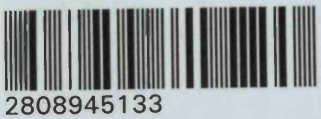

UNIVERSITY OF LONDON THESIS

Degree PhD Year 2006 Name of Author KITSOU Efstathia

Characterisation of the Physical and Functional Interaction of DRhoGEF2 with DMec2 via its PDZ domain

by

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ABSTRACT

Morphogenesis is the process which will define the final form of an organism by a series of complex cellular events such as cell division, shape changes and migration, events that require the coordinated modification of the cytoskeleton. The cytoskeleton is mainly regulated by the Rho family of small GTPases. The Guanine Nucleotide Exchange Factor, DRhoGEF2, is an activator of Rho1 and it is essential for morphogenetic cell shape changes.

Signalling through DRhoGEF2 seems to be restricted to a specific area in the cell. One major question in the field is the mechanism by which the activity of RhoGEFs is spatially and temporally limited. The multidomain nature of DRhoGEF2 provides the framework for a tight regulation and the participation in a protein network.

The activity of the distinct structural elements of DRhoGEF2 has not been completely elucidated. This thesis investigates the role of the PDZ domain for the function of DRhoGEF2. Preliminary results indicate that the PDZ domain acts as a positive regulator. In addition, an interaction has recently been discovered between the DRhoGEF2 PDZ domain and the novel protein DMec2. This thesis explores the functional significance of DMec2 and in particular its putative contribution to morphogenesis through its interaction with DRhoGEF2. Overexpression and elimination of DMec2 does not alter the actin nor microtubule cytoskeleton and ectopic expression does not produce any obvious phenotypes therefore its role remains obscure. Furthermore, DMec2 binds to the PDZ domain however there is no indication of a functional relevance of this interaction. This work suggests further study to explore the integration of signals by the PDZ domain of DRhoGEF2.

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LIST OF ABBREVIATIONS

A-P: anterior-posterior
APC: adenomatosis polipsis coli
BSA: bovine serum albumin
C1: phorbol ester/diacylglycerol-binding
C. elegans: *Caenorhabditis elegans*
CKII: casein kinase II
cta: concertina
Cyo: curly of Oster
da: daughterless
DAG: diacylglycerol
ddH_2O: double distilled water
DH: Dbl homology
dia: diaphanous
Dlg: discs large
Dr: drop
ECM: extracellular matrix
EMS: ethylmethane sulfonate
ey: eyeless
fog: folded gastrulation
FTases: farnesyl-transferases
GAP: guanine nucleotide activation protein
GDI: guanine nucleotide dissociation inhibitor
GEF: guanine nucleotide exchange factor
GFP: green fluorescent protein
GGTases: geranyl-geranyl transferases
hMSCs: human mesenchymal stem cells
IPTG: isopropyl-beta-D-thiogalactopyranoside
LARG: leukaemia associated RhoGEF
Leu: leucine
LPA: lysophosphatidic acid
N/D: Not defined
NP: nonidet P-40
OreR: Oregon R
PAK: p21 activated kinase
PBS: phosphate buffered saline
PBST: phosphate buffered saline tween
PCP: planar cell polarity
PCR: polymerase chain reaction
PDZ: post-synaptic density protein 95, discs large, zonula occludens 1
PFA: paraformaldehyde
PH: Pleckstrin homology
PI(4,5)P_2: phosphatidyl inositol 3 kinase
PIP_2: phosphatidyl inositol diphosphate
PKC: protein kinase C

PKN: protein kinase N
PLC: phospholipase C
PSD 95: post-synaptic density protein 95
Rg2: RhoGEF2
RGS: regulation of G protein subunit
Rho: Ras related protein
ROK: Rho kinase
S.D: Standard deviation
SEM: scanning electron micrograph
SH2: src homology 2
SH3: src homology 3
SRE: serum response element
SRF: serum response factor
TRITC: tetramethylrhodamine
TBS: Tris buffered saline
TCR: T-cell receptor
UAS: upstream activating sequence
WASP: Wiskott Aldrich Syndrome Protein
WAVE: Wasp Veprolin homologous protein
wt: wild type
ZO-1: zonula occludens 1

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To my grandfather's memory

I. INTRODUCTION

Chapter 1: INTRODUCTION

1. MORPHOGENESIS

Morphogenesis is the process by which the mature forms of a cell, tissue, organ, or organism develop. In the beginning the embryo consists of a large number of cells with the same genetic content. The development starts by the establishment of anterior-posterior and dorsal-ventral axes more or less perpendicular to each other (Leptin, 2005). The axis patterning determines regions within the embryo allowing specific transcription factors to differentiate the various cell groups and give rise to the germ layers (Leptin, 1995). During development the cells go through morphogenetic movements requiring a precise coordination of the cells' cytoskeletal and adhesive properties in order to change their shape, intercalate and migrate to give rise to the final form of an organism.

In order to function as a coordinated tissue, cells must have the right shape and structure to pack all together. Furthermore, differentiated cells have morphological features that reflect their specialised functions in the organs they are part of. Having various surface architectures the cells can carry out different specialised functions. Therefore, as cell shape is intrinsically related to cell function, it is necessary to understand the mechanism by which components of the cytoskeleton get reorganised during morphological modification.

1.1 Gastrulation

One process during which conspicuous cell shape changes occur is gastrulation. Gastrulation is a developmental process in embryos of multicellular organisms by which the presumptive mesoderm and endoderm move inside the ectoderm to form a three-layered embryo consisting of the ectoderm on the outside, the endoderm on the inside, and the mesoderm in between the ectoderm and endoderm. The mesoderm will form the future middle layer of the adult body plan, the endoderm will form the future lining of the gut, and the ectoderm will form the adult integument and nervous system.

A hallmark event during *Drosophila* gastrulation is invagination of the mesodermal precursor cells initiated by the formation of a ventral furrow (Fig. 1.1). Ventral furrow formation in *Drosophila* embryos occurs in a stripe about 10 cells wide along the ventral midline (Leptin, 1995). First the cell apices flatten, then the apical plasma membrane slowly constricts, followed by a random faster constriction resulting in formation of a shallow groove and compressing the cells' contents to the basal side. The cells assume a wedge shape as the apical side remains constricted, and the basal side extends. The cells then shorten along the apical-basal axis causing further basal extension and deepening of the furrow (Fig.1.1). At the end as the furrow closes, the invaginated mesodermal cells undergo an epithelial-to-mesenchymal transition (Leptin, 1995). One protein that is required for gastrulation is DRhoGEF2. The intriguing feature of the ventral furrow formation is that this constriction happens in a very limited number of cells while *DRhoGEF2* is ubiquitously expressed. One possibility for the localised cell shape changes is that DRhoGEF2 is shuttled apically and therefore activates Rho1 only locally.

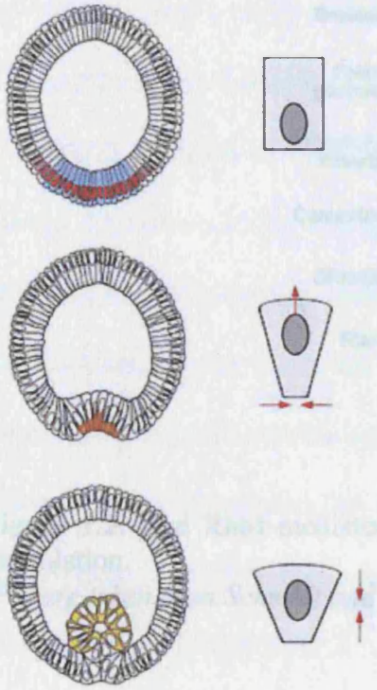


Figure 1. 1: Schematic representation of the cell shape changes as they occur in *Drosophila* gastrulation. *(Picture taken from Leptin, 1999)*

However, it is not known how DRhoGEF2 might be localised apically. One hypothesis is that it is recruited by β_H- spectrin, whose apical localisation becomes particularly strong during ventral furrow formation (Thomas and Kiehart, 1994). Another possibility is, being a multidomain protein, one of its domains, for instance the PDZ (Postsynaptic density protein/Discs Large/ Zonula occludens), PH (Pleckstrin Homology) or C1 domain (phorbol ester/diacylglycerol (DAG)-binding), could be targeting it to specific locations in the plasma membrane. Understanding how DRhoGEF2 gets localised is very important because this specific localisation could be important for restricting the activation of Rho GTPase to membrane subdomains, thereby eliciting specific cell shape changes to a limited area.

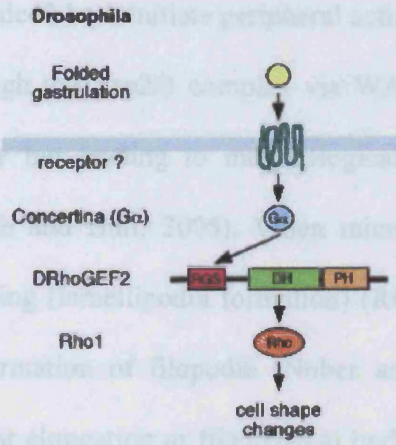


Figure 1.2: The Rho1-mediated pathway for cell shape changes during *Drosophila* gastrulation.
(Picture taken from Schmidt and Hall, 2002)

1.2 The actin cytoskeleton

The actin network provides the basis for the cellular architecture and a scaffold for the recruitment of regulatory factors all of which generate and maintain the cell form. The major component of the cytoskeleton is actin, an ATP-binding protein that exists as a globular monomer called G-actin and as a filamentous polymer called F-actin. Actin polymerisation starts with the nucleation of a few free actin monomers aided by the Arp2/3 complex and continues by the addition of more monomers in one direction so that the newly created filaments are bestowed with a polarity by virtue of the two ends with distinct biochemical properties (Welch and Mullins, 2002). Then the actin filaments are organized into bundles and networks. The Arp2/3 complex promotes

also the formation of branched actin filament networks (Welch and Mullins, 2002). The Rho family of small GTPases has a well established role in regulating the actin cytoskeleton. Rac and Cdc42 both initiate peripheral actin polymerisation (i.e. branched filament network) through the Arp2/3 complex via WAVE (a WASP family protein) and WASP respectively but leading to morphologically distinct protrusions at the plasma membrane (Jaffe and Hall, 2005). When microinjected into fibroblasts Rac induces membrane ruffling (lamellipodia formation) (Ridley et al., 1992). In contrast, Cdc42 promotes the formation of filopodia (Nobes and Hall, 1995). Rho activates formins to promote linear elongation or filaments at barbed ends (plus end of the actin filament where monomers of actin can be added) (Jaffe and Hall, 2005). When microinjected into fibroblasts activated Rho stimulates the formation of actin stress fibres and focal adhesions (Ridley and Hall, 1992).

Cell shape changes refer to the modification in one or two dimensions within an epithelial sheet and actin polymerisation is the driving force for these changes of the cellular form. In addition, actin polymerisation generates contractile structures at the cell cortex whereby bipolar assemblies of non muscle myosin II molecules can slide actin filaments over each other; this differential actomyosin contractility is supported by the spectrin network that recruits the regulators to the apical or basolateral domain (Schöck and Perrimon, 2002).

1.3 Microtubule-actin interactions in morphogenesis

Actin and microtubule filament systems coordinate dynamic processes such as cell shape changes and shape maintenance. These polarized cytoskeletal polymers assemble and disassemble rapidly, and interact with binding proteins and molecular motors.

The Rho family of small GTPases regulates both microtubules and actin (Whittman and Waterman-Storer, 2001). For example PAK activated by Cdc42/Rac can inactivate by phosphorylation members of the Op18/stathmin family which promote catastrophic disassembly and polymerisation of microtubules (Daub et al., 2001). Microtubules through interactions of their plus ends with proteins at the cell cortex such as CLIP-170 and EB-1 whose activity is indirectly regulated by Cdc42 can play a major role in defining cell shape and polarity (Jaffe and Hall, 2005).

Rho1 mediates formation of contractile actin structures, such as stress fibres (Etienne-Manneville and Hall, 2002), and at the same time promotes stabilisation of a sub-population of microtubules (Cook et al., 1998). Two key factors are known to function downstream of Rho1: Rho kinase, which promotes contractility by increasing phosphorylation of the regulatory light chain of myosin-II, and Diaphanous (mDia), which regulates actin polymerisation into bundles and also mediates microtubule stabilisation (Jaffe and Hall, 2005). In turn, the activity of Rho proteins is regulated directly or indirectly by microtubules and actin (Whittman and Waterman-Storer, 2001). For instance, microtubule disassembly activates Rho1 by a release .of the microtubule-bound Rho guanine nucleotide-exchange factor (GEF) GEF-H1 (Krendel et al., 2002).

2. MECHANICS AND BIOLOGICAL PROCESSES

Gastrulation is considered a biomechanical process in the sense that the cell reshaping is intricately dependent on the physical constrains imposed by the environment. During its course there are massive tissue rearrangements that could exert a force on the surrounding tissues or, conversely, the surrounding tissues or extracellular matrix (ECM) components could restrict the cell shape changes feeding back to the actin cytoskeleton.

How mechanical forces influence cell shape and consequently cellular processes has been gaining a lot of attention. Mechanotransduction is the process by which a mechanical stimulus exerted on a cell or tissue elicits a biochemical response (Shyy and Chien, 1997; Chicurel, et al., 1998). This happens through a coordination of biochemical signalling pathways with permutations of the cytoskeletal organisation. The change of the so called "tensegrity" architecture (Ingber, 2003) occurs when a mechanical force transduced directly to the underlying cytoskeleton via integrins, alters the force balance that exists between the cytoskeleton elements resulting in a change in their assembly and organisation (Alenghat and Ingber, 2002). Examples of processes influenced by forces that alter the cytoskeletal equilibrium are osteoblast differentiation (McBeath et al., 2004), remodelling of vascular endothelial cells due to shear stress caused by blood flow (Malek and Izumo, 1996; Nelson et al., 2003), modulation of fibroblast morphology during collagen matrix remodelling (Tamariz and Grinnell, 2002), and mechanical stress sensed by cardiac myocytes (Aikawa et al., 1999). In another model, mechanosensitive ion channels play a central role in influencing biochemical signalling pathways as a consequence of a change in the ion flux after

being stimulated by perturbed components of the extracellular matrix that deflect the plasma membrane (Gillespie and Walker, 2001). Processes such as hearing, touch, nociception and proprioception follow this model (García-Añoveros and Corey, 1997; Ernstrom and Chalfie, 2002).

Different kinds of force elicit different kinds of responses depending on the cell type. Mechanical signals can influence for example gene expression. One system where mechanical tension influences transcription is the migrating border cells that delaminate from the *Drosophila* follicular epithelium (Somogyi and Rørth, 2004). When the border cells are stretched MAL-D, a transcriptional cofactor for serum response factor (SRF), translocates from the cytoplasm to the nucleus. However, when these cells could not be stretched as happens in mutants of *slbo*, a gene required for migration and elongation, the nuclear translocation was blocked. The nuclear translocation and transcriptional activity of the mammalian homologue of MAL-D is also regulated by actin polymerisation (Miralles et al., 2003).

Another example showing that a force balance can direct transcriptional events in cells comes again from experiments in flies. In a study by Farge (2003) it was firstly shown that an artificial external mechanical compression of *Drosophila* embryos caused β-catenin/armadillo to move from the cell membrane, where it associates with cadherin, to the nucleus where it can activate expression of *twist*, which is required for the invagination and development of stomodeum. When β-catenin/armadillo was inhibited, this stress-induced expression of *twist* was suppressed. Secondly, the same process was studied under normal conditions during embryogenesis. In wild type embryos stomodeal cells express *twist* when the anterior-pole cells feel a compression

caused by the germ band extension. It was shown that if this compression was abolished by photo ablation of the dorsal epithelium so that the pushing of the extending germ band was not felt by the anterior-most cells then the stomodeal cells failed to switch on *twist* and no longer invaginated. Failure to invaginate also happens with *bicoid, nanos, torso*-like mutants that do not undergo germ band extension.

Rho GTPases can respond to mechanical forces and induce cell shape changes (Aoki et al., 1998; Aikawa et al., 1999; Numaguchi et al., 1999; Katsumi et al., 2002; Kole et al., 2004; Kaunas et al., 2005). For example, cell differentiation can be determined by the cell shape regulated by modulating endogenous Rho1 activity: in an activated Rho1-ROCK signalling pathway that generated an actin-myosin tension human mesenchymal stem cells (hMSCs) able to adhere and spread, underwent osteogenesis, while hMSCs expressing a dominant-negative Rho1, remained round and became adipocytes (McBeath et al., 2004). Endothelial cells experience shear stress due to the blood flow and this type of force has been shown to activate Rho GTPases (Li et al., 1999; Tzima, et al., 2001; 2002; 2003; Wojciak-Stothard and Ridley, 2003). Microtubules are also a potential cytoskeletal target influenced by mechanical forces. For instance, it was shown that in cultured smooth muscle cells external mechanical strain controls microtubule assembly which regulates membrane targeting of Rho GTPases (Putnam et al., 2003).

