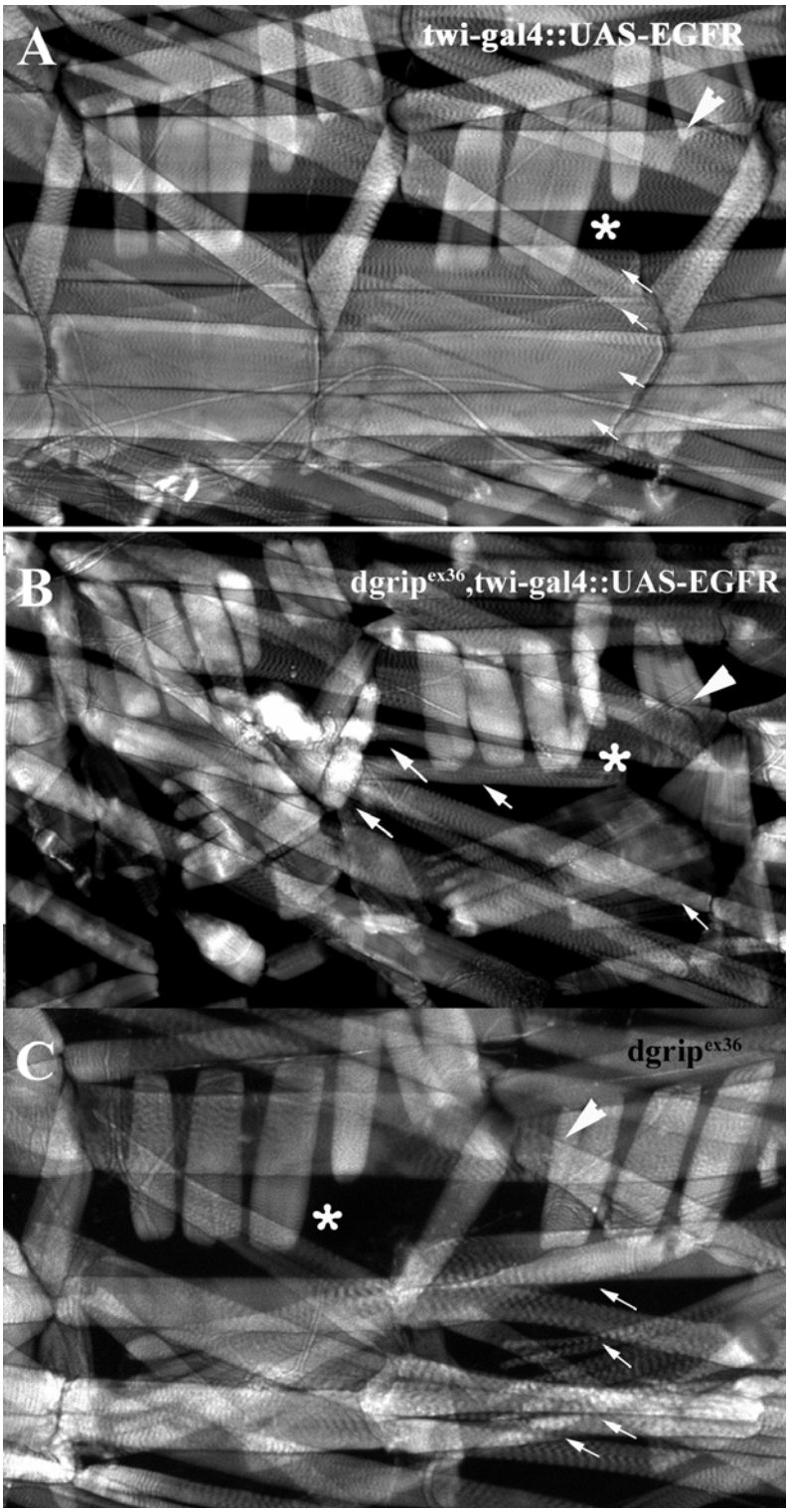


Figure 44: **A**. *twist-gal4*::*UAS-EGFR* male **B**. *dgrip*^{ex36}, *twist-gal4*::*UAS-EGFR* male. DGrip negative animals are sensitive to overexpression of the EGFR, leading to defects in muscles 4, 6, 7, 12 and 13 (arrows) as well as the LTM group of muscles (asterices). Only minor defects in the LTM group of muscles are visible in *twist-gal4*::*UAS-EGFR* males.

3.9 DGrip and TGFβ signalling.

Having also found members of the TGFβ pathway in both a yeast two-hybrid screen (Mad) and via the suppression-of-lethality screen (Baboon), mutants were examined to see if these signals played a role in muscle guidance. A point to consider was that the TGFβ signalling members identified were in fact members of different signalling cascades: Mad is involved in the downstream transduction of signals from the ligands Decapentaplegic (Dpp) [230] and Glass-bottom boat (Gbb) [231, 232], whereas Baboon is a receptor for a different ligand, dActivin [200], and its downstream transduction is mediated by a protein called Smox [233], and not by Mad [234, 235]. This of course begs the question: which if any of these two pathways are involved in muscle guidance?
Thus far, the mutant allele of Baboon, $babo^{32}$ was examined. This allele acted as a suppressor of DGripΔ1-3 mediated lethality (**Section 3.7.5.3**). Mutants of Baboon, and overexpressed members of the Baboon-dependent signalling cascade were characterised to understand the role Baboon has, if any, in muscle guidance.

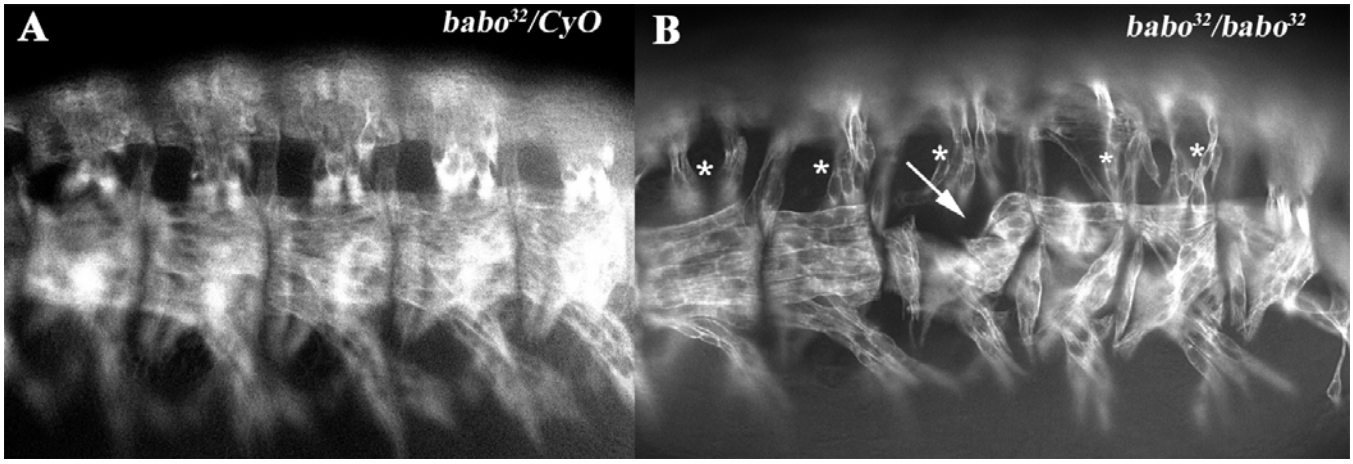


Figure 45: Preliminary result: $babo^{32}$ has a muscle guidance phenotype. Embryos from a $babo^{32}/CyO$ collection were stained with anti-muscle myosin. Two types of muscle morphologies were observed, one essentailly wild-type (**A**), putatively corresponding with $babo^{32}/CyO$ genotype, and animals (**B**), likely $babo^{32}/babo^{32}$, with marked defects in particularly LTM (asterisk) and VLM (arrow) formation. There were no $babo^{32}$ homozygous larvae produced by a $babo^{32}/CyO$, *act:GFP* stock, meaning these defects could not be examined in larvae.

It was examined if the $dgrip^{ex36}$ muscle defect could be enhanced by expressing the Baboon receptor in muscles using *twist-gal4*, as had been the case with both Robo2 and the EGFR.
Expression of a Baboon transgene with the driver *twist-gal4* was lethal in larvae, and thus embryos were examined for muscle defects. In embryos expression of a *UAS-Baboon* construct does in fact, enhance the $dgrip^{ex36}$ defect, but only in the VLM group of muscles.

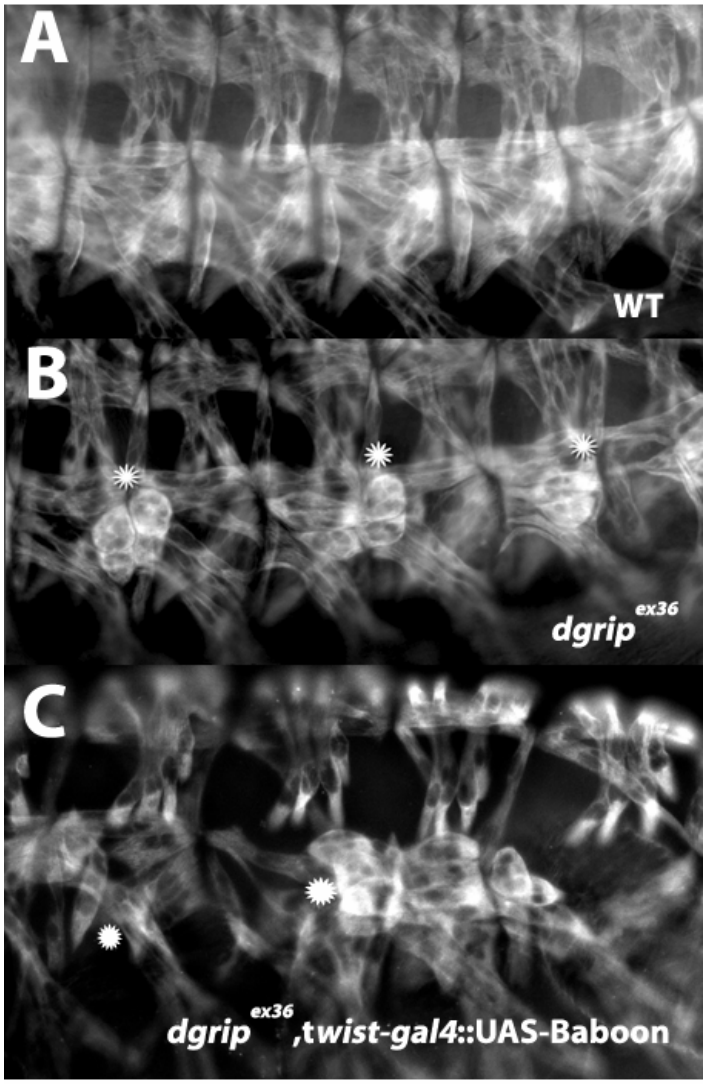


Figure 46: Expression of Baboon enhances the dgrip^{ex36} defect in the VLM group of muscles (asterices), but does not affect the LTM group of muscles

Overexpressing a downstream effector of Baboon-dependent signalling, Smox/dsmad2, in the *dgrip*^{ex36} background, did not increase the severity of the *dgrip*^{ex36} phenotype. The animals also survived to larval stages, where they were dissected. Both the lack of early lethality, and the lack of any enhancement of the *dgrip*^{ex36} phenotype indicate that Baboon receptor activation is necessary for Smox's function in muscle guidance. This also suggests that a ligand for Baboon is present in the embryonic muscular system, although if it is the known ligand dActvin, or one of the as yet uncharacterised TGFβ ligands such as the muscle-expressed TGFβ ligand myoglianin [236] is as yet untested.

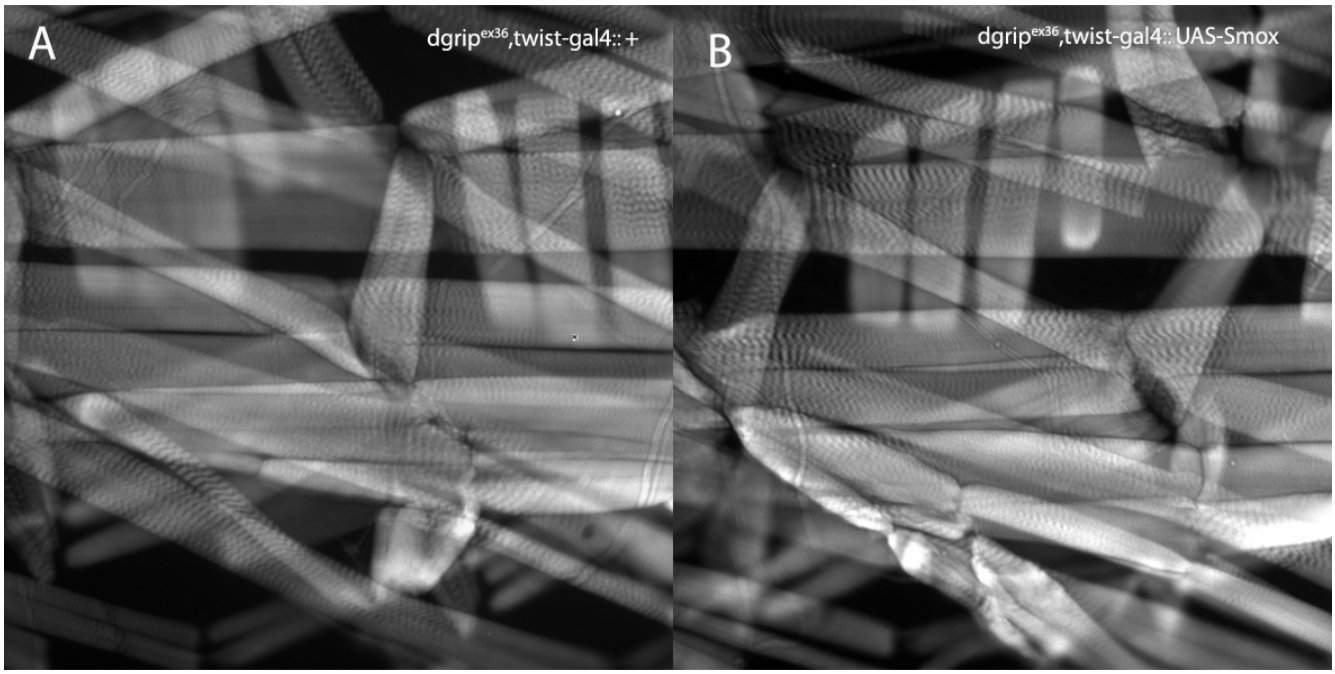


Figure 47: Smox expression does not make the *dgrip*^{ex36} phenotype more severe .**A**. Control animal, *dgrip*^{ex36}, *twist-gal4*. **B** Animals expressing a *UAS-smox* transgene in this background show the same muscle phenotype.

Unfortunately, reliable pMad immunostaining in muscles has not thus far been detected, meaning that markers of the state of activity for the Dpp and Gbb signalling pathways mediated by Mad phosphorylation can not be examined. It would very interesting to study, not only how Baboon-dependent muscle guidance interacts with either Echinoid or Robo2 dependent guidance, but if DGrip is even involved in the regulation of different inputs for TGFβ signalling as well.

3.10 Reduction of DGrip causes defects in presynaptic neurotransmitter release.

There are also indications that DGrip has a role in presynaptic processes at the *Drosophila* Neuromuscular Junction (NMJ). Due to the massive reorganisation of the postsynaptic muscle in *dgrip*^{ex36} mutants, the postsynapse was not accessible for electrophysiological techniques. Instead, *dgrip*^{ex36}, *twist-gal4*::*UAS-dgrip* male larvae were used to see if there was a measurable presynaptic defect. In these larvae, the muscle defect is rescued by *twist-gal4::UAS-dgrip*, allowing electrophysiology to be carried out, but the presynapse is still a *dgrip*^{ex36} mutant.
In such larvae, henceforth called '*dgrip*^{ex36} muscle-rescued' larvae, a strong defect in Ca^{2+} dependent synaptic release was found (Robert Kittel, personal communication). In *dgrip*^{ex36} muscle-rescued larvae in 1mM external [Ca^{2+}], the evoked junctional current (EJC) is potentiated by 50% over the appropriate control.
As the external calcium concentration is lowered, this effect becomes significantly stronger, to a point where in 0.2mM $[Ca^{2+}]_{ext}$ the *dgrip*^{ex36} muscle-rescued synapse is ten times more likely to release a vesicle in response to stimulation as the control junction. Curiously, the frequency of miniature EJCs was not altered in *dgrip*^{ex36} muscle-rescued larvae. These and other experiments conducted in the laboratory suggest that the defect in *dgrip*^{ex36} muscle-rescued synapses is primarily one of increased release probability, interestingly coupled with an increase in basal levels of vesicle cycling to replenish the releasable pools of synaptic vesicles (Robert Kittel).
This was confirmed by using a presynaptically-expressed RNAi transgene against DGrip (Manuela Schmidt, Robert Kittel) which produced a similar potentiation of EJCs, suggesting that zygotically expressed DGrip has a function in the presynapse. However, as overexpression or presynaptic rescue of *dgrip*^{ex36} muscle-rescued larvae with DGrip cDNA in the presynapse has the same, potentiating effect on EJCs when compared to controls, it is difficult to determine what isoform of DGrip may be responsible for presynaptic function, or if it is possible to rescue the *dgrip*^{ex36} presynaptic defect to normal levels of release (Robert Kittel, personal communication).
DGrip, as a protein with known functions in endocytic processes [113] and with a known distribution which includes endosomal compartments [130], may exert its effect on presynaptic release via changing endocytic trafficking. For this reason, presynaptic endocytotic compartments were examined for changes in appearance or distribution. Endosomes were counted using FYVE-GFP, a marker of early endosomes which intercalculates into PI(3)P-enriched membranes in a rab-5- and wortmannin-dependent manner [237]. This was presynaptically expressed in *dgrip*^{ex36} and wild-type junctions with two different presynaptic drivers (*elav-gal4*, *OK6-gal4*), to exclude artefacts arising from the use of the driver line.
Synapses on muscle 4 were chosen for this analysis, as this muscle's morphology is not disturbed in the *dgrip*^{ex36} mutant. This allows a clear comparison between wild-type and mutant neuromuscular synapses, without concern that the postsynaptic muscle morphology could influence the formation of presynaptic compartments.
Using both the *OK6-gal4* and *elav-gal4* driver lines, a significant increase was observed ($p<0.0005$, single-tailed t-test) in the number of FYVE-labelled compartments per bouton

in *dgrip*^{ex36} males compared to wild-type males (**Figure 48**), indicative of an increase in the number of early endosomal compartments.

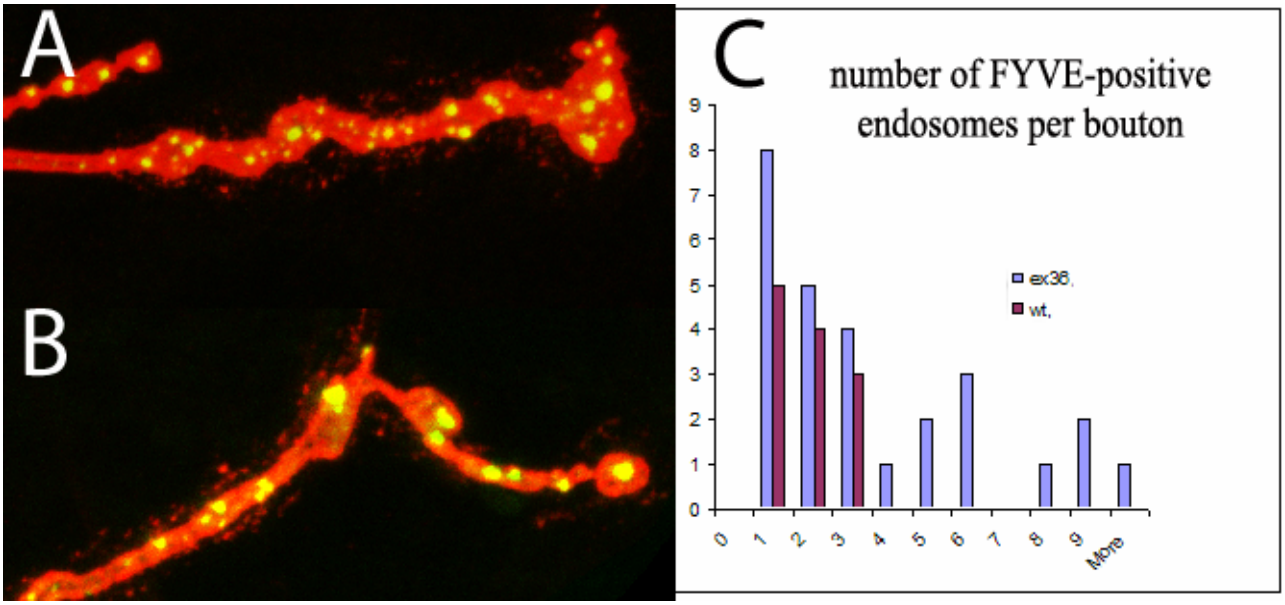


Figure 48: The number of FYVE-GFP positive endosomal compartments is increased in *dgrip*^{ex36} presynapses. Male larvae expressing the FYVE-GFP endosomal marker, driven by *OK6-gal4*, in (**A**) *dgrip*^{ex36} and (**B**) wild-type backgrounds. Larvae were immunostained for GFP and the presynaptic membrane marker horse radish peroxidase (HRP). **C:** Histogram showing distribution of number of FYVE-GFP positive punctae per bouton, assayed in synapses on muscle 4. In wild-type animals, the majority of boutons have 1 to 3 FYVE-GFP positive punctae, whereas *dgrip*^{ex36} boutons tend to have more.

Furthermore, electron microscopic investigation of wild-type, *dgrip*^{ex36} and *dgrip*^{ex36} muscle-rescued male larvae showed that both *dgrip*^{ex36} and *dgrip*^{ex36} muscle-rescue larvae show the appearance of large (150-300nm) vesicles in the presynapse without any disturbance of the number or distribution of synaptic vesicles (**Figure 49**, Carolin Wichmann, personal communication). There is not, as yet, clear evidence that these abnormally large vesicles are the same FYVE-GFP labelled vesicles observable under light microscopy, however there is a strong likelihood that these two phenomena are correlated.

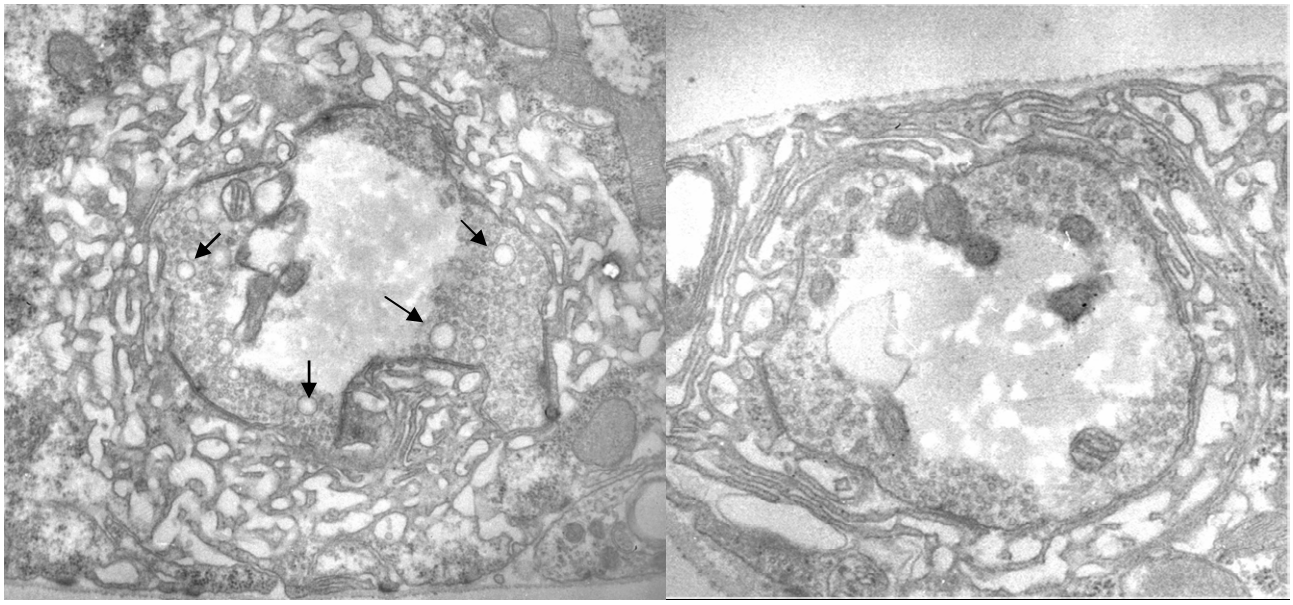


Figure 49: *dgrip*^{ex36} (left panel) and wild-type (right panel) boutons from third instar male larvae. Note the presence of abnormally large vesicles (arrows) in the *dgrip*^{ex36} presynapse (images courtesy of Carolin Wichmann). These vesicles are also observed in the *dgrip*^{ex36} muscle-rescued larvae used for electrophysiological recordings (not shown).

4.Discussion

4.1 DGrip is necessary for myotube guidance

While proteins of the mGRIP family have first been described in the context of synapse formation and plasticity for the clustering and/or transporting of synaptic proteins - most notably glutamate receptors - studies in mice have already indicated a much broader spectrum of functions for mGRIPs, even early in development [114, 115]. *Drosophila* Grip has a key function in the guidance of myotubes throughout the development of somatic muscles in the fly embryo. While the processes of myotube specification, and even that of muscle-apodeme adhesion, have been described in great detail [56-58, 150, 161, 162, 224, 236, 238, 239], the process of myotube guidance has not been extensively investigated, and besides a few studies, our understanding of the process is minimal. DGrip is one of very few factors specifically involved in this process described so far with homologues in both *Drosophila* and mammals [60, 150, 236, 239-241] although given the paucity of work on this process, more are likely to be uncovered.
The DGrip transcript and protein are predominantly expressed within a specific subset of embryonic muscles – the ventral longitudinal muscles (VLMs), although we are not able to positively exclude the existence of DGrip in other sets of muscles such as the lateral transverse muscles (LTMs). The VLM founder cells are determined in the posterior part of the segments and extend growth-cone like projections to target specific end-segment attachment sites where finally the muscles form stable connections in a tripartite complex with epidermal apodemes, the extracellular matrix and other VLMs [51-54, 68, 164, 242].
The elimination of DGrip function in *Drosophila* embryos removes the ability of the VLM group of muscles to guide themselves correctly to the segment borders. Instead, cells form abnormal morphologies and then often form ectopic contacts with other muscles (**Figure 7**). The lack of DGrip within the nascent myotubes is directly responsible for the defects observed, as this defect was rescued by expressing DGrip within the nascent VLMs, while epidermal expression had no rescuing effect.
In mouse, mGRIP1-negative animals develop abnormalities in dermo-epidermal junctions [115]. In the case of these mutants, the underlying defect was the mistrafficking of a extracellular matrix component, the cell adhesion molecule Fras1 [114, 115]. That the junction between epidermis and specific muscles is not properly formed in the *Drosophila grip* mutant, might on first sight hint towards an underlying defect in the stabilisation of attachment. However, this is unlikely, as defects in $dgrip^{ex36}$ are essentially limited to one muscle group. A specific loss of adhesion such as this would be unusual for cell adhesion mutants, but more compatible with a loss of guidance factors, where all pathways identified to date are muscle subgroup specific [60, 236]. The ability of *dgrip* mutants to form stable attachments seems intact, as VLMs formed integrin-positive attachment sites in their new positions (**Figure 8**) and were clearly firmly anchored, as *dgrip*-mutant larvae were capable of sustained locomotion without rupturing these muscle contacts.
As these observations already indicated that the $dgrip^{ex36}$ phenotype might not primarily be a muscle detachment phenotype, such as that shown for integrin mutants [51-54, 68, 163, 164, 172, 242, 243], the muscle guidance process was also studied in detail.

The expression of endogenous DGrip perfectly coincides with the time period for VLM motility, where the nascent myotubes sample epidermis in order to find their prospective contact points. The development of nascent VLMs was reconstructed in the relevant period [244]. In wild-type, all projections invariantly project anteriorly, towards their future attachment site, whilst DGrip is progressively enriched in the leading edge of these structures. In *dgrip* negative animals however, these extensions are unusually short and the direction of their projection appears essentially randomised.
The LTMs, a class of muscle which respond to a different set of guidance cues than VLMs [60], form ectopic extensions upon strong overexpression of the DGrip cDNA, and upon expression of a dominant active version of DGrip missing the first three PDZ domains. These ectopic extensions most often contacted the segment borders and became stabilised, as they are still easily identified in late larval development (**Figures 9, 16 and 18**).
The fact that DGrip mutant muscles exhibit aberrant behaviour and random polarisation of muscle extensions during the muscle guidance process is strongly suggestive that the final process, that of cell adhesion to the target, is not the cause of the muscle phenotype observed. The two remaining alternatives are thus an inability to recognise or respond to an attractive signal, or alternatively a general defect in muscle polarity as a whole. Since $dgrip^{ex36}$ muscles preserve the typical orientation of minus-end microtubulae (**Figure 10**), that they are later able to form the specialised membrane compartments needed for synapse formation (not shown) –itself a process requiring polarity determining molecules [47, 245-249] - and due to the direct interaction of DGrip with elements of the TGFβ, Robo and the EGFR signalling pathways, it seems unlikely that the basic polarity of these muscles is disturbed in the DGrip mutant. The much more probable explanation is a specific defect in terms of signal recognition, either mistrafficking of receptors, or lack of signalling from these receptors, processes which were investigated in more detail.
By its molecular nature as an intracellular adaptor molecule, DGrip might either organise signalling processes by clustering transmembrane receptors or alternatively the protein could work downstream of the signal reception, executing transporting events essential for directed muscle cell motility, or integrating inputs from several different cascades. Our data suggest that muscles which normally express no, or at least significantly less DGrip start, in the ectopic presence of DGrip, to sense distant guidance cues. How this sensitisation process in LTMs may work is not yet clear. Mammalian GRIP was first proposed to be simply a transport factor, trafficking molecules such as glutamate receptors and ephrins from intracellular compartments to the cell surface [159, 250-254]**,** and indeed, for cell adhesion molecules such as Fras1, this appears to be the case, as Fras1 is mislocalised in mGRIP1-/- animals [114]. However, it is clear from mammalian studies that mGRIPs are not only trafficking proteins. Further investigation of the mGRIP-GluR2/3 interaction suggests that mGRIP is associated with syntaxin-13 positive endosomal compartments [130] (amongst many others), and that mGRIP may act to protect endocytosed GluR2 complexes from lysosomal degradation [113]. Thus, an alternative role for DGrip in muscle guidance, rather than the organisation of membrane presentation for receptors, could also be the endocytosis of receptors, perhaps in order to organise their intracellular signalling, as described for TGFβ and RTKs [reviewed by 255]. Both hypotheses would be compatible with the punctate staining observed for

DGrip (**Figure 5**). The remainder of this work is devoted to the determination of putative members of the DGrip-dependent signalling pathway, and to the study of the molecular logic underlying DGrip's function in muscle guidance.