From the above presented evidence it is seen that in mammalian cells Rho GTPases have an established role in regulating the actin cytoskeleton due to a biochemical signal but also due to a physical force. In *Drosophila* there is an indication that the Rho1 activator, DRhoGEF2 interacts with a protein called DMec2 (K. Barrett,

unpublished data). The fact that Mec2 in *C. elegans* interacts with a mechanosensitive ion channel to relay a mechanical stimulus suggests the possibility that the interaction between DRhoGEF2 and DMec2 could provide a link between mechanical cues and actin regulation in *Drosophila.*

3. THE Rho FAMILY OF SMALL GTPases

3.1 Rho GTPases as Signalling Molecules

The Rho GTPases belong to the Ras superfamily of small GTPases that have been shown to regulate a variety of cellular processes. The primary structure of these proteins has been highly conserved throughout evolution, from yeast to humans showing a 50-55% homology to each other (Table 1.1), (Van Aelst and D' Souza-Schorey, 1997). Seven *Drosophila* Rho GTPases have been identified so far (Table 1.1). These include Rho1, Rac1, Rac2, Cdc42, RhoL, RhoBTB, and Mtl which are 70-90% identical in amino acid sequences to their mammalian orthologues (Lu, and Settleman, 1999). These GTPases are expressed throughout embryogenesis, and some are widely expressed in many tissues (such as Rho1) while others are restricted in the mesoderm, gut, and nervous system later in development (such as Rac1 and Cdc42) (Settleman, 2001).

Table 1.1

	Mammals	***S. cerevisiae***	***S. pombe***	***Drosophila***	***Dictyostilium***	***C. elegans***
Rho	Rho (A,B,C)	Rho1	Rho1	Rho1		RhoA
Rac	Rac (1,2,3)			Rac (1,2)	Rac (1A,1B,B)	Rac (1,2)
Cdc42	Cdc42, G25K Wrch-1	Cdc42	Cdc42	Cdc42		
Others	RhoD	Rho2	Rho2	RhoL	RacA	mig2
	RhoE/Rnd1	Rho3		Rho BTB	RacC	
	Rnd2	Rho4		Mtl		
	Rnd3					
	RhoG				RacD	
	TC10				RacE	
	TTF					
	Rap-1					

Table 1.1: Rho GTPases. Members of the Rho family are listed for mammals and for selected model organisms where their function has been analysed in most detail. Some of these are grouped into subfamilies based on their homology to mammalian Rho, Rac, Cdc42. Other members (Others) are not organised into subfamilies as homologues of the mammalian proteins have so far not been identified in other species. Rho2 in *S. cerevisiae* and *S. pombe* are homologues, however. [*Modified table and legend from Ridley, A.J, page 90, GTPases ed. Hall, 2000].*

Like all members of the Ras superfamily, the Rho GTPases function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state (Etienne-Manneville and Hall, 2002). The nucleotide state of Rho family proteins is regulated by three classes of regulatory proteins: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (Etienne-Manneville and Hall, 2002). GEFs catalyse the exchange of GDP for GTP by facilitating the release of GDP and transient stabilisation of the nucleotide-free protein. GAPs stimulate the relatively weak intrinsic GTP hydrolysing capacity of the Rho proteins, thereby enhancing their conversion to the GDP-bound form. GDIs preferentially bind to GDP-bound GTPases and prevent spontaneous and GEF-catalysed release of the nucleotide, thereby maintaining the

GTPases in the inactive state. Rho GDI appears to be a molecule capable of blocking the GTP binding/GTPase cycle at two points: at the GDP/GTP exchange step and at the GTP hydrolytic step (Van Alest and D' Souza-Schorey, 1997).

3.2 Activation of Rho GTPases

The upstream signalling pathways that lead to activation of the Rho GTPases are under intense investigation. Various receptors such as the seven transmembrane-domain family of receptors linked to heterotrimeric G protein (e.g. LPA, bradykinin, and bombesin), cytokine and adhesion receptors or growth factor receptors (e.g. PDGF, and insulin) may be required for activating Rho GTPases (Van Aelst and Souza-Schorey, 1997) to elicit a variety of cellular responses (Fig. 1.3). Other examples of receptors and ligands that lead to activation of Rho include the Plexin receptors and their ligands the Semaphorins which can activate Rho to induce neuronal specific outcomes (Hu, et al., 2001; Liu and Strittmatter, 2001; Swiercz et al., 2002). The T-cell receptor (TCR) can activate the Rho signalling pathway required for maturation from early to late pre-T-cell (Cantrell, 1994; Cleverley et al., 1999).

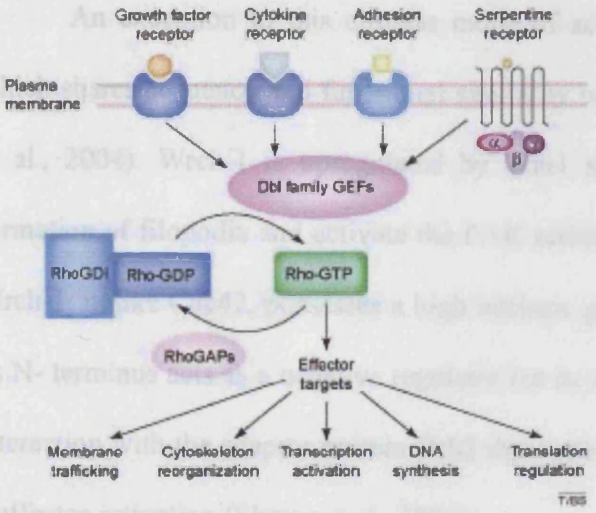


Figure 1.3: Upstream activation, regulation and downstream targets of the Rho signaling pathway. Various extracellular stimuli via different kind of receptors trigger the activation Rho GTPases and elicit specific responses. Rho cycles between an active and an inactive state which is regulated by GEFs, GAPs, and GDIs, *(figure taken from Zheng, 2001).*

Upon activation, Rho GTPases change their conformation thereby allowing the binding of different partner proteins. GEFs stimulate the dissociation of the tightly bound GDP nucleotide from the small GTP-binding protein in response to upstream signals. This reaction involves several stages (Fig. 1.4). First, the GEF forms a low affinity, docking complex with the GDP-bound small GTP-binding protein. Upon dissociation of GDP from this initial complex, a high affinity complex is formed consisting of GEF-small-GTP-binding-protein. This intermediate does not accumulate in the cell because it is rapidly dissociated by GTP (Cherfils and Chardin, 1999; Snyder et al., 2002). Thus GEFs can destabilise the strong interaction with GDP and stabilise the nucleotide-free small GTP-binding protein.

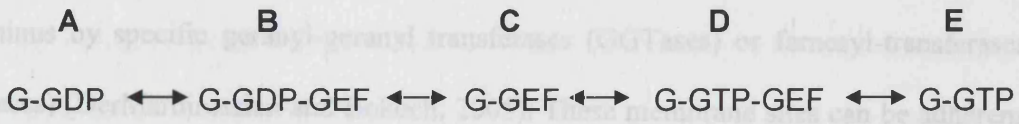


Figure 1.4: The guanine-nucleotide-excahange reaction. Small GTP-binding proteins and GEFs. The small GTP-binding proteins adopt different conformations in the complexes A, C, and E and possibly also at stages B and D.
(Figure and modified legend from Cherfils and Chardin, 1999).

An exception to this circular mode of activation of Rho GTPases is Wrch-1 which shares sequence and functional similarity with the Cdc42 small GTPase (Shutes et al., 2004). Wrch-1 is upregulated by Wnt1 signalling and it is able to promote formation of filopodia and activate the PAK serine/threonine kinase (Tao et al., 2001). Wrch-1, unlike Cdc42, possesses a high intrinsic guanine nucleotide exchange rate and its N- terminus acts as a negative regulator for its activity (Shutes et al., 2004); it is the interaction with the adaptor protein Grb2 that relieves this inhibition to promote Wrch-1 effector activation (Shutes et al., 2004).

3.3 Targeting of Rho proteins to their effectors.

The current theory is that Rho GTPases are primarily cytosolic and that they shuttle from the cytosol, where they are probably inactive, to specific membrane sites where they activate their effectors (Fukata & Kaibuchi, 2001). The Rho GDIs seem to have a crucial role in the translocation of the Rho GTPases between membranes and the cytoplasm. In resting cells, GDIs maintain Rho GTPases as soluble cytosolic proteins by masking their geranyl-geranyl membrane-targeting moiety present at the C-terminus (DerMardirossian and Bokoch, 2005). On activation the Rho GTPases are released from the GDI and targeted to the membrane microdomains through isoprenylated C-terminus by specific geranyl-geranyl transferases (GGTases) or farnesyl-transferases (FTases) (DerMardirossian and Bokoch, 2005). These membrane sites can be adherens junctions, cell-matrix adhesion sites, or intracellular membranes involved in vesicle targeting.

How do GTPases get to these specific intracellular sites and how are they retained there? GEFs could be recruiting GTPases to specific places of the plasma membrane through interaction with other proteins which are localised to the plasma membrane. For instance, PDZ-GEF1, a RhoGEF for the Rap1 GTPase (de Rooj et al., 1999), binds to β catenin and colocalises with β catenin at adherens junctions in MDCK epithelial cells (Kawajiri et al., 2000). GEFs contain domains that are involved in localisation of proteins to the plasma membrane such as PDZ domains (Jeleń et al., 2003) and PH domains. Tiam1, a GEF specific for Rac, contains both a PDZ and Pleckstrin homology (PH) domain and is localised to adherens juctions by virtue of its PH domain (Hordijk et al., 1997). Moreover, Tiam 1 localises to adherens junctions in epithelial MDCK cells but in migrating cells is found in lamellipodia (Sander et al., 1998). This provides evidence that the GTPases can be recruited to different subcellular locations depending on the conditions. RhoGEFs being multidomain proteins could be creating a compact signal transduction centre by recruiting to specific places both the GTPases and their downstream effectors.

Upon recruitment to the right place in the plasma membrane and activation to the GTP-bound form, GTPases undergo a conformational change that enables the interaction with so-called downstream effector targets, which contribute to the cellular response to GTPase activation. Many of the putative GTPase effector targets are protein kinases. For example, the Rho GTPase associates specifically with several identified protein kinases, including the PKC-related PKN (Watanabe et al., 1996; Amano et al., 1996) and PRK2 kinases (Quilliam et al., 1996), the ROK (Rho kinase) family of serine/threonine kinases (Vincent and Settleman, 1997), and the kinase called Citron

(Zhao and Manser, 2005) (Fig.1.5). The closely related Rac and Cdc42 GTPases associate with a distinct family of kinases referred to as PAK (p21-activated) kinases among other kinases, summarised in Fig.1.6.

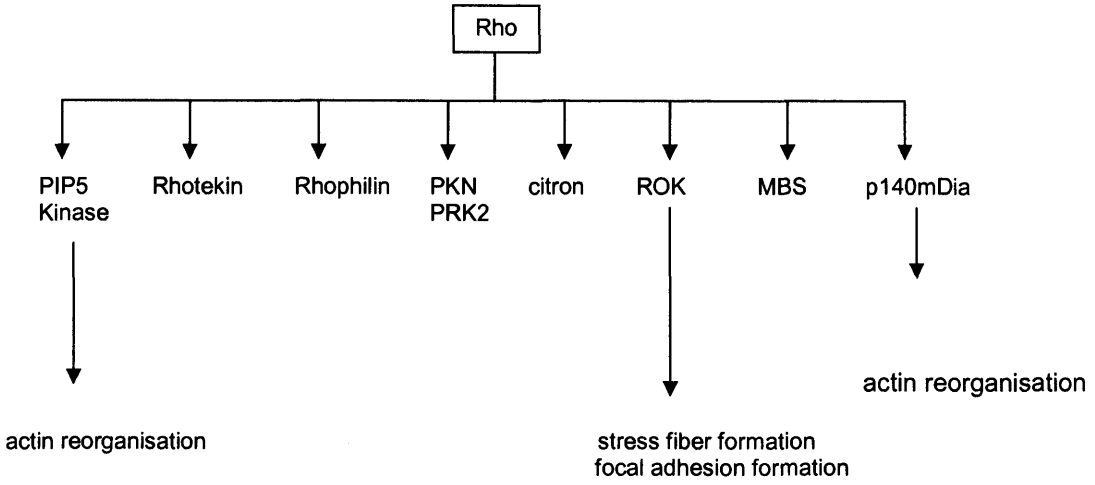


Figure 1.5: Mammalian targets of RhoA. The kinases PKN and PRK2, and the non-kinases Rhotekin and Rhophilin contain a homolgous Rho-binding motif, whereas ROK (Rho Kinase/ROKα and p160ROCK/ROKβ/ROCK II) and citron share a distict Rho-binding motif. MBS (myosin-binding subunit of myosin light chain phosphatase). The PIP5 kinase interaction may not be direct. *(Modified picture and legend from Van Aelst and D' Souza-Schorey, 1997).*

Different targets of Rho bind to different parts of the protein, which can be divided in three regions: the amino terminal part, aminoacids 23-40, called switch I, is the binding region for a class of effectors that include the kinase citron (Fujisawa et al., 1998). The second region spanning from amino acids 75-92, called switch II, is the binding region for yet another class of molecules, such as the non-kinase molecule, rhophilin (Fujisawa et al., 1998). A third region between amino acids 92-119 together with switch I and II, are required for a third type of target, which is the different isoforms of the kinase ROK (Fujisawa et al., 1998). It is possible that when one target

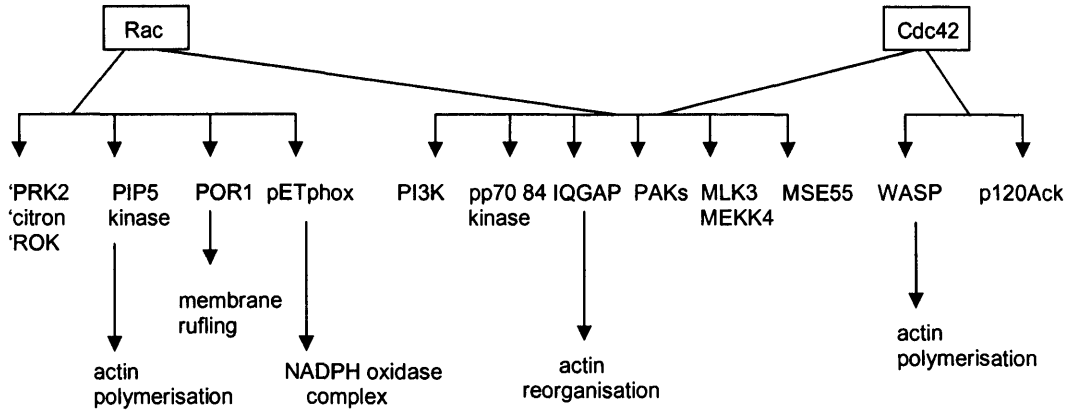


Figure 1.6: Mammalian targets of Rac and Cdc42. Rac and Cdc42 interact with a variety of common targets. The serine/threonine kinases belonging to the PAK family, MLK3, MEKK4, MSE55, and WASP share a common Rac/Cdc42 binding motifs; (POR1) partner of Rac; (IQGAP) GAP-containing Ile-Gln motifs. 'PRK2, 'citron, and 'ROK also interact with Rho. *(Modified picture and legend from Van Aelst and D' Souza-Schorey, 1997).*

is bound to Rho, it can mask the binding site of other targets, as in the case of PKN which when bound to Rho probably blocks the site involved in the binding with Diaphanous (Flynn et al., 1998; Maesaki et al., 1999). Many of these proteins exhibit specific interactions with a particular Rho GTPase, although a few of them appear to be shared among different Rho proteins. Thus it has been difficult to establish the mechanisms by which signalling specificity is achieved *in vivo*.

3.4 Biological Functions mediated by Rho GTPases

Rho proteins regulate many cellular processes such as differentiation (Takano et al., 1998), cell morphology (Moorman et al., 1999), cell motility and adhesion (Kaibuchi et al., 1999), phagocytosis (Chimini et al., 2000), cytokinesis (Prokopenko et

al., 2000), and smooth muscle contraction (Somlyo et al., 2000). Studies have shown not only how individual Rho family GTPases mediate multiple temporally and spatially distinct developmental processes but also how the coordinated action of multiple Rho GTPases can sometimes be used to accomplish a single morphogenetic process.

3.4.1 Rho and the early *Drosophila* embryo

The *Drosophila* embryo initially consists of a syncytial of individual nuclei that subsequently are going to be enveloped by plasma membrane recruited from the cytoplasm (Lecuit and Wieschaus, 2000). For cellularisation, a process that involves reorganisation of the actomyosin cytoskeleton Rho GTPases are required (Crawford et al., 1998).

After cellularisation is completed, a series of post blastoderm mitoses follow throughout embryogenesis that also require Rho GTPases probably because these regulate the function of cytoskeletal components such as actomyosin and microtubules which are necessary for the contractile ring of the mitotic furrow. It has been shown that Rho localises to the cleavage furrow and plays a crucial role in contractile ring function by activating at least three known effectors, ROK, Citron kinase, and mDia (Glotzer, 2001). Loss of Pebble, a Rho-specific GEF, results in failed cytokinesis events in the post blastoderm embryo leading to an accumulation of multinucleated cells (Prokopenko et al., 1999). Regulated Rho activity is required for cytokinesis as GAPs are also responsible for a well defined process (Lee et al., 2004).

3.4.2 Rho and development of the *Drosophila* embryo

Rho GTPases are involved in many developmental processes such as oogenesis, gastrulation, dorsal closure, muscle development, neural development, eye development and tissue polarity (Lu and Settleman, 1999). During embryogenesis, at the last stages of gastrulation, the dorsal surface of the embryo is covered by a thin layer of cells, referred to as the amnioserosa. During dorsal closure the amnioserosa is sealed by epidermal cells that stretch along the dorsal-ventral axis to meet the dorsal midline and undergo a zippering-like process. This does not involve either cell division or migration but is solely dependent on cell shape changes by some of the embryonic cells. Three distinct family GTPases are implicated in the cytoskeletal-mediated shape changes during this process (Harden et al., 1995; Jacinto et al., 2002). More precisely evidence points to a requirement for these GTPases in leading edge cell stretching (Woolner et al., 2005) through activation of distinct signalling pathways.

Rho GTPases have a role in various aspects of neural development (Luo, 2000; Govek et al., 2005). Significantly, the growth cone (tip of neurite) is an actin-rich dynamic structure that exhibits morphological and cytoskeletal features resembling filopodia, lamellipodia, and stress fibres of fibroblasts known to be regulated by the various Rho GTPases. In *Drosophila* Rho1 is involved in the cytokinesis of neuroblasts and in dendritic morphogenesis (Lee et al., 2000). In another study, expression of mutationally activated and inhibitory forms of Rac and Cdc42 in the developing fly nervous system revealed roles for these GTPases in establishing neuronal polarity, and in the outgrowth of neurites (Luo et al., 1994). Specifically, Rac has a precise role in

the initiation of axon outgrowth and axon elongation. In the same study, activated Cdc42 inhibited both axon and dendrite outgrowth.

In mature neurons Rho GTPases continue to play a role in the development of a fully functional nervous system. For example Rac1 is implicated in motor axon guidance (Kaufman et al., 1998), synaptogenesis (Allen et al., 2000), and in photoreceptor morphogenesis (Chang and Ready, 2000).

Following the completion of *Drosophila* embryogenesis, larval development begins during which epithelial polarization plays a particularly important role. Aside from the apical-basal polarity, epithelial cells organise themselves within tissues in such a way as to establish a so-called planar cell polarity (PCP) relative to the body axis and perpendicular to the anterior-posterior (A-P) axis. Examples of this are the appearance of distally pointing hairs in the wing blades of the fly as well as the hairs and bristles on the thorax and abdomen, and the chirality of regularly arrayed ommatidia. Rho GTPases play an essential role in the establishment of PCP in flies. Expression of mutationally activated and inhibitory forms of Rac1 in imaginal discs revealed Rac1 is essential for the proper assembly of cell adherens junctions as well as for the establishment of PCP, while Cdc42 was found to be required for epithelial cell shape changes but it is not required for actin assembly at adherens junctions (Eaton et al., 1995). Mutant tissue clones containing hypomorphic Rho1 alleles exhibit abnormal wing hair polarity, and in somatic eye clones harbouring such alleles, ommatidia are incorrectly oriented, while the position of photoreceptors is unaffected (Strutt et al., 1997). Genetic interaction studies established that Rho1 functions downstream of *frizzled,* encoding a G protein coupled receptor, and *dishevelled,* encoding for a

cytoplasmic signalling molecule, to mediate tissue polarity (Lu and Settleman, 1999). The roles of RhoGTPases in different developmental processes are summarised in Table 1.2.

Table 1.2

Developmental Processes	**Genes/Pathways involved**
Oogenesis: border cell Migration	Rac1
Oogenesis: transfer of nurse cells contents to oocytes	Rac1, Cdc42, RhoL
Gastrulation	Rho1
Dorsal closure	Rho1, Rac1, Cdc42
Muscle development	Rac1
Neural development	Rac1, Cdc42
Eye development	Rho1, Rac1, Rac2, Cdc42
Tissue polarity: wing development	Rac1, Cdc42, Rho1
Tissue polarity: eye development	Rho1

Table 1.2: Summary of Rho GTPase signalling components involved in various *Drosophila* developmental processes, *(updated and modified version from Lu and Settleman, 1999).*

3.5 Summary

To summarise so far, the Rho GTPase protein family influences diverse cellular and developmental events most commonly by regulating the actin cytoskeleton. The change in the architecture of a cell is a sequence of events: first a cue has to trigger the signaling pathway, this will cause the translocation, activation and retention of the Rho GTPase to the right place where it can interact with its effectors to cause morphological

permutations that reflect the function of the cell. Each of these steps is to a certain degree a regulatory step that dictates the outcome of the Rho protein activation. All evidence points to GEFs being the critical mediators of Rho GTPases activation. The following text considers GEFs and their role.

4. GUANINE NUCLEOTIDE EXCHANGE FACTORS FOR Rho GTPases

Guanine nucleotide exchange factors (GEFs) activate directly the Rho GTPases in response to extracellular signals in a specific spatio-temporal frame. Therefore, their timing (presence at the right place and activity) is crucial for many cellular processes. They activate Rho GTPases by exchanging GDP for GTP. GEFs that contain the DH (Dbl Homology) domain in tandem with the PH domain (Pleckstrin Homology) form the Dbl family. The DH domain is responsible for catalysing the exchange of GDP for GTP. Other than the DH/PH domain, they also contain a variety of other domains. The Dbl family of GEFs is composed of a large number of members, structurally very different from each other, with particular mechanisms of regulation participating in different signalling pathways.

There are also Rho GEFs called Dock without the DH/PH domain. These have two regions called DHR1 and DHR2 (Dock-Homology Region-1 and -2). It is probably the DHR2 domain which is sufficient for promoting guanine nucleotide exchange activity for this family of GEFs (Rossman et al., 2005). Certain members of this family also have additional domains such as PH, SH3, C2 or coiled coil regions (Rossman et al., 2005).

4.1 Structural features of GEFs

Rho GEFs are large proteins consisting of domains, 200-300 amino acid long, with catalytic activity; they also contain various other domains involved in oligomerisation, protein-protein interactions or membrane targeting as well as of regions whose functions remain unknown (Fig. 1.7). Dbl-family GEFs have a DH domain, which is necessary for GEF activity. DH domains have three conserved regions: CR1, CR2, and CR3, each 10-30 aminoacids long but for the rest they do not show homology between them. Although GEFs can activate the same GTPase they have very small (<20%) sequence identity (Schmidt and Hall, 2002).

Adjacent and C-terminal to the DH domain there is a Pleckstrin homology (PH) domain which binds to phosphorylated phosphoinositides. PH domains could affect the catalytic activity of the DH domain in GTPase binding (Rossman et al., 2002). In addition, they could be involved in recruiting GEFs to the appropriate intracellular location (Rossman et al., 2002). Aside the DH-PH module that is always present in the Dbl family members, GEFs contain additional functional domains that include SH2, SH3, Ser/Thr or Tyr kinase, Rho-GAP, Ca^{2+}-dependent lipid binding, coiled coil, cysteine-rich zinc butterfly motif, $G_{\beta\gamma}$, RGS, PDZ or additional PH domains (Fig. 1.7). These domains are involved in coupling GEFs to upstream receptors and signalling molecules, and in localising them to subcellular structures; moreover they could confer additional functions associated with GEFs.

A sub-family of RhoGEFs has been identified by virtue of the presence of a regulator of G protein signalling (RGS) domain (Fukuhara et al., 2001) that directly binds activated heterotrimeric G protein α subunits of the G_{12} family.

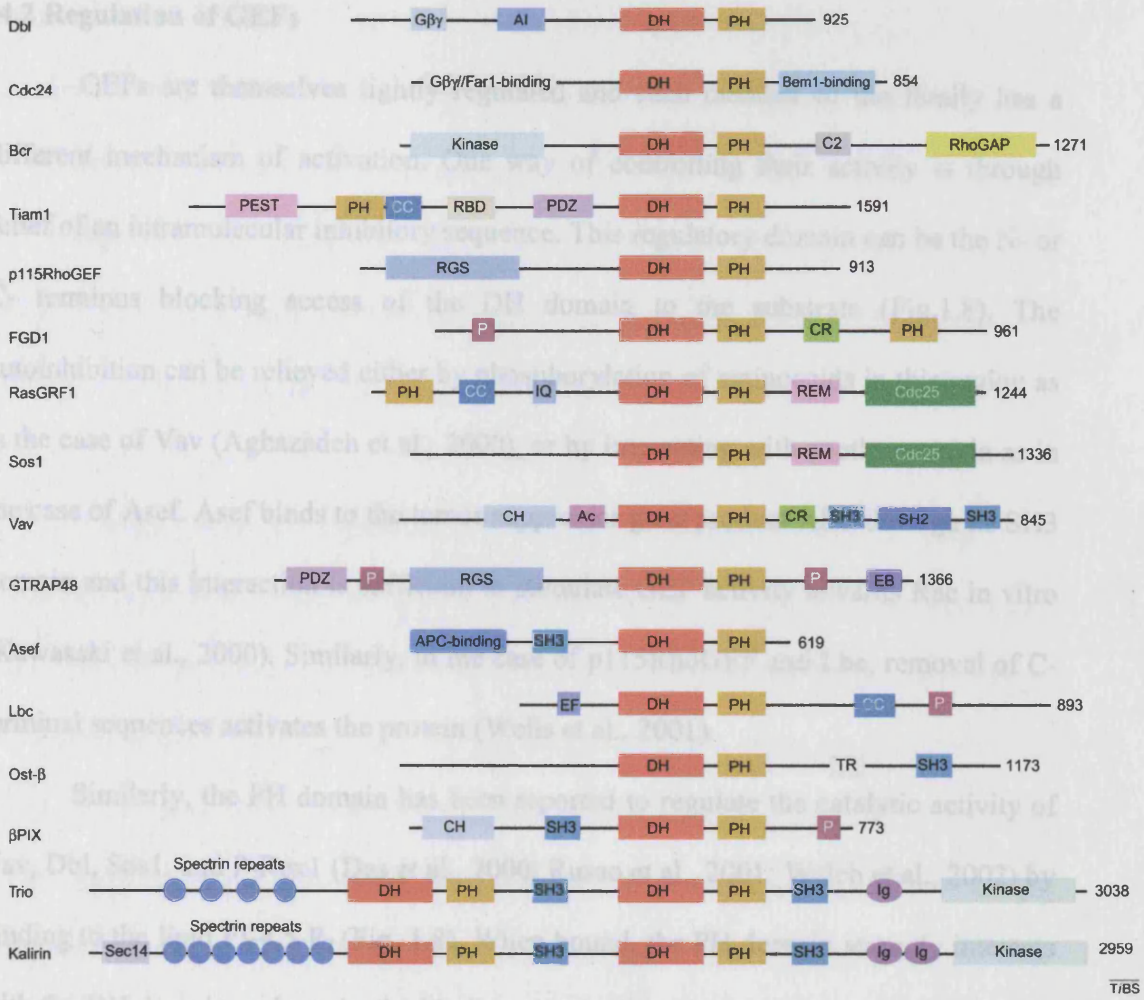


Figure 1.7: Schematic structures of representative mammalian Dbl family members. Note that the only Dbl family members that have PDZ domain are Tiam1, GTRAP (mouse homogue of the PDZ-RhoGEF not shown here) and LARG (not shown here). *(Picture taken from Zheng, 2001)*

In humans, three RGS domain-containing RhoGEFs have been described, namely p115-RhoGEF, PDZ-RhoGEF, and LARG. All three can be activated by α_{12} or α_{13} and are specific for RhoA but not the other Rho family GTPases Rac1 and Cdc42 (Hart et al., 1996; Rumenapp et al., 1999). DRhoGEF2 also belongs to the RGS subfamily of GEFs.

4.2 Regulation of GEFs

GEFs are themselves tightly regulated and each member of the family has a different mechanism of activation. One way of controlling their activity is through relief of an intramolecular inhibitory sequence. This regulatory domain can be the N- or C- terminus blocking access of the DH domain to the substrate (Fig.1.8). The autoinhibition can be relieved either by phosphorylation of aminoacids in this region as is the case of Vav (Aghazadeh et al., 2000), or by interacting with another protein as in the case of Asef. Asef binds to the tumor suppressor gene product APC through its SH3 domain and this interaction is sufficient to stimulate GEF activity towards Rac in vitro (Kawasaki et al., 2000). Similarly, in the case of p115RhoGEF and Lbc, removal of C-terminal sequences activates the protein (Wells et al., 2001).

Similarly, the PH domain has been reported to regulate the catalytic activity of Vav, Dbl, Sos1, and P-Rex1 (Das et al., 2000; Russo et al., 2001; Welch et al., 2002) by binding to the lipid PI-4,5-P_2 (Fig. 1.8). When bound, the PH domain strongly interacts with the DH domain and masks the binding site for Rac binding (Das et al., 2000). The autoinhibitory constraint imposed by the PH domain is relieved in response to activation by PI 3-kinase. PI-4,5-P_2 is converted to PI-3,4,5-P_3 the DH/PH interaction is weakened leaving DH domain free for binding to Rac. The Rho/Rac GEFs appear to be regulated by a variety of factors, including, lipid interactions and membrane localisation, indicating that diverse regulatory inputs may be utilised to promote their ability to activate Rho family GTPases (Van Aelst and Souza-Schorey, 1997).

Several GEFs are stimulated by phosphorylation (Crespo et al., 1997) or protein-protein interactions (Fig. 1.8). For example, in the case of p115RhoGEF,

stimulation of cells by LPA or thrombin induces release of the α_{13} subunit from the heterotrimeric G protein G_{13}, which subsequently binds to an RGS-like domain of p115RhoGEF. Upon binding α_{13} enhances the GEF activity of p115RhoGEF both *in vivo* and *in vitro* (Hart et al., 1998; Kozasa et al., 1998). The RGS domain is not always involved in the GEF regulation: interestingly, α_{13} interacts with and stimulates the GEF activity of Dbl *in vivo*, although it does not contain an RGS domain (Jin and Exton, 2000).

Another way of regulation is through GEF oligomerisation (Fig. 1.8). Oligomerisation is mediated through the DH domain and requires the conserved region CR2. The current view is that oligomerisation is perhaps important for generating larger signalling complexes that augment GTPase activation. The fact that mutants that can no longer oligomerise still possess GEF activity in vitro but are less potent at activating Cdc42 and Rho *in vivo* (Zhu et al., 2001) are consistent with this view. It has thus been suggested that oligomers of Dbl can recruit multiple Rho GTPases into a large complex, raising the possibility that this serves to activate co-ordinately several pathways (Zhu et al., 2001). Oligomerisation has been reported for RasGRF1 and RasGRF2 (Anborgh et al., 1999).