4.2 The DGrip PDZ domains are not independent modules

DGrip, as the majority of PDZ domain proteins, contains multiple PDZ domains arranged in tandem clusters. Yet rather than being independent protein-protein interaction domains, as was originally thought, interactions over PDZ domains have lately been demonstrated to be critically dependent on cooperative interactions from neighbouring PDZ domains present in the same tandem cluster [256].

The DGrip protein, far from being composed of PDZ domains functionally isolated from one another, critically relies on several PDZ domains in the protein for function in muscle guidance, and in other later processes. The fact that multiple domains are needed for function is suggestive of an integrative role for this protein, rather than a simple role in protein trafficking. This integrative behaviour potentially makes DGrip a very sensitive molecular switch, capable of quickly reacting to its molecular environment, and tuning the protein to different functions in different molecular milieus. These naturally impose interaction logic at the molecular level, allowing a multiplicity of functions in different, or even the same, tissue.

There are several mechanisms by which this can occur. On one hand this may be due to structural chaperoning, for example mGRIP PDZ5 is chaperoned by the proper folding of its tandem partner PDZ4, itself unlikely to be capable of binding conventional PDZ ligands [257]. A second option is via co-incidence detection. It was recently shown [256] that a fixed orientation between tandem PDZs 1 and 2 in PSD-95 was required for high affinity binding to its targets, and that a complex of the two PDZs had higher binding affinity than either PDZ domain alone. Intriguingly, a more recent study [122] showed a potential for allosteric conformational changes upon PDZ–ligand binding, potentially changing binding affinities of neighbouring domains on very short timescales. All of these mechanisms naturally impose a simple logic for the integration of ligand binding with functional output of PDZ domain proteins.

From the study of muscle guidance phenotypes in this thesis, an interaction logic for DGrip's seven PDZ domains in DGrip-dependent muscle guidance could be constructed, and interactors of these domains characterised.

That proteins involved in several different signalling cascades, including the TGFβ, Robo and EGFR dependent signalling cascades are all potential interactors with the DGrip protein (see below for a more detailed treatment of the topic), suggesting that DGrip is a protein with the ability to integrate signals from multiple receptors.

DGrip function in muscle guidance is reliant on the co-operation of several PDZ domains, the two most important for muscle function likely to be PDZs 3 and 7 (**Figures 22 and 26**). Rescue experiments with DGrip missing PDZs 6-7 (**Figure 14, 15 and 24**), or with point mutations of PDZs 7 (**Figure 26**) or 3 (**Figure 22 E**) only, showed an incomplete rescue function. DGripΔ1-3x7 (**Figure 22**) showed less rescue ability than either DGripx3 or DGripx7, suggesting that PDZs 3 and 7 are most important for DGrip function in embryogenesis, although they need not be the only active domains.

At least two other domains are involved in muscle function, in that constructs missing PDZs 1-3 (particularly, their ligand binding capacity) exhibit dominant active phenotypes, suggesting that DGrip is able to self-regulate. This impression was strengthened by the observation that a construct expressing PDZs 1-3 only was unable to repress muscle function, meaning it was unable to bind and deactivate the elements involved in DGrip-dependent signalling when removed from the context of an intact DGrip molecule.

Point mutations of PDZ1 and PDZ2 recapitulated the DGrpΔ1-3 phenotype in the LTM group of muscles (**Figure 21**), indicating that these domains function primarily as ligand binding domains. This experiment suggests that PDZs 1-3 do not act as repressors structurally (i.e. by covering other PDZ domains on the protein).

PDZ3, when mutated in a full length transgene, exhibited a mild loss-of-function phenotype, but was still able to provoke a weak dominant phenotype in muscles expressing endogenous DGrip (**Figure 22**). This could indicate one of two things; that the VLM group of muscles are inherently more sensitive to active forms of DGrip, or that DGrip-dependent regulation of muscle guidance is regulated differently in the two different muscle groups, a possibility due to the fact that putative interaction partners such as the Robo receptors are reported to be expressed only in the VLM group of muscles [60].

How is DGripΔ1-3 dominantly active? One possibility is that PDZs 1-3 bind a ligand and inactivate it independent of any other interactions carried out by the remainder of the DGrip protein. This was tested by expressing a construct containing only PDZs 1-3 in wild-type and $dgrip^{ex36}$ muscles. If PDZs1-3 could bind and inactivate ligands by itself, expression of PDZ1-3 would cause a loss-of-function phenotype when expressed in wild-type, or enhance the $dgrip^{ex36}$ phenotype. Neither of these phenomena occurred. Likewise, if PDZs1-3 were able to inactivate ligands simply by binding them and making them inaccessible to the muscle cell, then expression of PDZs 1-3 alone should be able to repress the action of DGripΔ1-3. It was not able to do so, instead enhancing the defect (**Figure 19**).

The most likely explanation was that PDZs 1-3 act as repressors only within the context of the intact DGrip gene. While some domains of DGrip would be responsible for a given pathway's repression, others would be responsible for the positive function of the same signalling pathway. This last could occur either by binding the same ligand, and placing them in a different interaction state by its position on the DGrip molecule, or alternatively by binding different members of the same complex.

Evidence supports the theory that DGrip binds elements of the same pathway, in that the dominant active DGripΔ1-3 phenotype is partially suppressed by the point mutation of the PDZ7 ligand binding surface (**Figure 26**). Mutations of PDZ6 however do not suppress the DGripΔ1-3 phenotype (**Figure 25**). This would suggest that the dominant active DGripΔ1-3 phenotype comes at least in part from an unregulated interaction over PDZ7.

Specific interactors of PDZ domains 1-3 and 7 were examined on the yeast-two-hybrid level, looking for interactors which were specifically abolished in point mutations of individual PDZ ligand-binding pockets. These were correlated with muscle guidance phenotypes found with these same point mutations.

A very interesting candidate was Echinoid, a cell adhesion molecule involved in antagonizing EGFR signalling. This interactor is treated in **Section 4.4**.

Two candidate molecules, Robo2 and Mad, showed an interaction with PDZs 1-3 which was abolished in point mutations of any one of these three domains (**Section 3.7.4**). Either these two proteins have, and require, affinity to all three PDZ domains, or their binding is sensitive to conformational changes in neighbouring domains.
This kind of structural intraction between PDZ domains is not unknown.Tandem arrangements of PDZs including short linker regions are very strongly preserved in evolution [256], suggesting that relative spacing or orientation of individual domains can have a functional effect on combinatorial binding. Thus, one can consider evolutionarily well-preserved clusters of PDZs as functional units. Doing so with DGrip, alignments for tandems in PDZ 1-3 and 4-5 would lead one to believe that PDZ 6 and 7 are more isolated from the other DGrip PDZ domains in their function - as they change their relative spacing to other PDZs- while in fact over the PDZ domain itself the conservation is the highest (**Figure 1**).
Thus, not all PDZs are fixed in orientation in tandem clusters, but the order is conserved over evolution, indicating that some conserved interactions require PDZ tandems, and others do not. In this study, one functional cluster, PDZ1-3, was identified which acted as a repressor of muscle function, apparently over PDZ 7.
Some domains of the DGrip protein appear to have no function in the muscle guidance process, as constructs missing PDZs 4-5 show no defects in their ability to rescue the muscle phenotype, and can be used at very high levels of expression (driven by *24B-gal4* at 29°C) to evoke the same overexpression phenotype as DGrip. As PDZ4-5 is the mGRIP glutamate receptor interacting region, one might then expect that some parts of the protein are not utilised for all functions, although how the transition from one state not requiring glutamate receptor interaction to its glutamate receptor regulating role is accomplished is unknown.
Interestingly, the molecular logic that is demonstrated for DGrip for muscle guidance is transferred in other functions of DGrip as well, treated in **Section 4.9** below. These results then show an interesting new aspect to DGrip function, which, given the relatively high level of conservation in structure and binding affinity (**Figure 1 and Table 1**), is likely to be conserved in mGRIP function as well.
Using the DGrip muscle guidance phenotype as a model system, it was determined that DGrip acts as an integrative molecule which relies on multiple PDZ interactions for function. This may force the reconsideration of mGRIP's molecular logic as well. It may then be instructive to consider interactions, such as those of mGRIP with the GluR2/3, within the context of its other interactors, a process which has started, but thus far lacks analysis of whether complexes such as mGRIP/GluR2/EphB2 [134, 136] are mutually exclusive with a complex like mGRIP/GluR2/LAR [132, 133] and if so whether they are functionally different in terms of glutamatergic transmission. With a finer understanding of any underlying molecular rules, the potentially very complex set of interactors can be greatly simplified, and even more so, a better understanding of the true role of mGRIPs in these processes can be developed.

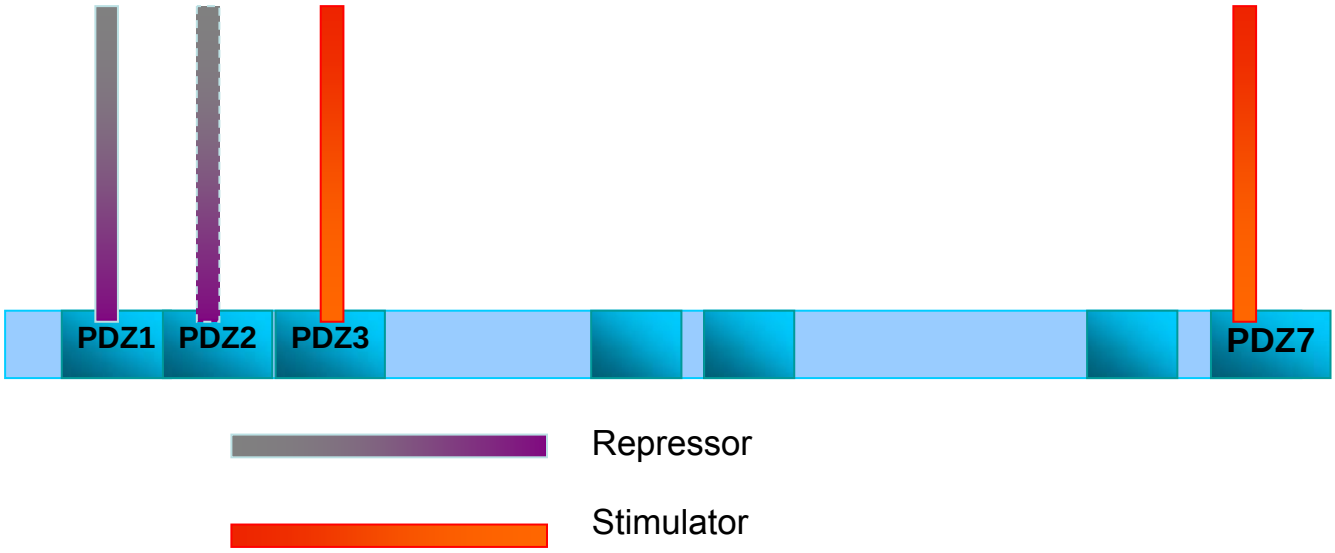


Figure D: Molecular logic of DGrip in the muscle guidance process.

4.3 The second interdomain is a non-essential interacting domain

A structural curiosity is why the second interdomain region, despite being unnecessary for rescue function by itself, appears necessary in the context of loss of other PDZ domains. Constructs missing this domain, and PDZs 6 and 7 (and to a lesser extent missing the second interdomain and PDZs 4 and 5) exhibit a more severe loss-of-function phenotype than mere loss of DGrip, as shown the *dgrip*^{ex36} mutant. This would suggest that constructs missing the second interdomain region, plus any of the neighbouring PDZ domains, are able to bind ligands but not to utilise them in the DGrip-dependent signalling pathway. This can be explained in different ways: 1) That more than one PDZ domain can bind the same ligand or at least the same complex allowing this inactive form of DGrip to sequester proteins for muscle guidance 2) That the second interdomain region executes a structural function only or has an interacting function with the same complex.
The possibility that the second interdomain region was responsible for binding to the motor protein kinesin heavy chain was examined. If this were the case, then mutations of this domain might produce a transport-inactive form of DGrip, which could still bind proteins, but not execute the necessary transport steps for function.
A positive interaction with the *Drosophila* conventional kinesin heavy chain, but not light chain (**Figure 28**) was observed in yeast two-hybrid tests which involved regions of the second interdomain. However, the genetic evidence (that constructs missing the second interdomain can execute normal DGrip function), is suggestive that the yeast two-hybrid result is not relevant for the muscle phenotype. Additional experiments, pulling down kinesin heavy chain from lysates of adult flies on a column of baculo-Grip-myc (not shown, described in **Materials and Methods**), did not deliver any conclusive results to date.
Another interaction which may provide an explanation of the DGripΔ6-7L phenotype would be that DGrip is in fact capable of dimerisation, which might rescue the ability of the mutant transgene to deal functionally with its ligands (explaining why this transgene

dos not provoke a phenotype in the wild-type background), but that dimerisation is not essential. The work done so far cannot exclude dimerisation as a contributing factor. FLIM/FRET experiments (**Figure 13**) between DGrip-GFP and DGrip-tdimer2 transgenes in larvae showed no strong resonant transfer between the two fluorophores, which naturally does not exclude dimerisation.
A conserved motif, just N-terminal of PDZ6, was found which in a mammalian study abolished dimerisation [156]. Making this point mutation in DGrip, like removal of the second interdomain itself, had no adverse effect on the rescue function of this transgene. Given that the dimerisation mode proposed would then have to be non-essential for rescue in an otherwise intact DGrip transgene, thus far the behaviour of the point mutation [156], could be consistent with the destruction of a non-essential motif. If one was to introduce this point mutation into the *UAS-GripΔ67* transgene, one would expect that it would then exhibit the same phenotype as *UAS-GripΔ67L*.
An instructive experiment to clarify the role (if any) of dimerisation in DGrip function would be to approach dimerisation biochemically- i.e. to express, for example, DGrip-GFP in the wild-type background and attempt to immunoprecipitate endogenous DGrip with an anti-GFP antibody.

4.4 DGrip interacts with Echinoid and the EGFR signalling pathway

In this work, the cell-adhesion molecule Echinoid was identified as a novel DGrip interactor. Echinoid is similar to, but not a member of, the L1-CAM family of cell adhesion molecules, for while possessing Immunogluobulin (Ig) and fibronectin type III (FNIII) domains, it lacks the ankyrin repeats in the cytoplasmic domain, characteristic of true L1-CAMs [217]. Thus far, four lines of evidence link Echinoid to DGrip:
1) Echinoid interacts with PDZ1-3 of DGrip in a yeast two-hybrid screen and this interaction can be specifically blocked by point mutations removing PDZ1 or 2 ligand binding (**Table 8**).
2) Two alleles of Echinoid, a putative loss-of-function allele ed^{SlH8} and a hypomorphic allele ed^{SlA1}, are able to suppress a pupal lethality caused by expressing dominant active form of DGrip, DGripΔ1-3, driven by *G14-gal4* (**Section 3.7.5.3 and Appendix 1**).
3) the genetic combination $dgrip^{ex36}$ (a DGrip null-mutant) with one mutant ed^{SlH8} chromosome leads to an increase in the severity of the $dgrip^{ex36}$ muscle phenotype, which affects muscles, such as the LTM group of muscles, which are not affected in a pure $dgrip^{ex36}$ mutant (**Figure 35**).
4) $dgrip^{ex36}$ muscles are not able to regulate overexpression of Echinoid in the same way that wild-type muscles can (**Figure 37**). Overexpression of Echinoid with *twist-gal4* elicits mild muscle phenotypes in wild-type. In $dgrip^{ex36}$ animals, these defects become much more severe, and begin to affect muscles not affected in pure $dgrip^{ex36}$ mutants.
Interestingly, not only DGripΔ1-3, but DGripx2, caused pupal lethality when expressed by *24B-gal4* (**Section 3.6**). The Echinoid allele ed^{SlH8} was a strong transheterozygous suppressor of DGripΔ1-3 mediated lethality (**Section 3.7.5.3**). In a yeast-two hybrid test of interaction, Echinoid interacted predominantly with PDZ2 (**Table 8**) and weakly with PDZ1, while also interacting with PDZ7. Given the proposed model of DGrip molecular

integration (**Section 4.4, Figure D**), it seems likely that Echinoid may be the interaction partner that confers this interaction logic on the DGrip molecule.

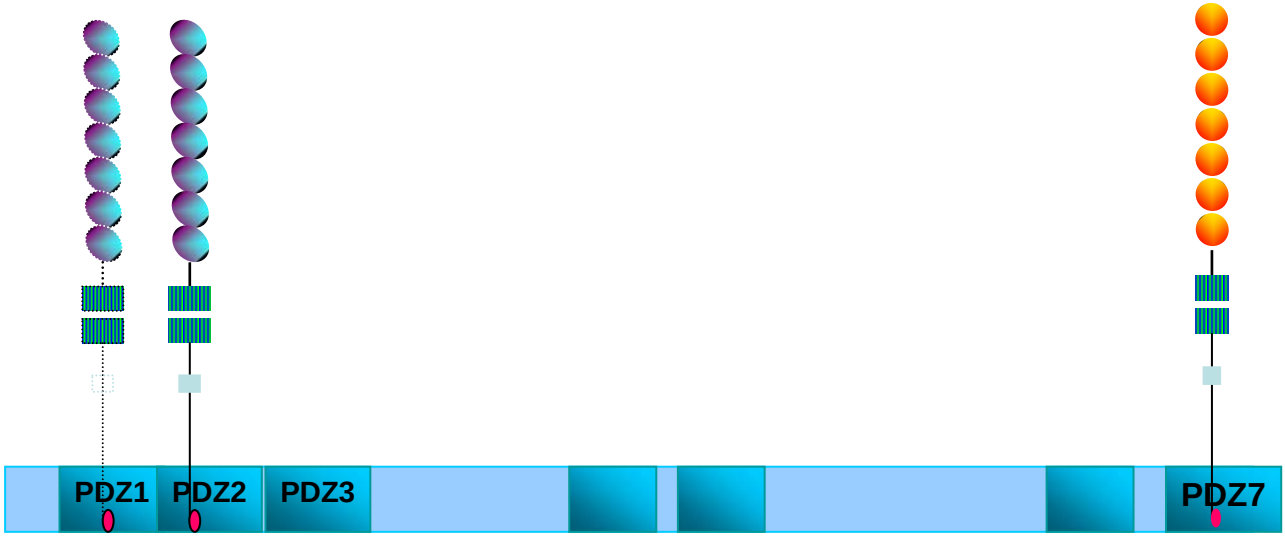


Figure E: A speculative model: Echinoid (6 Ig domains, 2 FNIII domains and a transmembrane domain) may be the interaction partner which mediates both repressive and active function of the DGrip gene. Binding to PDZ2 (and also PDZ1) may confer repression (perhaps by binding itself or other Ig-domain proteins such as Robo2, which also binds PDZs 1-3), while Echinoid bound to PDZ 7 may be active for the muscle guidance process.

With this in mind, the function of Echinoid in muscles was examined, to understand how this may interact with DGrip function. The ed^{SlH8} mutant animals have a mild muscle phenotype (**Figure 34**), which affects both the VLM and LTM groups of muscles. Stainings with an anti-Echinoid serum produced a staining pattern which appears to be perinuclear, although not restricited to muscle cells only (**Figure 38**). Staining with this antibody also produced a specific stain in the CNS, which had already been reported [213].

Echinoid has been studied within the context of other processes in *Drosophila*, namely in eye development and the patterning of bristles on the adult mesothorax. Although it was established that mutation of DGrip, and overexpression of DGrip variants could not interfere with either eye development or bristle patterning in any observable way (not shown), using those model systems it has been determined that Echinoid is a regulator of both the EGFR and Notch – dependent signalling pathways [213, 215, 216, 258, 259]. Although both Notch and EGFR signalling are involved in earlier stages of muscle development [238, 258-270], thus far I have concentrated on aspects of EGFR signalling in *dgrip* mutants. Echinoid interacts with the EGFR in both *cis* and *trans* [219] during R8 photoreceptor specification, and leads to reduced levels of EGFR activation, upstream of MAP kinase signalling. Echinoid binds the *Drosophila* L1-CAM Neuroglian in *trans*, and synergises with Neuroglian in repressing EGFR signalling, dependent on the presence of the Echinoid intracellular domain [218]. Two mechanisms for Echinoid-dependent repression of EGFR signalling have been proposed, firstly, that Echinoid, via homotypic

interactions, activates a novel pathway that antagonises EGFR signalling by regulating the activity of the TTK88 transcriptional repressor, the most downstream component of the EGFR pathway in the eye [214]. Curiously, Phyllopod, another putative DGrip interacting protein, is also a regulator of TTK88, being part a complex which conjugates TTK88 to ubiquitin [271]. An alternate method of EGFR repression was proposed in two papers [219, 220], which suggest that Echinoid represses EGFR by direct interaction, and that Echinoid could be tyrosine phosphorylated by the EGFR [220].

The role of EGFR-dependent signalling in the muscle guidance process has not been addressed before. It is known that EGFR-dependent signalling is required to establish a specific set of muscle precursors [151] and thus could be available to myoblasts in the process of muscle guidance. Later in muscle development, the EGFR is expressed in the apodemes, epidermal cells to which the somatic muscles attach [272].

It was important to ask if EGFR expression, driven by the pan-muscular driver *twist-gal4*, could change muscle morphology. In the wild-type background, EGFR expression had very little effect on somatic muscle morphology (**Figure 44**), whereas EGFR expression in $dgrip^{ex36}$ hemizygotic males showed significant muscle defects, over and above those found in pure $dgrip^{ex36}$ mutants. Many groups of muscles were affected, most interestingly, the LTM group of muscles, which show no morphological defects in $dgrip^{ex36}$ mutants. This suggests that in the absence of DGrip, EGFR signalling becomes misregulated, and also that DGrip may well be expressed in LTMs at a level below immunodetection.

Both $dgrip^{ex36}$ and wild-type embryos were examined for markers of EGFR activation to see if DGrip mutants do indeed have defects in EGFR-dependent signalling. One such marker of EGFR signalling is the activation of the Rolled/ERK (extracellular signal-regulated kinase)/MAPK (mitogen activated protein kinase) signalling pathway. Briefly summarised, the pathway from EGFR activation to ERK activation is dependent on several proteins, passing over the small GTPase Ras, Raf, the Band 14-3-4 protein Leonardo, and a complex of MEK and MP1 which in turn activates ERK, which can then phosphorylate cytoplasmic targets or is transposed to the nucleus [summarised in 273]. This can be visualised by an antibody specific for the doubly-phosphorylated form of ERK, (anti-dpERK), which has been used in many studies of *Drosophila* receptor-tyrosine kinase (RTK)-dependent signalling [227, 273-278]. It is, however, downstream of all known RTKs [227, 228], and as such needs to be genetically controlled to causatively link activation of one RTK with a specific dpERK signal.

dpERK staining has also previously been identified in both muscles and apodemes at embryonic stage 15 [228], and had not been positively associated with any RTK pathway, in either muscles or apodemes.

The known appearance of the EGFR in apodemes [272] was suggested as the likely activator of the ERK in that tissue. This hypothesis, that of EFGR signalling being involved in muscle-to-apodeme signalling, was further supported by work with the EGFR ligand, Vein. Vein is a secreted molecule produced by all embryonic somatic muscles, and contains a PEST domain, a single immunoglobulin domain and an EGF domain at its carboxyl terminus. Yarnitzky and co-workers observed that embryos lacking Vein were unable to complete the differentiation of the apodeme [150], in that the apodemes were able to express the primary markers of apodeme fate, Delilah and Alien, but that in *vein*

mutants, muscles were unable to induce a Ras-dependent pathway in apodemes, leading to the expression of a second, stronger wave of Delilah and β1-tubulin.
In the *dgrip*^{ex36} allele, all apodemes, not only those associated with the misformed muscles 6 and 7, expressed significantly lower levels of dpERK than *dgrip*^{ex36}/+ controls (**Figure 39**) and also significantly lower levels of Delilah, while another marker of apodemes, Alien, was not affected (**Figure 41**). This, as mentioned above, is the same result as obtained in *vein* mutants affecting EGFR-derived signalling in apodemes. This led to speculation that EGF signalling to the apodemes is defective in *dgrip*^{ex36} mutants.
Lack of EGF signalling to apodemes, however, did not appear to cause significant problems with muscle-apodeme adhesion, as *vein* mutants where apodeme differentiation is defective [150] were not embryonic lethal [279], and is as such compatible with the DGrip phenotype, which also exhibits robust muscle attachment during embryonic and larval life, despite lacking dpERK staining.
Curiously, *vein* mutants were not reported as having such strong muscle defects as those associated with the *dgrip*^{ex36} allele [150], indicating that Vein signalling to apodemes might not be the primary defect in DGrip mutants with respect to muscle guidance. Nevertheless, the question of EGFR signalling *per se* in DGrip mutants can be efficiently addressed in this model system.
As DGrip has been demonstrated to be expressed in muscles only, it would seem unlikely that DGrip is directly involved in the apodeme, although this was explicitly checked by staining for dpERK in *dgrip*^{ex36} mutant embryos where the muscle phenotype is rescued by muscle-specific rexpression of DGrip. Here, as expected, muscle-specific expression of DGrip is able to restore dpERK staining to the apodeme (**Figure 42**). Thus DGrip signals *in trans* to the apodeme, via an intermediate which may be Echinoid.
To understand this interaction better, it will also be necessary to examine Echinoid mutants and *UAS-Echinoid* variants in muscles, to understand if muscle-expressed Echinoid is in fact involved in the process of apodeme maturation.
Since specific expression of DGrip in muscles can restore normal levels of dpERK staining to *dgrip*^{ex36} mutants, the possibility is left that DGrip promotes signalling to the apodeme by its action in the muscle.
Several alternatives exist: 1) through the DGrip-Echinoid interaction, DGrip properly traffics Echinoid so that Echinoid on the muscle is able to present the Vein ligand (both Echinoid and Vein contain Ig-domains which in theory could bind one another) to the EGFR on the apodeme.
2) Echinoid acts as a repressor of EGFR signalling as suggested for the *Drosophila* eye [218] and that the DGrip-Echinoid interaction segregates Echinoid in such a way that it does not interfere with EGFR signalling.
3) Echinoid is in fact an enhancer of EGFR signalling in muscles and is not properly trafficked in *dgrip*^{ex36} mutants.
Interestingly, genetic analysis of Vein function in *Drosophila* wing-vein development suggested that the Vein becomes a dominant negative inhibitor of EGFR signalling when Vein's EGF motif was removed [280], an observation which Donaldson and co-workers took to imply that Vein's action on EGFRs was in fact mediated by an intermediary protein. The idea that Vein requires other, accessory, proteins for active signalling was also suggested by work where Vein was ectopically expressed in *Drosophila* wing discs,

where it is the major activating ligand for EGFR signalling [281]. Zecca and Struhl found that ectopic expression of Vein could not produce the same phenotype as a constitutively activated EGFR in these tissues, indicating that without accessory interactions, the Vein ligand may be inactive.
These theories can be approached using a simple assay system, that of looking for dpERK/Delilah staining in embryonic apodemes in *dgrip*^{ex36} mutants overexpressing *UAS-EGFR* (dominant active) in apodemes (to prove that the phenotype can be rescued by EGFR signalling), and then following dpERK staining in apodemes in response to alterations of all the members of the putative cascade.
Having established that full-length DGrip can restore dpERK to apodemes, it would be particularly interesting to repeat this assay with point-mutated DGrip variants, to understand which domains of DGrip (and thus, what interactors) are necessary for this signalling process.

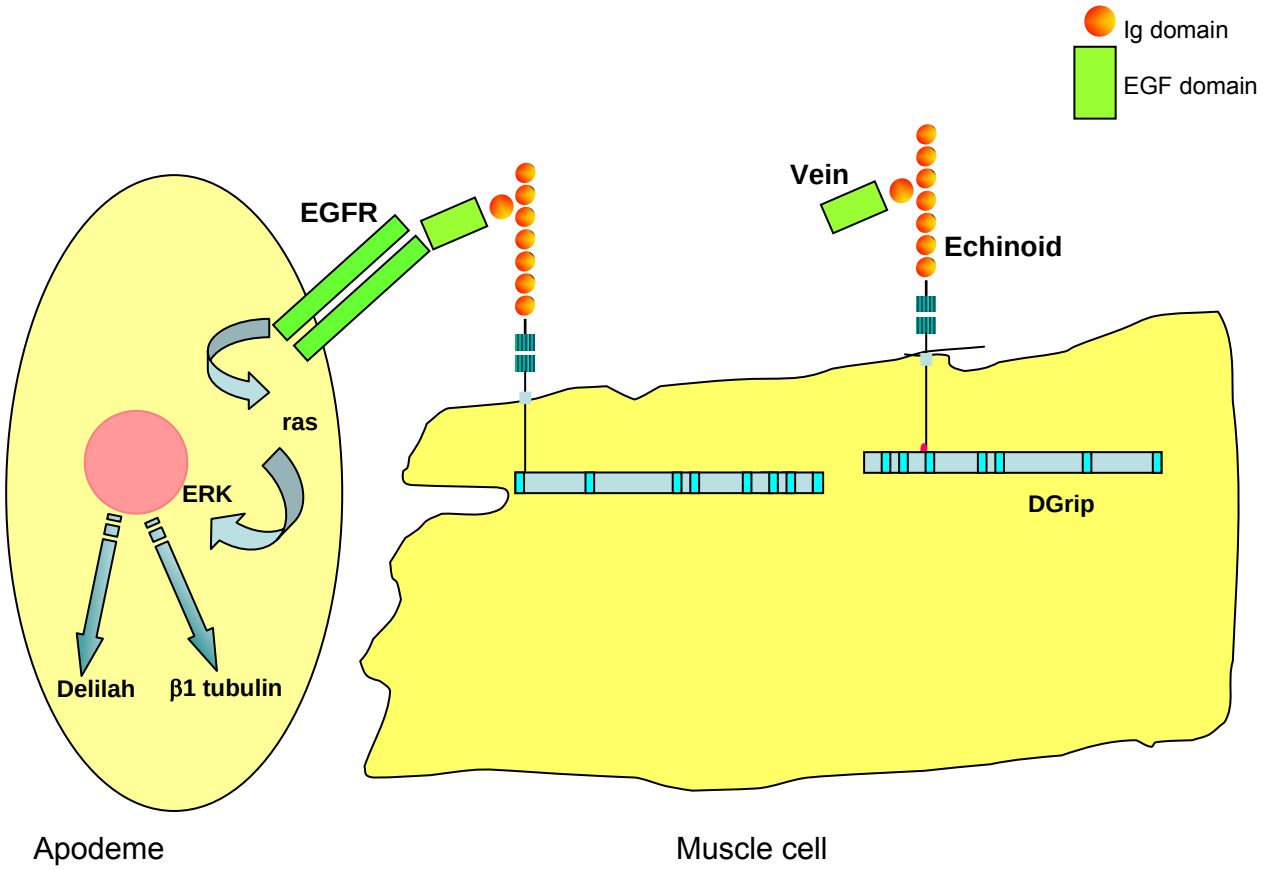


Figure F: Possible mechanism of muscle-apodeme signalling mediated by DGrip. See text above for more details.