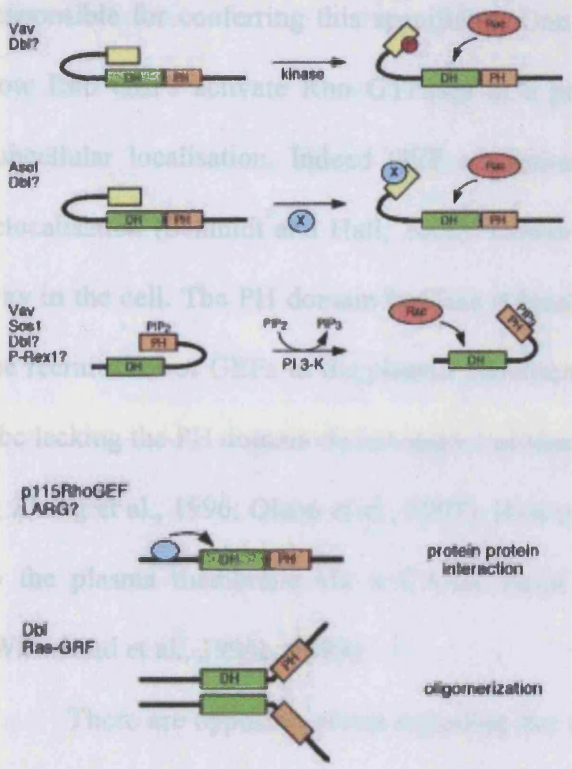


Figure 1.8: Schematic representation of GEF activation through (i) relief of intramolecular inhibitory sequences and (ii) through protein-protein interactions or oligomerization. *(Picture taken from Schmidt and Hall, 2002)*

Very little is known about how GEFs are turned off. One possibility is simple reversal of the activation mechanism through dephosphorylation, or disruption of protein-protein or protein-lipid interactions. However many proteins have been identified as GEF inhibitors such as Cbl-b or hSiah2 that suppress Vav (Bustelo et al., 1997; Germani et al., 1999). Tiam1 is inhibited by binding to nm23H1 (Otsuki et al., 2001); whereas p115RhoGEF is turned off by association with the HIV-1 gp41 protein (Zhang et al., 1999). The mechanism of the inhibition is not known.

4.3 Rho GEFs can act as signalling landmarks for the direction of the pathway

Rho GTPases regulate a grand variety of cellular functions raising the question how specificity is achieved. Rho GEFs being the direct activators could be one factor

responsible for conferring this specificity. One highly favoured hypothesis regarding how Rho GEFs activate Rho GTPases in a precise spatio-temporal manner is their subcellular localisation. Indeed GEF activation seems to be intimately linked with relocalisation (Schmidt and Hall, 2002). However it is not clear how GEFs find their way in the cell. The PH domain because it binds to phospholipids could be mediating the recruitment of GEFs to the plasma membrane. Mutants of Dbs, Dbl, Lsc, Lfc, and Lbc lacking the PH domain do not show transforming activity (Whitehead et al., 1995a, b; Zheng et al., 1996; Olson et al., 1997). However when Lfc and Dbs can be localised to the plasma membrane via a CAAX motif they re-acquire the *in vivo* activity (Whitehead et al., 1995b; 1999).

There are opposing views regarding the role of the PH domain as a membrane anchor as it has also been shown that the PH domain has a low binding affinity and little specificity for phospholipids indicating that these interactions are insufficient for membrane localisation (Snyder et al., 2001). In other cases such as that of Sos1, recruitment to tyrosine kinase receptors is mediated through adaptor proteins like Grb2 and Shc and not through its PH domain (Buday and Downward 1993; Gale et al., 1993; Skolnik et al., 1993).

The Tiam-1 and Ras-GRF are recruited to the plasma membrane in response to cellular activation by serum and calcium, respectively through a N-terminally located second PH domain (Buchsbaum et al.1996; Michiels et al., 1997). In other cases GEFs can be localised to the plasma membrane upon activation of receptors. For example, Vav is recruited to activated B- and T-cell receptors via its SH2/SH3 domains (Bustelo, 2000). Another GEF called Ephexin interacts with the receptor Ephrin A via its DH/PH

module (Shamah et al., 2001) and p115RhoGEF activated by binding to $G_{\alpha 13}$ linked to an activated heptahelical receptor is redistributed from the cytoplasm to the plasma membrane (Bhattacharyya and Wedegaertner, 2000).

In other cases Rho GEFs could interact with other proteins that can localise Rho: p115RhoGEF can bind to the heterotrimeric G protein after activation of the LPA heptahelical receptor and recruit Rho to the membrane (Seashotlz et al., 1999; Zheng, 2001). The localisation of PDZRhoGEF either to the cytoplasm or the tip of neurites determines the Rho response to LPA stimulation, which is either stress fibre formation or neurite retraction respectively (Togashi et al., 2000), demonstrating that the subcellular localization of the GEF and the activated GTPase can select the cellular response. If Rac1/Tiam 1 is localised at the E-cadherin adhesion sites and the cells attached to fibronectin, then cell-cell contacts are promoted. On the other hand if Rac1/Tiam1 is localised in the migratory edge and the cells are attached to collagen then cell migration is promoted (Price and Collard, 2001). In this case both the subcellular localisation of the Rho protein together with the exchange factor and the nature of the environmental conditions are crucial in balancing cell to cell adhesion and cell migration.

A distinct mechanism of regulation by localisation has been identified by Ect2/Pebble, and Net1 which contain nuclear localisation signals within the N-terminus and through import into the nucleus they are sequestered away from their substrate (Prokopenko et al., 1999; Tatsumoto et al., 1999; Schmidt and Hall, 2002). Table 1.3 summarizes some reported examples of interactions involved in GEF regulation.

Table 1.3

GEF	Interacting molecule	Interacting/phosphorylated domain	Effect on GEF function
Vav	syc/src kinases	phosphorylation of N terminus	relief of autoinhibition, activation
	PI-3,4,5P_3	PH domain	relief of autoinhibition, activation
	adaptors and receptors	SH2/SH3, other domains	membrane recruitment
	SOCS1	N terminus	ubiquitination
	Cbl-b	C terminus	inhibition
	hSiah2	C terminus	inhibition
Dbl	Ack1	phosphorylation	activation
	PI-4,5P_2, PI-3,4,5P_3	PH domain	inhibition
	$G\alpha_{13}$	N.D.	activation
	$G\beta\gamma$	N terminus	activation ?
	Dbl	DH domain (oligomerization)	potentiation of GEF activity
	N.D.	PH domain	recruitment to stress fibers
Sos1	PI-3,4,5P_3	PH domain	relief of autoinhibition, activation
	E3b1, Eps8	C terminus	activation
	N.D.	PH domain	membrane recruitment
P-REX1	PI-3,4,5,P_3	PH domain	activation
	$G\beta\gamma$	N.D.	activation
Asef	APC	N terminus	activation, relief of autoinhibition ?
p115RhoGEF	$G\alpha_{13}$	RGS-like domain	activation and membrane recruitment
	HIV-1 gp41	C terminus	inhibition
LARG	$G\alpha_{12/13}$	RGS-like domain	N.D.
	IGF-1 receptor	PDZ domain	membrane recruitment, activation ?
Lbc	N.D.	PH domain	recruitment to stress fibers
RasGRF	RasGRF	DH domain (oligomerization)	activation, potentiation ?
Dbs	N.D.	PH domain	membrane recruitment
Lfc	N.D.	PH domain	membrane recruitment
	tubulin	PH domain	recruitment to microtubules
p190RhoGEF	tubulin	C terminus	recruitment to microtubules
Tiam1	N.D.	N terminal PH domain + adjacent sequences	membrane recruitment
	PKC, CamKII	phosphorylation	activation
	PI-3,4P_2, PI-3,4,5P_3	N terminal PH domain	activation
	nm23H1	N terminus	inhibition
	CD44	N-terminal PH domain + adjacent sequences	membrane recruitment, activation
	ankyrin	N-terminal PH domain + adjacent sequences	membrane recruitment, activation
Ephexin	EphA4, other EphAs	DH-PH module	membrane recruitment, activation
Pix	Cat/Git/PKL	C terminus	recruitment to endosomal membranes ?
Ect2	Cdc2 ?	phosphorylation	activation ?
Trio	LAR		recruitment ? activation ?
	filamin	PH domain	recruitment to actin ?

Table 1.3: Interactions involved in GEF regulation
(Table taken from Schmidt and Hall, 2002).

An interesting case demonstrating that protein platforms are necessary for channelling a signal towards a specific downstream target of Rho is the scaffold protein CNK1 which interacts with two Rho-specific GEFs, Net1 and p115RhoGEF linking them to components of the Rho-dependent JNK MAP kinase cascade (Jaffe et al., 2005). This could indicate that in the cell an uneven distribution of protein complexes could compartmentalise a subset of outputs of the Rho signalling pathway.

4.4 Various biological functions of GEFs

RhoGEFs acting as signal integrators to activate Rho GTPases participate in many cellular functions. Rho GTPases play a major role in regulating cytoskeletal changes during neuronal morphogenesis (Luo, 2000; Dickson, 2001). Several GEFs have been involved in the regulation of various neuronal processes by the localised activation of their counterpart GTPases. Tiam1, a Rac-specific GEF, is involved in neuronal polarization, and is implicated in the axon formation process (Kunda et al., 2001). The *Drosophila* homolog of Tiam1, Still life, is involved in synaptic development (Sone et al., 1997). Still life (Rac/Rho GEF) is also involved in proper synaptic function (Sone et al., 1997) and Trio and Ephexin have been shown to play essential roles in activating Rho GTPases during growth cone guidance (Steven et al., 1998; Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000; Wahl et al., 2000; Shamh et al., 2001). Trio (Rac-specific GEF) is also required for normal axonal pathfinding in the central and peripheral nervous systems of developing embryos, as well as in the photoreceptors of the adult eye (Luo, 2000). One

candidate GEF that might be controlling the formation of dendritic spines is Kalirin specific for Rac (Luo et al., 1996; Nakayama et al., 2000; Penzes et al., 2001). Studies in mouse embryos implicated Trio and Obscurin in the control of skeletal muscle development (O'Brien et al., 2000).

Mammalian GEFs play a role in immune responses. For example Vav downstream of T-cell receptors is required to stimulate Rac-mediated actin reorganization, which contributes to activation of the transcription factor NF-AT to produce the cytokine interleukin-2 (Holsinger et al., 1998; Bustello, 2000). In neutrophils, a different GEF, P-Rex-1, has been shown to control Rac-mediated NADPH oxidase activation (Welch et al., 2002).

4.5 RhoGEFs control the cell morphology

RhoGEFs relay a signal from various receptors to Rho GTPases thereby inducing cell shape changes. Controlled activity of RhoGEFs is required for correct regulation of the actin cytoskeleton by the Rho GTPases.

There are several studies that demonstrate the important role RhoGEFs have in the regulation of morphology of various cell types. For example RhoGEF Pebble is transducing a signal from the Heartless receptor to induce cell shape changes during migration of mesodermal cells of the *Drosophila* gastrula (Schumacher et al., 2004). The neuronal RhoGEF Kalirin-7 has been shown to regulate dendritic morphogenesis in response to NMDA receptor through activation of Rac1 (Penzes et al., 2001). Further studies on the functional role of RhoGEFs have shown that the mammalian LARG and PDZ-RhoGEF by controlling the cell shape of neurons can affect axon guidance and

cell migration and growth cone collapse in response to semaphoring-plexin interaction (Perrot et al., 2002; Swiercz et al., 2002). Finally, a recent study showed that the Rho-specific GEF Lfc interacts with neurabin/spinophilin in response to activation of NMDA receptors and by virtue of this interaction can regulate dendritic spine morphology (Ryan et al., 2005).

4.6 The Rho-specific exchange factor DRhoGEF2

A genetic screen in *Drosophila* was conducted in order to identify important regulators of the Rho signalling pathway. The overexpression of two copies of Rho in the fly eye using the GMR promoter causes a rough eye phenotype (Hariharan et al., 1995). This rough eye phenotype was used as a basis to screen for suppressors and enhancers of the Rho-induced effect. Flies were fed with a chemical mutagen (EMS) and crossed with GMR-Rho^{1}-Rho^{3} (Hariharan et al., 1995). Several lines of suppressors and enhancers were identified. One of the suppressors was found to be a Rho specific exchange factor named DRhoGEF2 (Barrett et al., 1997). DRhoGEF2 belongs to the Dbl family of nucleotide exchange factors, thus it has the DH and PH domains required for Rho activation. In addition it has a PDZ domain near its amino terminus whose function is not known. DRhoGEF2 has also two more domains, the G-protein Regulation Subunit (RGS) and a C1 domain. There are three mammalian orthologues to DRhoGEF2: p115RhoGEF that has also an RGS domain but not a PDZ domain, PDZRhoGEF (also known as KIAA0382 or ArhGEF12) and LARG (Fig. 1.9). Both PDZ-RhoGEF and LARG have a PDZ domain and an RGS domain but not a C1

domain (Fukuhara et al., 2000; Wells et al., 2001; Schmidt and Hall 2002). DRhoGEF2 is a specific exchange factor for Rho, since it modifies only GMR-Rho and not GMR-Rac1, GMR-Rac2, GMR-Cdc42.

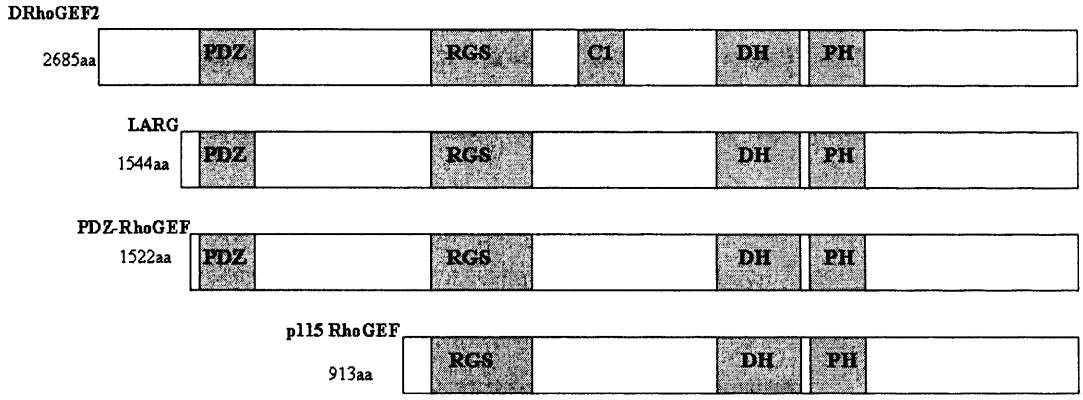


Figure 1.9: Schematic representation of DRhoGEF2 protein with the putative domains and its mammalian orthologues.

In situ hybridisation of DRhoGEF2 mRNA in embryos and Northern blots showed that the mRNA is maternally loaded and expressed ubiquitously and at low levels (Barrett et al., 1997). The maternal product of DRhoGEF2 is required during gastrulation. Embryos derived from germ line clones of cells in which DRhoGEF2 had been mutated do not form a ventral furrow and the anterior and posterior midgut primordial tissues do not invaginate. As a result the embryos become wrinkled due to a failure in germ band extension and die. Transverse sections of DRhoGEF2 embryos demonstrate that the cell shape changes required for gastrulation do not occur (Fig.1.10). Only a small number of

cells are able to constrict their apical side and a few nuclei migrate to the basal side but the overall constrictions and cell shape changes fail (Barrett et al., 1997; Häcker and Perrimon, 1998). The interesting thing about this process is how DRhoGEF2 being ubiquitously expressed directs these shape changes to only a subset of cells. One possibility is that it is recruited to the apical membrane at a specific moment to restrict the effects of Rho1. This could occur via its PDZ domain which has been shown to be important for the localisation of many proteins involved in epithelial polarisation (Bilder, 2001).

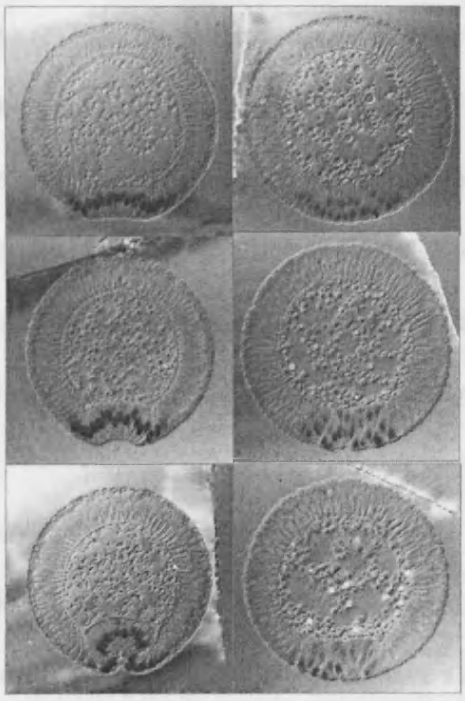


Figure 1.10: Transverse sections of 50% egg length of wild type (left column) and DRhoGEF2$^{4.1}$ embryos (right column) stained with anti-Twist to mark the nuclei of presumptive mesodermal cells. In wild type embryos there is a well choreographed movement of the nuclei and the formation of the ventral furrow. In the mutant embryos nuclear migration is not coordinated and not followed by ventral furrow formation. *(Picture taken from Barrett et al., 1997)*

5. GTPase-ACTIVATING PROTEINS: not just inactivators of Rho GTPases

Another class of enzymes that regulates Rho protein activity is the one composed of the GTPase-activating proteins (GAPs) which catalyze GTP hydrolysis bringing Rho to its GDP-bound inactive state. The domain that confers the GAP activity consists of approximately 200-300 amino acids (Scheffzek et al., 1996; Rittinger et al., 1997) but sequences outside the GAP domain influence also the function of the catalytic domain (Molnár et al., 2001). This is consistent with the fact that individual RhoGAPs exhibit different kinetic properties in the interaction with Rho1 GTPase suggesting a unique mechanism for each particular interaction as a result of distinct structural requirements (Zhang and Zheng, 1998). RhoGAPs conceal the effector-binding region of the GTPase by making contact with switch I and II region of the substrate and stabilize the transition state of GTP-hydrolysis reaction by providing an essential arginine residue into the GTPase active site (Rittinger et al., 1997) thereby terminating Rho protein activity. Probably the interaction of RhoGAPs with the Rho GTPases happens at the plasma membrane as membrane-associated GAPs increase the intrinsic rate of hydrolysis only on prenylated and thus membrane localized Rac1 and Rho1 (Molnár et al., 2001).