To return to the muscle guidance process itself, EGFR signalling was examined to see if it could also be involved in muscle guidance. Muscle guidance is a process which is defective in *dgrip*^{ex36} mutants long before apodeme maturation should take place [150]. Interestingly, muscle-specific expression of *UAS-EGFR* only produced a phenotype in

dgrip^{ex36} animals, not in the wild-type background, and was able to increase the severity of the *dgrip*^{ex36} phenotype (**Figure 44**).
This indicates that perhaps DGrip-regulated EGFR signalling does have a role not only in the final stages of muscle-induced apodeme differentiation, as described above, but also in guiding muscle motility. The fact that reduction in the amount of the Vein protein in *dgrip*^{ex36} mutants also causes muscle guidance defects (**Figure 43**) raises the intriguing question if Vein is capable of signalling to EGFRs on the same cell to regulate the muscle's motility. It seems unlikey that DGrip in muscles directly interacts with the EGFR, as the C-terminus of the EGFR did not interact with PDZs1-3 or 7 in a yeast two-hybrid test (**Section 3.7.4**). Antibody stainings looking for Vein protein in *dgrip*^{ex36} mutants would then be able to indicate if DGrip is required for Vein trafficking to the cell's motile edge during this process.
EGFRs have been described in cell-culture systems as important regulators of cell motility and movement. EGFR dependent signalling is required for two processes vital for motility, both the dissolution of focal adhesions via ERK [282], and the actual organisation of motility, regulated by EGFR-induced phospholipase C (PLC) activity [282-286]. It is thus possible to speculate that either one of these processes, either dissolution of focal adhesions during the guidance process, or organisation of motility may be defective in *dgrip* animals. *In vivo* time-lapse imaging experiments of *dgrip*^{ex36} muscles, if possible, may be able to distinguish between these two specific processes.
This Echinoid-DGrip interaction however, may not, and given the complexity of DGrip PDZ-ligand binding interactions needed for muscle function, is likely not, the full explanation of the DGrip muscle phenotype. For this reason, other pathways which interacted with DGrip signalling were studied, to see if they are also involved in muscle guidance.

4.5 Does DGrip interact in multiple signalling pathways?

In this work, interactors were found which represent members of three signalling pathways, namely; Mad (mothers against Dpp, the *Drosophila* smad1/5 homologue [287])as a PDZ1-3 specific interactor in yeast-two-hybrid and Baboon (the *Drosophila* activin receptor [200, 288]) as a strong repressor of DGripΔ1-3-mediated pupal lethality, both members of the TGFβ signalling cascade, the Robo2 receptor, and Echinoid and Phyllopod, treated above, members of the EGFR signalling cascade. Mutants of DGrip may then produce a very complex, hybrid phenotype between all these signals.
One important question to ask is whether all these three pathways are active during the process of muscle guidance, or whether DGrip acts in concert with these pathways in different tissues and timepoints. From the data recovered from the suppression-of-lethality screen performed with the *UAS-dgripΔ1-3* transgene, it would appear that members of the EGFR cascade (namely, Echinoid) and from the TGF-β signalling cascade (Baboon) are also active in DGrip-dependent processes in later development, and several strands of evidence are presented here to suggest that they may be active in the muscle guidance process as well. Further experiments would be well advised to see if it is possible to recreate the *dgrip*^{ex36} phenotype by combination of mutants of these pathways. The way in which these proteins may interact with one another (for example, both Echinoid and

Robos are Ig domain proteins which serve as heterophillic cell adhesion molecules [172, 214], as well as signalling molecules), may provide the missing information for DGrip-dependent regulation of muscle guidance.

4.5.1 Robo signalling

Robo receptors have a strong and specific muscle guidance phenotype, strongly reminiscent of DGrip in terms of both phenotype and expression pattern in somatic muscles [60]. However, the DGrip muscle phenotype has not been demonstrated to be due to misregulation or trafficking of the Robo receptors alone.
For example, while the DGripΔ1-3 phenotype in the LTM muscles is reminiscent of ectopic Robo1 or Robo2 expression- closer inspection shows that it is not identical, as DGripΔ1-3 LTMs, while bending toward the segment border, also form multiple ramifications, rather than simply the one misguided projection as do Robo overexpressers [60]. While HA-Robo 1 or 2 do not colocalise with DGrip-GFP in mammalian cell culture (**Figure 30**), Robo2, which has a C-terminal PDZ ligand motif, interacted with DGrip PDZs1-3 and 7 on a yeast two-hybrid level. This interaction was abolished by point mutation of the PDZ1-3 and 7 ligand binding pockets (**Table 8**).
Interestingly, repression, in as much as the *24B-gal4*::*UAS-Robo2* phenotype in LTMs is changed from their normal overexpression phenotype, does occur in the $dgrip^{ex36}$ mutant (**Figure 29**), while leading to a increased severity of the misguidance phenotype in muscles which span the segment, such as muscles 12, 13 and 4. These segment spanning muscles are all known to express Robos 1 and 2 [60] as well as DGrip (**Figure 5**).
An instructive experiment would be to look for the repression of the DGripΔ1-3 muscle phenotype in animals deficient for the Robo ligand, Slit. In this way, it could be seen ask if any part of this overexpression phenotype is dependent on Slit signalling.

4.5.2 TGFβ signalling

The Transforming Growth Factors β family of signalling molecules are involved in many developmental processes as well as maintenance and signalling functions in the mature animal. In mammals there are three subgroups of ligands that fall under this category: the bone morphogenic proteins (BMPs), TGFβ/activins and lastly Glial-derived neurotrophic factors (GDNFs), a more distant relative of this family. To date, there are seven identified TGFβ ligands expressed in *Drosophila,* of which five have been characterised to a varying degree [289]: three well characterised ligands; *decapentaplegic (dpp), screw (scw)* and *glass bottom boat (gbb)*, all homologues of BMPs, two activin-like ligands, *dActivin (dAct)* and *activin-like-protein (alp*) who have also been studied in functional terms. Two ligands as yet uncharacterised in biological or genetic terms are *maverick (mav),* equidistant between BMPs and TGFβ-activins, and *myoglianin (myo)* [290] a relative of mammalian BMP-11.
A large amount of functional information about TGFβ family receptors is known, largely from studies in *Drosophila*. The receptor complex consists of two types of serine/threonine kinases, the Type I and Type II receptors. A dimeric (or perhaps, trimeric [291, 292]) complex of Type I and Type II receptors are formed upon binding a ligand

homo- or hetero-dimer. Type II receptors are constitutively active kinases and phosphorylate the Type I receptor upon receptor-ligand complex formation [293]. There are three known Type I receptors, the Dpp/BMP receptors Thickveins and Saxophone and a recently identified activin receptor Baboon. Two Type II receptors are known, called Punt and Wishful Thinking. Each of the Type I receptors is particularly responsive to a certain ligand, whereas the Type II receptors are capable of dimerising with and transducing signals from all Type I receptors.

As Baboon was also isolated as a DGrip interactor (**Section 3.7.5.3**), signalling from its effector molecule, Smox was examined. Smox (smad on X/ dsmad2), only functions downstream of *Drosophila* activin signalling, and not from Dpp or BMP signalling, which signals over Mad [200, 230, 234, 294, 295]. As there is no phospho-specific antibody for Smox, it was not determined if endogenous Smox is activated in somatic muscles. High levels of *smox* mRNA are reported in stage 13 mesoderm, including the somatic musculature [200], suggesting that Smox protein would be available to somatic muscles during the muscle guidance process. The expression of the Baboon receptor in somatic musculature is unknown.

Preliminary examination of $babo^{32}$ mutants (**Figure 45**), suggest that *babo* mutants may have defects in the somatic musculature, more strongly affecting the LTM group than the VLM group of muscles.

Ectopic Baboon expression in muscles using *twist-gal4* causes severe defects in somatic musculature in $dgrip^{ex36}$ mutants (**Figure 46**). However, expression of the downstream effector of Baboon signalling, Smox, exerts no effect on muscle morphology (**Figure 47**). This indicates that ectopic Baboon in the muscle is activated by an endogenous ligand.

The receptor Saxophone was found as candidate interactor in a yeast two-hybrid screen with DGrip. However, although it interacted with PDZs 1-3, this interaction was not abolished by point mutation of any of these PDZ domains. Either Saxophone interacts with DGrip in an atypical manner, or the interaction with PDZ1-3 was not specific (**Table 8**).

Its downstream signalling partner, Mad, interacted with PDZ1-3 and the interaction was abolished by point mutation of any one of the these three PDZ domains (**Table 8**). Perhaps this indicates an underlying sensitivity of this interaction to the conformation of neighbouring PDZ domains. The role of either Mad or Saxophone in muscle guidance is yet to be established. Interestingly, Smads like Mad are phosphorylated on the C-terminal SSxS-COOH motif [200]- if Mad were the actually a DGrip ligand, then pMad would likely have a different interaction status with DGrip than in its unphosphorylated state.

4.6 Is DGrip's role in trafficking or endocytotic signalling?

At this point, it cannot be determined if DGrip is purely a trafficking protein, acting as a kind of 'smart adaptor' to bring signalling proteins to the membrane, or whether DGrip may act downstream of receptor trafficking, to modulate the signals of these proteins, or even to process these receptors themselves, as is known for the mGRIP-GluR2 interaction [113].

It is not yet clear if the endocytosis of TGFβ receptors is strictly required for their signalling, and different lines of evidence exist: clonal analysis indicates that the *Dpp* target *spalt* can still be expressed in endocytosis-defective wing discs [296], however this may yet be the consequence of perdurance of proteins (in this case α-adaptin) formed before the clone was established. Experiments using dominant-negative Rab5 in the same tissue has been shown to interfere with Dpp signalling. It is also known that TGFβ signalling requires a protein called SARA (**s**mad **a**nchor for **r**eceptor **a**ctivation), which is endosomally located [297, 298]. The SARA protein is required to recruit the so-called R-Smads, in *Drosophila* Smox/dsmad2 (activin-like pathway [200, 233]) and Mad (Dpp/BMP pathway) to the activated receptor complex, before TGFβ dependent gene transcription can take place. This would suggest that the endosomal localisation of the TGFβ signalling complex would be obligatory [294, 299-304].
If the interaction with both TGFβ receptors (Saxophone, Baboon) and downstream effectors (Mad) could be shown *in vivo*, the most likely compartment for these two proteins to be utilised in a functional complex would then be the endosome.
Likewise, EGFR signalling is known to be modulated by endocytosis. The gene Phyllopod, a putative DGrip interactor (**Sections 3.7.4 and 3.7.5.3**), acts as a ubiquitin ligase [271, 305] and is as such an endosomal protein.
Both the EGFR and at least one receptor in the TGFβ pathway, Thickveins, are downregulated by Hrs-dependent ubiquitinisation and degradation [153]. In mammalian cell cultures, it has been shown that the amount of PLC activation is reduced by endocytosis, as the affinity of the EGFR receptor for the ligand TGF-α is reduced in endosomes while the EGF-EGFR interaction is not altered [306]. Furthermore, endocytosis has been proposed to enhance ERK2 activation [226]. Thus, the mode of action of EGFR receptors can be changed by endocytic activity, and appears to be regulated by EGF itself [307].
Immunolocalisation of DGrip in endosomal compartments in *Drosophila* has thus far been unsuccessful, although DGrip-GFP did partially colocalise with endosomal markers in Cos-7 cells [147]. The punctate pattern of DGrip immunoreactivity observed during muscular development is strongly reminiscent of vesicular structures, but they were not identified by co-immunostaining with antibodies against endosomal markers such as Hrs (gift of Hugo Bellen, not shown) or Rab5 (gift of Marcos Gonzales-Gaitan, not shown).
Likewise, examination of DGrip-tdimer2 and FYVE-GFP co-expression in larval muscles was not indicative of endosomal localisation (not shown). A biochemical approach to look at DGrip's cellular distribution in embryonic muscle may well be the most instructive for our question, to either eliminate or include DGrip endosomal location as a factor in DGrip-dependent signalling.
An interesting idea might be, when the DGrip-dependent signalling pathway is better characterised, to look at markers of this pathway (for example dpERK) in animals with inducible expression of dominant negative Rab5, or even under sucrose application [226] which should block endocytosis in these tissues. Should these markers of DGrip activation still be expressed, one could surmise that endocytosis is not required for this process.

4.7 The role of DGrip in other *Drosophila* tissues

In addition to the muscle guidance phenotype, several other processes where DGrip expression is necessary were identified. Our laboratory has studied a presynaptic defect in neurotransmitter release in some detail (see **Section 3.10**). There, *dgrip*^{ex36} presynapses, as well as animals expressing presynaptically-driven DGrip RNAi, have much higher evoked release of neurotransmitter than their respective controls, leading to a higher quantal content, most likely stemming from an increase in release probability at presynaptic terminals (Robert Kittel).

It is not yet clear where DGrip is active, either in a role as an endocytotic protein, or as a trafficking protein. mGRIPs have ben reported to be present in central nervous presynapses [117], but their roles there are unknown. Given that mammalian GRIPs, or splice variants thereof, have been proposed to be involved in trafficking [103, 112, 137], endocytosis [104, 112, 113], cytoskeletal binding [308] , cell cycle [309] and even transcription [140, 310], potentially DGrip-bound complexes could mediate all steps from membrane bound signalling to switching on downstream transcription, not an easy task to dissect. As Mint, a putative DGrip interactor, is known to interact with Munc-18 in mammals [190], the weak interaction of the Munc-18 homologue, Ras Opposite (*rop*) [211], during the suppression of DGripΔ1-3 mediated lethality screen (**Section 3.7.5.3**) was an encouraging result- although much work remains to show that this interaction is valid.

Some preliminary data also suggest that presynaptically expressed DGripΔ1-3 (which is dominant active during muscle guidance) caused a strong increase in the size of evoked junctional currents (Robert Kittel, personal communication). At this point it cannot be distinguished between this being a dominant negative or dominant active phenomenon in this system. It would, however, be interesting to see if overexpression of DGripΔ1-3x7 shows that, as in other processes, mutation of PDZ7 represses DGripΔ1-3 activity. In that case, regardless of whether the presynaptic activity of DGripΔ1-3 is negative or positive, a similar molecular logic is at play.

Presynaptic motor terminals showed significantly more FYVE-GFP endosomal compartments than wild-type controls (**Figure 48**). Likewise, enlarged presysnaptic vescicular compartments can be observed on the electron microscopic level. However, whether the increase in endosomal compartments is the causative factor for the increase in release probability, or merely an epiphenomenon associated with bulk modes of endocytosis in a highly potentiated synapse is not yet clear. Two possible explanations of the observed increase in release probability, coupled with a proliferation of early endosomal compartments, are as follows: Firstly, DGrip may have a role in endosomal trafficking. Mutants of endosomal factors such as *rab5* have been shown to affect endo- and exocytosis rates and to decrease the evoked neurotransmitter release probability at the *Drosophila* NMJ [237], possibly by changing the protein complement on synaptic vesicles. DGrip may thus have role in 'conditioning' synaptic vesicles, by exchanging or modifying vesicle proteins for less Ca^{2+} sensitive forms. In that case, the early endosomal compartments observed may be indicative of a block in a necessary endosomal processing step used for synaptic vesicles.

Alternately, the endosomal compartments observed may be merely the result of high demand put on the synapse by sustained high levels of release. In this theory, DGrip would act in the stages before vesicle fusion, perhaps as a factor trafficking Ca^{2+} channels, or directly involved in the modification of release probability via some other mechanism, perhaps by interaction with members of the fusion apparatus. The two alternatives are summarised in **Figure G** below.

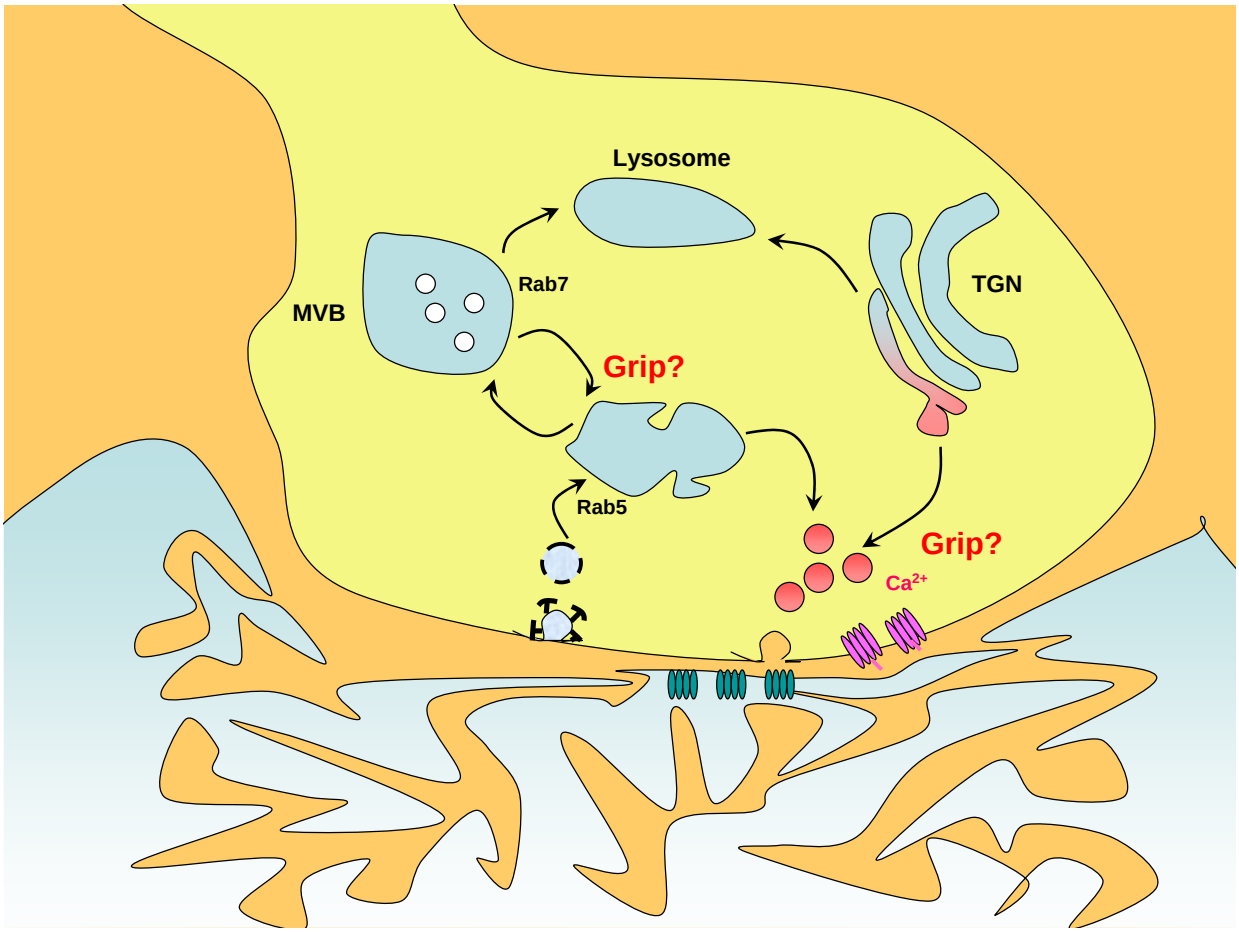


Figure G: DGrip may act on the presynaptic endocytic pathway, either because it has a role in endocytic trafficking or processing, or because it directly influences Ca^{2+}- dependent release.

By looking at the rescue ability of mutant transgenes in two later processes, (**Section 3.6**) it is clear that some of DGrip's molecular logic is transferred to later, poorly understood functions of DGrip in the genesis of adult flies. Two defects were screened, male pupal lethality and adult abdominal malformation, which occur upon the loss of DGrip in *dgrip*^{ex36} mutants. These defects can be fully rescued by full-length DGrip expressed by the driver *24B-gal4*, and by transgenes without functional PDZs 7, 6 or 1.
DGripΔ1-3 causes pupal lethality, which like the muscle defect caused by DGripΔ1-3, can be repressed by mutation of PDZ7. This may indicate a similar spectrum of interaction partners, or at the least of interaction logic in this later process (**Table 7**). As two mutant genes which strongly interacted to suppress *G14-gal4*::*UAS-GripΔ1-3* pupal lethality,

Baboon and Echinoid (**Section 3.7.5.3**), can both evoke defects in muscle guidance (**Sections 3.8 and 3.9**), it seems more suggestive that the same complex can be involved in both the muscle guidance and dominant pupal lethal processes.
However (**Section 3.6**), not all PDZ domains are utilised in every DGrip-dependent process. This indicates, as one might expect, that apart from some core interactions (possibly with Echinoid or Baboon) not all interactors are conserved for all processes.
On an interaction level, three major signalling pathways which DGrip may be involved in were identified. These could explain other phenotypes other than muscle guidance. DGrip is not an obligate member any of these signalling processes. For example, EGFR signalling takes place in many tissues where DGrip exerts no effect, as a rough eye phenotype, characteristic of EGFR signalling mutants in the eye, could not be induced with eye-specific expression of either DGrip or DGripΔ1-3, nor could a rough eye phenotype be seen in *dgrip*^{ex36} mutants. Likewise, DGrip is also not a necessary interaction partner for all functions of the transmembrane protein Echinoid, as no CNS hyperplasia is found in *dgrip*^{ex36} embryos as described for echinoid mutants [213].
This is also the case for Robo signalling, as Robo signalling in muscles has been shown to be biphasic, where the Slit ligand is repulsive for migrating myoblast precursors, while attractive during the muscle guidance process [60]. DGrip mutants only affect this second process.
This would suggest that DGrip executes its role only in specific tissues or in specific processes. The concept of DGrip as a tissue-specific interactor adds an interesting dimension to DGrip function, as DGrip may well then act as a ‘smart adaptor’, its function defined by the combination of interaction partners available to it. Further studies of DGrip and its interactors may thus be of use to understand how it regulates its many known functions in mammals, and how it makes the transition from one function to another within the context of glutamatergic neurotransmssion.

5. Summary

Here, the identification and characterisation of the *Drosophila* homologue of the Glutamate Receptor Interacting Protein, DGrip is described. Mutants of DGrip show four scorable defects, a specific loss of response to guidance cues in a subgroup of embryonic muscles, a strong potentiation of evoked release at the larval neuromuscular junction, pupal lethality, and adult abdominal malformation. Using the muscle guidance phenotype to assay functional domains of this protein, a dominant active form, DGripΔ1-3, was identified. The dominant activity could be linked to ligands binding PDZs 1 and 2, and dominant activity was repressed in DGripΔ1-3x7, where a specific point mutation of the PDZ7 ligand binding pocket was made. This and other data obtained this study suggests a model where at least four PDZ domains are required to bind ligands for DGrip's function in muscle guidance. PDZs 1 and 2 bind repressors, while PDZs 3 and 7 appear necessary for function.

Correlating interaction with the point mutations of PDZ ligand binding pockets made above, it was shown that interaction of putative interactors was abolished by specific point mutation of PDZ domains' ligand binding pockets.

One candidate gene, Echinoid, interacts with PDZs 1, 2 and 7. Combining functional data from the structure-function study above with this data suggests a model where Echinoid binding to PDZs 1 and 2 may be repressive in muscle guidance, but that if Echinoid binds to PDZ 7, it is able to execute its function.

Using the dominant active DGripΔ1-3 as a screening tool, two strong interactors suppressing DGripΔ1-3-mediated pupal lethality, Baboon and Echinoid, were found. Both these proteins also had a role in the process of muscle guidance, as mutants showed specific muscle defects and ectopic expression was able to derange somatic musculature. Echinoid is a regulator of EGFR signalling. In *dgrip* mutants, it was shown that muscular expression of DGrip is required for EGFR-dependent signalling *in trans* to apodemes, and that muscle-expressed EGFR causes defects in muscle morphology only in the $dgrip^{ex36}$ background, indicating that DGrip is needed for EGFR regulation in muscles as well as apodemes.

This study demonstrates that DGrip is a molecule which relies on cooperative interactions over many of its PDZ domains to integrate function. It does so by interacting with at least three novel signalling pathways (Robo, TGFβ and EGFR), and may function as the integration point of all these signals. The same molecular logic and several interaction partners are then transferred to later functions of DGrip. Due to the conservation of DGrip with mGRIP, this logic may also be indicative of functional organisation of mGRIPs as well. The study of this integrative function based on the structure function data obtained here may prove most rewarding: A deeper insight may be gained into the role complimentary interactions over different PDZ domains play in the regulation of such processes as GluR presentation in mammals.

6. Abbreviations

GRIP	glutamate receptor interacting protein
ABP	AMPA receptor binding protein
PDZ	PSD-95/Discs-large/ZO-1 domain
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
NMDA	N-methyl D-aspartate
GluR	glutamate receptor
CNS	central nervous system
LTP	long term potentiation
EJC	evoked junctional current
TGF	transforming growth factor
FGF	fibroblast growth factor
EGFR	epidermal growth factor receptor
MAPK/ERK	mitogen activated protein kinase /extracellular signal-regulated kinase
MEK	MAP-erk kinase
MP1	MEK partner 1
PLC	phospholipase C
Ig	immunoglobulin
FNIII	fibronectin type III
Hrs	hepatocyte growth factor regulated tyrosine kinase substrate
RTK	receptor tyrosine kinase
GFP	green fluorescent protein
NMJ	neuromuscular junction
ECM	extracellular matrix
VLM	ventral longitudinal muscle(s)
LTM	lateral transverse muscle(s)
UAS	upstream activating sequence
FRET	Forster resonance energy transfer
FLIM	fluorescent lifetime imaging

7. References

1. Gardner, E.J. and M.D. Gardner, *Further evidence for maternal effect and modifiers of tumorous head genes in natural populations of Drosophila melanogaster.* Cancer Res, 1953. **13**(10): p. 689-93.
2. Gardner, E.J. and A.M. Hansen, *Reconsideration of maternal effect transfer by injection in Drosophila melanogaster.* Genetics, 1962. **47**: p. 847-52.
3. Levitan, M. and R. Schiller, *Further evidence that the chromosome breakage factor in Drosophila robusta involves a maternal effect.* Genetics, 1963. **48**: p. 1231-8.
4. Tsien, H.C. and J.M. Wattiaux, *Effect of maternal age on DNA and RNA content of Drosophila eggs.* Nat New Biol, 1971. **230**(13): p. 147-8.
5. Bellotto, M., et al., *Maternal-effect loci involved in Drosophila oogenesis and embryogenesis: P element-induced mutations on the third chromosome.* Int J Dev Biol, 2002. **46**(1): p. 149-57.
6. Knirr, S., et al., *Somatic mesoderm differentiation and the development of a subset of pericardial cells depend on the not enough muscles (nem) locus, which contains the inscuteable gene and the intron located gene, skittles.* Mech Dev, 1997. **67**(1): p. 69-81.
7. Saint, R. and B. Patterson, *Zygotic transcription and cell proliferation during Drosophila embryogenesis.* Genetica, 1993. **90**(2-3): p. 157-63.
8. Carmena, M., et al., *Dosage dependence of maternal contribution to somatic cell division in Drosophila melanogaster.* Development, 1991. **113**(4): p. 1357-64.
9. Perrimon, N., et al., *X-linked female-sterile loci in Drosophila melanogaster.* Genetics, 1986. **113**(3): p. 695-712.
10. Cronmiller, C. and T.W. Cline, *The relationship of relative gene dose to the complex phenotype of the daughterless locus in Drosophila.* Dev Genet, 1986. **7**(4): p. 205-21.
11. Clark, A.G., *Natural selection with nuclear and cytoplasmic transmission. II. Tests with Drosophila from diverse populations.* Genetics, 1985. **111**(1): p. 97-112.
12. Payre, F., C. Yanicostas, and A. Vincent, *Serendipity delta, a Drosophila zinc finger protein present in embryonic nuclei at the onset of zygotic gene transcription.* Dev Biol, 1989. **136**(2): p. 469-80.
13. O'Farrell, P.H., et al., *Directing cell division during development.* Science, 1989. **246**(4930): p. 635-40.
14. Keshishian, H., et al., *The drosophila neuromuscular junction: a model system for studying synaptic development and function.* Annu Rev Neurosci, 1996. **19**: p. 545-75.
15. Brand, A.H. and N. Perrimon, *Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.* Development, 1993. **118**(2): p. 401--415.
16. Truman, J.W. and M. Bate, *Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster.* Dev Biol, 1988. **125**(1): p. 145-57.
17. Jan, L.Y. and Y.N. Jan, *L-glutamate as an excitatory transmitter at the Drosophila larval neuromuscular junction.* J Physiol, 1976. **262**(1): p. 215-36.
18. Johansen, J., et al., *Stereotypic morphology of glutamatergic synapses on identified muscle cells of Drosophila larvae.* J Neurosci, 1989. **9**(2): p. 710-25.
19. Zhong, Y. and L.A. Pena, *A novel synaptic transmission mediated by a PACAP-like neuropeptide in Drosophila.* Neuron, 1995. **14**(3): p. 527-36.
20. Sparks, G.M., S. Dasari, and R.L. Cooper, *Actions of MDMA at glutamatergic neuromuscular junctions.* Neurosci Res, 2004. **48**(4): p. 431-8.
21. Nishikawa, K. and Y. Kidokoro, *Octopamine inhibits synaptic transmission at the larval neuromuscular junction in Drosophila melanogaster.* Brain Res, 1999. **837**(1-2): p. 67-74.
22. McLarnon, J.G. and D.M. Quastel, *A voltage clamp study of the glutamate responsive neuromuscular junction in Drosophila melanogaster.* Can J Physiol Pharmacol, 1988. **66**(4): p. 321-7.
23. Broadie, K.S. and M. Bate, *Development of larval muscle properties in the embryonic myotubes of Drosophila melanogaster.* J Neurosci, 1993. **13**(1): p. 167-80.
24. Broadie, K.S. and M. Bate, *Development of the embryonic neuromuscular synapse of Drosophila melanogaster.* J Neurosci, 1993. **13**(1): p. 144-66.
25. Bate, M. and K. Broadie, *Wiring by fly: the neuromuscular system of the Drosophila embryo.* Neuron, 1995. **15**(3): p. 513-25.
26. Sigrist, S.J., et al., *Experience-dependent strengthening of Drosophila neuromuscular junctions.* J Neurosci, 2003. **23**(16): p. 6546-56.