The *Drosophila* genome has 64 genes encoding GAPs for Ras superfamily members of which 21 genes are for the Rho subfamily of small GTPases (Bernards, 2003). Other than the GAP domain the RhoGAP proteins have also other domains that include C1, C2, SH2, SH3, PH, PDZ, and PTB domains which might be involved in the regulation of these proteins by various mechanisms such as protein or lipid interactions,

phosphorylation/dephosphorylation, subcellular localization and proteolytic degradation (Bernards and Settleman, 2004).

Since there is a functional interplay between the various Rho GTPases their activity has to be modulated. Certain GAPs act as effectors of Rho GTPases for example RhoE/Rnd activate p190 RhoGAP which inactivates Rho1 (Wennerberg et al., 2003). p190 RhoGAP can also be stimulated by Src family phosphorylation induced by cadherin engagement (Noren et al., 2003). In addition to cadherins, integrin activation (Arthur et al., 2000) and growth factor can promote phosphorylation of p190 RhoGAP by Src family kinases (Ellis et al., 1990). In the nervous system a class of GAPs responds to Slit-Robo signaling having an effect on neuronal migration (Wong et al., 2001).

In conclusion, RhoGAPs by inhibiting Rho1 participate in various cellular processes such as migration (Arthur and Burridge, 2001), morphogenetic movements during development (Brouns et al., 2000; 2001), cell differentiation such as adipogenesis versus myogenesis (Sordella et al., 2003).

6. PDZ domains

PDZ domains are one of the most commonly found protein-protein interaction domains in organisms from bacteria to humans. PDZ is an acronym from the initial letters of the Postsynaptic density protein 95 (PSD-95), Discs large (Dlg), Zonula occludens-1 (ZO-1) the first identified proteins containing this motif (Jeleń et al., 2003). PDZ domain proteins can be classified into three principal families according to their modular organisation (Fig.1.11). The first family contains proteins consisting

entirely of PDZ domains. The number of PDZ domains can vary from two (Na^+/H^+ exchanger regulatory factor) to more than ten in certain proteins (Nourry et al., 2003). The MAGUKs (membrane associated guanylate kinases, including PSD-95, Dlg, and ZO-1), which contain PDZ domains (one or three), one SH3 domain, and a guanylate kinase domain (GuK) comprise a second family (Nourry et al., 2003). The third family encompasses proteins that contain PDZ domains as well as other protein domains, such as ankyrin, LIM, L27, C2, PH, WW, DEP and LRR domains (Nourry et al., 2003). All of these proteins act as adaptors that hold receptors and signalling molecules in large molecular complexes. Other PDZ proteins do not serve as functional units (transducisomes) but serve as enzymes and as such, can directly participate in signalling events. PDZ proteins are often associated with cell adhesion molecules, G protein-coupled receptors, and receptor tyrosine kinases (Nourry et al., 2003).

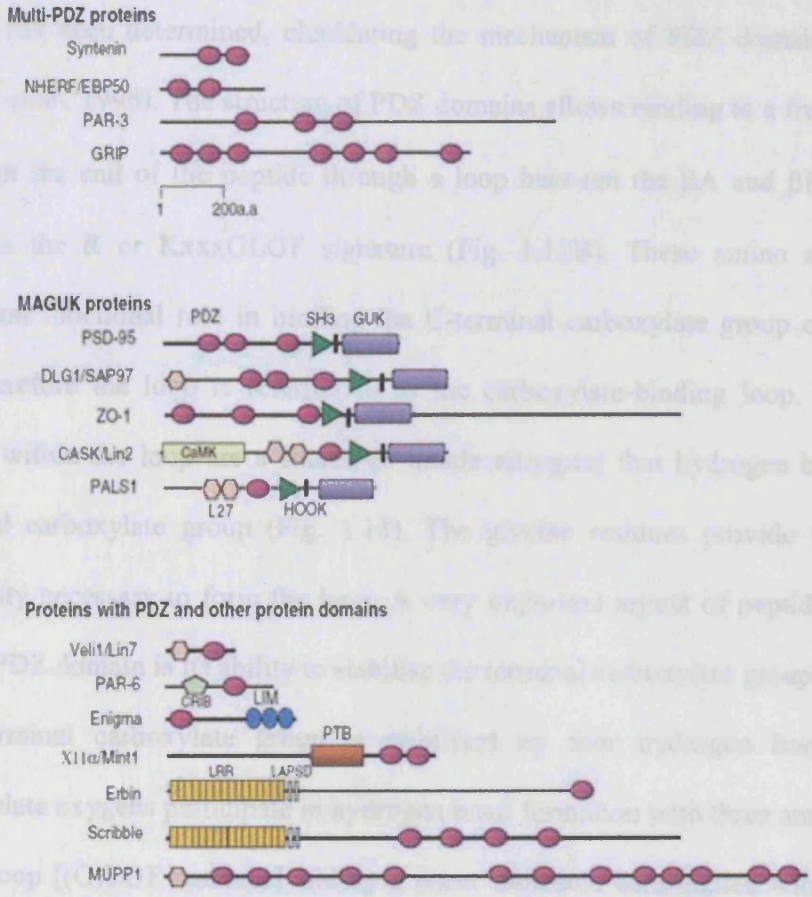


Figure 1.11: PDZ domain proteins classified according to their modular organization. (*Picture taken from Nourry et al., 2003)*

6.1 Structure and Binding specificities of PDZ domains

PDZ domains are small peptides of 80 to 90 amino acids length. The secondary structure forms six β strands (βA to βF) and two α-helices, αA and αB, arranged in a way so that N- and C-termini are next to each other thereby resulting in a spherical

shape (Nourry et al., 2003) (Fig.1.12A). The X-ray crystallographic structure of the third PDZ domain from PSD-95 in complex with and in the absence of its peptide ligand has been determined, elucidating the mechanism of PDZ domain interactions (Doyle et al., 1996). The structure of PDZ domains allows binding to a free carboxylate group at the end of the peptide through a loop between the βA and βB strands that contains the R or KxxxGLGF signature (Fig. 1.12B). These amino acids play an important functional role in binding the C-terminal carboxylate group of the peptide and therefore the loop is referred to as the carboxylate-binding loop. The residues GLGF within the loop are a source of amide nitrogens that hydrogen bond with the terminal carboxylate group (Fig. 1.13). The glycine residues provide the structural flexibility necessary to form the loop. A very important aspect of peptide recognition by the PDZ domain is its ability to stabilise the terminal carboxylate group of the target. The terminal carboxylate group is stabilised by four hydrogen bonds: the two carboxylate oxygens participate in hydrogen bond formation with three amide nitrogens in the loop [(G)LGF residues] and by a water molecule coordinated with the R or K charged residues (Fig. 1.13).

A

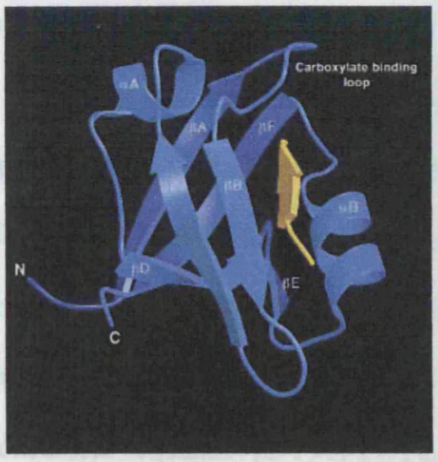
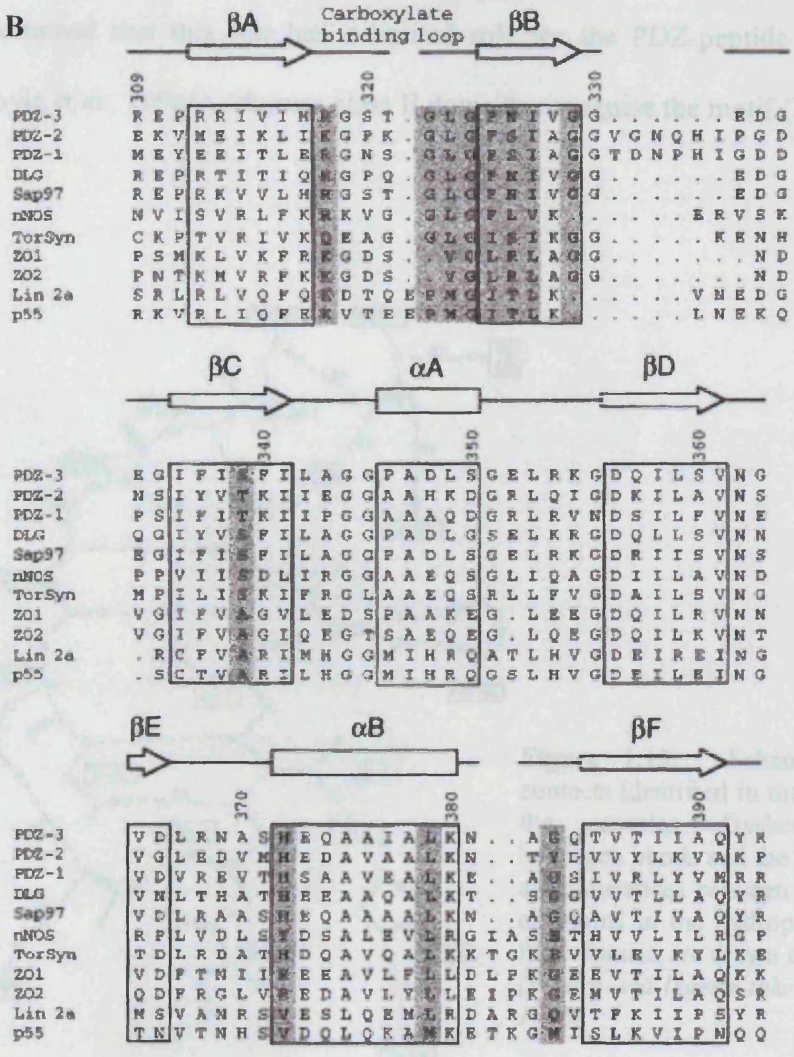


Figure 1.12: (A) Ribbon Diagram Showing the Three-Dimensional Fold of the PDZ-3 Domain from PSD-95 Corresponding to Residues 309-393. The peptide (yellow) inserts between the βB strand and the αB helix and forms an antiparallel β sheet with βB. The connecting loop between βA and βB is involved in binding the peptide C-terminus and therefore is designated the carboxylate-loop.
(B) Sequence alignment of Selected PDZ domains *(Pictures and legends taken from Doyle et al., 1996)*

B



The side chain of the C-terminal amino acid points into a large pocket formed by hydrophobic residues.

The PDZ domains have been classified into three classes according their specificity for C-terminal peptides. Songyang et al. (1997) using a degenerate peptide library determined which carboxyl-terminal residues were crucially required for protein interaction with the PDZ domain. They categorised PDZ domains into two classes on the basis of target sequence specificity. Class I domains bind to peptides with the consensus sequence (S/T)-X-(V/I/L) [X denoting any amino acid as it has been determined that this one has a limited role for the PDZ-peptide binding specificity (Doyle et al., 1996)]; whereas class II domains recognise the motif (F/Y)-X-(F/V/A).

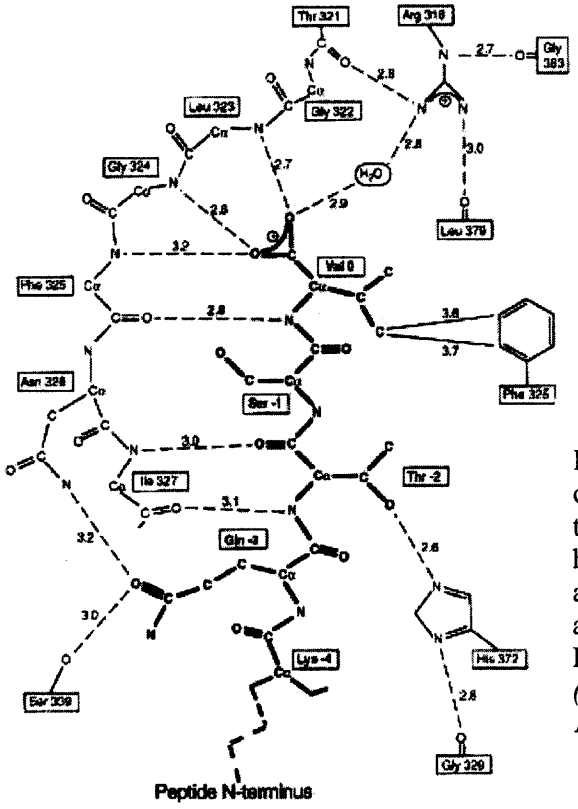


Figure 1.13: Schematic view of the contacts identified in the crystal structure of the complex. Dashed lines represent hydrogen bonds and the two closest atom-to-atom distances between Val0 side chain and all atoms in the hydrophobic pocket of the PDZ domain are drawn as solid black lines. *(Figure and legend taken from Doyle et al., 1996)*

Finally, there is Class III PDZ domains that prefer a DXV motif. PDZ domains participate in at least four different classes of interaction: recognition of carboxyl-terminal motifs in peptides, recognition of internal motifs in peptides, PDZ-PDZ dimerization, and recognition of lipids (Nourry et al., 2003) (Fig. 1.14).

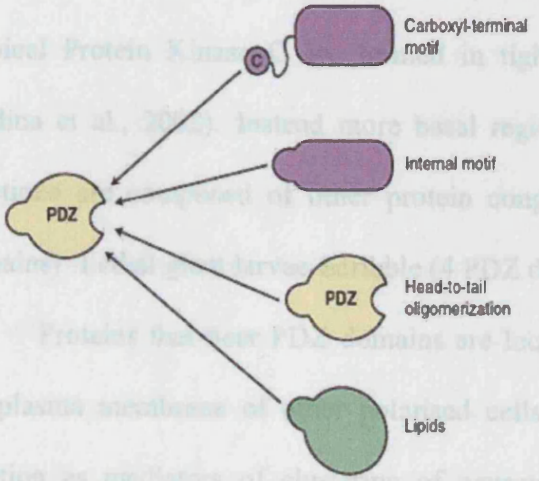


Figure 1.14: Possible PDZ interaction modes. PDZ domains participate in at least four different classes of interaction: recognition of carboxyl-terminal motifs in peptides, recognition of internal motifs in peptides, PDZ-PDZ dimerization, and recognition of lipids. (*Pictures taken from Nourry et al., 2003)*

6.2 Functions of PDZ Proteins

PDZ domains often serve as scaffolds of protein complexes at the plasma membrane. They are important in transporting and targeting of different proteins to the sites of cellular signalling thus assuring localisation and organisation of both receptors and downstream effectors to proper regions of the cell. For instance, proteins that bear PDZ domains together with proteins having different functional activities are involved

in asymmetric distribution of protein complexes necessary for epithelial polarity (Bilder et al., 2003; Tanentzapf and Tepass 2003).

Different regions of the cytocortex have characteristics specific to that area because they are composed of protein complexes which differ from area to area. For example various protein complexes such as Crumbs-Stardust (1 PDZ domain)-Discs Lost (4 PDZ domains), and Cdc42-Par6 (1 PDZ domain)-Bazooka (3 PDZ domains)-atypical Protein Kinase C are formed in tight junctions (Doe, 2001; Ohno, 2001; Medina et al., 2002). Instead more basal regions of the cell membrane like septate junctions are composed of other protein congregates such as Discs Large (3 PDZ domains) -Lethal giant larvae-Scribble (4 PDZ domains) (Bilder et al., 2000).

Proteins that bear PDZ domains are localised at specific subcellular sites near the plasma membrane of other polarised cells as well such as neurons where they function as mediators of clustering of neurotransmitter receptors and ion channels (Sheng and Sala, 2001).

PDZ domains are very important for the assembly of protein complexes and therefore for building networks necessary for the cross-talk between molecules. An interesting feature of the PDZ domains is that they are versatile in choosing their partners being able to form heterodimers or to interact either with a Carboxy-terminal motif or with an internal one, or even with lipids. Their function is also quite unpredictable as they can act as localised scaffolds but also as mediators of the trafficking of their binding targets; however what characterises most PDZ domains is that most of them are localised to highly restricted regions of the cytocortex (Ponting et al., 1997). Despite the fact they are widespread it is still unclear how they are localised

to microdomains of the cell membrane and how they discriminate among the many possible binding partners.

6.3 Diseases involving PDZ proteins

PDZ proteins have a central role during development. For instance loss of PDZ containing protein Shroom that binds to actin and localises in the adherens junctions, causes failure of the neural tube to close, leading to exencephaly, acrania, and spina bifida in mice (Hildebrand, and Soriano, 1999). Disruption of *cask* or *dlg*, two PDZ proteins involved in epithelial polarity, lead to craniofacial dysmorphogenesis (Laverty, and Wilson, 1998; Caruana and Bernstein, 2001).

Since PDZ domains play an important role in maintaining tissue integrity it is implied that their disruption would cause an effect on signalling pathways or on the cytoskeleton leading to cancers. For example mutated function of Scribble, another protein for epithelial polarity, in *Drosophila* affects not only cell polarity but also causes cell proliferation and tumorigenesis in imaginal discs (Bilder et al., 2000). In addition *Scribble* mutant mice develop severe neural tube defects (Murdoch et al., 2003). Another PDZ domain-containing protein implicated in cancer is Syntenin which promotes cell migration in metastatic human breast and gastric cancer lines (Koo et al., 2002). It has been shown that expression levels of syntenin correlate with invasive and metastatic potential in these cell lines. Moreover, syntenin-transfected cells migrated more actively, showing cell surface extensions, suggestive of an effect on the actin cytoskeleton (Koo et al., 2002).

To summarise, PDZ domains are involved in tumorigenesis, cell migration and metastasis as various PDZ containing proteins participate in cytoskeletal reorganisation in cancer. Therefore understanding how PDZ domains assemble and regulate protein networks will also help in drug design for therapeutic reasons.

6.4 Summary

Proteins that bear PDZ domains are often localised at specific subcellular sites near the plasma membrane of polarised cells, such as epithelial and endothelial cells and neurons. Among the many functions that PDZ proteins can have, very often they play a central role in establishing and maintaining epithelial polarity. Thus, PDZ proteins can serve as a hub of different protein complexes that convey signals from cell surface molecules to the interior, participating in signalling cascades and construction of the cytoarchitecture. This is a study of the function of the PDZ domain of DRhoGEF2 as well as an analysis of its interaction with a novel protein called DMec2.

7. MEC2

The *Drosophila* Mec-2 has yet to be characterised and its function remains unknown. It is homologous to the *C. elegans* Mec-2 and the human protein called stomatin. Stomatin was originally isolated from erythrocyte membranes and it was shown to form mainly dimers and a small amount of higher oligomers (Snyers et al., 1998; Hiebl-Dirschmied et al., 1991). This protein is apparently absent from the red cell membrane of patients suffering from overhydrated hereditary stomatocytosis, a form of

autosomal dominant haemolytic anemia (Stewart and Argent, 1992; Stewart et al., 1993; Stewart, 1997). Stomatin has been associated with cell membrane microdomains, called lipid rafts (Mairhofer M., et al., 2002; Snyers et al., 1999). Lipid rafts are detergent-resistant, low density regions of the membrane that are thought to be important in sequestering protein complexes (Moffett, et al., 2000; Hooper, 1999). In addition, these microdomains are relatively rich in cholesterol, and sphingolipids. Stomatin is postulated to play a role similar to that of caveolin, i.e. to regulate the formation and maintenance of membrane domains. Stomatin and caveolin have been shown to share structural similarities (Hooper, 1999; Tavernarakis et al., 1999; Moffett, et al., 2000).

There is also a *C. elegans* orthologue of stomatin called Mec2 involved in modulation of ion channels. Saturation genetic screens in *C. elegans* for touch-insensitive mutants have identified several genes needed for the function of the touch receptor neurons. Four genes (the stomatin-like protein MEC-2, MEC-4, MEC-10, and the paraoxonase-like protein MEC-6) encode membrane-associated proteins that interact with each other and form an amiloride-sensitive sodium channel complex (Goodman et al., 2002; Chelur et al., 2002). The central portion of the integral membrane protein MEC-2 contains a stomatin-like region with 64% identity to the human stomatin that is highly conserved from bacteria to mammals. MEC-2 interacts with the MEC-4 subunit of the degenerin channel (DEG/ENa$^+$ channel) through its stomatin-like region, which therefore acts as a protein binding domain; this binding allows non-stomatin domains of MEC-2 to regulate channel activity (Zhang et al., 2004). The sequences that lie N- and C-terminal to the stomatin-like region are unique

to MEC-2 with the specific C-terminal domain being required for self association. Two other proteins needed for touch neuron function, the α-tubulin MEC-12, and the β-tubulin MEC-7, are needed to form touch neuron-specific 15-filament microtubules (García-Añoveros and Corey, 1997). Additionally, MEC-5, MEC-1 and MEC-9 are components of the extracellular matrix (Ernstrom and Chalfie, 2002).

All these components are thought to form the mechanotransduction machinery in *C. elegans* involved in the transduction of external forces to the interior of the cell. This working model implicates the involvement of an ion channel that opens or closes in response to the movement of the extracellular matrix and the microtubule network relative to each other. The link in *C. elegans* that is thought to relay the consequent deflection of the membrane to the microtubule network, or vice versa, is Mec-2 (Fig. 1.15).

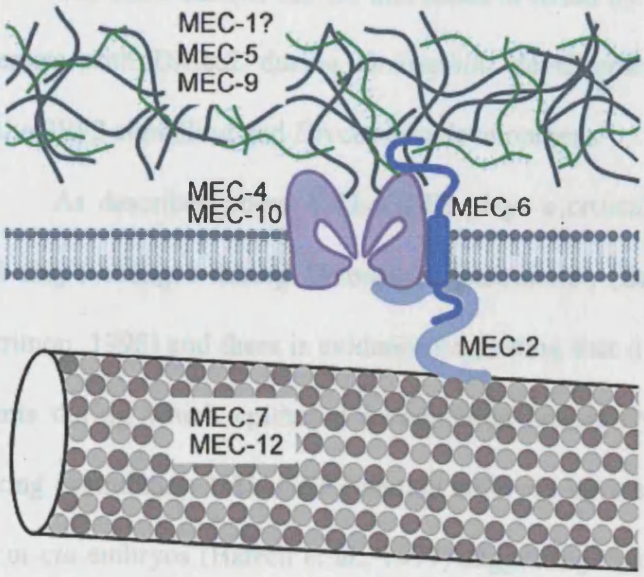


Figure 1.15: Schematic representation of the mechanotransduction machinery in *C. elegans* composed of ECM components, an ion channel, and the microtubule network. *(Picture taken from Sukharey and Corey, 2004)*

Little is known about the molecular mechanism of mechanotransduction which is of great importance for a variety of processes in a diverse array of tissues. Apart from touch sensation, mechanotransduction is also involved in numerous other biological processes including hearing, and shear stress in vascular endothelial cells. Many questions arise regarding the involvement of forces at the cellular level. Can the mechanism that underlies the transduction of mechanical cues be similar whether these regard intrinsic or extrinsic forces? Can it consist of the same components? Can we study mechanotransduction using as a model the process of epithelial invagination? Finally, is it possible that DMec-2 is relaying mechanical information to modify the cortical actin cytoskeleton?

8. HYPOTHESIS AND AIMS OF THE THESIS

The work carried out for this thesis is based on the hypothesis that DRhoGEF2 interacts with DMec2 during *Drosophila* development and this interaction affects DRhoGEF2 signalling and *Drosophila* development.

As described above DRhoGEF2 plays a critical role for the well orchestrated cell shape changes during *Drosophila* gastrulation (Barrett et al., 1997; Häcker and Perrimon, 1998) and there is evidence suggesting that it is used in other morphogenetic events during which epithelial invagination occurs (Nikolaidou and Barrett, 2004). During gastrulation *DRhoGEF2* mutant embryos have a much stronger phenotype than *fog* or *cta* embryos (Barrett et al., 1997) suggesting that there are other signals feeding into or out of DRhoGEF2. DRhoGEF2 has multiple domains whose function is more or

less known or inferred from other studies. However, there are no available data regarding the function of its PDZ domain. Preliminary yeast two-hybrid data suggest that DRhoGEF2 may interact via its PDZ domain with DMec2. This interaction could provide the link between mechanotransduction and Rho signalling pathway. All these factors make the interaction between DMec2 and DRhoGEF2 a very interesting one to study.

In order to prove or disprove the hypothesis set above, this study looks to achieve two main aims. The first is to characterise the function of DMec2 in *Drosophila* development and the second is to provide a better understanding of the relationship between DRhoGEF2 and DMec2.

This work is divided in three areas:

1) Analysis of the role of the PDZ domain for the function of DRhoGEF2 (Chapter 3).
2) Analysis of DMec2 loss and overexpression (Chapter 4).
3) Analysis of DMec2 interaction with DRhoGEF2 (Chapter 5).

II. MATERIALS AND METHODS

Chapter 2:MATERIALS AND METHODS

1. BIOCHEMISTRY

1.1 Protein Expression and Purification

The wild type PDZ domain of DRhoGEF2 and a mutated form of it were obtained separately as recombinant proteins expressed in *Escherichia coli*. For the mutated PDZ the amino acids tyrosine and methionine in the inside of the binding pocket were changed for a leucine and a phenylalanine respectively by site-directed mutagenesis to alter the binding affinity for the substrate. Using the pET cloning system, a fragment encoding the above mentioned PDZ domains was digested from pBluescript II SK(+) (Stratagene) and subcloned into the pET28c (Novagen) between *SmaI* and *NotI* sites for PDZ wild type and mutated form and between *EcoRI* and *NotI* for the Discs Large PDZ domain, to create an N-terminal fusion with a His-tag sequence and T7-tag sequence. The verified fusion construct was then transformed into BL21 (DE3) *E.coli* strain (Stratagene) and the bacterial cultures were in Luria-Bertani medium supplemented with 100μg ml^{-1} ampicillin. The cultures for the wild type PDZ domain and the PDZ domain of Discs Large were incubated overnight at 37^0C, instead the culture for the mutated form of PDZ was incubated at 30^0C. The following day the overnight cultures were diluted 1:50 and let seed for 2 hours. Protein expression was induced at OD_{600}= 0.4-0.6 with 0.5 mM isopropylthiogalactopyranoside (IPTG) and the cultures were incubated for 2 hours at 37^0C. Cells were harvested by centrifugation at 10000xg for 10min and the pellet was immediately used for protein purification. The

pellet was resuspended in BugBuster Protein Extraction Reagent (Novagen) at room temperature. Benzonase Nuclease was added and the cell suspension was incubated on a shaking platform for 20min at room temperature. The resulting cell lysate was centrifuged at 16000xg for 20min at 4^0C to remove insoluble cell debris. The subsequent steps were followed for the wild type PDZ domain as well as for its mutated form. The supernatant containing the soluble fraction, was applied to Ni-NTA agarose column (Novagen) pre-equilibrated with 1X Binding buffer (8X= 4M NaCl, 160mM Tris-HCl, 40mM imidazole, pH 7.9). Binding of the soluble His-tag-PDZ fusion protein to the matrix was achieved by gently rocking the column and incubating for 5min after this time centrifugation followed at 1000x g. The flowthrough fraction was collected and the matrix was washed extensively with 1X Binding buffer and subsequently with 1X Washing Buffer (8X= 4M NaCl, 160mM Tris-HCl, 480mM imidazole pH 7.9). The His-6-PDZ fusion was released with elution buffer (4X= 2M NaCl, 80mM Tris-HCl, 4M imidazole pH 7.9). The purified proteins were analyzed by SDS-PAGE. Pure PDZ appeared on the gel as a single band corresponding to a molecular weight of approximately 30KDa, in agreement with the calculated value of 23KDa. The PDZ domain of Discs Large was found in the inclusion body fraction. The inclusion bodies were purified under denaturing conditions at room temperature. The inclusion body fraction was solubilized in 1X Binding buffer including 6M urea. The recombinant protein was the dialyzed against 1X Dialysis Buffer (50X= 1M Tris-HCl, pH 8.5) to allow its refolding to occur. The purified protein was analyzed by SDS-PAGE. Pure Discs Large PDZ domain appeared on the gel as a single band corresponding to a

molecular weight of approximately 30KDa, in agreement with the calculated value of 23KDa.

1.2 Protein extraction from adult flies eyes

Thirty flies (15 males and 15 females) of each transgenic line were collected and put in an eppendorff tube on dry ice for 10 mins. The flies were tipped on the pad under the dissecting microscope and decapitated. The heads were immediately put on dry ice and lysed in 40μl of modified RIPA buffer (150mM NaCl; 50mM NaF; 20mM HEPES, pH 8; 1% Triton X-100; 0.1% SDS; 0.5% sodium deoxycholate) containing a cocktail of protease inhibitors (Roche) in 1:1000 dilution. After lysis the eyes were left on ice for 10 minutes and then centrifuged at maximum speed for 5 min, at 4^0C.
25μl of the supernatant were removed into a clean eppendorff tube and to it 7μl of 5x sample buffer were added. The sample was boiled at 98^0C for 2 min and run on the gel for the western blot analysis.

1.3 Bradford method

BioRad Protein Assay solution was diluted 1:5 with ddH_2O and 1ml of that was added to2 μl of each sample in plastic cuvettes (10x4x45mm from Starsted). The colour intensity was measured using a spectrophotometer (Varian CARY 50 Bio UV-visible) at 580nm and the Bradford Assay Software. The program creates a linear curve from the standards and calculates the total protein concentration of each sample.
The results were then compared with the standard curve. The BioRad Protein Assay solution is a dye reagent concentrate, containing coomassie brilliant blue G-250 dye

that changes colour on binding to the protein. The protein used as a concentration standard was 0.1% Bovine Serum Albumin (BSA).

1.4 Co-Immunoprecipitation Experiments

SR+ or S2 cells were washed three times with ice-cold PBS (Gibco) and were lysed on ice with IP buffer (NP-40 1%; Tris 25mM, pH=7.4; NaCl 150mM) containing a 1:1000 dilution of a cocktail of protease inhibitors (Roche). Nuclei were discarded after centrifugation at 10,000g for 10 min. Sepharose G-beads that have been previously washed three times with PBS were incubated for 1 hour at 4^0C with T7-antibody (Novagen) and mixed with recombinant protein. Lysates were incubated for 2 h at 4^0C with the beads. Immunoprecipitates were collected and washed with the IP buffer containing a cocktail of protease inhibitors. Immunoprecipitated proteins were eluted with SDS sample buffer and were analysed by 10% SDS-PAGE.

1.5 SDS-PAGE

SDS-PAGE was performed on the immunoprecipitated proteins and cell lysates. For Western blotting, samples were transferred to Immobilon (Millipore) by the wet method. Following the transfer the membrane was blocked with 5% non-fat dry milk in TBS Tween buffer (10mM TRIS, pH = 8; 150mM NaCl; 0.1% Tween) for 1 h at 37 ^{0}C and incubated with primary antibody anti-c-myc(9E10) (Santa Cruz Biotechnology, Inc.) at 1:1000 dilution or with anti-T7 (Novagen), at 1:5000 dilution in 5% non-fat dry milk in TBS Tween overnight at 4^o on shaking platform. Antibody binding was detected using the ECL Western blot detection system (Amersham Corp.)

2. MOLECULAR BIOLOGY

2.1 5'RACE

To obtain the 5' end of the DMec2 cDNA 5' RACE PCR (Smart Race, Clonetech) was carried out. The oligonucleotides Race_out and Race_in and Race_2nd (Table 2.1) were used in 35 rounds of PCR to amplify the gene. The RACE product was characterized by cloning the fragment directly into pCR-Blunt cloning vector (Invitrogen) and transforming into TOP10 Cells (Invitrogen). Different independent clones were picked for diagnostic digest. Once the clone containing the insert was identified, it was sequenced. Database searches were performed using the BLAST network server and confirmed that we had the full-length mec-2 cDNA.