27. Sigrist, S.J., et al., *The postsynaptic glutamate receptor subunit DGluR-IIA mediates long-term plasticity in Drosophila.* J Neurosci, 2002. **22**(17): p. 7362-72.
28. Sigrist, S.J., et al., *Postsynaptic translation affects the efficacy and morphology of neuromuscular junctions.* Nature, 2000. **405**(6790): p. 1062-5.
29. Aravamudan, B., et al., *Drosophila UNC-13 is essential for synaptic transmission.* Nat Neurosci, 1999. **2**(11): p. 965-71.
30. Broadie, K., et al., *Syntaxin and synaptobrevin function downstream of vesicle docking in Drosophila.* Neuron, 1995. **15**(3): p. 663-73.
31. Broadie, K.S., *Regulation of the synaptic vesicle cycle in Drosophila.* Biochem Soc Trans, 1996. **24**(3): p. 639-45.
32. Littleton, J.T., et al., *Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly.* Neuron, 1998. **21**(2): p. 401-13.
33. Nie, Z., et al., *Overexpression of cysteine-string proteins in Drosophila reveals interactions with syntaxin.* J Neurosci, 1999. **19**(23): p. 10270-9.
34. Rieckhof, G.E., et al., *Presynaptic N-type calcium channels regulate synaptic growth.* J Biol Chem, 2003. **278**(42): p. 41099-108.
35. Risinger, C., et al., *Evolutionary conservation of synaptosome-associated protein 25 kDa (SNAP-25) shown by Drosophila and Torpedo cDNA clones.* J Biol Chem, 1993. **268**(32): p. 24408-14.
36. Sanyal, S., et al., *Genetic interaction between shibire and comatose mutations in Drosophila suggest a role for snap-receptor complex assembly and disassembly for maintenance of synaptic vesicle cycling.* Neurosci Lett, 2001. **311**(1): p. 21-4.
37. Schulze, K.L., et al., *Genetic and electrophysiological studies of Drosophila syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission.* Cell, 1995. **80**(2): p. 311-20.
38. Schulze, K.L., et al., *rop, a Drosophila homolog of yeast Sec1 and vertebrate n-Sec1/Munc-18 proteins, is a negative regulator of neurotransmitter release in vivo.* Neuron, 1994. **13**(5): p. 1099-108.
39. Wu, M.N. and H.J. Bellen, *Genetic dissection of synaptic transmission in Drosophila.* Curr Opin Neurobiol, 1997. **7**(5): p. 624-30.
40. Wu, M.N., et al., *ROP, the Drosophila Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner.* Embo J, 1998. **17**(1): p. 127-39.
41. Wu, M.N., et al., *The ROP-syntaxin interaction inhibits neurotransmitter release.* Eur J Cell Biol, 2001. **80**(2): p. 196-9.
42. Currie, D.A., J.W. Truman, and S.J. Burden, *Drosophila glutamate receptor RNA expression in embryonic and larval muscle fibers.* Dev Dyn, 1995. **203**(3): p. 311-6.
43. Marrus, S.B. and A. DiAntonio, *Preferential localization of glutamate receptors opposite sites of high presynaptic release.* Curr Biol, 2004. **14**(11): p. 924-31.
44. Marrus, S.B., et al., *Differential localization of glutamate receptor subunits at the Drosophila neuromuscular junction.* J Neurosci, 2004. **24**(6): p. 1406-15.
45. Schuster, C.M., et al., *Molecular cloning of an invertebrate glutamate receptor subunit expressed in Drosophila muscle.* Science, 1991. **254**(5028): p. 112-4.
46. Koh, Y.H., et al., *Regulation of DLG localization at synapses by CaMKII-dependent phosphorylation.* Cell, 1999. **98**(3): p. 353-63.
47. Mathew, D., et al., *Recruitment of scribble to the synaptic scaffolding complex requires GUK-holder, a novel DLG binding protein.* Curr Biol, 2002. **12**(7): p. 531-9.
48. Schuster, C.M., et al., *Genetic dissection of structural and functional components of synaptic plasticity. II. Fasciclin II controls presynaptic structural plasticity.* Neuron, 1996. **17**(4): p. 655-67.
49. Schuster, C.M., et al., *Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth.* Neuron, 1996. **17**(4): p. 641-54.
50. Bloor, J.W. and N.H. Brown, *Genetic analysis of the Drosophila alphaPS2 integrin subunit reveals discrete adhesive, morphogenetic and sarcomeric functions.* Genetics, 1998. **148**(3): p. 1127-42.
51. Bogaert, T., N. Brown, and M. Wilcox, *The Drosophila PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments.* Cell, 1987. **51**(6): p. 929-40.
52. Brown, N.H., *Cell-cell adhesion via the ECM: integrin genetics in fly and worm.* Matrix Biol, 2000. **19**(3): p. 191-201.

53. Martin-Bermudo, M.D., O.M. Dunin-Borkowski, and N.H. Brown, *Specificity of PS integrin function during embryogenesis resides in the alpha subunit extracellular domain.* Embo J, 1997. **16**(14): p. 4184-93.
54. Martin-Bermudo, M.D., O.M. Dunin-Borkowski, and N.H. Brown, *Modulation of integrin activity is vital for morphogenesis.* J Cell Biol, 1998. **141**(4): p. 1073-81.
55. Carmena, A., et al., *Combinatorial signaling codes for the progressive determination of cell fates in the Drosophila embryonic mesoderm.* Genes Dev, 1998. **12** (24): p. 3910-22.
56. Rau, A., et al., *rolling pebbles (rols) is required in Drosophila muscle precursors for recruitment of myoblasts for fusion.* Development, 2001. **128**(24): p. 5061-73.
57. Ruiz-Gomez, M., et al., *Drosophila dumbfounded: a myoblast attractant essential for fusion.* Cell, 2000. **102**(2): p. 189-98.
58. Rushton, E., et al., *Mutations in a novel gene, myoblast city, provide evidence in support of the founder cell hypothesis for Drosophila muscle development.* Development, 1995. **121**(7): p. 1979-88.
59. Strunkelnberg, M., et al., *rst and its paralogue kirre act redundantly during embryonic muscle development in Drosophila.* Development, 2001. **128**(21): p. 4229-39.
60. Kramer, S.G., et al., *Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration.* Science, 2001. **292**(5517): p. 737-40.
61. Steigemann, P., et al., *Heparan sulfate proteoglycan syndecan promotes axonal and myotube guidance by slit/robo signaling.* Curr Biol, 2004. **14**(3): p. 225-30.
62. Kramer, S.G., et al., *Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration.* Science, 2001. **292**: p. 737-740.
63. Becker, S., et al., *Reciprocal signaling between Drosophila epidermal muscle attachment cells and their corresponding muscles.* Development, 1997. **124**(13): p. 2615-22.
64. Frommer, G., et al., *Epidermal egr-like zinc finger protein of Drosophila participates in myotube guidance.* EMBO Journal, 1996. **15**(7): p. 1642-9.
65. Volk, T. and K. VijayRaghavan, *A central role for epidermal segment border cells in the induction of muscle patterning in the Drosophila embryo.* Development, 1994. **120**(1): p. 59-70.
66. Vorbrüggen, G. and H. Jackle, *Epidermal muscle attachment site-specific target gene expression and interference with myotube guidance in response to ectopic stripe expression in the developing Drosophila epidermis.* PNAS, 1997. **94**(16): p. 8606-11.
67. Huber, A.B., et al., *Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance.* Annu Rev Neurosci, 2003. **26**: p. 509-63.
68. Bunch, T., et al., *The PS2 integrin ligand tiggrin is required for proper muscle function in Drosophila.* Development, 1998. **125**(9): p. 1679-89.
69. Brown, N.H., et al., *Talin is essential for integrin function in Drosophila.* Dev Cell, 2002. **3**(4): p. 569-79.
70. Martin-Bermudo, M.D., *Integrins modulate the Egfr signaling pathway to regulate tendon cell differentiation in the Drosophila embryo.* Development, 2000. **127**(12): p. 2607-15.
71. Martin-Bermudo, M.D. and N.H. Brown, *The localized assembly of extracellular matrix integrin ligands requires cell-cell contact.* J Cell Sci, 2000. **113 Pt 21**: p. 3715-23.
72. Martin-Bermudo, M.D. and N.H. Brown, *Intracellular signals direct integrin localization to sites of function in embryonic muscles.* J Cell Biol, 1996. **134**(1): p. 217-26.
73. Brower, D.L., et al., *Nonequivalent requirements for PS1 and PS2 integrin at cell attachments in Drosophila: genetic analysis of the alpha PS1 integrin subunit.* Development, 1995. **121**(5): p. 1311-20.
74. Dong, H., et al., *GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors.* Nature, 1997. **386**(6622): p. 279-84.
75. Heckmann, M. and J. Dudel, *Recordings of glutamate-gated ion channels in outside-out patches from Drosophila larval muscle.* Neurosci Lett, 1995. **196**(1-2): p. 53-6.
76. Ultsch, A., et al., *Glutamate receptors of Drosophila melanogaster: cloning of a kainate-selective subunit expressed in the central nervous system.* Proc Natl Acad Sci U S A, 1992. **89**(21): p. 10484-8.
77. Schenk, U., et al., *A novel pathway for presynaptic mitogen-activated kinase activation via AMPA receptors.* J Neurosci, 2005. **25**(7): p. 1654-63.
78. Wang, Y. and J.P. Durkin, *alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, but not N-methyl-D-aspartate, activates mitogen-activated protein kinase through G-protein beta gamma subunits in rat cortical neurons.* J Biol Chem, 1995. **270**(39): p. 22783-7.

79. Wang, J.Q., et al., *Glutamate signaling to Ras-MAPK in striatal neurons: mechanisms for inducible gene expression and plasticity.* Mol Neurobiol, 2004. **29**(1): p. 1-14.
80. Henley, J.M., *Proteins interactions implicated in AMPA receptor trafficking: a clear destination and an improving route map.* Neurosci Res, 2003. **45**(3): p. 243-54.
81. Bliss, T.V. and G.L. Collingridge, *A synaptic model of memory: long-term potentiation in the hippocampus.* Nature, 1993. **361**(6407): p. 31-9.
82. Isaac, J.T., R.A. Nicoll, and R.C. Malenka, *Evidence for silent synapses: implications for the expression of LTP.* Neuron, 1995. **15**(2): p. 427-34.
83. Rumpel, S., H. Hatt, and K. Gottmann, *Silent synapses in the developing rat visual cortex: evidence for postsynaptic expression of synaptic plasticity.* J Neurosci, 1998. **18**(21): p. 8863-74.
84. He, Y., W.G. Janssen, and J.H. Morrison, *Synaptic coexistence of AMPA and NMDA receptors in the rat hippocampus: a postembedding immunogold study.* J Neurosci Res, 1998. **54**(4): p. 444-9.
85. Petralia, R.S., et al., *Selective acquisition of AMPA receptors over postnatal development suggests a molecular basis for silent synapses.* Nat Neurosci, 1999. **2**(1): p. 31-6.
86. Nicoll, R.A. and R.C. Malenka, *Expression mechanisms underlying NMDA receptor-dependent long-term potentiation.* Ann N Y Acad Sci, 1999. **868**: p. 515-25.
87. Liao, D., et al., *Regulation of morphological postsynaptic silent synapses in developing hippocampal neurons.* Nat Neurosci, 1999. **2**(1): p. 37-43.
88. Takumi, Y., et al., *Different modes of expression of AMPA and NMDA receptors in hippocampal synapses.* Nat Neurosci, 1999. **2**(7): p. 618-24.
89. Ali, D.W., R.R. Buss, and P. Drapeau, *Properties of miniature glutamatergic EPSCs in neurons of the locomotor regions of the developing zebrafish.* J Neurophysiol, 2000. **83**(1): p. 181-91.
90. Song, I. and R.L. Huganir, *Regulation of AMPA receptors during synaptic plasticity.* Trends Neurosci, 2002. **25**(11): p. 578-88.
91. Zhang, C., et al., *Expression of glutamate receptor subunit genes during development of the mouse retina.* Neuroreport, 1996. **8**(1): p. 335-40.
92. Hirbec, H., et al., *The PDZ proteins PICK1, GRIP, and syntenin bind multiple glutamate receptor subtypes. Analysis of PDZ binding motifs.* J Biol Chem, 2002. **277**(18): p. 15221-4.
93. Kawahara, Y., et al., *GluR4c, an alternative splicing isoform of GluR4, is abundantly expressed in the adult human brain.* Brain Res Mol Brain Res, 2004. **127**(1-2): p. 150-5.
94. Ravindranathan, A., T.N. Parks, and M.S. Rao, *New isoforms of the chick glutamate receptor subunit GluR4: molecular cloning, regional expression and developmental analysis.* Brain Res Mol Brain Res, 1997. **50**(1-2): p. 143-53.
95. d'Aldin, C., et al., *Antisense oligonucleotides to the GluR2 AMPA receptor subunit modify excitatory synaptic transmission in vivo.* Brain Res Mol Brain Res, 1998. **55**(1): p. 151-64.
96. Sans, N., et al., *Aberrant formation of glutamate receptor complexes in hippocampal neurons of mice lacking the GluR2 AMPA receptor subunit.* J Neurosci, 2003. **23**(28): p. 9367-73.
97. Borgdorff, A.J. and D. Choquet, *Regulation of AMPA receptor lateral movements.* Nature, 2002. **417**(6889): p. 649-53.
98. Tardin, C., et al., *Direct imaging of lateral movements of AMPA receptors inside synapses.* Embo J, 2003. **22**(18): p. 4656-65.
99. Richmond, S.A., et al., *Localization of the glutamate receptor subunit GluR1 on the surface of living and within cultured hippocampal neurons.* Neuroscience, 1996. **75**(1): p. 69-82.
100. Hayashi, Y., et al., *Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction.* Science, 2000. **287**(5461): p. 2262-7.
101. Shi, S., et al., *Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons.* Cell, 2001. **105**(3): p. 331-43.
102. Matsuda, S., S. Mikawa, and H. Hirai, *Phosphorylation of serine-880 in GluR2 by protein kinase C prevents its C terminus from binding with glutamate receptor-interacting protein.* J Neurochem, 1999. **73**(4): p. 1765-8.
103. Seidenman, K.J., et al., *Glutamate receptor subunit 2 Serine 880 phosphorylation modulates synaptic transmission and mediates plasticity in CA1 pyramidal cells.* J Neurosci, 2003. **23**(27): p. 9220-8.
104. Xia, J., et al., *Cerebellar long-term depression requires PKC-regulated interactions between GluR2/3 and PDZ domain-containing proteins.* Neuron, 2000. **28**(2): p. 499-510.
105. Lee, S.H., A. Simonetta, and M. Sheng, *Subunit rules governing the sorting of internalized AMPA receptors in hippocampal neurons.* Neuron, 2004. **43**(2): p. 221-36.

106. Lee, S.H., et al., *Biochemical and morphological characterization of an intracellular membrane compartment containing AMPA receptors.* Neuropharmacology, 2001. **41**(6): p. 680-92.
107. Ehlers, M.D., *Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting.* Neuron, 2000. **28**(2): p. 511-25.
108. Dong, H., et al., *Characterization of the Glutamate Receptor-Interacting Proteins GRIP1 and GRIP2.* J. Neurosci, 1999. **19**(16): p. 6930-6941.
109. Srivastava, S., et al., *Novel anchorage of GluR2/3 to the postsynaptic density by the AMPA receptor-binding protein ABP.* Neuron, 1998. **21**(3): p. 581-91.
110. Srivastava, S. and E.B. Ziff, *ABP: a novel AMPA receptor binding protein.* Ann N Y Acad Sci, 1999. **868**: p. 561-4.
111. Wyszynski, M., et al., *Association of AMPA receptors with a subset of glutamate receptor-interacting protein in vivo.* J Neurosci, 1999. **19**(15): p. 6528-37.
112. Braithwaite, S.P., H. Xia, and R.C. Malenka, *Differential roles for NSF and GRIP/ABP in AMPA receptor cycling.* Proc Natl Acad Sci U S A, 2002. **99**(10): p. 7096-101.
113. Osten, P., et al., *Mutagenesis reveals a role for ABP/GRIP binding to GluR2 in synaptic surface accumulation of the AMPA receptor.* Neuron, 2000. **27**(2): p. 313-25.
114. Takamiya, K., et al., *A direct functional link between the multi-PDZ domain protein GRIP1 and the Fraser syndrome protein Fras1.* Nat Genet, 2004. **36**(2): p. 172-7.
115. Bladt, F., et al., *Epidermolysis bullosa and embryonic lethality in mice lacking the multi-PDZ domain protein GRIP1.* Proc Natl Acad Sci U S A, 2002. **99**(10): p. 6816-21.
116. Burette, A., et al., *Characterization of glutamate receptor interacting protein-immunopositive neurons in cerebellum and cerebral cortex of the albino rat.* J Comp Neurol, 1999. **411**(4): p. 601-12.
117. Charych, E.I., et al., *A four PDZ domain-containing splice variant form of GRIP1 is localized in GABAergic and glutamatergic synapses in the brain.* J Biol Chem, 2004. **279**(37): p. 38978-90.
118. Cotrufo, T., et al., *Effects of neurotrophins on synaptic protein expression in the visual cortex of dark-reared rats.* J Neurosci, 2003. **23**(9): p. 3566-71.
119. Kittler, J.T., I.L. Arancibia-Carcamo, and S.J. Moss, *Association of GRIP1 with a GABA(A) receptor associated protein suggests a role for GRIP1 at inhibitory synapses.* Biochem Pharmacol, 2004. **68**(8): p. 1649-54.
120. Bezprozvanny, I. and A. Maximov, *Classification of PDZ domains.* FEBS Lett, 2001. **509**(3): p. 457-62.
121. Bilder, D., *PDZ proteins and polarity: functions from the fly.* Trends Genet, 2001. **17**(9): p. 511-9.
122. Fuentes, E.J., C.J. Der, and A.L. Lee, *Ligand-dependent dynamics and intramolecular signaling in a PDZ domain.* J Mol Biol, 2004. **335**(4): p. 1105-15.
123. Kennedy, M.B., *Origin of PDZ (DHR, GLGF) domains.* Trends Biochem Sci, 1995. **20**(9): p. 350.
124. Zhang, M. and W. Wang, *Organization of signaling complexes by PDZ-domain scaffold proteins.* Acc Chem Res, 2003. **36**(7): p. 530-8.
125. Sheng, M. and C. Sala, *PDZ domains and the organization of supramolecular complexes.* Annu Rev Neurosci, 2001. **24**: p. 1-29.
126. Tsunoda, S. and C.S. Zuker, *The organization of INAD-signaling complexes by a multivalent PDZ domain protein in Drosophila photoreceptor cells ensures sensitivity and speed of signaling.* Cell Calcium, 1999. **26**(5): p. 165-71.
127. Meyer, G., et al., *The complexity of PDZ domain-mediated interactions at glutamatergic synapses: a case study on neuroligin.* Neuropharmacology, 2004. **47**(5): p. 724-33.
128. Zeth, K., *Structural analysis of DegS, a stress sensor of the bacterial periplasm.* FEBS Lett, 2004. **569**(1-3): p. 351-8.
129. Lin, D., et al., *A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity.* Nat Cell Biol, 2000. **2**(8): p. 540-7.
130. Wyszynski, M., et al., *Biochemical and immunocytochemical characterization of GRIP, a putative AMPA receptor anchoring protein, in rat brain.* Neuropharmacology, 1998. **37**(10-11): p. 1335-44.
131. Hirbec, H., et al., *Rapid and differential regulation of AMPA and kainate receptors at hippocampal mossy fibre synapses by PICK1 and GRIP.* Neuron, 2003. **37**(4): p. 625-38.
132. Baran, R. and Y. Jin, *Getting a GRIP on liprins.* Neuron, 2002. **34**(1): p. 1-2.
133. Wyszynski, M., et al., *Interaction between GRIP and liprin-alpha/SYD2 is required for AMPA receptor targeting.* Neuron, 2002. **34**(1): p. 39-52.

134. Bruckner, K., et al., *EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains.* Neuron, 1999. **22**(3): p. 511-24.
135. Contractor, A., et al., *Trans-synaptic Eph receptor-ephrin signaling in hippocampal mossy fiber LTP.* Science, 2002. **296**(5574): p. 1864-9.
136. Lin, D., et al., *The carboxyl terminus of B class ephrins constitutes a PDZ domain binding motif.* J Biol Chem, 1999. **274**(6): p. 3726-33.
137. DeSouza, S., et al., *Differential palmitoylation directs the AMPA receptor-binding protein ABP to spines or to intracellular clusters.* J Neurosci, 2002. **22**(9): p. 3493-503.
138. Yamazaki, M., et al., *Differential palmitoylation of two mouse glutamate receptor interacting protein 1 forms with different N-terminal sequences.* Neurosci Lett, 2001. **304**(1-2): p. 81-4.
139. Setou, M., et al., *Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA Receptor-containing vesicle transport.* Science, 2000. **288**: p. 1796-1802.
140. Yu, G., et al., *Evidence that GRIP, a PDZ-domain protein which is expressed in the embryonic forebrain, co-activates transcription with DLX homeodomain proteins.* Brain Res Dev Brain Res, 2001. **130**(2): p. 217-30.
141. Yang, J.T., R.A. Laymon, and L.S. Goldstein, *A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analyses.* Cell, 1989. **56**(5): p. 879-89.
142. Gauger, A.K. and L.S. Goldstein, *The Drosophila kinesin light chain. Primary structure and interaction with kinesin heavy chain.* J Biol Chem, 1993. **268**(18): p. 13657-66.
143. Keleman, K., et al., *Comm sorts robo to control axon guidance at the Drosophila midline.* Cell, 2002. **110**(4): p. 415-27.
144. Rajagopalan, S., et al., *Crossing the midline: roles and regulation of Robo receptors.* Neuron, 2000. **28**(3): p. 767-77.
145. Rajagopalan, S., et al., *Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the Drosophila CNS.* Cell, 2000. **103**(7): p. 1033-45.
146. LifeTechnologies, *BAC-TO-BAC Baculovirus Expression Systems*. 1999.
147. Swan, L.E., et al., *A Glutamate Receptor-Interacting Protein homolog organizes muscle guidance in Drosophila.* Genes Dev, 2004. **18**(2): p. 223--237.
148. Clark, I., et al., *Transient posterior localization of a kinesin fusion protein reflects anteroposterior polarity of the Drosophila oocyte.* Curr Biol, 1994. **4**(4): p. 289-300.
149. Clark, I.E., L.Y. Jan, and Y.N. Jan, *Reciprocal localization of Nod and kinesin fusion proteins indicates microtubule polarity in the Drosophila oocyte, epithelium, neuron and muscle.* Development, 1997. **124**(2): p. 461-70.
150. Yarnitzky, T., L. Min, and T. Volk, *The Drosophila neuregulin homolog Vein mediates inductive interactions between myotubes and their epidermal attachment cells.* Genes Dev., 1997. **11**: p. 2691-2700.
151. Yarnitzky, T., L. Min, and T. Volk, *An interplay between two EGF-receptor ligands, Vein and Spitz, is required for the formation of a subset of muscle precursors in Drosophila.* Mech Dev, 1998. **79**(1-2): p. 73-82.
152. Lin, D.M. and C.S. Goodman, *Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance.* Neuron, 1994. **13**(3): p. 507-23.
153. Jekely, G. and P. Rorth, *Hrs mediates downregulation of multiple signalling receptors in Drosophila.* EMBO Rep, 2003. **4**(12): p. 1163-8.
154. Griffith, L.C., *Receptor clustering: nothing succeeds like success.* Curr Biol, 2004. **14**(11): p. R413-5.
155. Vaccaro, P. and L. Dente, *PDZ domains: troubles in classification.* FEBS Lett, 2002. **512**(1-3): p. 345-9.
156. Im, Y.J., et al., *Crystal structure of GRIP1 PDZ6-peptide complex reveals the structural basis for class II PDZ target recognition and PDZ domain-mediated multimerization.* J Biol Chem, 2003. **278**(10): p. 8501-7.
157. Zhang, Q., J.S. Fan, and M. Zhang, *Interdomain chaperoning between PSD-95, Dlg, and Zo-1 (PDZ) domains of glutamate receptor-interacting proteins.* J Biol Chem, 2001. **276**(46): p. 43216-20.
158. Feng, W., et al., *PDZ7 of glutamate receptor interacting protein binds to its target via a novel hydrophobic surface area.* J Biol Chem, 2002. **277**(43): p. 41140-6.
159. Torres R, F.B., Dong H, Staudinger J, Olson EN, Huganir RL, Bredt DS, Gale NW, Yancopoulos GD, *PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ligands.* Neuron, 1998. **21**: p. 1453-1463.

160. Qin, G., et al., *Four different subunits are essential for expressing the synaptic glutamate receptor at neuromuscular junctions of Drosophila.* J. Neurosci, 2005. **in press**.
161. Baylies, M. and M. Bate, *twist: a myogenic switch in Drosophila.* Science, 1996. **272**(5267): p. 1481-4.
162. Vorbruggen, G. and H. Jackle, *Epidermal muscle attachment site-specific target gene expression and interference with myotube guidance in response to ectopic stripe expression in the developing Drosophila epidermis.* PNAS, 1997. **94**(16): p. 8606-11.
163. Bunch, T.A. and D.L. Brower, *Drosophila cell adhesion molecules.* Curr Top Dev Biol, 1993. **28**: p. 81-123.
164. Bunch, T.A., et al., *Characterization of mutant alleles of myospheroid, the gene encoding the beta subunit of the Drosophila PS integrins.* Genetics, 1992. **132**(2): p. 519-28.
165. Bilder, D. and N. Perrimon, *Localization of apical epithelial determinants by the basolateral PDZ protein Scribble.* Nature, 2000. **403**(6770): p. 676-80.
166. Wodarz, A., et al., *Bazooka provides an apical cue for Inscuteable localization in Drosophila neuroblasts.* Nature, 1999. **402**(6761): p. 544-7.
167. Feng, W., et al., *Tandem PDZ repeats in glutamate receptor-interacting proteins have a novel mode of PDZ domain-mediated target binding.* Nat Struct Biol, 2003. **10**(11): p. 972-8.
168. Ehrlich, I. and R. Malinow, *Postsynaptic density 95 controls AMPA receptor incorporation during long-term potentiation and experience-driven synaptic plasticity.* J Neurosci, 2004. **24**(4): p. 916-27.
169. Setou, M., et al., *Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites.* Nature, 2002. **417**(6884): p. 83-7.
170. Brose, K., et al., *Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance.* Cell, 1999. **96**(6): p. 795-806.
171. Englund, C., et al., *Attractive and repulsive functions of Slit are mediated by different receptors in the Drosophila trachea.* Development, 2002. **129**(21): p. 4941-51.
172. Hivert, B., et al., *Robo1 and Robo2 are homophilic binding molecules that promote axonal growth.* Mol Cell Neurosci, 2002. **21**(4): p. 534-45.
173. Kidd, T., K.S. Bland, and C.S. Goodman, *Slit is the midline repellent for the robo receptor in Drosophila.* Cell, 1999. **96**(6): p. 785-94.
174. Kidd, T., et al., *Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors.* Cell, 1998. **92**(2): p. 205-15.
175. Lee, J.S., R. Ray, and C.B. Chien, *Cloning and expression of three zebrafish roundabout homologs suggest roles in axon guidance and cell migration.* Dev Dyn, 2001. **221**(2): p. 216-30.
176. Rusch, J. and D. Van Vactor, *New Roundabouts send axons into the Fas lane.* Neuron, 2000. **28**(3): p. 637-40.
177. Seeger, M., et al., *Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline.* Neuron, 1993. **10**(3): p. 409-26.
178. Simpson, J.H., et al., *Short-range and long-range guidance by slit and its Robo receptors. Robo and Robo2 play distinct roles in midline guidance.* Neuron, 2000. **28**(3): p. 753-66.
179. Zallen, J.A., B.A. Yi, and C.I. Bargmann, *The conserved immunoglobulin superfamily member SAX-3/Robo directs multiple aspects of axon guidance in C. elegans.* Cell, 1998. **92**(2): p. 217-27.
180. Biederer, T., et al., *Regulation of APP-dependent transcription complexes by Mint/X11s: differential functions of Mint isoforms.* J Neurosci, 2002. **22**(17): p. 7340-51.
181. Biederer, T. and T.C. Suedhof, *Mints as adaptors.* J. Biol. Chem, 2000. **275**(51): p. 39803-6.
182. Borg, J.-P., et al., *Molecular analysis of the X11-mlin-2/CASK complex in brain.* J. Neurosci., 1999. **194**(4): p. 1307-1316.
183. Ferguson, E.L. and H.R. Horvitz, *Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode Caenorhabditis elegans.* Genetics, 1985. **110**: p. 17-72.
184. Maximov, A. and I. Bezprozvanny, *Synaptic targeting of N-type calcium channels in hippocampal neurons.* J. Neurosci., 2002. **22**(16): p. 6939-6952.
185. Yi, B.A., et al., *Controlling potassium channel activities: Interplay between the membrane and intracellular factors.* Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11016-23.
186. Zamponi, G.W., *Regulation of presynaptic calcium channels by synaptic proteins.* J. Pharm. Sci, 2003. **92**: p. 79-83.
187. Ho, A., et al., *A role for Mints in transmitter release: Mint 1 knockout mice exhibit impaired GABAergic synaptic transmission.* Proc. Natl. Acad. Sci., 2003. **100**(3): p. 1409-1414.

188. Mori, A., et al., *Alteration of methamphetamine-induced striatal dopamine release in mint-1 knockout mice.* Neurosci Res, 2002. **43**(3): p. 251-7.
189. Okamoto, M., T. Matsuyama, and M. Sugita, *Ultrastructural localization of mint1 at synapses in mouse hippocampus.* Eur J Neurosci, 2000. **12**(8): p. 3067-72.
190. Okamoto, M. and T.C. Sudhof, *Mints, Munc18-interacting proteins in synaptic vesicle exocytosis.* J Biol Chem, 1997. **272**(50): p. 31459-64.
191. Zhang, W., et al., *Mint1, a Munc-18-interacting protein, is expressed in insulin-secreting beta-cells.* Biochem Biophys Res Commun, 2004. **320**(3): p. 717-21.
192. Tomita, S., et al., *Interaction of a neuron-spcific protein containing PDZ domains with Alzheimer's amyloid pecursor protein.* J. Biol. Chem, 1999. **274**: p. 2243-2254.
193. Mueller, H.T., et al., *Modulation of amyloid precursor protein metabolism by X11alpha /Mint-1. A deletion analysis of protein-protein interaction domains.* J Biol Chem, 2000. **275**(50): p. 39302-6.
194. King, G.D., K. Cherian, and R.S. Turner, *X11alpha impairs gamma- but not beta-cleavage of amyloid precursor protein.* J Neurochem, 2004. **88**(4): p. 971-82.
195. Borg, J.-P., et al., *The phosphotyrosine domains of X11 and FE65 bind to distinct sites on the YEPNTY motif of amyloid precursor protein.* Mol. Cell Biol., 1996. **16**: p. 6229-6241.
196. Whitfield, C.W., et al., *Basolateral localization of the Caenorhabditis elegans Epidermal Growth Factor Receptor in epithelial cells by the PDZ protein LIN-10.* Mol. Biol. Cell, 1999. **10**: p. 2087-2100.
197. Simske, J.S., et al., *LET-23 receptor localization by the cell junction protein LIN-7 during C.elegans vulval induction.* Cell, 1996. **85**: p. 195-204.
198. Stricker, N.L. and R.L. Huganir, *The PDZ domains of mLin-10 regulated its trans-Golgi network targeting and the surface expression of AMPA receptors.* Neuropharmacology, 2003. **45**: p. 837-848.
199. Hase, M., et al., *Expresion and characterization of the Drosophila X11-like/Mint protein during neural development.* J. Neurochem, 2002. **81**: p. 1223-1232.
200. Brummel, T., et al., *The Drosophila activin receptor baboon signals through dSmad2 and controls cell proliferation but not patterning during larval development.* Genes Dev, 1999. **13**(1): p. 98-111.
201. Shishido, E., M. Takeichi, and A. Nose, *Drosophila synapse formation: regulation by transmembrane protein with leu-rich repeats, CAPRICIOUS.* Science, 1998. **280**(5372): p. 2118--2121.
202. Golembo, M., E. Raz, and B.Z. Shilo, *The Drosophila embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm.* Development, 1996. **122**(11): p. 3363--3370.
203. Ghazi, A., S. Anant, and K. VijayRaghavan, *Apterous mediates development of direct flight muscles autonomously and indirect flight muscles through epidermal cues.* Development, 2000. **127**(24): p. 5309--5318.
204. Koh, Y.H., et al., *Regulation of DLG localization at synapses by CaMKII-dependent phosphorylation.* Cell, 1999. **98**(3): p. 353--363.
205. Gustafson, K. and G.L. Boulianne, *Distinct expression patterns detected within individual tissues by the GAL4 enhancer trap technique.* Genome, 1996. **39**(1): p. 174--182.
206. Shayan, A.J., H.L. Atwood, and, *Synaptic ultrastructure in nerve terminals of Drosophila larvae overexpressing the learning gene dunce.* J. Neurobiol., 2000. **43**(1): p. 89--97.
207. Davis, G.W., C.M. Schuster, and C.S. Goodman, *Genetic analysis of the mechanisms controlling target selection: target-derived Fasciclin II regulates the pattern of synapse formation.* Neuron, 1997. **9**(3): p. 561-573.
208. Marques, G., et al., *The Drosophila BMP type II receptor Wishful Thinking regulates neuromuscular synapse morphology and function.* Neuron, 2002. **33**(4): p. 529--543.
209. Schuster, C.M., et al., *Genetic dissection of structural and functional components of synaptic plasticity.* Neuron, 1996. **17**(4): p. 641--654.
210. Harrison, S.D., et al., *Mutations in the Drosophila Rop gene suggest a function in general secretion and synaptic transmission.* Neuron, 1994. **13**(3): p. 555-66.
211. Salzberg, A., et al., *The Drosophila Ras2 and Rop gene pair: a dual homology with a yeast Ras-like gene and a suppressor of its loss-of-function phenotype.* Development, 1993. **117**(4): p. 1309-19.
212. Schwarz, T.L., *Genetic analysis of neurotransmitter release at the synapse.* Curr Opin Neurobiol, 1994. **4**(5): p. 633-9.
213. Ahmed, A., et al., *Echinoid mutants exhibit neurogenic phenotypes and show synergistic interactions with the Notch signaling pathway.* Development, 2003. **130**(25): p. 6295-304.

214. Bai, J., et al., *The cell adhesion molecule Echinoid defines a new pathway that antagonizes the Drosophila EGF receptor signaling pathway.* Development, 2001. **128**(4): p. 591-601.
215. Chandra, S., A. Ahmed, and H. Vaessin, *The Drosophila IgC2 domain protein Friend-of-Echinoid, a paralogue of Echinoid, limits the number of sensory organ precursors in the wing disc and interacts with the Notch signaling pathway.* Dev Biol, 2003. **256**(2): p. 302-16.
216. Escudero, L.M., et al., *Echinoid synergizes with the Notch signaling pathway in Drosophila mesothorax bristle patterning.* Development, 2003. **130**(25): p. 6305-16.
217. Hortsch, M., *Drosophila Echinoid is an antagonist of Egfr signalling, but is not a member of the L1-type family of cell adhesion molecules.* Development, 2003. **130**(22): p. 5295.
218. Islam, R., et al., *Neuroglian activates Echinoid to antagonize the Drosophila EGF receptor signaling pathway.* Development, 2003. **130**(10): p. 2051-9.
219. Rawlins, E.L., N.M. White, and A.P. Jarman, *Echinoid limits R8 photoreceptor specification by inhibiting inappropriate EGF receptor signalling within R8 equivalence groups.* Development, 2003. **130**(16): p. 3715-24.
220. Spencer, S.A. and R.L. Cagan, *Echinoid is essential for regulation of Egfr signaling and R8 formation during Drosophila eye development.* Development, 2003. **130**(16): p. 3725-33.
221. de Belle, J.S., M.B. Sokolowski, and A.J. Hilliker, *Genetic analysis of the foraging microregion of Drosophila melanogaster.* Genome, 1993. **36**(1): p. 94-101.
222. Chang, H.C., et al., *phyllopod functions in the fate determination of a subset of photoreceptors in Drosophila.* Cell, 1995. **80**(3): p. 463-72.
223. Ghiglione, C., et al., *Mechanism of inhibition of the Drosophila and mammalian EGF receptors by the transmembrane protein Kekkon 1.* Development, 2003. **130**(18): p. 4483-93.
224. Jackson Hamlet, M.R. and L.A. Perkins, *Analysis of corkscrew signalling in Drosophila epidermal growth factor receptor pathway during myogenesis.* Genetics, 2001. **159**: p. 1073-1087.
225. Wassarman, D.A., M. Therrien, and G.M. Rubin, *The Ras signaling pathway in Drosophila.* Curr Opin Genet Dev, 1995. **5**(1): p. 44-50.
226. Xue, L. and J. Lucocq, *ERK2 signalling from internalised epidermal growth factor receptor in broken A431 cells.* Cell Signal, 1998. **10**(5): p. 339-48.
227. Gabay, L., R. Seger, and B.-Z. Shilo, *In Situ activation pattern of Drosophila EGF receptor pathway during development.* Science, 1997. **277**: p. 1103-1106.
228. Gabay, L., R. Seger, and B.-Z. Shilo, *MAP kinase in situ atlas during Drosophila embryogenesis.* Development, 1997. **124**: p. 3535-3541.
229. Goubeaud, A., et al., *The Drosophila gene alien is expressed in the muscle attachment sites during embryogenesis and encodes a protein highly conserved between plants, Drosophila and vertebrates.* Mech Dev, 1996. **57**(1): p. 59-68.
230. Kim, J., et al., *Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic.* Nature, 1997. **388**(6639): p. 304-8.
231. Rawson, J.M., et al., *Drosophila neuromuscular synapse assembly and function require the TGF-beta type I receptor saxophone and the transcription factor Mad.* J Neurobiol, 2003. **55**(2): p. 134-50.
232. Hoffmann, F.M., *Transforming growth factor-beta-related genes in Drosophila and vertebrate development.* Curr Opin Cell Biol, 1991. **3**(6): p. 947-52.
233. Henderson, K.D. and D.J. Andrew, *Identification of a novel Drosophila SMAD on the X chromosome.* Biochem Biophys Res Commun, 1998. **252**(1): p. 195-201.
234. Das, P., et al., *Drosophila dSmad2 and Atr-I transmit activin/TGFbeta signals.* Genes Cells, 1999. **4**(2): p. 123-34.
235. Graff, J.M., A. Bansal, and D.A. Melton, *Xenopus Mad proteins transduce distinct subsets of signals for the TGF beta superfamily.* Cell, 1996. **85**(4): p. 479-87.
236. Lo, P.C. and M. Frasch, *Sequence and expression of myoglianin, a novel Drosophila gene of the TGF-beta superfamily.* Mech Dev, 1999. **86**(1-2): p. 171-5.
237. Wucherpfennig, T., M. Wilsch-Brauninger, and M. Gonzalez-Gaitan, *Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release.* J Cell Biol, 2003. **161**(3): p. 609-24.
238. Artero, R., et al., *Notch and Ras signaling pathway effector genes expressed in fusion competent and founder cells during Drosophila myogenesis.* Development, 2003. **130**(25): p. 6257-72.
239. Frommer, G., et al., *Epidermal egr-like zinc finger protein of Drosophila participates in myotube guidance.* EMBO Journal, 1996. **15**(7): p. 1642-9.

240. Dworak, H., et al., *Characterization of Drosophila hibris, a gene related to human nephrin.* Development, 2001. **128**(21): p. 4265-76.
241. Fromm, L. and M. Rhode, *Neuregulin-1 induces expression of Egr-1 and activates acetylcholine receptor transcription through an Egr-1-binding site.* J Mol Biol, 2004. **339**(3): p. 483-94.
242. Prokop, A., et al., *Absence of PS integrins or laminin A affects extracellular adhesion, but not intracellular assembly, of hemiadherens and neuromuscular junctions in Drosophila embryos.* Dev Biol, 1998. **196**(1): p. 58-76.
243. Martin-Bermudo and Brown, *The localized assembly of extracellular matrix integrin ligands requires cell-cell contact.* Journal of Cell Science, 2000. **113**: p. 3715-3723.
244. Bate, M., *The embryonic development of larval muscles in Drosophila.* Development, 1990. **110**(3): p. 791-804.
245. Bachmann, A., et al., *Cell type-specific recruitment of Drosophila Lin-7 to distinct MAGUK-based protein complexes defines novel roles for Sdt and Dlg-S97.* J Cell Sci, 2004. **117**(Pt 10): p. 1899-909.
246. Cantera, R., et al., *Muscle structure and innervation are affected by loss of Dorsal in the fruit fly, Drosophila melanogaster.* Mol Cell Neurosci, 1999. **13**(2): p. 131-41.
247. Caruana, G. and A. Bernstein, *Craniofacial dysmorphogenesis including cleft palate in mice with an insertional mutation in the discs large gene.* Mol Cell Biol, 2001. **21**(5): p. 1475-83.
248. Roche, J.P., et al., *Regulation of synaptic plasticity and synaptic vesicle dynamics by the PDZ protein Scribble.* J Neurosci, 2002. **22**(15): p. 6471-9.
249. Ruiz-Canada, C., et al., *New synaptic bouton formation is disrupted by misregulation of microtubule stability in aPKC mutants.* Neuron, 2004. **42**(4): p. 567-80.
250. Bruckner, K., et al., *EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains.* Neuron, 1999. **22**(3): p. 511-24.
251. Hirbec, F., Lauri, Braithwaite, Coussen, Mulle, Dev, Couthino, Meyer, Isaac, Colingridge, Henley, *Rapid and Differential regulation of AMPA and Kainate receptors at hippocampal mossy fibre synapses by PICK1 and GRIP.* Neuron, 2003. **37**: p. 625-638.
252. Braithwaite, e.a., *Differential roles for NSF and GRIP/ABP in AMPA receptor cycling.*
253. Dong, H., et al., *Characterization of the glutamate receptor-interacting proteins GRIP1 and GRIP2.* J Neurosci, 1999. **19**(16): p. 6930-41.
254. Wyszynski, K., Yang, and Sheng, *Biochemical and Immuncytochemical characterization of GRIP, a putative AMPA receptor anchoring protein, in rat brain.* Neuropharmacology, 1998. **37**: p. 1335-1344.
255. Seto ES, Bellen HJ, and T. Lloyd, *When cell biology meets development: endocytic regulation of signalling pathways.* Genes and Development, 2002. **16**: p. 1314-1336.
256. Long, J., F, et al., *Supramodular structure and synergistic target binding of the N-terminal tandem PDZ domains of PSD-95.* J Mol Biol., 2003. **327**(1): p. 203-14.
257. Feng, W., et al., *Tandem PDZ repeats in glutamate receptor-interacting proteins have a novel mode of PDZ domain-mediated target binding.* Nat Struct Biol, 2003. **10**(11): p. 972-8.
258. Anant, S., S. Roy, and K. VijayRaghavan, *Twist and Notch negatively regulate adult muscle differentiation in Drosophila.* Development, 1998. **125**(8): p. 1361-9.
259. Carmena, A., et al., *Reciprocal regulatory interactions between the Notch and Ras signaling pathways in the Drosophila embryonic mesoderm.* Dev Biol, 2002. **244**(2): p. 226-42.
260. Artero, R.D., I. Castanon, and M.K. Baylies, *The immunoglobulin-like protein Hibris functions as a dose-dependent regulator of myoblast fusion and is differentially controlled by Ras and Notch signaling.* Development, 2001. **128**(21): p. 4251-64.
261. Bour, B.A., et al., *Drosophila SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion.* Genes Dev, 2000. **14**(12): p. 1498-511.
262. Brennan, K., M. Baylies, and A.M. Arias, *Repression by Notch is required before Wingless signalling during muscle progenitor cell development in Drosophila.* Curr Biol, 1999. **9**(13): p. 707-10.
263. Cossu, G., S. Tajbakhsh, and M. Buckingham, *How is myogenesis initiated in the embryo?* Trends Genet, 1996. **12**(6): p. 218-23.
264. Dutta, D., et al., *Founder myoblasts and fibre number during adult myogenesis in Drosophila.* Development, 2004. **131**(15): p. 3761-72.
265. Fuerstenberg, S. and E. Giniger, *Multiple roles for notch in Drosophila myogenesis.* Dev Biol, 1998. **201**(1): p. 66-77.

266. Giebel, B., *The notch signaling pathway is required to specify muscle progenitor cells in Drosophila.* Mech Dev, 1999. **86**(1-2): p. 137-45.
267. Jordan, K.C., et al., *The homeobox gene mirror links EGF signalling to embryonic dorso-ventral axis formation through notch activation.* Nat Genet, 2000. **24**(4): p. 429-33.
268. Rusconi, J.C. and V. Corbin, *Evidence for a novel Notch pathway required for muscle precursor selection in Drosophila.* Mech Dev, 1998. **79**(1-2): p. 39-50.
269. Stute, C., et al., *Myoblast determination in the somatic and visceral mesoderm depends on Notch signalling as well as on milliways(mili(Alk)) as receptor for Jeb signalling.* Development, 2004. **131**(4): p. 743-54.
270. Tapanes-Castillo, A. and M.K. Baylies, *Notch signaling patterns Drosophila mesodermal segments by regulating the bHLH transcription factor twist.* Development, 2004. **131**(10): p. 2359-72.
271. Li, S., C. Xu, and R.W. Carthew, *Phyllopod acts as an adaptor protein to link the sina ubiquitin ligase to the substrate protein tramtrack.* Mol Cell Biol, 2002. **22**(19): p. 6854-65.
272. Zak, N.B., et al., *Localization of the DER/flb protein in embryos: implications on the faint little ball lethal phenotype.* Development, 1990. **109**(4): p. 865-74.
273. Koh, Y.H., et al., *The Ras1-mitogen-activated protein kinase signal transduction pathway regulates synaptic plasticity through fasciclin II-mediated cell adhesion.* J Neurosci, 2002. **22**(7): p. 2496-504.
274. Amin, A., Y. Li, and R. Finkelstein, *Hedgehog activates the EGF receptor pathway during Drosophila head development.* Development, 1999. **126**(12): p. 2623-30.
275. Bergmann, A., et al., *Regulation of cell number by MAPK-dependent control of apoptosis: a mechanism for trophic survival signaling.* Dev Cell, 2002. **2**(2): p. 159-70.
276. Freeman, M., *Complexity of EGF receptor signalling revealed in Drosophila.* Curr Opin Genet Dev, 1998. **8**(4): p. 407-11.
277. Wang, X., L.P. Weng, and Q. Yu, *Specific inhibition of FGF-induced MAPK activation by the receptor-like protein tyrosine phosphatase LAR.* Oncogene, 2000. **19**(19): p. 2346-53.
278. Yang, L. and N.E. Baker, *Role of the EGFR/Ras/Raf pathway in specification of photoreceptor cells in the Drosophila retina.* Development, 2001. **128**(7): p. 1183-91.
279. Simcox, A.A., et al., *Molecular, phenotypic, and expression analysis of vein, a gene required for growth of the Drosophila wing disc.* Dev Biol, 1996. **177**(2): p. 475-89.
280. Donaldson, T., et al., *Regulation of the Drosophila epidermal growth factor-ligand vein is mediated by multiple domains.* Genetics, 2004. **167**(2): p. 687-98.
281. Zecca, M. and G. Struhl, *Subdivision of the Drosophila wing imaginal disc by EGFR-mediated signaling.* Development, 2002. **129**(6): p. 1357-68.
282. Xie, H., et al., *EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLCgamma signaling pathway.* J Cell Sci, 1998. **111 (Pt 5)**: p. 615-24.
283. Kassis, J., et al., *A role for phospholipase C-gamma-mediated signaling in tumor cell invasion.* Clin Cancer Res, 1999. **5**(8): p. 2251-60.
284. Chen, P., et al., *Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but mitogen-activated protein kinase activity is not sufficient for induced cell movement.* J Cell Biol, 1994. **127**(3): p. 847-57.
285. Chen, P., H. Xie, and A. Wells, *Mitogenic signaling from the egf receptor is attenuated by a phospholipase C-gamma/protein kinase C feedback mechanism.* Mol Biol Cell, 1996. **7**(6): p. 871-81.
286. Khoshyomn, S., et al., *Inhibition of phospholipase C-gamma1 activation blocks glioma cell motility and invasion of fetal rat brain aggregates.* Neurosurgery, 1999. **44**(3): p. 568-77; discussion 577-8.
287. Liu, F., et al., *A human Mad protein acting as a BMP-regulated transcriptional activator.* Nature, 1996. **381**(6583): p. 620-3.
288. Yang, M., et al., *Genome-wide microarray analysis of TGFbeta signaling in the Drosophila brain.* BMC Dev Biol, 2004. **4**(1): p. 14.
289. Zheng, X. et. al, *TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the Drosophila brain.* Cell, 2003. **112**: p. 303-315.
290. Lo, P.C.H. and M. Frasch, *Sequence and expression pattern of myoglianin, a novel Drosophila gene of the TGF-b superfamily.* 1999.
291. McCabe, B.D., et al., *The BMP homolg Gbb provides a retrograde signal that regulates synaptic growth at the Drosophila neuromuscular junction.* Neuron, 2003. **39**: p. 241-254.

292. Nguyen, M., et al., *Interpretation of a BMP activity gradient in Drosophila embryos depends on synergistic signaling by to type I receptors, SAX and TKV.* Cell, 1998. **95**: p. 495-506.
293. Wrana, J.L., et al., *Mechanism of activation of the TGF-beta receptor.* Nature, 1994. **370**(6488): p. 341-7.
294. Hayes, S., A. Chawla, and S. Corvera, *TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2.* J Cell Biol, 2002. **158**(7): p. 1239-49.
295. Nakao, A., et al., *Identification of Smad2, a human Mad-related protein in the transforming growth factor beta signaling pathway.* J Biol Chem, 1997. **272**(5): p. 2896-900.
296. Gonzalez-Gaitan, M. and H. Jaeckle, *The range of spalt-activating Dpp signalling is reduced in endocytosis-defective Drosophila wing discs.* Mech Dev, 1999. **87**: p. 143-151.
297. Tzukazaki, T., et al., *SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor.* Cell, 1998. **95**: p. 779-791.
298. Itoh, F., et al., *The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGFbeta/Smad signalling.* Genes Cells, 2002. **7**: p. 321-331.
299. Di Guglielmo, G.M., et al., *Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover.* Nat Cell Biol, 2003. **5**(5): p. 410-21.
300. Gillooly, D.J., C. Raiborg, and H. Stenmark, *Phosphatidylinositol 3-phosphate is found in microdomains of early endosomes.* Histochem Cell Biol, 2003. **120**(6): p. 445-53.
301. Hu, Y., et al., *SARA, a FYVE domain protein, affects Rab5-mediated endocytosis.* J Cell Sci, 2002. **115**(Pt 24): p. 4755-63.
302. Itoh, F., et al., *The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-beta/Smad signalling.* Genes Cells, 2002. **7**(3): p. 321-31.
303. Lin, H.K., S. Bergmann, and P.P. Pandolfi, *Cytoplasmic PML function in TGF-beta signalling.* Nature, 2004. **431**(7005): p. 205-11.
304. Panopoulou, E., et al., *Early endosomal regulation of Smad-dependent signaling in endothelial cells.* J Biol Chem, 2002. **277**(20): p. 18046-52.
305. Tang, A.H., et al., *PHYL acts to down-regulate TTK88, a transcriptional repressor of neuronal cell fates, by a SINA-dependent mechanism.* Cell, 1997. **90**(3): p. 459-67.
306. Haugh, J.M., et al., *Effect of epidermal growth factor receptor internalization on regulation of the phospholipase C-gamma1 signaling pathway.* J Biol Chem, 1999. **274**(13): p. 8958-65.
307. Xie, J., et al., *Novel biphasic traffic of endocytosed EGF to recycling and degradative compartments in lacrimal gland acinar cells.* J Cell Physiol, 2004. **199**(1): p. 108-25.
308. Seog, D.H., *Glutamate receptor-interacting protein 1 protein binds to the microtubule-associated protein.* Biosci Biotechnol Biochem, 2004. **68**(8): p. 1808-10.
309. Jonason, A.S., S.M. Baker, and J.B. Sweasy, *Interaction of DNA polymerase beta with GRIP1 during meiosis.* Chromosoma, 2001. **110**(6): p. 402-10.
310. Nakata, A., et al., *GRIP1tau, a novel PDZ domain-containing transcriptional activator, cooperates with the testis-specific transcription elongation factor SII-T1.* Genes Cells, 2004. **9**(11): p. 1125-35.

Appendix I:

Results and fly stocks for DGripΔ1-3 lethality screen

Data is presented as follows:
Gene name
name of mutation or construct
source of fly line

The following columns (green header) indicate the number of animals collected for each genotype in the final experiment (**Section 3.7.5**).

In red are two columns which give the total percentage of flies with the escaper genotype (*G14-gal4;mutant chromosome*; UAS-dgripΔ1-3*) and the negative control genotype which should not produce adult flies: *G14-gal4, balancer chromosome; UAS-dgripΔ1-3*.

The % mendelian column gives the percentage of the expected mendelian ratio of *G14-gal4;*;UAS-dgripΔ1-3* flies, if the mutant chromosome was fully able to rescue DGripΔ1-3 mediated lethality.

Interactors are colour coded: *Orange* are weak interactors 5-30%
Yellow are strong interactors >30%

Experiments were repeated either from the same stock or from a newly produced stock as indicated, and the results of that experiment shown immediately below the first entry for that gene.

Gene	construct	contact	G14;*;D13	G14; *; MKR	G14;bal; D13	G14;bal; MKR	CyO;*;D13	CyO;*;MKRS	CyO;bal;D13	CyO;bal;MKR	Dead pupae	G14,*,D13	G14,bal,D13	%Mendelian
bazooka	baz4/FM7c	Jaeckle Lab	1	31	1	51	26	21	50	26	78	0	0	3
CG 32677 mint2	L(1)6PP19	Bloomington	1	15	0	18	25	20	29	22	79	0	0	4
Dlg	Dlg m 52 /Fm6	Ulrich Thomas	0	25		30	49	27	65	33	88	0	0	0
Grip	UASGrip D67		0	6	0	53	47	45	38	52	72	0	0	0
Jun kinase (basket)	UAS-bsk.DN	Bloomington	0	23	0	63	78	52	83	72	54	0	0	0
rhophillin	l(1)GO442	Jaeckle Lab	0	92	1	75	80	46	79	45	108	0	0	0
Stardust	[1] sdt[N5]/FM7c	Bloomington	0	31	2	33	19	23	23	21	72	0	1	0
stardust	Df(1)HA11	Bloomington	0	56	0	40	49	29	27	25	65	0	0	0
Stardust	KG01701	Bloomington	1	78	2	83	68	62	82	55	144	0	0	1
X11L	Df(1)BK10 /FM7c	Bloomington	2	42	0	59	31	35	50	50	78	1	0	5
Mad	y wUAS.Mad.N;Glab/Sm6a	Stuart Newfeld	0	14	0	56	54	61	47	64	60	0	0	0
UAS-Grip1-3 OE	UAS-Grip1-3 OE		0	40	0	54	55	40	43	50	83	0	0	0
Ras 85 D	UAS-Ras85D N17	Bloomington	1	35	1	51	35	20	36	21	56	0	0	3

gene	genotype	source of stock	G14,*,D13	G14,*,MKRS	G14,Cyo,D13	G14,Cyo,MKRS	Cyo-GFP,*,D13	Cyo-GFP,*,MKRS	Dead pupae	G14,*,D13	G14,Cyo,D13	% Mendelia
APKC	aPKC/CyO		0	72	2	32	47	56	49	0	1	0
baboon	w[*]; babo[32]/CyO	Bloomington	35	87	0	60	69	61	47	10	0	58
	retest from new independant stock		33	35	3	33	41	36	32	15	1	93
baboon	y[1] w[67c23]; P{w[+mC]=lacW}babo[k16912]/CyO	Bloomington	0	90	2	84	110	88	55	0	0	0
Dpp	cn[1] P{ry[+t7.2]=PZ}dpp[10638]/CyO; ry[506]	Bloomington	0	74	1	51	82	10	65	0	0	0
echinoid	ed(slA12) FRT40A/CyO wg-lacz	J-C Hsu	4	66	0	46	57	59	67	1	0	8
	retest from same stock above		13	48	0	47	68	82	129	3	0	20
echinoid	ed[slH8]FRT40A/CyO wg-lacz	J-C Hsu	26	43	0	34	50	46	46	11	0	64
	retest from new independant stock		39	44	1	44	49	43	36	15	0	91
echinoid	P{lacW}edk01102	J-C Hsu	1	59	0	56	51	76	69	0	0	2
EGFR	.P{UAS-Egfr.DN)	Bloomington	0	35	1	44	49	43	36	0	0	0
EGFR	Egfr[f24]/T(2;3)TSTL, CyO: TM6B, Tb[1	Bloomington	0	32	0	31	34	29	37	0	0	0
FGFR heartless	UAS-htl.DN	Bloomington	1	49	0	40	50	43	53	0	0	3
grip	UAS-Grip-D45		1	65	0	45	42	43	92	0	0	2
gurken	grk2B6,b,pr cn wx bw/CyO	Siegfried Roth	0	42	0	31	66	51	35	0	0	0
jra	n[1] Jra[IA109] bw[1] sp[1]/CyO	Bloomington	1	107	1	84	98	35	84	0	0	1
Jun kinase (basket)	bsk[2] cn[1] bw[1] sp[1]/CyO	Bloomington	0	106	12	75	85	41	120	0	3	0
Jun kinase (basket)	UAS-bsk	Bloomington	1	48	0	51	47	56	60	0	0	2
lesswright	P{ry}lwr[05486] /CyO	Bloomington	1	63	2	39	41	46	83	0	1	2
LGL	Yw lgl(1) /CyO		1	81	0	56	74	71	83	0	0	2
mad	w; MadB1 FRT40A/CyO	Siegfried Roth	0	49	1	59	67	32	45	0	0	0
phyllopod	phyl[2245]/CyO	Bloomington	3	38	0	26	33	40	48	2	0	10
	retest from same stock above		1	51	0	49	48	64	58	0	0	2
pka-c1	w[*]; Pka-C1[DN]/CyO	Bloomington	0	20	0	11	7	6	25	0	0	0
Rack1	yw; P{EPgy2}Rack1[EY00128]/CyO	Bloomington	0	41	0	36	56	50	44	0	0	0
Ras 85 D	UAS-Ras85D.K	Bloomington	2	42	0	53	59	49	55	1	0	5
	retest from same stock above		0	88	3	40	96	88	78	0	1	0
Ras64B	UAS-Ras64B.V14}1/CyO	Bloomington	1	0	1	145	186	128	50	0	0	1
robo1	Robo q1-15/CyO		0	45	0	47	29	49	37	0	0	0
robo2	UAS-HA Robo2	Barry Dickson	0	34	0	41	57	77	64	0	0	0
robo3	Robo3 (1)/CyO		0	29	0	28	42	50	57	0	0	0
Sara	f(2R)Egfr3, cn[1] bw[1] sp[1]/CyO	Bloomington	0	114	0	82	42	10	120	0	0	0
Sax	UAS-Sax		0	123	0	51	121	88	171	0	0	0
Sax	UAS-Sax DN		0	74	0	62	77	56	67	0	0	0
tkv	FRT tkv a12 FRT40A/CyO	Siegfried Roth	1	135	0	91	137	95	156	0	0	1
dad	y w; UAS.Dad416+4	Stuart Newfeld	0	2	0	63	75	81	62	0	0	0
gbb	gbb1, dp, cn, bev/cyo wg lacz	Mike O´Connor	0	57	0	47	46	53	41	0	0	0
gbb	gbb2,dp, cm/Cyo-wg lacz	Mike O´Connor	0	23	0	22	28	32	42	0	0	0
RhoL	P{UAS-RhoL.N25} (DN) /Cyo	Gerd Vorbrueggen	3	62	0	3	57	85	36	1	0	7
smox	UAS.dSmad2 v1d2	Mike O´Connor	0	44	0	39	47	54	56	0	0	0
Rho	UAS-Rho V12/Cyo GFP	Gerd Vorbrueggen	0	0	0	43	51	38	34	0	0	0
Robo3	UAS-Robo3		3	42	0	21	83	76	76	1	0	6

gene	genotype	source of stock	G14,*,D13	Cyo,*,D13	G14,MKRS,D	Cyo,MKRS,D13	Dead pupae	G14,*,D13	G14,MKRS	%Mendelian
Ago, Ras64B	Df(3L)Exel9000, P+PBac{XP5.RB3}Exel9000/TM6B, Tb[1	Bloomington	5	135	6	127	159	1	1	5
akt1	ry[506] P{ry[+t7.2]=PZ}Akt1[04226]/TM3, ry[RK] Sb[1] Ser[1]	Bloomington	0	12	0	8	22	0	0	0
CG5053	GE28110 (homozygous viable, no muscle phenotype)	GenExis, Korea	4	95	4	104	78	1	1	6
Dlg	UAS-Dlg RNAi		3	146	4	149	168	1	1	3
FGF branchless	P{PZ}bnl[00857]/TM3, Sb[1]	Bloomington	1	160	2	138	174	0	0	1
FGFR heartless	htl[AB42]/TM3, P{ry[+t7.2]=ftz/lacC}SC1, ry[RK] Sb[1] Ser[1]	Bloomington	0	135	8	138	132	0	2	0
grip	UAS-Grip-C13S		10	156	13	139	136	2	3	9
Magi	UAS-GFP-Dmagi /tm6 or hom		1	89	1	127	145	0	0	1
Med	w/+;;e med1 FRT82b/tm3,sb	Siegfried Roth	2	106	7	99	151	1	2	2
p50 RhoGAP	P50 rho-GAP									0
punt	ry[506] P{ry[+t7.2]=PZ}put[10460]/TM3, ry[RK] Sb[1] Ser[1]	Bloomington	2	159	4	176	104	0	1	2
Ras 85 D	sev[14]; Ras85D[e2F]/TM3, Sb[1]	Bloomington	1	140	4	120	127	0	1	1
RhoGAPp190	UAS-RhoGAPp190-RNAi	Bloomington	2	113	16	107	150	1	4	2
rhomboid	yw hsflp122; rhode11FRT80/Tm6	Siegfried Roth	5	136	15	138	197	1	3	4
robo1	UAS-HA-Robo	Barry Dickson	0	55	2	47	82	0	1	0
Rop (ras opposite)	bw[1]; Rop[G27] st[1]/TM6B, Tb[+]	Bloomington	6	128	1	97	68	2	0	8
	Rop retest from same stock above		15	189	5	174	191	3	1	10
Sax	UAS-Sax A		1	121	6	152	131	0	1	1
wit	witHA5	Hermann Aberle	2	64	1	51	110	1	0	4
wit	bw[1]; wit[A12] st[1]/TM6B, Tb[1]	Hermann Aberle	1	82	4	81	81	0	2	2
med	UAS.Med 5-13	Stuart Newfeld								0
Ephrin RTK	PUAS-ephDN	Rich Dearborn Jr.	0	111	0	121	155	0	0	0
baboon	UAS babo 1a3 strong	Mike O´Connor								0
baboon	UAS babo 9b3 weak	Mike O´Connor								0
RhoL	UAS-RhoL.V20	Gerd Vorbrueggen	0	187	4	184	211	0	1	0
smox	UAS.dSmad2 6e3	Mike O´Connor	5	100	5	93	109	2	2	6
trio	trio8/Tm6,Sb,Ser	Bloomington	0	29	1	40	44	0	1	0

Appendix II:

Published paper:

Swan LE, Wichmann C, Prange U, Schmid A, Schmidt M, Schwarz T, Ponimaskin E, Madeo F, Vorbruggen G, Sigrist SJ. *A glutamate receptor-interacting protein homolog organises muscle guidance in Drosophila*. Genes Dev. 2004 Jan 15;18(2):223-37.