2.2 Generation of DMec2 Constructs

cDNAs encoding DMec-2_{myc} were generated by PCR using the mec-2 cDNA as template. Five different constructs of mec-2 tagged with myc were constructed. In DMec-2_{myc} the myc epitope (EQKLISEEDL) was inserted at the amino or carboxy-terminus by PCR. The forward primers N1-N4 (Table 2.1) and the reverse primer N_rev (Table 2.1) were designed for the generation of the amino-tagged constructs. For the carboxy-terminus the forward primer C_for (Table 2.1) was designed while the reverse primer was C_rev (Table 2.1). A *Sal I* cloning site and a translational initiation sequence were introduced by the primers immediately upstream of the epitope myc tag sequence for the amino tagged contstructs. The resulting products for the amino-terminus were cloned into the *Sal* I and the *Nde* I site of a pBluescript SK I (Stratagene) plasmid with already cloned full length MEC-2. The carboxy-terminus product was

cloned into a *MscI* and *NotI* sites of pBluescript SK I plasmid with already cloned full length MEC-2. The authenticity of all contructs was subsequently confirmed by sequencing. The tagged constructs were subsequently cloned between *KpnI* and *NotI* sites of pcDNA3 (Invitrogen) and finally subcloned into pUAST vector between *KpnI* and *XbaI* for expression in *Drosophila* cells using the UAS/GAL4 system.

The point mutations *T348A* [codon 348 mutated to *GCC* using primer Mut1(T-A) (forward primer) and Mut1/2 (reverse primer), Table 2.1) and the *L350A* [codon 350 mutated to *CCA* using primer Mut2(L-A) (forward primer) and Mut1/2 (reverse primer), Table 2.1)] were introduced by PCR with Pfu polymerase (Stratagene) in order to generate two mutant forms of DMec2 subcloned into pUAST vector using the cloning strategy as described above. That the desired mutations occurred was checked by sequencing.

Table 2.1 Primers used for the generation of DMec2 constructs

Sequence	Name
GGCGATGTGATGATGAAG	Race_out
GACCCGTGACCCCAAAGCAGAA	Race_in
GTGACCACCCATTCCATG	Race_2nd
GAGCGTCGACAAAATGGAACAAAAACTCATCTCAGAAGAGG ATCTGGAGCCGCACCAGGATTCG	N1
GAGCGTCGACAAAATGGAACAAAAACTCATCTCAGAAGAGG ATCTGCGCAACTCTGGGCCGGCC	N2
GAGCGTCGACAAAATGGAACAAAAACTCATCTCAGAAGAGG ATCTGGTCAACATGGGCGCCGCC	N3
GAGCGTCGACAAAATGGAACAAAAACTCATCTCAGAAGAGG ATCTGGGCGCCGCCGGCATGGCA	N4
GCCGGAAGATGATCGCCCGC	N_rev
CCGTATTTGGCCAAATATGC	C_for

GACCGGCAGCGGCCGCTCACAGATCCTCTTCTGAGATGAGTT TTTGTTCTAGGTTGGTTTTTCGGCCA	C_rev
CGCCCTAGATTCAAATCATAGGTTGGCTTTCGGCCAAGCGTC CAGTGCG	Mut1 (T-A)
CGCCCTAGATTCAAATCATGGGTTGGTTTTCGGCCAAGCGTCC AGTGCG	Mut2 (L-A)
GCCCATGGAGCTGCTGACTCCGTATTTGGCCAAATATG	Mut1/2

2.3 Inverse PCR for Recovery of sequences flanking *piggyBac* elements

Fly genomic DNA was recovered from 30 flies and ground in 400 µl of Buffer A (100mM Tris-HCl, pH= 7.5; 100mM EDTA; 100mM NaCl; 0.5%SDS). The solution was incubated at 65^{0}C for 30 min and subsequently 800µl LiCl/KAc solution (2.5v:1v) was added. The new solution was incubated for 15min at RT and then spun for 15 min. 1ml of supernatant was transferred into a new tube and respun for 10min. The clean supernatant was transferred into a new tube and 600µl of isopropanol were added, mixed and the solution was spun for 15 min at maximum speed. The supernatant was aspirated away, pulsed, aspirated, washed with 70% ethanol and let to dry. The pellet was resuspended in 150µl TE and stored at -20^{0}C.

Separate digestions with Sau3A I (Promega) and HinP1 I (New England Biolabs) followed and the digested DNA was ligated with T4 ligase (New England Biolabs).

The ligated DNA was used for the inverse PCR.

10µl of ligated DNA were used for the first round of PCR together with 2mM each dNTP, 1µM forward primer, 1µM reverse primer, 1x Taq Buffer, 2 units Taq polymerase (Sigma) and ddH_2O. Run on the following PCR program:

1) 95^{0}C for 5min

2) 95^0C for 30 sec
55^0C for 1min
72^0C for 2 min
Go to 2x 34
3) 72^0C for 10min

The first PCR product was diluted 1:10 and the second PCR round followed using the same program as above with the second round primers. Purified second round PCR product was cloned into pGEM-T easy vector (Promega) in a 3:1 insert to vector ratio.

Table 2.2 Primers used for Inverse PCR on *piggyBac* DMec2 lines

Sequence	Name
GACGCATGATTATCTTTTACGTGAC	5F1
TGACACTTACCGCATTGACA	5R1
GCGATGACGAGCTTGTTGGTG	5F2
TCCAAGCGGCGACTGAGATG	5R2
CAACATGACTGTTTTTAAAGTACAAA	3F1
GTGAGAAACAACTTTGGCACATATC	3R1
CCTCGATATACAGACCGATAAAAC	3F2
TGCATTTGCCTTTCGCCTTAT	3R2

2.4 Screening by PCR EMS and X-ray treated flies

Four Ethylmethane Sulfonate (EMS) –induced mutant lines of DRhoGEF2 and six X-ray induced mutant lines of DRhoGEF2 were screened by PCR for mutation in the PDZ domain. First, the PCR conditions were set using as a template genomic DNA from wild type flies. For this three positive controls were used: i) genomic DNA with primers map6 and map 7, ii) genomic DNA with control primers, iii) plasmid DNA with experimental primers (EXSEQ1-5 and New1 and New2) (Table2.3). For the screening, DNA was extracted from stage 15-16 homozygous embryos as selected for the absence of GFP expression. In order to verify that no heterozygous, GFP expressing

embryos were selected by mistake GFP primers (Table 2.3) were used as controls. The purified PCR product was cloned into pGEM-T easy vector (Promega) and sent for sequencing (MWG, Biotech).

Table 2.3 Primers used to screen EMS and X-ray treated flies

Sequence	Name	Exons covered
GCTGCCAGATATCAAGATG	EXSEQ1	3,4
CCAAATCTCCTCAGACCA	EXSEQ2	4,5
GTAGCCTCAATCTGACTC	EXSEQ3	4,5
GCTTAATGAGCCTGTGCA	EXSEQ4	6,7
ATGGAGATTTCGCCTTCG	EXSEQ5	6,7
GGTGTCCGCTCTGACCCATTAGTATAG	Map6	9,10,11
GCGCAAGCCATACATATTCCAATGCC	Map7	9.10,11
CCTCTCATAGATGATTGAGCTGGCAATCCGC	New1	3,4
ACGCCTCGAGAAACCGCTACCTAGAAATCCCC	New2	4,5
GGAGTGGTCCCAGTTCTTGTT	GFP_for	N/A
TCTGGTAAAAGGACAGGGCCAT	GFP_rev	N/A

3. CELL BIOLOGY

3.1 Cell transfections and Fixation

S2R+ cells were maintained in Schneider's Drosophila medium containing 10% fetal calf serum (FCS), penicillin and streptomycin from Invitrogen (Carlsbad, CA). For transfections cells were seeded at 10^6 cells ml^{-1}. Transfection reagents Fugene (Roche Molecular) or Cellfectin (Invitrogen) were used at a concentration of 3 µl μg^{-1} or 10 µl μg^{-1} respectively of plasmid DNA (0.5µg). Transfections took place after 24 hours, and were maintained in the Schneider's Drosophila Medium containing 10% fetal calf serum (FCS), penicillin and streptomycin for 3 days until fixation or co-immunoprecipitation experiments. Cellular extracts for immunoblotting were prepared

in lysis buffer (NP-40 1%, Tris 25mM, pH=7.4, NaCl 150mM). For immunofluorescence studies S2 cells were plated on glass bottom 35mm microwell slide (Nunc) coated with concanavalin A 0.5mg/ml and washed with acid. S2R+ cells were plated on the same slides that have been previously coated with fetal calf serum for 2 hours at 37 °C. The cells were then fixed with 4% paraformaldehyde (PFA). For immunofluorescence studies to visualise DRhoGEF2 and DRhoGEF2ΔPDZ localisation S2R+/S2 cells were rinsed in HL3 buffer (70mM NaCl, 5Mm KCl, 1.5mM $CaCl_2$, 20mM $MgCl_2$, 10Mm $NaHCO_3$, 5mM trehalose, 115Mm sucrose, and 5Mm HEPES [pH 7.2] and fixed for 10min with 10% paraformaldehyde (Sigma) in HL3 buffer; for experiments in which microtubules were visualized and Mec2 at microtubule plus ends, cells were fixed for 10 min in a prechilled mixture (to -80°) of 3.2% paraformaldehyde in methanol. This fixation protocol was essential to preserve microtubule tip association. The cells were then washed and permeabilized with 0.1% Triton X-100 in PBS (PBST), blocked in 5% normal goat serum in PBST, and treated with primary antibodies in the same solution for 1 hr.

3.2 Immunofluorescence

To visualize F-actin, the cells were plated on serum-coated slides for 1-2 hr before fixation in 4% paraformaldehyde in PBS for 20 min and stained them with TRITC-labeled phalloidin (Sigma) (Table 2.4). To visualize $DMec2_{myc}$ myc antibody (Santa Cruz Biotechnology Inc.) was used 1:150 for immunofluorescence (Table 2.4). DRhoGEF2 antibody was used 1:500 (Table 2.4). Anti α-tubulin antibody was used at

1:1000 (Table 2.4). Anti-clathrin (abcam) was used in 1:1000 dilution (Table 2.4). After being washed, the cells were stained with secondary antibodies (Alexa 488, Alexa 568, Alexa 350 at a dilution 1:250). After being washed in PBST, the cells were rinsed in distilled water and mounted in fuorescence mounting medium (Dako Cytomation, Carpinteria, CA).

Table 2.4 Antibodies used for western blots and immunofluorescence

Antigen	Source	Obtained from	Dilution
α-myc	Mouse	Santa Cruz Biotechnology	1:150 (immunostaining) 1:1000 (western)
α-tubulin	Rat	Zymed Lab. Inc	1:1000 (immunostaining)
α-tubulin	Mouse	Sigma	1:5000 (western)
α-T7	Mouse	Novagen	1:5000 (western)
α-RhoGEF2	Rabbit	Rogers, S.	1:100 (immunostaining)
α-clathrin	Mouse	Abcam Ltd.	1:1000 (immunostaining)
Phalloidin-TRITC	*Amanita phalloides*	Sigma	1:5000 (immunostaining)
α-stomatin	Rabbit	Stewart, G.W	1:1000(western)

3.3 RNAi experiment

Briefly $2x10^6$ cells in 10μl serum free Schneider's medium were added to 0.3μg ds RNA against DMec2 in an eppendorf tube, centrifuged at 1,200 rpm for 1min, then incubated at room temperature for 30 min before adding 300μl of more medium with serum and antibiotics. Cells were grown for 6 days at 23^0C before being harvested for microscopic analysis or western blotting. Controls cells without being treated with ds RNA were grown in parallel. For the microscopic analysis cells were fixed for 10min in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed twice in PBS with

0.1% PBST, stained overnight at 4^0C with anti-tubulin (DM1A; Sigma) or TRITC-phalloidin (Sigma) in PBST with 3% bovine serum albumin, stained for 10 min in PBS with secondary antibody (Alexa, 568) for the anti-tubulim treated cells and washed with PBS.

3.4 Shear Stress Experiment

Laminar shear stress was applied to cells for various times ranging from 5 to 20 min using parallel plate flow chambers set in series in a closed circulating system with 0% CO_2 at 37 ^{0}C. Parallel plate flow chambers were custom-made a Glaxo Wellcome, and circulation of the medium was produced by a peristaltic pump (Masterlex) calibrated to deliver a shear stress of 3 dyn/cm^2. The chambers were assembled as described previously (Houston et al., 1999). The level of shear stress chosen for the experiments corresponded to the physiological level of shear stress in venous vessels (Morawietz et al., 2000). Static controls were performed on cells not subjected to shear stress.

4. DEVELOPMENTAL BIOLOGY AND GENETICS

4.1 Fly husbandry

Males and females were crossed for each genetic experiment following the standard mating procedure (Ashburner 1989; Greenspan 1997). The fly lines were kept and used under the standard methods (Asburner 1989; Greenspan 1997). The fly food used was prepared by organic 210gr of molasses (Potter's) 32gr of Agar (Sigma), 210gr of cornmeal (William Lilco and Sons), 100gr of baker's yeast (Westwood

International), 90ml of Nipagin (p-hydroxybenzoic methyl acid ester from Sigma) and 32 ml of propionic acid (Sigma), for every 3L batch of food.

4.2 Preparation of egg-laying plates

375ml of ddH_2O and 11.25gr of agar (Sigma) were put into a 500ml pyrex beaker, and boiled in a microwave or an autoclave to dissolve the agar. In a 200ml conical pyrex flask 12.5gr of sucrose (Merck) and 125ml of organic apple juice (Tesco) were also brought to boil. Afterwards, the two solutions were mixed and microwaved for 5 minutes on high power. The mixture was then left to cool down to 50°C and poured into 60mm bacterial plates (Sarstedt). The plates were left to cool until their content solidified and then were put into plastic bags or wrapped in parafilm and stored at 4°C.

4.3 Preparation of yeast paste

Yeast paste was prepared by mixing granules of yeast (Westwood International) with tap water, almost in 1:1 weight to volume ratio, until the yeast granules were dissolved and the mixture became a paste. Yeast paste was kept at 4°C.

4.4 Collection and dechorination of embryos

Wine vinegar (Tesco) was spread by a brush on egg-laying plates and a small amount of yeast paste was applied to the plate. The plate was then placed on the opening of the cage, a 250ml aerated beaker containing flies. The flies laid their eggs

on the plate. After the appropriate collection time, the plate was replaced with a fresh one.

To remove the outer chorion layer of the embryo the embryos were placed in a small basket with a porous bottom and the basket was placed in a 50ml beaker containing 20ml of 50% sodium hypochlorite (BDH), which was then incubated at room temperature for exactly 2 minutes. Subsequently, the embryos were washed thoroughly with water.

4.5 Study of embryogenesis of four DRhoGEF2 transgenic lines

125 DRhoGEF2 transgenic embryos from 4 different lines were collected and placed only- agar egg-laying plates. The plates with the embryos were incubated at 25°C and scored every 24 hours for three days. The dead embryos, chorions and larvae that hatched from each plate were counted.

4.6 Balancers

Balancers allow maintenance of the mutation, viability of the line and provide a marker to identify in which of the two chromosomes of the homologous pair the mutation is carried (Ashburner 1989; Greenspan 1997). Balancers are chromosomes that have undergone significant rearrangements caused by big inversions in most regions of the chromosome that prevent recombination between homologous chromosomes (Ashburner 1989; Greenspan 1997). Balancer chromosomes also possess scorable, usually dominant, markers (Table 2.5)

Table 2.5 Balancer lines

Names	Genotype	Source
FM7c	y[1]arm[4]w[*]/FM7c, P{ry[+t7.2]=ftz/lacC}YH1	Bloomington 616
FM7d	P{ry[+t7.2]=ftz/lacC}YH1, oc[1]ptg[1]/C(1)DX, y[1]f[1]	Bloomington 5267
FM7i	P{ry[+t7.2]=ftz/lacC}YH1, w[+]/C(1)DX, y[1]f[1]	Bloomington 5438
FM6/CyO	RpS5a[2]/FM6;noc[Sco]1(2)37 Ca[4]pr[1]/CyO	Bloomington 438
FM7a/CyO	FM7a,1(1)TW24[1]/oc[1]ptg[3] 1(1)TW[cs];CyO/1(2)DTS91[1]	Bloomington 4558

4.7 Ectopic gene expression

Ectopic expression of genes is achieved by the use of transgenes. This transgene would carry the gene of interest under a specific promoter, (Table 2.6). The transgenic lines used here had the gene of interest under the control of the yeast Upstream Activation Sequence (UAS). Introduction of only this transgene into a fly is inert, unless the gene that activates the promoter, the yeast gene GAL4 is introduced, which is usually downstream of a fly tissue specific promoter. When the fly tissue specific promoter is activated, it activates the expression of the GAL4. GAL4 then binds to the UAS sequence of the second transgene and activates the expression of the gene of interest in the expression domain of the promoter of the GAL4 transgene (Table 2.6) (Brand and Perrimon 1993; Brand et al., 1994; Phelps and Brand, 1998).