A Glutamate Receptor–Interacting Protein homolog organizes muscle guidance in *Drosophila*

Laura E. Swan,[1,5] Carolin Wichmann,[1,5] Ulrike Prange,[1] Andreas Schmid,[1] Manuela Schmidt,[1] Tobias Schwarz,[2] Evgeni Ponimaskin,[2] Frank Madeo,[3] Gerd Vorbrüggen,[4] and Stephan J. Sigrist[1,6]

[1]European Neuroscience Institute Göttingen, Max-Planck-Society, 37073 Göttingen, Germany; [2]Department of Neuro and Sensory Physiology, Medical School, University of Göttingen, 37073 Göttingen, Germany; [3]Institute for Physiological Chemistry, University of Tübingen, 72076 Tübingen, Germany; [4]Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany

During *Drosophila* embryogenesis, developing muscles extend growth-cone–like structures to navigate toward specific epidermal attachment sites. Here, we show that the homolog of Glutamate Receptor–Interacting Proteins (DGrip) acts as a key component of proper muscle guidance. Mutations in *dgrip* impair patterning of ventral longitudinal muscles (VLMs), whereas lateral transverse muscles (LTMs) that attach to intrasegmental attachment sites develop normally. Myoblast fusion, stabilization of muscle contacts, and general muscle function are not impaired in the absence of DGrip. Instead, the proper formation of cellular extensions during guidance fails in *dgrip* mutant VLMs. DGrip protein concentrates at the ends of VLMs while these muscles guide toward segment border attachment sites. Conversely, LTMs overexpressing DGrip form ectopic cellular extensions that can cause attachment of these muscles to other muscles at segment borders. Our data suggest that DGrip participates in the reception of an attractive signal that emanates from the epidermal attachment sites to direct the motility of developing muscles. This *dgrip* phenotype should be valuable to study mechanistic principles of Grip function.

[*Keywords*: *dgrip*; muscle; muscle guidance; Glutamate Receptor–Interacting Protein; *Drosophila*]

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The establishment of specialized cell–cell junctions plays a determining role in the formation of mature patterned organs in all multicellular organisms. The most prominent examples are synaptic connections, which are formed either between neurons or between neurons and other cells, for example, muscles. Cells form extensions such as growth cones, lamellipodia, or filopodia, which they use to sense specific guidance cues and to finally anchor at the relevant target cells. In *Drosophila*, developing muscles grow growth-cone–like projections to navigate toward specific epidermal attachment sites (Baylies et al. 1998; Volk 1999). *Drosophila* muscles are grouped into two categories. One muscle type, which includes the lateral transverse muscles (LTMs), is characterized by single muscle fibers attaching to a single epidermal tendon cell. The other type, indirectly attaching muscles, including the ventral longitudinal muscles (VLMs), converges with several muscle fibers on single tendon cell, recruiting extracellular matrix, to which they adhere (Prokop et al. 1998). Recent evidence showed that these tendon cells, also called apodemes, are the source of secreted Slit protein. Slit is sensed as a positive guidance cue by Robo receptors expressed in the nascent VLMs. Furthermore, experimentally induced overexpression of Robo receptors causes LTMs to extend toward Slit expressing tendon sites (Kramer et al. 2001). In addition to the Robo/Slit-system controlling VLM guidance, the Derailed receptor tyrosine kinase controls LTM guidance (Callahan et al. 1996). Interestingly, both systems also have firmly established roles in axonal guidance processes, suggesting a common mechanistic basis for cellular motility of muscles and neurons.

Guidance processes are controlled by a diverse array of signaling proteins, with spatiotemporal activity that is subject to subtle regulation (Dickson 2002; Huber et al. 2003). How the cellular metabolism of such supramolecular signaling complexes is organized is subject of intense investigation. Proteins containing PDZ domains, a protein–protein interaction domain of ~90 amino acids (Willott et al. 1993; Jesaitis and Goodenough 1994), recruit components of a signaling network into larger molecular complexes in order to allow rapid and specific intracellular signaling (Bilder 2001; Sheng and Sala 2001). GRIP family proteins (GRIP1 and ABP/GRIP2)

[5]These authors contributed equally to this work.
[6]Corresponding author.
E-MAIL ssigris@gwdg.de; FAX 49-551-3912346.
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contain six or seven PDZ domains in tandem. They were first identified via an interaction of their fifth PDZ domain with the C-terminal sequence (ESVKI) of the GluR2 AMPA receptor subunit (Dong et al. 1997; Srivastava et al. 1998; Wyszynski et al. 1999) and are suggested to participate in the synaptic localization of AMPA receptors. Interfering with the interaction between GRIPs and GluR2/3 prevents AMPA receptor recruitment to the synapse in vitro (Dong et al. 1997; Osten et al. 2000; Xia et al. 2000). GRIP has also been identified as a binding partner of both ephrin receptors and ligands (Torres et al. 1998; Brückner et al. 1999; Lin et al. 1999; Contractor et al. 2002), ARF-GAP GIT1 (Ko et al. 2003), the kinesin motor protein KIF1A, and liprin-α (Ko et al. 2003; Wyszynski et al. 2002). Despite this information, the cell biological basis of GRIP function is only poorly understood. Biochemically, GRIP1 is slightly enriched in synaptic preparations but also is strongly expressed in intracellular compartments, including putative transport vesicles for glutamate receptors (Wyszynski et al. 1998, 2002; Dong et al. 1999a). GRIPs have been suggested to mediate (1) the transport of glutamate receptors directly (Dong et al. 1997; Wyszynski et al. 2002), (2) the stabilization of receptors within postsynaptic densities (Osten et al. 2000), or (3) the stabilization of intracellular stores and/or participation in sorting decisions for the destruction or recycling of internalized receptors (Shi et al. 2001; Hirbec et al. 2003). Genetic analysis in mice has shown that GRIP1 function is already required early during development, because a GRIP1 knockout was embryonic lethal at day 12 and the embryos suffered from defects in junction formation between dermis and epidermis (Bladt et al. 2002).

In the present study, we present evidence that CG14447, the single GRIP homolog in *Drosophila* and therefore named DGrip, participates in muscle development during embryogenesis. Loss of *dgrip* function caused severe defects in VLM but not LTM patterning. DGrip is required for the guidance of developing VLMs toward the apodemes. Other processes such as myoblast fusion, stabilization of muscle attachments, and muscle function per se are not affected in *dgrip* mutant embryos and larvae. Mesodermal expression of DGrip using transgenes rescued the *dgrip* mutant phenotype. Consistent with its specific function in VLM guidance, DGrip protein progressively concentrates at the ends of these muscles as they establish contact to their target position. Furthermore, when DGrip was overexpressed within embryonic mesoderm, LTMs were guided toward ectopic attachment sites at segment borders. The DGrip protein therefore appears to be used by a subset of muscles to direct their motility, likely by transporting and/or localizing signaling components of a novel pathway.

Results

Drosophila *contains a single GRIP homolog that is specifically expressed in developing muscles*

As previously noted (Littleton and Ganetzky 2000), the *Drosophila* genome encodes a single GRIP homolog (CG14447). CG14447 is represented by several embryonic cDNA isolates (see Materials and Methods; Rubin et al. 2000), which all predict the same protein sequence. Comparison of this sequence with mouse GRIP1 in respect to both position and sequence of PDZ domains clearly identifies CG14447 as a GRIP family member (Fig. 1).

To examine the expression pattern of *dgrip*, an in situ hybridization against *dgrip* was performed on *Drosophila* embryos (Fig. 1). Until germ band extension, no *dgrip* expression was detected (Fig. 1A). From stage 10 onward, staining in the posterior half of the segments (Fig. 1C–F, arrowheads) is observed, indicating expression in developing somatic muscles (Baylies et al. 1998). In fact, in stage 16 embryos, a strong *dgrip* mRNA expression is observed specifically within muscles (Fig. 1G, arrowhead), whereas at this stage neither epidermis (Fig. 1G, arrow) nor central nervous system (CNS; Fig. 1F, arrow) seem to express *dgrip* mRNA.

DGrip mutants show strong patterning defects of VLMs

The *dgrip* locus maps to position 5C10 on chromosome X (Adams et al. 2000). To generate mutations, we made use of the P-element insertion P(GT1)BG01736 (Kimmerly et al. 1996) located 2 kb downstream of the DGrip stop codon. Upon remobilization of the P-element, the small deletion $dgrip^{ex36}$ was recovered, in which the whole transcription unit but no other annotated gene is deleted (Fig. 2A). Alternatively, we started from P(KG)02862 (Roseman et al. 1995), which is inserted just upstream of the *dgrip* transcription start and recovered $dgrip^{ex122}$, in which the first exon, including the predicted start codon of the *dgrip* locus, is deleted. Individuals of the genotype $dgrip^{ex36}$/Y, $dgrip^{ex122}$/Y, and $dgrip^{ex36}/dgrip^{ex122}$ were semilethal. Precise excisions of the parental P-lines instead were fully viable and did not present any of the phenotypes observed in $dgrip^{ex36}$ and $dgrip^{ex122}$ (data not shown). Embryos hemizygous for both $dgrip^{ex36}$ and $dgrip^{ex122}$ were negative for *dgrip* mRNA in the in situ hybridization (data not shown). To examine protein expression in *dgrip* mutant embryos, a polyclonal antibody against PDZ domains 6 and 7 of the protein was affinity-purified (see Materials and Methods). The DGrip encoding cDNAs predict a protein of 112 kD. Consistently, Western blot analysis of wild-type *Drosophila* embryo extracts (stage 10–17) probed with our antibody detected a single band of ~110 to 120 kD apparent size, which comigrated with recombinant DGrip expressed in insect cells. In contrast, embryo extracts derived from a $dgrip^{ex122}$ homozygous strain were negative for DGrip protein on Western blot (Fig. 2B). We therefore conclude that both $dgrip^{ex36}$ and $dgrip^{ex122}$ represent protein null alleles of the *dgrip* locus. Consistently, both alleles resulted in identical phenotypes (see below). Moreover, phenotypes were identical in $dgrip^{ex122}$/Y irrespective of whether animals were obtained from $dgrip^{ex122}$ homozygous or heterozygous mothers. Examining this together with the in situ results

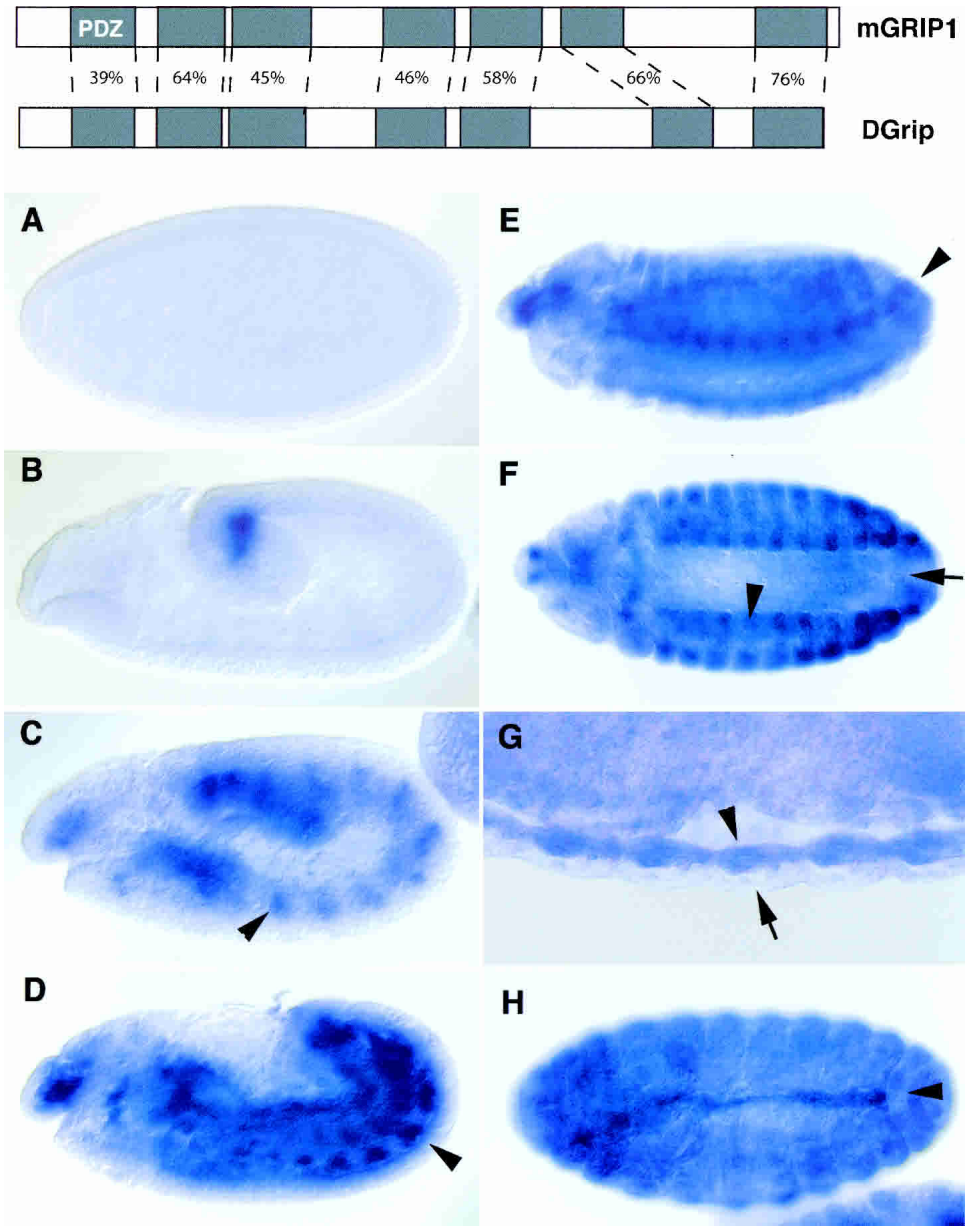

Figure 1. Embryo in situ hybridization for *dgrip*. (*Top*) Comparison between mouse GRIP1 and *Drosophila* protein CG14447 (DGrip), which both encode seven individually conserved PDZ domains. Sequence similarity between corresponding PDZ domains is indicated in percentages. (*A–H*) In situ hybridization of *dgrip*. Embryonic stages shown are as follows: 3 (*A*), 6 (*B*), 10 (*C*), 12 (*D*), 13/14 (*E* and *F*; *F* shows ventral view), 16 (*G*; dorsal view of ventral longitudinal muscles [VLMs]), and 17 (*H*; dorsal view). The *dgrip* mRNA accumulates in the precursors of the VLMs (*C–F*, *arrowheads*), resulting in a strong expression within VLMs after the formation of attachments (*G*, *arrowhead*). Epidermis (*G*, *arrow*) and CNS (*F*, *arrow*) do not show detectable *dgrip* expression. At stage 17 (*H*), cardioblasts of the dorsal vessel show strong expression of *dgrip* mRNA (*arrowhead*) and of DGrip protein (data not shown). $dgrip^{ex36}$/Y embryos did not express any detectable *dgrip* mRNA (data not shown).

(Fig. 1), we therefore conclude that no maternal activity of DGrip is present and that both alleles thus establish true DGrip null situations in embryos.

To examine the embryonic development of mesoderm in the absence of DGrip activity, the somatic muscle pattern of mutant embryos was visualized by myosin stainings. Control embryos showed the typical pattern of somatic muscles (Fig. 2C, WT). However, an abnormal patterning of the VLMs was easily detected in embryos of the genotype $dgrip^{ex36}$/Y, $dgrip^{ex122}$/Y (Fig. 2C, asterisks), and $dgrip^{ex36}/dgrip^{ex122}$ (data not shown). The defective VLMs of the mutants appeared rounded instead of stretched between the attachment sites at the segment borders. Mesodermal expression of DGrip using the *24B-gal4* (Fig. 3D) or *twist-gal4* driver (data not shown) rescued the VLM patterning defect of $dgrip^{ex36}$/Y (Fig. 3C)

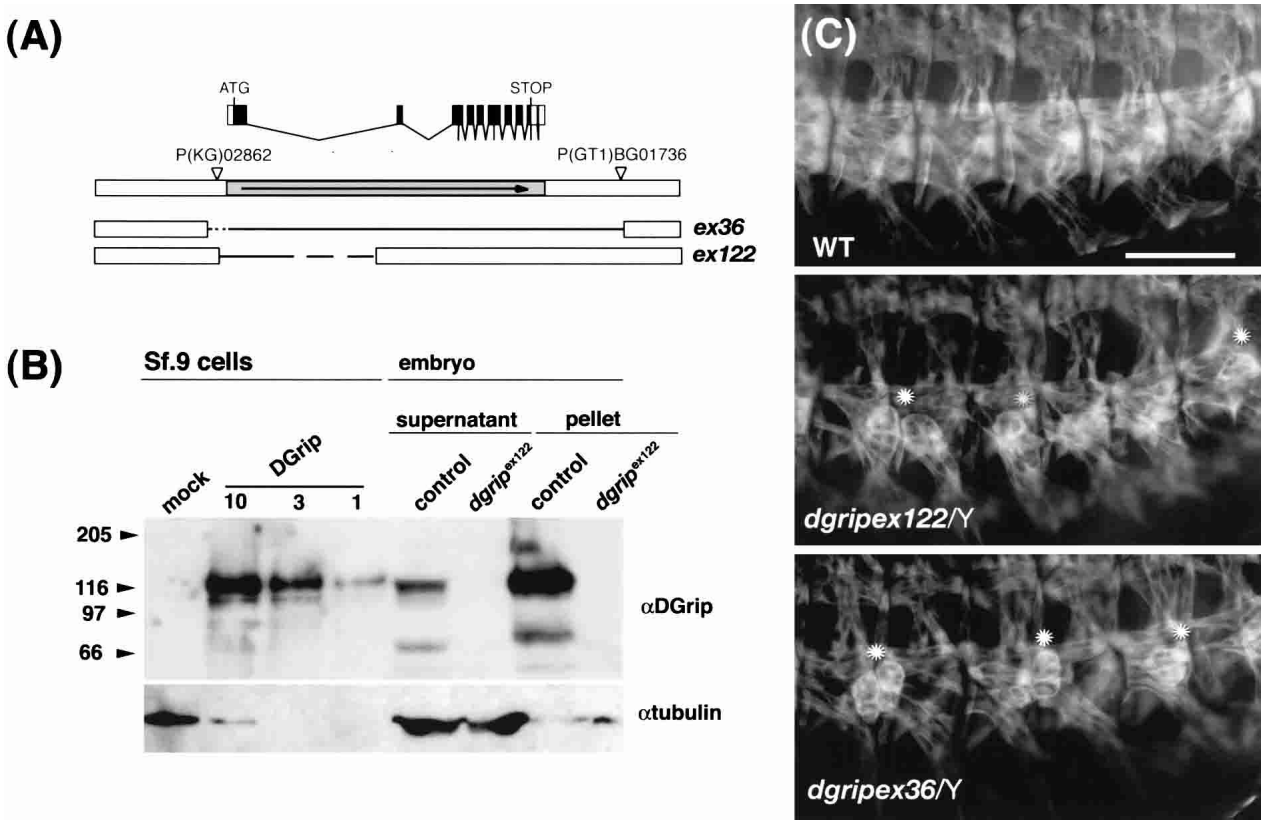

Figure 2. Specific muscle defects in *dgrip* mutants. (*A*) Genetic analysis of *dgrip*. By mobilization of transposon P(GT1)BG01736 2 kb downstream of the DGrip stop, deficiency $dgrip^{ex36}$ eliminating the full *dgrip* ORF was recovered. P(KG)02862, located 500 bp upstream of the DGrip start codon, gave deficiency $dgrip^{ex122}$, which deletes the first exon of DGrip, including the putative start codon. Both deficiencies resulted in identical phenotypes, which were rescued by mesodermal expression of DGrip using the *24B-gal4* or *twist-gal4* driver lines. Precise excisions of the parental P-lines gave wild-type phenotypes. (*B*) Western blot probed with affinity purified anti-DGrip (*top*, see Materials and Methods) and anti-Tubulin as a loading control (*bottom*). Embryos (5 to 15 h) were homogenized under low-detergent conditions ("supernatant") and the pellet was solubilized under high detergent ("pellet"); for details, see Materials and Methods. A band of ~120 kD (predicted size for DGrip 112 kD) is detected in wild-type embryo extracts, whereas no signal is present in identically produced embryonic extracts from $dgrip^{ex122}$ homozygous flies, proving this band represents DGrip. Embryonic DGrip comigrated with DGrip recombinantly expressed in Sf.9 cells (mock: untransfected Sf.9 cells; 10, 3, and 1: dilution series from a *dgrip* transfected Sf.9 cell extract). DGrip was especially enriched in the pellet fraction after high-detergent extraction. (*C*) Muscle myosin labeling visualizing the somatic muscle pattern in several hemisegments of late stage 16 embryos. Shown are lateral images of whole-mount embryos. Muscle patterning of ventral longitudinal muscles (VLMs) is clearly defective in the two independent alleles, $dgrip^{ex122}$ and $dgrip^{ex36}$. Asterisks mark groups of misattached VLMs in *dgrip* mutants. Bar, 60 μm.

to the wild-type muscle pattern. We similarly tested the epidermal *gal4* driver lines, *engrailed-gal4* (A. Brand, University of Cambridge, Cambridge, UK) and *stripe-gal4* (G. Morata, University of Madrid, Madrid, Spain), with the latter specifically expressing in tendon cells. Driving *dgrip* expression with these both lines did not rescue muscle defects of $dgrip^{ex36}$/Y (data not shown). These results indicate that *dgrip* is required in the developing muscle but not in the epidermis for proper muscle guidance.

Muscle patterning defects in the absence of DGrip were further characterized on the cellular level by using confocal microscopy. Figure 3 shows that in *dgrip* mutants, the VLMs differed markedly from the elongated cylindrical appearance of wild-type VLMs (Fig. 3A) and consistently failed to attach at both segment borders (Fig. 3B). The mutant VLMs appeared atypically compact and rounded. Such strongly affected VLMs position themselves randomly more at either the anterior or posterior segment end. In weaker cases, while still attached to both segment borders, the mutant VLMs appeared irregularly shaped and did not align in register at the segment borders, a defect not observed for wild-type VLMs. We observed that > 95% of all VLMs 6/7 were affected in *dgrip* mutant embryos, with 40% of the cells of this VLM type showing a full "rounding up" of the muscle cells. VLMs 12/13 were affected to 80% with ~10% fully rounded up. Defects within other muscles apart from the VLMs were less obvious in *dgrip* mutants. With a frequency of ~5%, another type of indirectly attaching muscle, muscle 8, was strongly affected (Fig. 3B, asterisk). Some defects in segment border attachment were also recognized in other muscles: muscle 4, the ventral oblique muscles 14 and 30, and the dorsal muscles 1 and

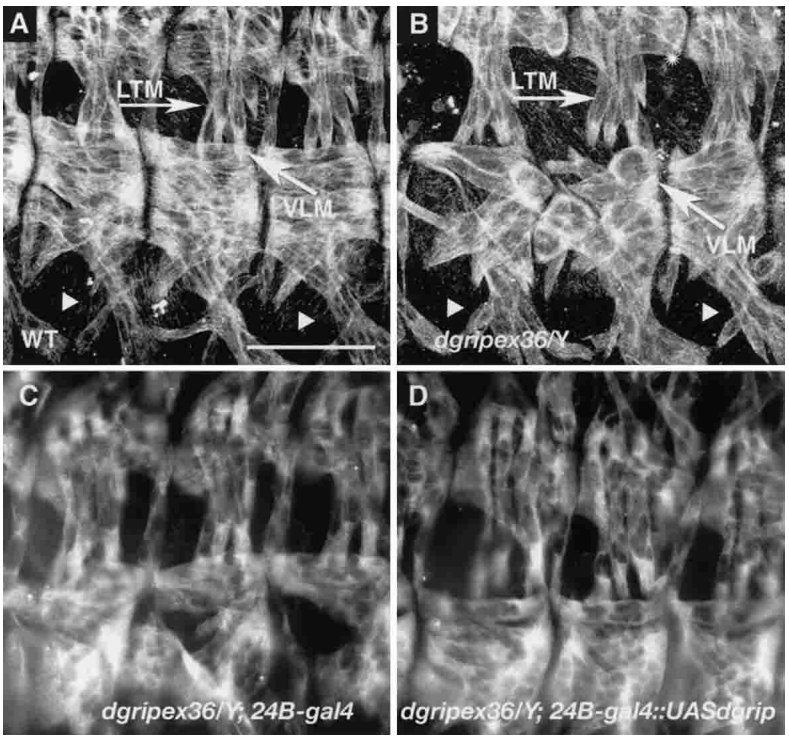

Figure 3. DGrip is essential for patterning ventral longitudinal muscles (VLMs) but not lateral transverse muscles. All images show lateral views on three hemisegments in muscle myosin stainings of late stage 16 embryos. (*A*, *B*) Confocal images projected through all muscle layers. Muscles in the VLM area (thick *arrows*) often round up and no longer attach to both segment borders in *dgrip*^{ex36}/Y (*B*). Lateral transverse muscles (thin *arrows*, cf. *A* and *B*) and ventral oblique muscles (*arrowheads*) are not affected. Occasionally, muscle 8 is also defective (*asterisk* in *B*). Bar, 50 µm. (*C*, *D*) Reexpression of DGrip using a weakly-expressing copy of *UAS-dgrip* in the *dgrip* mutant background (*D*: *dgrip*^{ex36}/Y; *24B-gal4*::*UAS-dgrip*) rescues the VLM defects obvious in *dgrip* mutants (*C*: *dgrip*^{ex36}/Y; *24B-gal4*).

2 showed milder defects in ~10% to 20% of the cells counted. Importantly however, we find directly attaching muscles such as the LTMs to be absolutely unaffected in *dgrip* mutants (Fig. 3A,B, arrows). In summary, we conclude that DGrip represents an essential component needed to establish the correct patterning within VLMs and to a lesser extent in other indirectly associating muscles during *Drosophila* embryogenesis.

In the absence of DGrip, muscles differentiate properly and stably attach at ectopic positions

Defective muscle adhesion, such as that found after interfering with integrin function, often results in muscle detachment. This effect is caused in response to contractile force in the affected muscle, a condition usually fatal in late embryogenesis (MacKrell et al. 1988; Leptin et al. 1989; Brown 1994; Bökel and Brown 2002). However, *dgrip*^{ex36}/Y, *dgrip*^{ex122}/Y, and *dgrip*^{ex36}/*dgrip*^{ex122} individuals develop into larvae, which maintained the defective VLM pattern observed in embryos (Fig. 4B,C). Within these animals, the embryonically affected muscles had obviously grown and elongated throughout larval development. No sign of muscle degeneration was recognizable. Affected VLMs had produced ectopic intrasegmental attachment sites (Fig. 4B,C, arrowheads) instead of the normal intersegmental attachments in wild type (Fig. 4A, arrowheads). Microscopic inspection of *dgrip* mutant larvae showed that these attachments form at the inner layer of muscles and not to the epidermis, showing that in the absence of DGrip function, muscle–muscle junctions are formed. Such muscle–muscle junctions, instead of normal tripartite muscle–muscle–epidermis junctions, have also been reported for other mutants such as *kakapo* (Prokop et al. 1998). Both in embryos and larvae, even the most strongly affected *dgrip* mutant VLMs form multiple extensions (Fig. 4D, arrowheads), implying that DGrip-deficient muscles still seek attachments.

That *dgrip*-deficient larvae were capable of sustained locomotion and the absence of detached muscles strongly suggest that muscle attachments were functional in this mutant. Based on phalloidin stainings (Fig. 4A–C) and electron microscopy (C. Wichmann and S.J. Sigrist, unpubl.), the defective VLMs had normal organization of the contractile apparatus as well. Moreover, escaping *dgrip*^{ex36}/Y, *dgrip*^{ex122}/Y, or *dgrip*^{ex36}/*dgrip*^{ex122} adults showed a shrunken abdomen (Fig. 4J) and defective head posture, phenotypes likely due to adult muscle patterning defects. However, no other defects—in particular, no signs of a general impairment of cell adhesion—were observed in these animals.

Drosophila muscles are highly differentiated concerning their attachment site and shape (Bate 1990; Jagla et al. 2001). Fate changes among VLM founder cells (Knirr et al. 1999) could therefore be responsible for the observed muscle phenotype. To examine this possibility, we monitored the VLM pattern in *dgrip* mutant embryos by staining with the VLM-specific differentiation marker Vestigial (Bate and Rushton 1993). Vestigial was expressed even in the most strongly affected muscles of *dgrip*^{ex36}/Y, indicating that the muscles develop according to their proper fate (data not shown). Furthermore, we made use of *5053-gal4*, which specifically drives expression in VLM 12 from stage 12 on (Ritzenthaler et al.

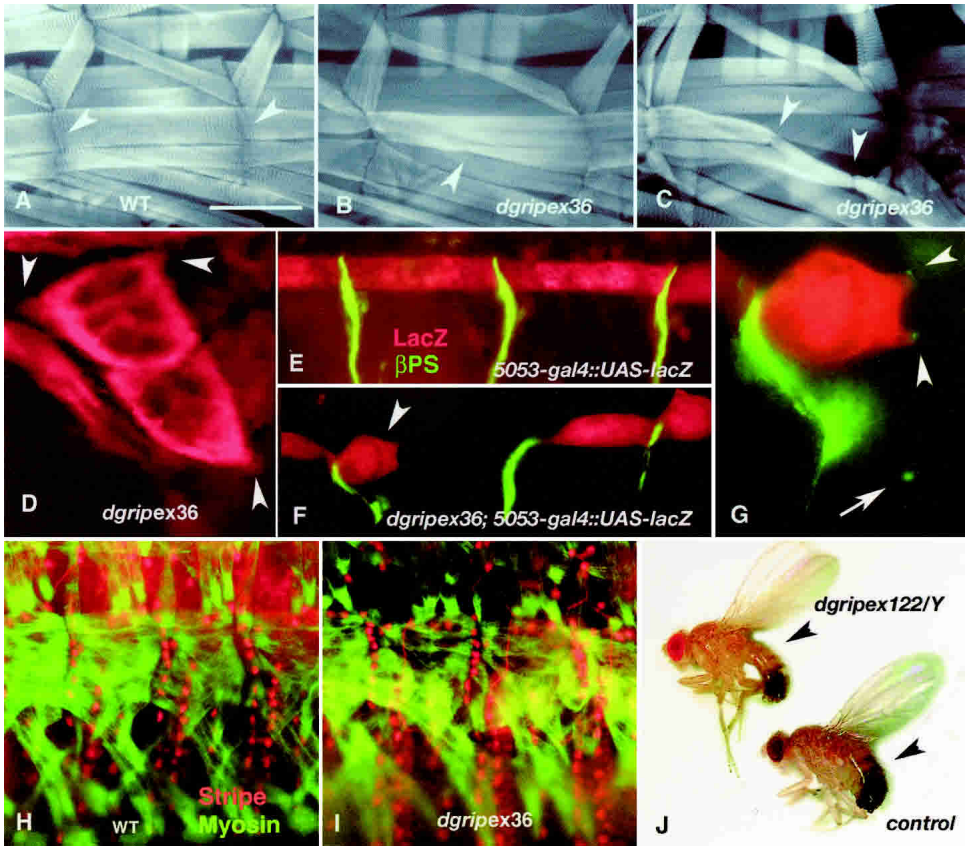

Figure 4. Muscle attachment per se is not affected in *dgrip* mutants. One hemisegment of third instar larvae in wild type (*A*) and $dgrip^{ex36}$/Y (*B*, *C*), fillet preparations stained with rhodamine-coupled phalloidin. In *dgrip* mutants, muscles differentiate a contractile apparatus, and no sign of detachment can be observed. Instead, ectopic muscle–muscle contacts form from misguided ventral longitudinal muscles (VLMs; *B*, *C*, *arrowheads*). Bar, 200 µm. (*D*) Confocal image of misguided VLMs in $dgrip^{ex36}$/Y; *arrowheads* point to cytoplasmic extensions, indicating the formation of muscle attachments in several directions. (*E–G*), Stage 17 embryos stained for β-Gal to show muscle 12 and βPS-integrin in control (*E*) and $dgrip^{ex36}$ (*F*, *G*) background. In *dgrip* mutants, one muscle 12 has not achieved segment border attachment (*F*, *arrowhead*). The ectopic muscle contacts from this misguided muscle are integrin positive (*G*, *arrowheads*; magnification of *F*). The lower integrin spot (*arrow*) in *G* likely represents an ectopic attachment between unlabeled muscles. (*H–I*) Stage 17 embryos costained with the apodeme marker Stripe (red) and with myosin (green) in *dgrip* mutants (*I*). Apodemes were differentiated as in wild type (*H*), and muscle pattern was highly impaired (*I*). (*J*) Adult $dgrip^{ex122}$ male showing a shrunken abdomen (*arrowheads*) as typical for all *dgrip* mutant escapers. Bars, 10 µm (*D*), 30 µm (*E*), and 40 µm (*H*).

2000). Expression of β-Galactosidase (β-Gal) driven by *5053-gal4* develops normally in *dgrip* mutant embryos (Fig. 4F,G). Collectively, these data show that VLMs still develop proper fate in the absence of *dgrip* activity. In *dgrip* mutants, muscle 12 frequently attached to ectopic positions, as shown by βPS-integrin staining (Fig. 4E–G, arrowheads in 4G), a marker of muscle attachment sites (Brown 2000). Thus, VLMs lacking DGrip activity are able to attach to ectopic intrasegmental attachment sites by contacting other muscles.

To evaluate myoblast fusion, the number of nuclei was determined in *dgrip* mutant muscles 12 of stage-17 embryos after Hoechst staining. The number of nuclei was only slightly lower in $dgrip^{ex36}$/Y than in wild type (8.7 ± 1.7 versus 10.0 ± 2.0, respectively; $P < 0.08$). Moreover, because correct muscle attachment is observed in the absence of myoblast fusion (Rushton et al. 1995), a defect in myoblast fusion can be excluded as the primary cause of the muscle defects observed.

Finally, we also examined possible differentiation defects within apodemes. In mutants for the transcription factor *stripe*, such defects provoke defective attachment of somatic muscles (Frommer et al. 1996; Becker et al. 1997) somewhat similar to those observed in *dgrip* mutants. However, neither *dgrip* mRNA (Fig. 1) nor protein (Fig. 6A, arrow in the inset) was detectable in the epidermis throughout the period of attachment formation. Given that DGrip is specifically expressed within the affected muscles and that the defect can be rescued by purely mesodermal expression, a direct role of DGrip in apodeme differentiation appears very unlikely. To positively exclude a role of epidermal cells with respect to the observed phenotype, we stained for the apodeme differentiation markers Delilah (data not shown) and Stripe (Fig. 4H,I) in *dgrip* mutant embryos of stage 17. Both the number and position of apodemes are unchanged in *dgrip* mutants (Fig. 4I) compared with wild type (Fig. 4H). Consistent with the formation of pure muscle–muscle junc-

tions by displaced VLMs, no sign of additional apodemes attached to VLMs was observed in *dgrip* mutants.

In summary, these results establish that muscle cell differentiation, myoblast fusion, and epidermal attachment sites are not affected by the lack of *dgrip* activity, leaving the option that the mutant muscles are misguided and fail to properly reach their target sites.

Defective guidance behavior of dgrip *mutant muscles*

To explore the possibility that DGrip may function in the guidance of developing VLMs toward their individual epidermal attachment sites, we followed the development of individual VLMs in *dgrip* mutants. For this task we labeled VLM 12 by expressing *UAS-lacZ* with the aid of the *5053-gal4* driver (Ritzenthaler et al. 2000).

The developing muscle 12 precursor was first observed at stage 12 in both control and *dgrip* mutant embryos. Throughout further development, muscles then expand by the integration of naïve myoblasts (Rushton et al. 1995). In control embryos of late stage 13, the growing muscle is still found in the posterior portion of the hemisegments, extending a single cellular extension invariably toward anterior (Fig. 5A–C). Already at stage 13, VLMs are apparently defective in the absence of DGrip (Fig. 5D–F). Specifically in *dgrip* mutants, we observed cellular extensions that were pointing in "wrong directions" (Fig. 5F). Moreover, muscle 12 precursor cells in *dgrip* mutant embryos often appeared bipolar, forming extensions in both anterior and posterior direction (Fig. 5D,E). In wild type, extensions extend further in stage 14 until they finally contact the epidermal attachment sites at the anterior border of the segment at late stage 14/early stage 15 (Fig. 5G,H). In *dgrip* mutants of this stage (Fig. 5I,J), cell extensions often appear collapsed, or if developed, they miss their proper target sites at the segment borders (Fig. 5J). This observation is consistent with the fact that muscles finally are often unable to form proper contact with its normal attachment site. Our results thus explain the appearance of the misattached rounded muscles that are observed in the *dgrip* mutants from stage 16 onward (cf. Fig. 5M,N, and Figs. 2C, 3B). As mentioned before, in *dgrip* mutant embryos the patterning of VLM 12 is somewhat less severely affected than that of VLMs 6/7. There was no muscle 6/7–specific driver available to explore the possibility of very likely even more penetrant guidance defects of this muscle pair

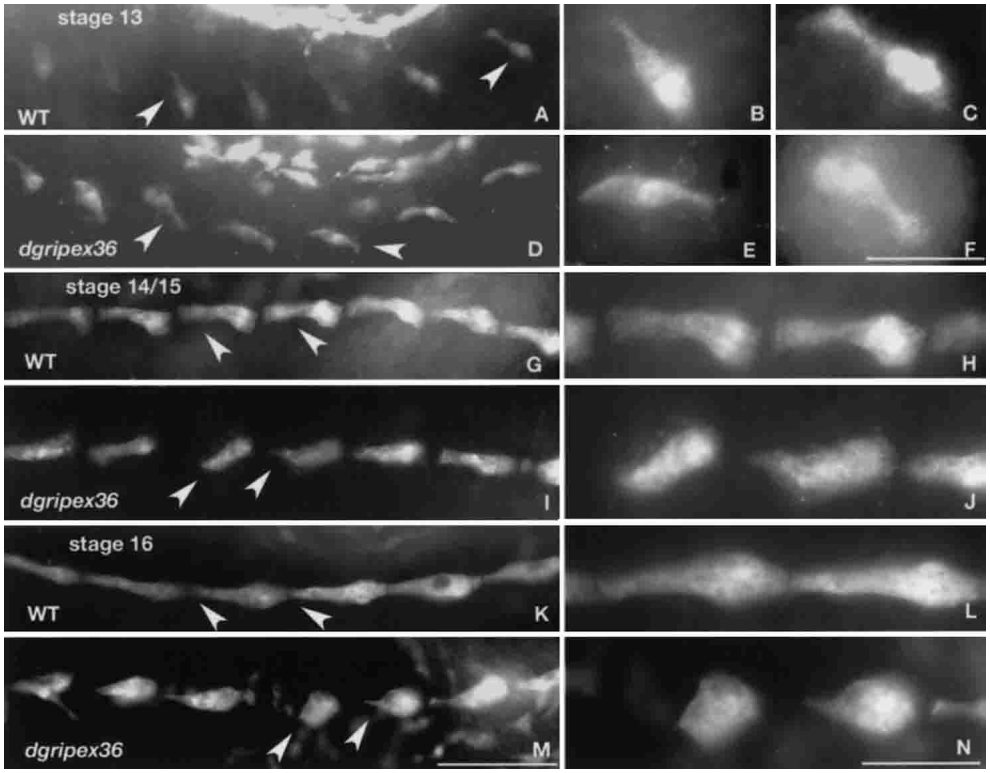

Figure 5. Defective guidance in ventral longitudinal muscles (VLMs) of *dgrip* mutant embryos. Segment border guidance of muscle 12, which is visualized in *Drosophila* embryogenesis by using *5053-gal4* to express β-Gal specifically in this somatic muscle. Out-of-focus staining derives from expression within visceral muscles. Shown are pictures from embryos in stage 13 (*A–F*), late stage 14/early 15 (*G–J*), and stage 16 (*K–N*); *right* panels show magnifications of individual muscles. In control embryos, developing muscles extend projections anteriorly starting in stage 13 (*A*, *arrowheads*, *B*, *C*) which broaden and establish first proper contact to the anterior segment border in stage14/15 (*G*, *arrowheads*, *H*). In stage 16 (*K*, *L*), VLM 12 is fully attached. In *dgrip* mutant embryos, from stage 13 extensions are unusual in shape and often project in wrong directions (*D–F*, *arrowheads*). Consequently, many *dgrip* mutant muscles fail to establish proper contact (*I*, *J*; *M*, *N*). Bars, 20 μm (*F*), 60 μm (*M*), and 25 μm (*N*).

in the *dgrip* mutant. We conclude that the cellular behavior of *dgrip* mutant muscles becomes aberrant significantly before the developmental time point at which segment border attachment normally is established. In *dgrip* mutants, directionality of the cellular extensions normally mediating guidance seems essentially randomized. We conclude this as cells extending in anterior or posterior direction as well as "bipolar" cells are observed in similar quantities. Consistently, *dgrip* mutant VLMs are found attached to either the anterior or posterior segment border only (Figs. 2C, 3B; for an example of an anterior attachment, see Fig. 4F). We also observed that VLMs in *dgrip* mutants often stretch over segment borders (data not shown), indicating that the muscles have missed their attachment sites at the segment borders. These results are consistent with our interpretation that prior to any attachment, during stage 13, *dgrip* mutant muscles fail to respond to attractive cues by directing the outgrowth of cellular extensions (Fig. 5D). Our results thus suggest a direct role of DGrip to mediate the response to an essential attractive signal within the developing muscles. This attractive signal seems to emanate from the segment border in order to direct and/or stabilize cellular extensions of developing muscles.

DGrip accumulates in discrete compartments at muscle ends

To correlate the embryonic expression with the mutant phenotype, we stained embryos for DGrip protein expression by using our anti-DGrip antibody. In immunostainings of both $dgrip^{ex36}$/Y and $dgrip^{ex122}$/Y embryos, no signal was observed (data not shown), proving the specificity of our DGrip antibody in embryo stainings.

We first looked at stage-16 embryos, in which muscles are fully attached. Here, DGrip protein is localized to both anterior and posterior edge of the VLMs, where the muscles are in contact with their attachment sites (Fig. 6A, inset, and Fig. 6B, arrowhead; for a costaining with muscle myosin, see Fig. 6F). Weaker expression of DGrip is also detected in the contact regions of more dorsal muscles attaching to the segment border (Fig. 6A, arrows), whereas no DGrip expression could be detected at the contact sites of LTMs and other directly attaching muscles. This agrees with the mRNA distribution of *dgrip*, which also shows strong staining in the VLM region (Fig. 1E,F, arrowheads). A GFP-tagged variant of DGrip expressed using *24B-gal4* rescued the *dgrip* phenotype (data not shown) and was found to accumulate at muscle edges of VLMs as well (Fig. 6G, arrowhead). We also analyzed the temporal profile of DGrip expression in embryonic muscles. Consistent with the distribution of the *dgrip* transcript, DGrip protein is absent from early embryos and is first detected in developing mesoderm from stage 13 on (data not shown). Obviously already in early stage 14, DGrip starts accumulating at both anterior and posterior muscle end (Fig. 6C). The protein then progressively concentrates (Fig. 6C–E) to become very sharply localized there in stage 16 (Fig. 6A,B,E). To examine DGrip expression early in an identified VLM, we again used *5053-gal4* to stain muscle 12 (muscle myosin is not yet expressed in these early stages). Even before proper attachment of muscle 12 at the segment border is established, staining at both the posterior and anterior end of muscle 12 is observed (Fig. 6H,J,L, see arrowheads in J). As expected, DGrip is sharply localized in stage 16 (Fig. 6I,K,M, see arrowheads in K,M). It should be noted that DGrip staining is certainly not restricted to the labeled VLM.

DGrip staining in embryonic muscle appears as discrete punctae, suggesting that the protein accumulates in distinct intracellular compartments (Fig. 6D,E, magnifications). To learn about its subcellular distribution, we expressed DGrip in COS-7 cells. Here, DGrip was also found expressed in discrete punctae (Fig. 6N–P), which in terms of size and distribution appeared very similar to DGrip punctae of embryonic muscles. Colocalization experiments using established markers for intracellular compartments showed a substantial overlap with markers labeling the endocytic compartment (Fig. 6N). In contrast, no overlap with markers of endoplasmic reticulum (Fig. 6O), Golgi (Fig. 6P), cell membrane, or other organelles as lysosomes or mitochondria (data not shown) was observed.

The presented data show that DGrip is expressed in those muscles, which are affected by the absence of the gene product. The site of DGrip localization is in agreement with the argument that the protein participates in the process of muscle guidance, possibly executing its function in an endosomal compartment. Consistent with DGrip having a transient function needed for embryonic muscle patterning, DGrip expression at muscle ends vanishes in postembryonic development (data not shown).

Overexpression of DGrip: ectopic cellular extensions on directly attaching muscles

Our study so far shows that DGrip is essential to mediate a motility response in the VLMs toward the anterior segment border. This is based on the finding that in the absence of the DGrip, the cellular extensions of developing VLMs no longer form properly. In contrast, the motility of LTMs is undisturbed in the absence of DGrip, consistent with the observation that these muscles do not seem to normally express DGrip. We thus asked whether an ectopic activity of DGrip would influence muscle motility. To achieve *dgrip* expression in all muscle cells, we expressed the gene in response to either *24B-gal4* or *twist-gal4*, both driving expression in all myogenic cells of *Drosophila* embryos, together with two copies of *UAS-dgrip*. DGrip overexpression in muscles was confirmed by immunofluorescence stainings using the anti-DGrip antibody. In embryos overexpressing the *dgrip* gene by using either *24B-gal4* (Fig. 7A–E) or *twist-gal4* (data not shown), muscle morphology was only slightly affected in VLMs (Fig. 7B, arrowhead) and other indirectly attaching muscles. Similarly, direct overexpression of DGrip in VLM 12 using *5053-gal4* was without phenotypic consequence (data not

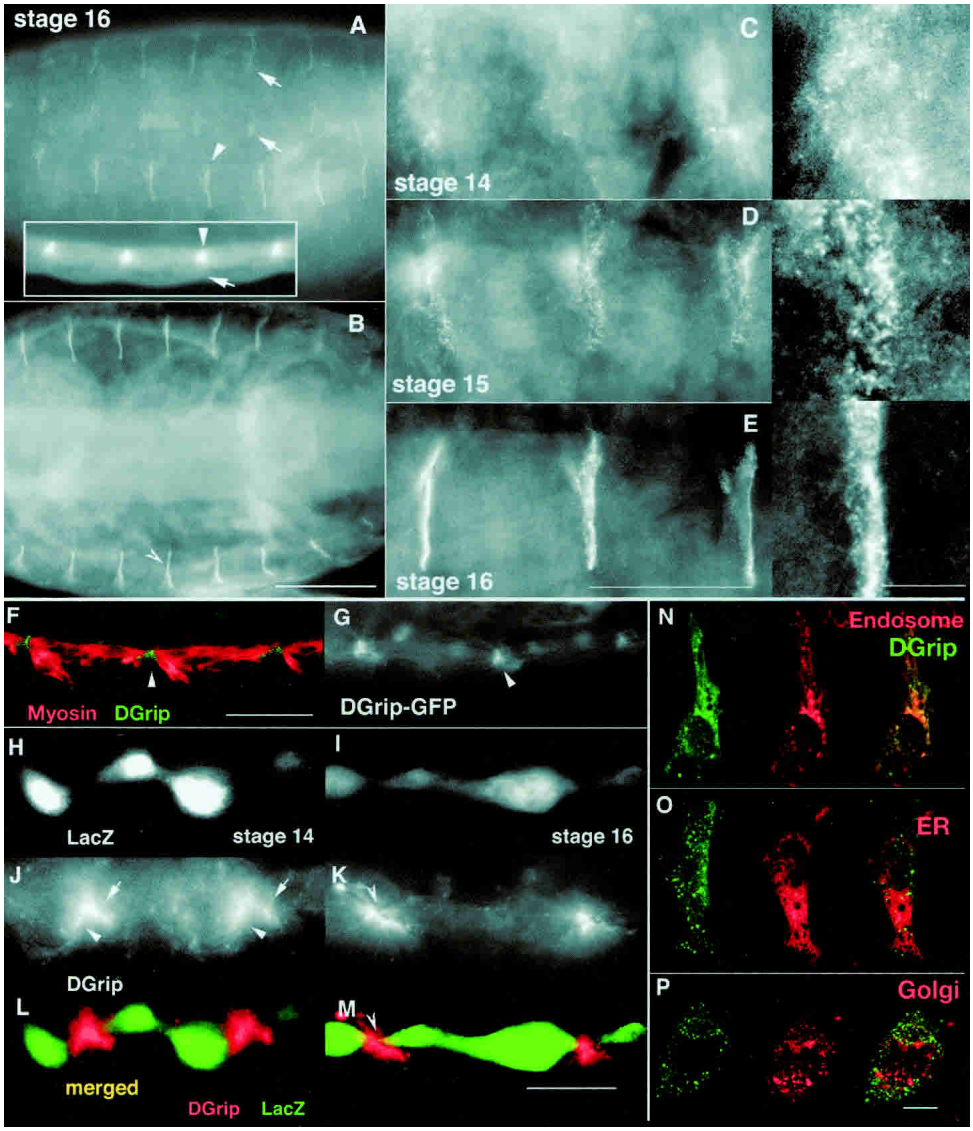

Figure 6. Expression of DGrip throughout muscle development. (*A–M*) Immunostainings of *Drosophila* embryos using an affinity-purified antibody against PDZ domains 6 and 7 of DGrip (see Materials and Methods). (*A*, *B*) Stage 16 wild-type embryos in lateral (*A*) and ventral (*B*) perspective. DGrip is strongly expressed at contact sites of ventral longitudinal muscles (VLMs; *A*, *B*, *arrowheads*) and is weaker at segmental attachments sites of dorsal muscles attaching at the segment border (*A*, *arrows*). DGrip is not observed in muscles making contacts away from the segment border. (*A*, *inset*) Vertical perspective on VLMs shows that DGrip expresses specifically in both anterior and posterior end of VLM muscles (*arrowhead*) but not in the epidermis (*arrow*). (*B*) Bar, 80 µm. (*C–E*) Higher magnification of DGrip expression in the VLM region of stage 14 (*C*), 15 (*D*), and 16 (*E*). (*Insets*) Further magnification. DGrip progressively accumulates at the contact sites of the muscles. (*E*) Bar, 30 µm; (*inset*) 3 µm. (*F*) Costaining of DGrip with muscle myosin in stage 16. DGrip expression is confined to segment border attachment sites (*arrowhead*); bar, 20 µm. (*G*) GFP-tagged DGrip expressed in mesoderm using *24B-gal4* is enriched at both anterior and posterior ends of muscle (*arrowhead*). (*H–M*) Costaining between endogenous DGrip (*J*, *K*) and β-Gal (*H*, *I*) specifically expressed in muscle 12 using *5053-gal4* together with *UAS-lacZ*. In stage 14, DGrip concentrates in both anterior (*H*, *J*, *L*, *arrowheads* in *J*) and posterior ends (*H*, *J*, *L*, *arrows* in *J*) of extending muscle 12, which has not yet established contact with the anterior segment border. In stage 16 (*I*, *K*, *M*), muscle 12 is firmly attached and DGrip expression is strongly concentrated at the segment border. (*M*) Bar, 30 µm. (*N–P*) COS 7 cell cotransfected with DGrip and compartment markers. DGrip localizes to intracellular punctae, which in size and distribution are similar to the DGrip punctae observed in embryonic muscles (*D*, *inset*). DGrip in COS cells overlaps with endosomal markers (*N*). No colocalization to ER (*O*) or Golgi (*P*) was observed.

shown). In contrast, LTMs (Fig.7A,C–E) were very sensitive with respect to DGrip overexpression. Such LTMs adopted an irregular morphology (Fig. 7A; for controls, see Fig. 3A), bent to attach at the segment border (Fig. 7A, arrowheads), or produced thin cellular extensions, which connected them to the segment borders (Fig. 7C,D). In controls, such cellular extensions formed by LTMs or bending of whole LTMs toward segment bor-

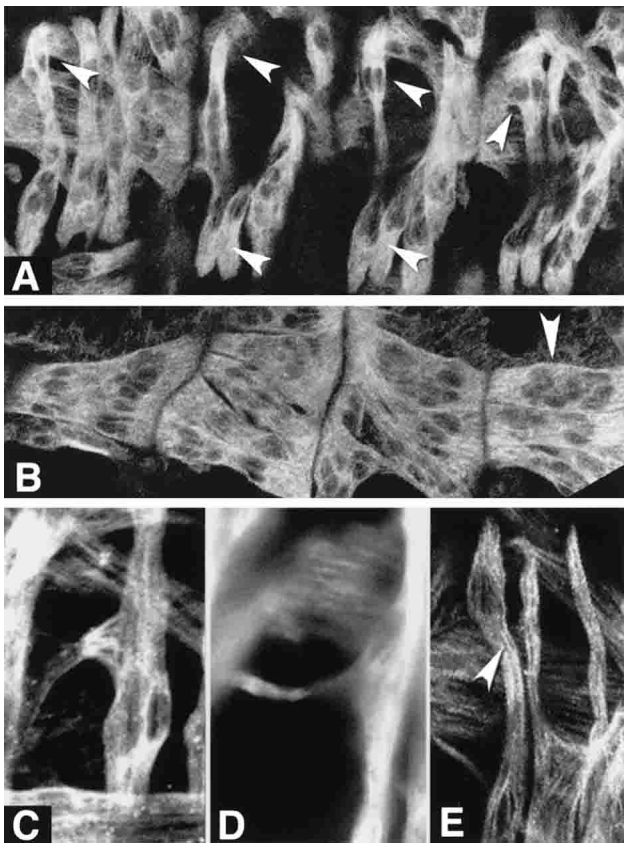

Figure 7. DGrip overexpression provokes segment border attachment of lateral transverse muscles (LTMs). All images show anti-muscle myosin stainings at stage 17 of DGrip overexpressing embryos (*24B-gal4::2xUAS-dgrip*). (*A*, *B*) Confocal projections of hemisegments A1 to A4 from one individual embryo showing the LTM (*A*) or VLM (*B*) region. Although LTMs are normally oriented upright in the segments and appear bar-like (for a control embryo, see Fig. 3A), after DGrip overexpression LTMs often fully bend and attach at segment borders (*A*, *arrowheads*). Alternatively, these muscles form thin projections (*C–E*, *arrowheads*), which attach to the segment borders. In VLMs overexpressing DGrip, neither ectopic projections nor defective choice of attachment sites were observed (*B*). (*A*) Bar, 30 μm.

ders were never observed, neither was formation of ectopic cellular extensions from directly attaching muscles overexpressing DGrip. Thus, ectopic expression of DGrip can efficiently provoke LTMs to attach to other muscles at the segment border. Muscles that display both direct and indirect attachment modes, such as the ventral oblique and acute muscles, display misrouted processes at the directly attaching end of the muscle, whereas the indirectly attaching ends of these muscles are essentially unaffected as for VLMs. Conversely, in the *dgrip* mutant we specifically find the indirectly attaching ends of these muscles to be affected (data not shown). No sign of ectopic tendon cell differentiation was observed in embryos overexpressing *dgrip* in developing muscles, indicating that misattached muscles were misguided toward preexisting segment border tendon cells (data not shown).

We conclude that DGrip is a key player in organizing different patterns of muscle attachment between different groups of muscles. The factor is both necessary and sufficient to promote the formation of muscle cell extensions, which we implicate in sensing and reacting toward a guidance signal expressed at the segment border.

Discussion

In this study we provide evidence that the only member of the GRIP family in *Drosophila* (DGrip) organizes cellular motility in order to allow proper attachment site guidance in embryogenesis. This is intriguing, as proteins of the GRIP family so far have mainly been described in the context of synapse formation and plasticity, and are considered to be involved in the clustering and/or transporting of synaptic proteins, most importantly glutamate receptors.

Drosophila *Grip is both necessary and sufficient to direct developing muscles toward segment borders*

The DGrip transcript and protein are strongly expressed within a specific subset of embryonic muscles of *Drosophila*, the VLMs. VLM founder cells are born in the posterior part of each segment, and they extend growth-cone–like structures in the anterior direction to target to specific attachment sites corresponding to the segment borders. At these sites, the VLMs form stable connections in a tripartite complex with apodemes and other VLMs (Fig.5; Bate 1990; Bunch et al. 1998; Prokop et al. 1998; Martin-Bermudo and Brown 2000). In the absence of DGrip activity, cellular extensions of the VLMs are abnormal "from the beginning." Consequently, VLMs fail to attach at the segment borders, but instead form pure muscle–muscle contacts irrespective of their position within the segment. Often the muscles fail to recognize the segment borders. All aspects of the *dgrip* mutant phenotype were fully rescued in response to DGrip expression from a *dgrip* cDNA-containing transgene in the developing VLMs of *dgrip* mutant embryos. This result unambiguously establishes that the lack of *dgrip* activity within the growing muscle is directly responsible for the defects observed.

Elimination of GRIP1 in mice results in embryonic lethality (Bladt et al. 2002) associated with defective dermoepidermal junctions. These results were interpreted to indicate that the architecture of this contact requires PDZ domain interactions mediated through GRIP1, in order to maintain proper cell adhesion. The contact between epidermis and specific muscles is not properly formed in *Drosophila* embryos mutant for *dgrip*. This observation on first sight might hint toward a defect in the stabilization of cell adhesion in *Drosophila dgrip* mutants as well. However, escaping adult *Drosophila* from *dgrip* null alleles showed no signs of adhesion loss (Fig. 4J). Moreover, defects in *dgrip* mutants are limited to one muscle group in a way that argues against cell

adhesion defects and favors a role of *dgrip* in muscle guidance, in which the pathways identified to date are found to act in a muscle subgroup–specific manner (Callahan et al. 1996; Kramer et al. 2001). Consistently, the mechanical attachment of muscles in *dgrip* mutants is unaffected, because the mutant VLMs form integrin-expressing attachment sites. Furthermore, the attachment was stable upon contraction, as the *dgrip* mutant larvae were able to locomote robustly.

Our findings exclude the possibility that the phenotypes of *dgrip* mutants are due to an effect on cell adhesion properties in the process of stabilizing the muscle attachment sites versus upcoming muscular contraction force. Instead, we provide direct evidence that the motility of VLMs is specifically affected in the absence of DGrip, by visualizing the morphological development of VLMs during guidance (Fig.5). Wild-type VLMs form growth-cone–like extensions invariantly projecting in the anterior direction (Fig. 5A–C). However, in *dgrip* mutant muscles, the direction of cellular extensions appears randomized from the beginning, and often extensions appear collapsed (Fig. 5D–F). Furthermore, upon overexpression of DGrip, ectopic cellular extensions form specifically from LTMs, which normally do not express the protein. These aberrant extensions frequently contacted and anchored at the segment borders (Fig. 7), where obviously they became stabilized as still they are detected in late larval muscles (data not shown).

Does DGrip organize a novel signaling pathway controlling muscle motility?

Our data imply that DGrip mediates a motility response within developing muscles toward an attractive signal expressed at the segment border. It has been reported that Robo receptors are required to extend toward Slit-expressing muscle attachment sites at segment borders (Kramer et al. 2001). Loss of Robo-Slit function eliminates segment border attachment in VLMs, whereas overexpression of Robos leads to segment border attachment in LTMs. Moreover, we see that Robo receptors are expressed at the edges of developing muscles in a spatiotemporal pattern very similar to the expression profile of DGrip (data not shown). Because of these obvious parallels between Robo/Slit and DGrip, we extensively addressed a potential interaction of these factors by genetic and biochemical means. No evidence for a functional or physical interaction could be obtained. It therefore appears most likely that DGrip organizes the response to a novel signal working in parallel to the Robo/Slit-system. The finding that DGrip overexpression provokes changes in LTMs, whereas Robos are reported to be absent from these muscles (Kramer et al. 2001), also argues in this direction. In principle, DGrip could be involved in the execution of a signaling event, or alternatively, it might be important for the stabilization of first interactions pioneered, for example, by Robo/Slit signaling. Because *dgrip* mutant muscles show defective extensions early during muscle guidance and, secondly, overexpression of DGrip directly causes the formation of cellular extensions, we favor the first alternative.

VLM-type muscles by far show the strongest defects within *dgrip* mutants, affecting ~100% of VLMs 6 and 7. However, other indirectly attaching muscles did show defects as well. Although the defects were weaker in these cells than in VLMs, they clearly were significant in comparison to control animals. Consistently, although DGrip expression seems strongest at VLM attachment sites, the contacts of more dorsal muscles, which also attach indirectly, also express the protein. A similar situation, characterized by VLMs being most affected and expressing the most DGrip between the indirectly attaching muscles, is reported for the Robo/Slit muscle guidance pathway (Kramer et al. 2001). It might be that spatiotemporal specificities in the development of the VLMs make this particular muscle group especially dependent on robust guidance signaling between the indirectly attaching muscles. *Drosophila* muscle guidance has not so far been subject to saturating genetic analysis and besides few seminal studies (Volk and VijayRaghavan 1994; Frommer et al. 1996; Becker et al. 1997; Vorbrüggen and Jäckle 1997; Kramer et al. 2001), our understanding of the process is still rather poor. In several other models of cellular motility, for example, growth cone migration, distinct pathways partially working in parallel have also been identified (for review, see Huber et al. 2003).

Mechanistic analysis of GRIP family proteins in Drosophila

Even in the complete absence of myoblast fusion, muscle founder cells still form properly attached mini-muscles (Rushton et al. 1995). Hereby, the initial polarization of these specific muscle precursors seemingly does not depend on tendon cells. However, the tendon cells provide essential guidance cues that direct muscle extension (Bate 1990; Frommer et al. 1996). It is essentially unknown, how cellular polarity is organized throughout the time course of guidance and subsequent muscle attachment. Most likely the polarized transport of relevant proteins toward the "active muscle ends" is important already early within muscle guidance (Yarnitzky et al. 1997). In fact, developing muscles display a polarized microtubule network with the + ends facing the attachment sites (Clark et al. 1997).

DGrip appears concentrated at ends of muscle cells (Fig. 6H,J,L) before any proper attachment between the muscle and its prospective attachment site is established. As an intracellular adaptor molecule, DGrip might organize signaling processes, for example, by clustering transmembrane receptors, or it might act downstream of the actual signaling processes, for example, by executing transporting events that are essential for directed muscle cell motility. In fact, the correct targeting of the EGF receptor ligand Vein to the site of muscle tendon attachment has been shown to be an essential step in organizing proper muscle pattern (Strumpf and Volk 1998). Our data suggest that after supplying DGrip

to muscles that normally do not express the protein, they start to a sense distant guidance cue, which in turn causes the formation of cellular extensions. DGrip thus might switch on dormant receptors in muscles, for example, by mediating their transport to relevant cellular locations. Interestingly, DGrip has been suggested to control the transport of transmembrane receptors and signaling molecules, such as glutamate receptors and ephrins, from intracellular compartments to the cell surface (Torres et al. 1998; Wyszynski et al. 1998; Brückner et al. 1999; Dong et al. 1999b; Braithwaite et al. 2002; Hirbec et al. 2003). In *Drosophila* muscles we find DGrip localizing to discrete punctae similar to punctae formed by DGrip in culture cells. Colocalization experiments in culture cells showed that DGrip punctae often colocalized with endosomal markers, whereas no colocalizations with ER, Golgi, plasma membrane, lysosomal, or mitochondrial markers were observed. We thus favor the hypothesis that DGrip mediates signaling throughout muscle motility by regulating the endosomal trafficking of receptor complexes. Specialized proteins regulating signaling by endosomal trafficking have recently emerged key players in animal development (for review, see Piddini and Vincent 2003). Regulation of membrane protein composition by GRIPs might be subtle, as different receptor populations such as AMPA/Kainate receptors have been suggested to be regulated by GRIP in opposing manners (Hirbec et al. 2003). Palmitoylation close to the N-terminal end has been described for the Grip family members GRIP1b and pABP-L, and is suggested to control their intracellular distribution (DeSouza et al. 2002; Yamazaki et al. 2001). Indeed, the absolute N terminus of DGrip contains a conserved cysteine residue at position 13 and is similar to the N-terminal sequences demonstrated to mediate palmitoylation of GRIP1b and pABP-L. Our first experimental data in fact suggest post-translational modification of DGrip with palmitate.

The highly penetrant embryonic phenotype of DGrip presented in this study should thus be especially well suited to further study mechanisms of GRIP function in the genetically well-tractable *Drosophila* model.

Materials and methods

Genetics

For *dgrip* mutagenesis, P(KG)02862 (Roseman et al. 1995) or P(GT1)BG01736 (Kimmerly et al. 1996) were crossed to Δ2-3-Transposase for P-element mobilization. Deficiencies $dgrip^{ex36}$ and $dgrip^{ex122}$ were identified and mapped by using genomic PCR from hemizygous mutant larvae (detailed information on demand). To identify *dgrip* mutant embryos, the corresponding alleles were balanced over *FM7-ftz-lacZ* (Heitzler 1997) and negatively identified in β-Gal and Sex-lethal (Bopp et al. 1991) costainings. For larvae, we balanced over *FM7-Act-GFP* and sorted male, non-GFP larvae under a fluorescence binocular (MZFLIII, Leica). For visualization of muscle 12 in *dgrip* mutants, recombinant $dgrip^{ex36}$, *UAS-lacZ* was balanced over *FM7-ftz-lacZ* and virgins crossed to *5053-gal4* males (Ritzenthaler et al. 2000). In rescue experiments, $dgrip^{ex36}$ or $dgrip^{ex122}$ was either recombined with *twist-gal4* (Yin and Frasch 1998) or combined with *24B-gal4* (Brand and Perrimon 1993) and virgins crossed with homozygous *UAS-dgrip* males. A more weakly expressing *UAS-dgrip* line was chosen, and rescued animals were identified as above. For DGrip overexpression, a recombination of two strongly expressing *UAS-dgrip* lines (overexpression scored by anti-DGrip immunostainings; data not shown) was crossed to *24B-gal4* or *twist-gal4*.

Molecular biology

The following EST clones encoding *dgrip* have been isolated in the Berkeley *Drosophila* Genome Project: RE14068, RE32265, RE70628, and RE44067. All contain identical 5′ and 3′sequence. RE14068 is fully sequenced (NCBI access no. AAL68270) and was used for conceptual translation of DGrip. For pUAST-*dgrip*, the DGrip ORF was amplified from EST RE14068 (BDGP, Research Genetics) by using primers 5′-ATACAAGATCTCAA GATGAAACTGTGGAAATCG-3′ and 5′-AGTACTCGAGGC TCGGTAAAGAATACAGGA-3′ and cloned BglII/XhoI into the pUAST trangenesis vector (Brand and Perrimon 1993). To express C-terminally GFP-tagged DGrip, we amplified using 5′-ATACAAGATCTCAAGATGA AACTGTGGAAATCG-3′ and 5′-CATCTCGAGAGAGCGCTGCATGATCATCTCG-3′ omitting the DGrip stop codon, subcloned BglII/XhoI into pEGFP-N1 (Clontech), and then cloned BglII/NotI into pUAST. All constructs were confirmed by double-strand sequencing and transgenic flies produced by using standard procedures.

Antibody production, affinity purification, and immunodetection

For immunogen purification, a fragment encoding PDZ domains 6 and 7 of DGrip was amplified by using primers 5′-GC GCCTCGAGCATGGGTGCTCCCACAAGCACAG-3′ and 5′-GACTCTAGACGGGCAAAAGCATCACTCAG-3′, subcloned into pDNR-1 (Clontech) XhoI/XbaI, and then cloned XhoI/NotI into pGex4T-3 (Pharmacia). The GST-fusion was expressed in BL21 cells and purified on GSH-agarose (Pharmacia), following the instructions of the manufacturer. Purified protein was injected into rabbits (BioGenes). For affinity-purification of sera, a XhoI/XbaI fragment from the pDNR-1-PDZ6-7 construct above was blunted at the 3′ end with Klenow enzyme and ligated into the $(His)_6$-tag vector pQE-32 (Qiagen), cut SalI/HindIII where the HindIII was also treated with Klenow. Recombinant protein was expressed in XL1-blue cells and purified on Talon resin (Clontech). One milligram of the $(His)_6$-fusion was immobilized on a column and used for affinity-purification. Specificity of sera was tested by immunostaining and Western blotting on *dgrip* mutant embryos.

For immunoblotting, 5- to 15-h-old *Drosophila* embryos were dechorionated and homogenized in lysis buffer (50 mM HEPES, 60 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% Triton X-100, 0.2% Nonidet P-40, 10% glycerol) with protease inhibitor "complete mini" (Roche) added. After centrifugation, supernatant ("supernatant", Fig. 2B) was harvested, and the pellet ("pellet" Fig. 2B) was extracted with a more stringent lysis buffer (see above but 2% Triton X-100, 2% Nonidet P-40). Samples were run on an 8% PAA gel, transferred to nitrocellulose membrane (BioRad), and probed with the anti-DGrip antibody (1 : 500) followed by ECL-detection (Amersham).

Recombinant expression and immunohistochemistry in cell culture

For baculovirus expression, the DGrip ORF was amplified as for pUAST-*dgrip*, cloned into pFastBac1 (Invitrogen), and trans-

formed into DH5αBAC cells. A positive baculovirus clone was selected with PCR and transfected into Sf.9 cells by using the BAC-to-BAC expression system (Invitrogen). For expression in mammalian cells, *dgrip* was cloned BglII/XhoI from pUAST-*dgrip* and inserted into pEGFP-N1 (Clontech) to produce p*dgrip*-EGFP. COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1% penicillin/streptomycin on coverslips. For the colocalization studies, cells were transiently transfected by using Lipofectamine2000 (Invitrogen). The following plasmids were used for transfection in combination with p*dgrip*-EGFP: pEYFP-Actin, pEYFP-Endo, pEYFP-Mito, and pEYFP-ER (all Clontech). For antibody stainings, cells were fixed 24 h after transfection with 4% PFA for 5 min, incubated in –20°C methanol for 10 min, permeabilized by treatment with high-salt PBS (20 mM NaPi at pH 7.4; 500 mM NaCl) containing 0.3% Triton X100 followed by an incubation with 10% normal goat serum, and then incubated for 1 h with primary antibody. Antibodies used were as follows: anti-MPR300 antibody for Golgi-labeling (Hybridoma Bank, Hopkins University, Iowa) and mouse anti-lamp1 antibody (Stefan Höning, Zentrum für Biochemie und Molekulare Zellbiologie, Göttingen) for lysosome-labeling. Cy3 and Cy5 (Molecular Probes) were used as secondary antibodies. Cells were monitored under a confocal laser-scan microscope LSM510 with META modul (Zeiss). Live imaging was performed in Tyrode solution (150 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$ at pH 7.4).

Stainings

Immunocytochemistry on embryos and larvae was essentially performed as described (Lin and Goodman 1994). Antibodies were usually preadsorbed to 0 to 4 h *Drosophila* embryos. Primary antibodies were as follows: FMM5A muscle myosin monoclonal (Christoph Schuster, 1 : 100), mouse anti-βPS-integrin (Nigg Brown, 1 : 100), rat anti-Delilah and guinea pig anti-Stripe (Becker et al. 1997; 1 : 500), monoclonal anti-Sex Lethal (DSHB, 1 : 500), rabbit anti-β-Gal (Cappel, 1 : 500), and monoclonal anti-β-Gal (Promega, 1 : 500). Secondary antibodies were Cy3- and FITC- conjugated goat anti-mouse and goat anti-rabbit FAB (Dianova), used at 1 : 200.

Phalloidin-TRITC (Sigma) was used to visualize F-actin. Previously immunostained or freshly fixed larvae were incubated for 30 min in PBS/0.5%Triton-TX-100 and 5% normal goat serum, protected from the light. Larvae were then rinsed and mounted as normal. Confocal images were taken on a Leica TCS NT system with a 63-fold objective (1.3 NA), 4× frame averaging, and a ΔZ of 600 nm, and image stacks were projected in maximal intensity mode.

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References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., et al. 2000. The genome sequence of *Drosophila melanogaster*. *Science* **287:** 2185–2195.

Bate, M. 1990. The embryonic development of larval muscles in *Drosophila*. *Development* **110:** 791–804.

Bate, M. and Rushton, E. 1993. Myogenesis and muscle patterning in *Drosophila*. *C. R. Acad. Sci. III* **316:** 1047–1061.

Baylies, M.K., Bate, M., and Ruiz Gomez, M. 1998. Myogenesis: A view from *Drosophila*. *Cell* **93:** 921–927.

Becker, S., Pasca, G., Strumpf, D., Min, L., and Volk, T. 1997. Reciprocal signaling between *Drosophila* epidermal muscle attachment cells and their corresponding muscles. *Development* **124:** 2615–2622.

Bilder, D. 2001. PDZ proteins and polarity: Functions from the fly. *Trends Genet.* **17:** 511–519.

Bladt, F., Tafuri, A., Gelkop, S., Langille, L., and Pawson, T. 2002. Epidermolysis bullosa and embryonic lethality in mice lacking the multi-PDZ domain protein GRIP1. *Proc. Natl. Acad. Sci.* **99:** 6816–6821.

Bökel, C. and Brown, N.H. 2002. Integrins in development: Moving on, responding to, and sticking to the extracellular matrix. *Dev. Cell* **3:** 311–321.

Bopp, D., Bell, L.R., Cline, T.W., and Schedl, P. 1991. Developmental distribution of female-specific sex lethal proteins in *Drosophila melanogaster*. *Genes & Dev.* **5:** 403–415.

Braithwaite, S.P., Xia, H., and Malenka, R.C. 2002. Differential roles for NSF and GRIP/ABP in AMPA receptor cycling. *Proc. Natl. Acad. Sci.* **99:** 7096–7101.

Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118:** 401–415.

Brown, N.H. 1994. Null mutations in the α PS2 and β PS integrin subunit genes have distinct phenotypes. *Development* **120:** 1221–1231.

———. 2000. Cell-cell adhesion via the ECM: Integrin genetics in fly and worm. *Matrix Biol.* **19:** 191–201.

Brückner, K., Pablo Labrador, J., Scheiffele, P., Herb, A., Seeburg, P.H., and Klein, R. 1999. EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains. *Neuron* **22:** 511–524.

Bunch, T., Graner, M., Fessler, L., Fessler, J., Schneider, K., Kerschen, A., Choy, L., Burgess, B., and Brower, D. 1998. The PS2 integrin ligand tiggrin is required for proper muscle function in *Drosophila*. *Development* **125:** 1679–1689.

Callahan, C.A., Bonkovsky, J.L., Scully, A.L., and Thomas, J.B. 1996. derailed is required for muscle attachment site selection in *Drosophila*. *Development* **122:** 2761–2767.

Clark, I.E., Jan, L.Y., and Jan, Y.N. 1997. Reciprocal localization of Nod and kinesin fusion proteins indicates microtubule polarity in the *Drosophila* oocyte, epithelium, neuron and muscle. *Development* **124:** 461–470.

Contractor, A., Rogers, C., Maron, C., Henkemeyer, M., Swanson, G.T., and Heinemann, S. 2002. Trans-synaptic Eph receptor-ephrin signalling in hippocampal mossy-fibre LTP. *Science* **296:** 1864–1869.

DeSouza, S., Fu, J., States, B.A., and Ziff, E.B. 2002. Differential palmitoylation directs the AMPA receptor-binding protein ABP to spines or to intracellular clusters. *J. Neurosci.* **22:** 3493–3503.

Dickson, B.J. 2002. Molecular mechanisms of axon guidance. *Science* **298:** 1959–1964.

Dong, H., O'Brien, R.J., Fung, E.T., Lanahan, A.A., Worley, P.F.,

and Huganir, R.L. 1997. GRIP: A synaptic PDZ domain-containing protein that interacts with AMPA receptors. *Nature* **386:** 279–284.

Dong, H., Zhang, P., Liao, D., and Huganir, R.L. 1999a. Characterization, expression, and distribution of GRIP protein. *Ann. N. Y. Acad. Sci.* **868:** 535–540.

Dong, H., Zhang, P., Song, I., Petralia, R.S., Liao, D., and Huganir, R.L. 1999b. Characterization of the glutamate receptor–interacting proteins GRIP1 and GRIP2. *J. Neurosci.* **19:** 6930–6941.

Frommer, G., Vorbrüggen, G., Pasca, G., Jackle, H., and Volk, T. 1996. Epidermal egr-like zinc finger protein of *Drosophila* participates in myotube guidance. *EMBO J.* **15:** 1642–1649.

Heitzler, P. 1997. New FM7 versions from Strasbourg. *DIS* **80:** 103.

Hirbec, H., Francis, J.C., Lauri, S.E., Braithwaite, S.P., Coussen, F., Mulle, C., Dev, K.K., Couthino, V., Meyer, G., Isaac, J.T.R., et al. 2003. Rapid and Differential regulation of AMPA and Kainate receptors at hippocampal mossy fibre synapses by PICK1 and GRIP. *Neuron* **37:** 625–638.

Huber, A.B., Kolodkin, A.L., Ginty, D.D., and Cloutier, J.F. 2003. Signaling at the growth cone: Ligand-receptor complexes and the control of axon growth and guidance. *Ann. Rev. Neurosci.* **26:** 509–563.

Jagla, K., Bellard, M., and Frasch, M. 2001. A cluster of *Drosophila* homeobox genes involved in mesoderm differentiation programs. *Bioessays* **23:** 125–133.

Jesaitis, L.A. and Goodenough, D.A. 1994. Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the *Drosophila* discs-large tumor suppressor protein. *J. Cell Biol.* **24:** 949–961

Kimmerly, W., Stultz, K., Lewis, S., Lewis, K., Lustre, V., Romero, R., Benke, J., Sun, D., Shirley, G., Martin, C., et al. 1996. A P1-based physical map of the *Drosophila* euchromatic genome. *Genome Res.* **6:** 414–430.

Knirr, S., Azpiazu, N., and Frasch, M. 1999. The role of the NK-homeobox gene slouch (S59) in somatic muscle patterning. *Development* **126:** 4525–4535.

Ko, J., Kim, S., Valtschanoff, J.G., Shin, H., Lee, J.-R., Sheng, M., Premont, R.T., Weinberg, R.J., and Kim, E. 2003. Interaction between Liprin-a and GIT1 is required for AMPA receptor targeting. *J. Neurosci.* **23:** 1667–1677.

Kramer, S.G., Kidd, T., Simpson, J.H., and Goodman, C.S. 2001. Switching repulsion to attraction: Changing responses to slit during transition in mesoderm migration. *Science* **292:** 737–740.

Leptin, M., Bogaert, T., Lehmenn, R., and Wilcox, M. 1989. The function of PS integrins during *Drosophila* embryogenesis. *Cell* **56:** 401–408.

Lin, D.M. and Goodman, C.S. 1994. Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* **13:** 507–523.

Lin, D., Gish, G.D., Songyang, Z., and Pawson, T. 1999. The carboxyl terminus of B class ephrins constitutes a PDZ domain binding motif. *J. Biol. Chem.* **274:** 3726–3733.

Littleton, J.T. and Ganetzky, B. 2000. Ion channels and synaptic organization: Analysis of the *Drosophila* genome. *Neuron* **26:** 35–43.

MacKrell, A.J., Blumberg, B., Haynes, S.R., and Fessler, J.H. 1988. The lethal myospheroid gene of *Drosophila* encodes a membrane protein homologous to vertebrate integrin β subunits. *Proc. Natl. Acad. Sci.* **85:** 2633–2637.

Martin-Bermudo, M.D. and Brown, N.H. 2000. The localized assembly of extracellular matrix integrin ligands requires cell–cell contact. *J. Cell Sci.* **113:** 3715–3723.

Osten, P., Khatri, L., Perez, J.L., Köhr, G., Giese, G., Daly, C., Shulz, T.W., Wensky, A., Lee, L.M., and Ziff, E.B. 2000. Mutagenesis reveals a role for ABP/GRIP binding to GluR2 in synaptic surface accumulation of the AMPA receptor. *Neuron* **27:** 315–325.

Piddini, E. and Vincent, J.P. 2003. Modulation of developmental signals by endocytosis: Different means and many ends. *Curr. Opin. Cell. Biol.* **15:** 474–481

Prokop, A., Martin-Bermudo, M.D., Bate, M., and Brown, N.H. 1998. Absence of PS integrins or laminin A affects extracellular adhesion, but not intracellular assembly, of hemiadherens and neuromuscular junctions in *Drosophila* embryos. *Dev. Biol.* **196:** 58–76.

Ritzenthaler, S., Suzuki, E., and Chiba, A. 2000. Postsynaptic filopodia in muscle cells interact with innervating motoneuron axons. *Nat. Neurosci.* **3:** 1012–1017.

Roseman, R.R., Johnson, E.A., Rodesch, C.K., Bjerke, M., Nagoshi, R.N., and Geyer, P.K. 1995. A P element containing suppressor of hairy-wing binding regions has novel properties for mutagenesis in *Drosophila melanogaster*. *Genetics* **141:** 1061–1074.

Rubin, G.M., Hong, L., Brokstein, P., Evans-Holm, M., Frise, E., Stapleton, M., and Harvey, D.A. 2000. A *Drosophila* complementary DNA resource. *Science* **287:** 2222–2224.

Rushton, E., Drysdale, R., Abmayr, S.M., Michelson, A.M., and Bate, M. 1995. Mutations in a novel gene, myoblast city, provide evidence in support of the founder cell hypothesis of *Drosophia* muscle development. *Development* **121:** 1979–1988.

Sheng, M. and Sala, C. 2001. PDZ domains and the organization of supramolecular complexes. *Ann. Rev. Neurosci.* **24:** 1–29.

Shi, S., Hayashi, Y., Esteban, J.A., and Malinow, R. 2001. Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell* **105:** 331–343.

Srivastava, S., Osten, P., Vilim, F.S., Khatri, L., Inman, G., States, B., Daly, C., DeSouza, S., Abagyan, R., Valtschanoff, J.G., et al. 1998. Novel anchorage of GluR2/3 to the postsynaptic density by the AMPA receptor-binding protein ABP. *Neuron* **21:** 581–591.

Strumpf, D. and Volk, T. 1998. Kakapo, a novel cytoskeletal-associated protein is essential for the restricted localization of the neuregulin-like factor, vein, at the muscle-tendon junction site. *J. Cell Biol.* **143:** 1259–1270.

Torres, R., Firestein, B.L., Dong, H., Staudinger, J., Olson, E.N., Huganir, R.L., Bredt, D.S., Gale, N.W., and Yancopoulos, G.D. 1998. PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ligands. *Neuron* **21:** 1453–1463.

Volk, T. 1999. Singling out *Drosophila* tendon cells: A dialogue between two distinct cell types. *Trends Genet.* **15:** 448–453.

Volk, T. and VijayRaghavan, K. 1994. A central role for epidermal segment border cells in the induction of muscle patterning in the *Drosophila* embryo. *Development* **120:** 59–70.

Vorbrüggen, G. and Jäckle, H. 1997. Epidermal muscle attachment site-specific target gene expression and interference with myotube guidance in response to ectopic stripe expression in the developing *Drosophila* epidermis. *Proc. Natl. Acad. Sci.* **94:** 8606–8611.

Willott, E., Balda, M.S., Fanning, A.S., Jameson, B., Van Itallie, C., and Anderson, J.M. 1993. The tight junction protein ZO-1 is homologous to the *Drosophila* discs–large tumor suppressor protein of septate junctions. *Proc. Natl. Acad. Sci.* **90:** 7834–7838.

Wyszynski, M., Kim, E., Yang, F.C., and Sheng, M. 1998. Biochemical and immunocytochemical characterization of GRIP, a putative AMPA receptor anchoring protein, in rat brain. *Neuropharmacology* **37:** 1335–1344.

Wyszynski, M., Valtschanoff, J.G., Naisbitt, S., Dunah, A.W., Kim, E., Standaert, D.G., Weinberg, R., and Sheng, M. 1999. Association of AMPA receptors with a subset of glutamate receptor- interacting protein in vivo. *J. Neurosci.* **19:** 6528–6537.

Wyszynski, M., Kim, E., Dunah, A.W., Passafaro, M., Valtschanoff, J.G., Serra-Pages, C., Streuli, M., Weinberg, R.J., and Sheng, M. 2002. Interaction between GRIP and Liprina/SYD2 is required for AMPA receptor targeting. *Neuron* **34:** 39–52.

Xia, J., Chung, H.J., Wihler, C., Huganir, R.L., and Linden, D.J. 2000. Cerebellar long-term depression requires PKC-regulated interactions between GluR2/3 and PDZ domain–containing proteins. *Neuron* **28:** 499–510.

Yamazaki, M., Fukaya, M., Abe, M., Ikeno, K., Kakizaki, T., Watanabe, M., and Sakimura, K. 2001. Differential palmitoylation of two mouse glutamate receptor interacting protein 1 forms with different N-terminal sequences. *Neurosci. Lett.* **304:** 81–84.

Yarnitzky, T., Min, L., and Volk, T. 1997. The *Drosophila* neuregulin homolog Vein mediates inductive interactions between myotubes and their epidermal attachment cells. *Genes & Dev.* **11:** 2691–2700.

Yin, Z. and Frasch, M. 1998. Regulation and function of tinman during dorsal mesoderm induction and heart specification in *Drosophila*. *Dev. Genet.* **22:** 187–200.

Appendix III:

Curriculum Vitae, Laura Swan

LAURA ELIZABETH SWAN

Curriculum vitae

- DATE AND PLACE OF BIRTH: 4th February 1979, Launceston, Tasmania (Australia)
- EDUCATION:
    - 1996: Secondary- Scotch Oakburn College Launceston, Dux of years 8,9,10,11 and 12. Tasmanian Certificate of Education score 99/100 (A+)
    - 1997-1999: Tertiary- Monash University, Melbourne, Australia. BSc (Sci. Schol.) Accelerated Degree (entry level second year) majoring in Theoretical Physics and Mathematics. Sir John Monash Scholarship.
    - Dec 1998- Jan 1999: Australian National University, Canberra, Australia. Science Summer School (Scholarship): Study on electrical properties of C_{60} carbon spheres (Buckyballs).
    - 1999: Monash University, Melbourne, Australia. First Class Honours in Theoretical Physics. Thesis: Electrical properties of small radius Carbon Nanotubes
    - Jan 2000- Sept 2000: Lab experience with Assoc. Prof. Stephen Robinson, Dept. of Psychology, Monash University, Melbourne: Topic: Variation of iron-binding capacity of female rats with menstrual cycle.
    - Sept 2000 – until present: Masters-PhD programme in Neuroscience, University of Goettingen/Max-Planck Institute for Biophysical Chemistry, Goettingen, Germany.
    - Sept 2001- until present: PhD thesis, Neuroplasticity Department, European Neuroscience Institute. Supervisor, Dr Stephan Sigrist, Co-Supervisors: Prof Nils Brose, Prof Erwin Neher. Topic: Synaptogenesis and morphological outgrowth of the Drosophila Neuromuscular system.

- WORK EXPERIENCE:
    - 1997-2000: College tutor in mathematics and physics (Mannix College, Melbourne, Australia)
    - 1998-2000: University tutor in Physics (Monash University, Melbourne, Australia)
    - 2001-present: German-English translation for textbooks (Physiology Dept, University of Goettingen, Goettingen, Germany)

- POSTERS:
    - 13. Neurobiologischer Doktorandenworkshop, Martinsried 26-29 Jun 2002: **Swan L**, Prange U. and Sigrist, S.: *The Glutamate receptor binding protein homologue organises myotube guidance.*

    - Cold Spring Harbor meeting on Neurobiology of Drosophila: 1-5 Oct 2003: Sigrist, S.J., Wichmann, C., Schmidt, M., Schmid, A. and **Swan, L.**: *The Drosophila Glutamate Receptor Binding Protein homolog organizes myotube guidance*

    - Third International Meeting on Protein and Membrane Transport in the Secretory Pathway, Goettingen 7-9 Oct 2004: Wichmann, C, **Swan, LE**, Kittel, R, Schmidt, M, Wenzel, D, Heckmann, M and Sigrist, SJ: Drosophila Glutamate Receptor Interacting Protein acts in the presynaptic endocytic pathway

    - Sixth Meeting of the German Neuroscience Society, Goettingen 17-20 Feb 2005: **Swan LE** and Sigrist SJ.: *A Structure-function characterization of the Glutamate Receptor-Interacting Protein Grip*

- PUBLICATIONS:
    - **Swan LE**, Wichmann C, Prange U, Schmid A, Schmidt M, Schwarz T, Ponimaskin E, Madeo F, Vorbruggen G, Sigrist SJ. A glutamate receptor-interacting protein homolog organizes muscle guidance in Drosophila. Genes Dev. 2004 Jan 15;18(2):223-37

    - Kole MH,**Swan L**, Fuchs E. The antidepressant tianeptine persistently modulates glutamate receptor currents of the hippocampal CA3 commissural associational synapse in chronically stressed rats. Eur J Neurosci. 2002 Sep;16(5):807-16

    - Bishop GM, **Swan L.E.**, Robinson SR. Altered cellular distribution of iron in rat cerebral cortex during the oestrous cycle.J Neural Transm. 2004 Feb;111(2):159-65.