Table 2.6 GAL4 lines

Name	Genotype	Comments
VP-16-V32	Mata4-Gal4 VP16V32Ap{w^+}	Maternal Gal4 driver
Eyeless	recessive	Eye Gal 4 driver
MS1096	X chromosome	Wing Gal driver
Tubulin	Tubulin-Gal4/TM3	Ubiquitous driver
Daughterless	recessive	Ubiquitous driver

4.8 *Drosophila* lines used

Table 2.7: Wild type, mutants DMec2 and DRhoGEF2 alleles

Name	Genotype	Comments	Source	Reference
OregoR	+/+		Sally Leevers	Lindsley and Zimm, 1992)
18428	w[118]PBac{w+[Mc]= WH}Mec2[f01352]	piggyBac insertion	Bloomington	unpublished
18965	w[118]PBac{w+[Mc]= WH}Mec2[f06342]	piggyBac insertion	Bloomington	unpublished
$RhoGEF2^{4.1}$	$RhoGEF2^{4.1}$/CyOftzlacZ	EMS allele Null	Kathy Barrett	Barrett et al., 1997
$RhoGEF2^{1.1}$	$RhoGEF2^{1.1}$/CyOftzlacZ	EMS allele Null	Kathy Barrett	Barrett et al., 1997
$RhoGEF2^{6.1}$	$RhoGEF2^{6.1}$/CyOftzlacZ	P-element excision	Kathy Barrett	Nikolaidou and Barrett, 2004

4.9 Wing mounting

Adult flies were collected and placed in SH buffer (3 parts ethanol and 1 part glycerol). Wings were removed by holding the fly from the thorax and pulling the wing gently from the hinge with No5 tweezers in SH buffer. The wings were then placed in water and rinsed twice, then placed in ethanol. 30µl of Euparal mountant (Agar) were

placed on a microscopic slide (BDH) for 8-12 wings. The tweezers were then placed underneath the wings, any drops of ethanol carried on the tweezers were removed by a tissue and the wings were placed in the Euparal drop on the slide. Wings that became folded were spread by the tweezers without damaging then and any bubbles in the mountant were removed. A glass coverslip (22x50mm or 18x24mm from BDH depending on the number of wings on the slide) was carefully placed on top of the wings to avoid making bubbles. The slide was then placed on a hot plate at 60^0C for 24 hours with a weight on top of the cover slip to keep the wings flat. The wings were then visualised and images were collected on a high resolution microscope.

4.10 Scanning Electron Microscopy (SEM) for adult eyes

- Preparation of the fly eyes

Females of the appropriate genotype were transferred to vials with fresh food without yeast and left for 24 hrs to clear their eyes. The eyes were checked with a dissecting microscope and they were placed in small glass vials in 25% ethanol. The flies were then dehydrated for 12 hours through ethanol series each of 25%, 50%, 75%, 100%. The wash in 100% ethanol was repeated twice. The flies can be kept in 100% ethanol for at least one month. The ethanol was then substituted by 100% amyl acetate (Sigma) and the flies were left in this solution overnight. The amyl acetate was then removed and replaced with fresh amyl acetate.

- Drying

After dehydration the eyes were dessicated in the critical point dryer with the help of Mark Turmaine in the electron microscopy suite of the Anatomy and Developmental Biology Department of the University College London.

Polypropylene containers (Agar) that were thoroughly cleaned were labeled with a diamond knife and placed in Petri dish containing amyl acetate. The flies were transferred to the containers, which were wiped gently and placed in the "boat". The metal grills were placed on the top of the containers in the boat and the boat placed in the critical point drier. Critical point drying was carried out according to the manufacturers' instructions.

- Mounting

The flies were mounted with the left eye uppermost using a dissecting microscope, on 12mm double sided sticky carbon tabs (Agar) on 0.5'' stubs (Agar). The eyes were arranged at the edge of the circular stub and looking upwards. Any dust on the eyes was carefully removed with a fine paint-brush. Mounted stubs were stored with silica gel in a vacuum.

- Gold coating

For SEMs the flies needed to be coated with gold. Six stubs were placed into the electrospray coater and manufacturer's instructions were followed.

- Collection of data

To collect the data usually two stubs of flies, maximum of four, were placed in the specific plates of the scanning electron microscope and a vacuum was generated inside the cylinder of the microscope. The brightness and the contrast were equilibrated in order to avoid charging and saturation. Using the microscope knobs, the fly eye

could be removed or placed at any angle to achieve the best field of view. All the eyes on the stub were observed (25-40 per genotype and photographs of the most representative eyes per genotype were taken.

4.11 Sectioning of adult eyes

- Fixation:

The dissected tissue was fixed into 0.5ml pre-cooled 2% glutaraldehyde in 0.1M PO_4 (0.2M Na_2HPO_4 and 0.2M NaH_2PO_4 in a 72/28 ratio respectively and then added the same volume of water).1ml of 1% osmium was added in 0.1M PO_4. The tissue was incubated on ice for 30 min. The glutaraldehyde/osmium mixture was removed as much as necessary so that the tissue was still covered. The tissue was washed with cold PO_4. The tube was filled with PO_4. The PO_4 was removed, leaving enough to keep the tissue covered, replaced with 0.5ml of osmium buffer and incubated for 2 hours on ice.

- Dehydration:

The tissue was rinsed with 0.1M PO_4 and dehydrated by adding on ice 30% ethanol for 10 min (the tissue remained covered with liquid at all times). Dehydration by ethanol series followed as described below:

50% ethanol for 10min

70% ethanol for 10 min

90% ethanol for 10min

100% ethanol for 10 min twice.

The ethanol was replaced with propylene oxide for 10 min at room temperature, twice. An equal volume of Durcupan resin (soft resin ~100ml= Resin A: 54gr,

Hardener B: 44.5gr, Accelerator C: 2.5gr, Plastciser D: 10gr. Mixed thoroughly for a long time until the colour was homogeneous and there were no fiber like structures in the solution) to propylene oxide was added and mixed well. Incubated overnight at room temperature.

- Embedding:

The resin was placed in the moulds and one specimen per mould was added. The tissue was oriented so that a flat surface was resting on the bottom of the mould very close to the edge to be cut and in the right orientation. The resin was baked at 70^0C for exactly 36 hours.

- Sectioning:

The sectioning was performed in a microtome which was set to cut 1 micron sections. Sets of 10 sections were put into a separate drop of water on multispot, coated, microscope slides (C.A. Hendley Ltd). The water was dried from the slide on a heater set at >70^0C. The sections were stained in toluidine blue solution (toluidine blue mixed dissolved with borax to 1% final concentration for each in water. Filtered before use for 3 min. Visualised and photographed on high resolution microscope.

4.12 Genetic crosses for rescue experiment

For the rescue experiment the following crosses were set:

w; If/CyO;MKRS/TM6BHu x w;+; da-GAL4, w; If/+; da-GAL4/TM6BHu x w; CyO/+; da-GAL4/TM6BHu, w; If/CyO;da-GAL4, w;Sp/Cyoftz; Dr/TM3ftzlacZ x w; 6.1/CyO; MKRS/TM5BHu, w;6.1/CyOftz; Dr/TM6BHu, w; 6.1/CyOftz/Dr/TM6BHu x

w; If/Cyoftz; da-GAL4, w; 6.1/CyOftz; da-GAL4/TM6BHu x w; 6.1/CyOftz; da-GAL4/TM6BHu, w;6.1/CyOftz; Dr/da-GAL4 x w; 4.1/CyO; transgenic line.

w; 6.1/4.1; Dr/transgenic line was compared to w; 6.1/4.1; da-Gal4/transgenic line.

w; 4.1/CyO; MKRS/TM6BHu x w; CyO/Sp;transgenic line, w; 4.1/CyO; transgenic line/TM6BHu (inter se), w; 4.1/CyO/transgenic line (inter se), w; Sp/CyOftz; Dr/TM3ftz x w; 4.1/CyO; MKRS/TM6BHu, w; 4.1/CyOftz; Dr/TM6BHu (inter se), w; 4.1/CyOftz; Dr/TM6BHu x w; CyO/Sp; transgenic line, w; 6.1/CyO; da-GAL4 x w; 4.1/CyO; Dr/transgenic line.

w; 6.1/4.1; Dr/da-Gal4 was compared to w; 6.1/4.1;transgenic line/da-GAL4

w; If/CyO; MKRS/TM6BHu x w; +; tub-GAL4/TM3Sb, w; If/+; TM6BHu/tub-GAL4 x w; Cyo/+; TM6BHu/tub-GAL4, w; If/CyO; tub-GAL4/TM6BHu (inter se), w; 6.1/CyOftz; Dr/TM6BHu x w; If/CyO; tub-GAL4/TM6BHu, w; 6.1/CyO; Dr/tub-GAL4 x w; 4.1/CyO;transgenic line. w; 6.1/4.1; Dr/transgenic line was compared to w; 6.1/4.1; tub-GAL4/transgenic line.

w; 6.1/CyO; tub-GAL4/TM3 x w; 4.1/CyO; transgenic line/Dr

w; 6.1/CyO; tub-GAL4/+ was compared to w; 6.1/CyO; tub-GAL4/transgenic line

w; Sp/Cyoftz; Dr/TM3ftz x w; 1.1/CyO;+, w; 1.1/CyOftz; Dr/+ x w; 1.1/CyOftz; TM3ftz/+, w; 1.1/CyOftz; Dr/+ x w; 1.1/CyOftz; TM3ftz/+, w; 1.1/CyOftz; Dr/TM3ftz (inter se), w; 1.1/CyOftz; Dr/TM3ftz x w; If/CyO; da-GAL4, w; 1.1/CyO; da-GAL4 (inter se), w; 1.1/CyO; da-GAL4/TM3ftz x w; 1.1/CyO; da-GAL4/TM3ftz

w; 1.1/CyO; Dr/daGAL4 x w; 4.1/CyO; transgenic line

w; 1.1/4.1; Dr/transgenic line was compared to w; 1.1/4.1; da-GAL4/transgenic line

w; 1.1/CyO; da-GAL4 x w; 4.1/CyO; Dr/transgenic line w;1.1/4.1; da/GAL4/transgenic line was compared to w;1.1/4.1; da-GAL4/Dr

III. RESULTS CHAPTER 3

Chapter 3: STUDY OF THE FUNCTION OF THE PDZ DOMAIN

1. INTRODUCTION

Rho guanine exchange factors (GEFs) activate GTPases by catalyzing the exchange of GDP by GTP (Erickson and Cerione, 2004). Because GEFs interact directly with their effector GTPases (Snyder et al., 2000) Rho GEF localisation can determine the spatial pattern of GTPase activity (Gulli and Peter, 2001). This localization is regulated by mechanisms that are not the same for all members of the Rho GEF family, however, as these possess different domains that can interact with various proteins and phospholipids which can determine their targeting (Rossman et al., 2005).

For example, the Ras-specific GEF, Son-of-sevenless, appears to be regulated in part by recruitment to the plasma membrane via the binding of the adaptor protein, Grb2, to an activated receptor tyrosine kinase (Egan et al., 1993). Other GEFs utilise other regulatory mechanisms. Indeed, several previously described Rho GEFs can be activated by deletion of regions of the protein outside of the conserved Dbl domain, suggesting that GEF activity in these proteins is normally repressed (Whitehead et al., 1997). Moreover, most of the Rho GEF proteins contain various additional domains that have been implicated in signal transduction, suggesting that they may be regulated by diverse inputs (Whitehead et al., 1997). For example the GEF activity of the Vav protein is regulated both by diacylglycerol binding (Gulbins et al., 1994) and by direct tyrosine phosphorylation (Crespo et al., 1997).

There is evidence suggesting that DRhoGEF2 is used during epithelial invagination events such as gastrulation (Barrett, et al., 1997), salivary gland formation and imaginal disc folding (Nikolaidou and Barrett, 2004) and thus it is potentially responsible for selecting the outcome of Rho1 activation. However, it is not known how DRhoGEF2 function is regulated.

DRhoGEF2 is a multidomain protein of 297 kDa belonging to the Dbl family of oncogenes. As all members of the family possesses a Pleckstrin homology (PH) domain, adjacent and C-terminal to the Dbl homology (DH) domain (Häcker and Perrimon, 1998). The tandem DH-PH domains represent the structural module responsible for catalysing the GDP-GTP exchange reaction of Rho1 protein (Snyder et al., 2002). In addition, the PH domain is thought to serve as a membrane-targeting signal (Bottomley et al., 1998). DRhoGEF2 has also an RGS domain that, by homology to the mammalian and *C. elegans* pathway, is presumed to interact with the G_α subunit of a heterotrimeric G protein (Ross and Wilkie, 2000; Fukuhara et al., 2001), called Concertina (Cta) (Parks and Wieschaus, 1991). In its central region it contains a Phorbol Ester Binding (C1) domain, which is very similar to the C1 domain in Protein Kinase C (PKC) (Kang et al., 2006). In PKC this domain binds to phorbol ester Diacylglycerol (DAG) and activates the kinase (Benjamin, 2000). Similar to the PH domain, this motif could promote membrane association of DRhoGEF2 via lipid interaction and activation in response to specific signals. Lastly, at the amino-terminal, there is a PDZ domain whose function has not been characterized yet.

The PDZ domain is a widespread modular protein motif and has been implicated in the recruitment of signalling molecules to the plasma membrane by binding to the

carboxyl terminus of transmembrane receptors (Jeleń et al., 2003) or by forming heterodimers with other PDZ domain-containing proteins (Fan and Zhang, 2002). Therefore, the DRhoGEF2-PDZ domain might link the DRhoGEF2 to similar proteins present in invaginating tissues.

The presence of three different regulatory domains might provide a mechanism to activate DRhoGEF2 to different levels in response to different signals; for example, C1 or PH domain could be involved in keeping the cell shape throughout the epithelium while the PDZ domain could mediate discrete signals present locally in the invaginating tissues.

This chapter is an analysis of the functional role *in vivo* of the PDZ domain of DRhoGEF2. Studying the function of PDZ domain would provide a better insight into the Rho signalling pathway and a better understanding of the function and regulation of DRhoGEF2.

2. RESULTS

2.1 Looking for PDZ fly mutants

To address the function of the PDZ domain it was necessary to have mutant flies for this part of the protein in order to compare them with wild type flies. Therefore, in order to study the role of the PDZ domain for the function of DRhoGEF2, ten different lines of flies harboring ethyl methane sulfonate (EMS) and X-ray mutations (gift of H. Bellen, Baylor College of Medicine, Houston) were screened by PCR with the aim of finding mutations in the PDZ domain coding region. Flies that

harboured mutations and were balanced with a modified *CyO* balancer chromosome consisting of green fluorescent protein (GFP) driven indirectly by a Kruppel (Kr) promoter, via the yeast GAL4-UAS regulatory system (Casso et al., 2000), were used to isolate homozygous DNA. GFP fluorescence could be seen in embryos as early as the germ band extension stage (stages 9-12); expression faded markedly during germ band retraction (stages 13 and 14), but increased again at the end of embryogenesis, culminating in strong expression in the Bolvig's organs, and the posterior spiracles. In addition, GFP fluorescence was observed in macrophages which distributed in a punctuate pattern. Expression of GFP could also be seen in larvae, pupae, and adults. Homozygous potentially mutant progeny were scored using a Leica Fluorescent Dissecting Microscope with a long pass GFP Endow filter cube and selected by the absence of the marker at stage 15-16 for extraction of genomic DNA.

Firstly, PCR conditions had to be tested and optimised for every set of primers. The gene sequence, encoding the PDZ domain, spans from exon 3 to exon 6 (Fig. 3.1A). Four sets of primers giving a PCR product of 300-600bp, were designed to cover exons and intron-exon boundaries spanning from exon 3 to exon 6 in order to cover the genomic fragment encoding the PDZ domain. As a control to check the absence of contamination with the balancer chromosome, primers specific for the GFP sequence were used as well with the same DNA samples. PCR products with the GFP primers were absent confirming the purity of the DNA preparation. The PCR products were purified, cloned into pGEM-T Easy vector (Invitrogen) and sequenced (MWG, using primers T7 and T3). Sequencing of the coding region of the Drosophila RhoGEF2-PDZ locus from each of the ten available lines of flies treated with ethylmethane sulfonate

and X-ray did not show discrepancies between the generated sequence and the known DRhoGEF2 (accession number AF032870) (Fig.3.1A). Few point changes were observed in intron sequences. In conclusion, DNA sequence analysis of the EMS and X-ray treated flies revealed no mutation in the PDZ domain.

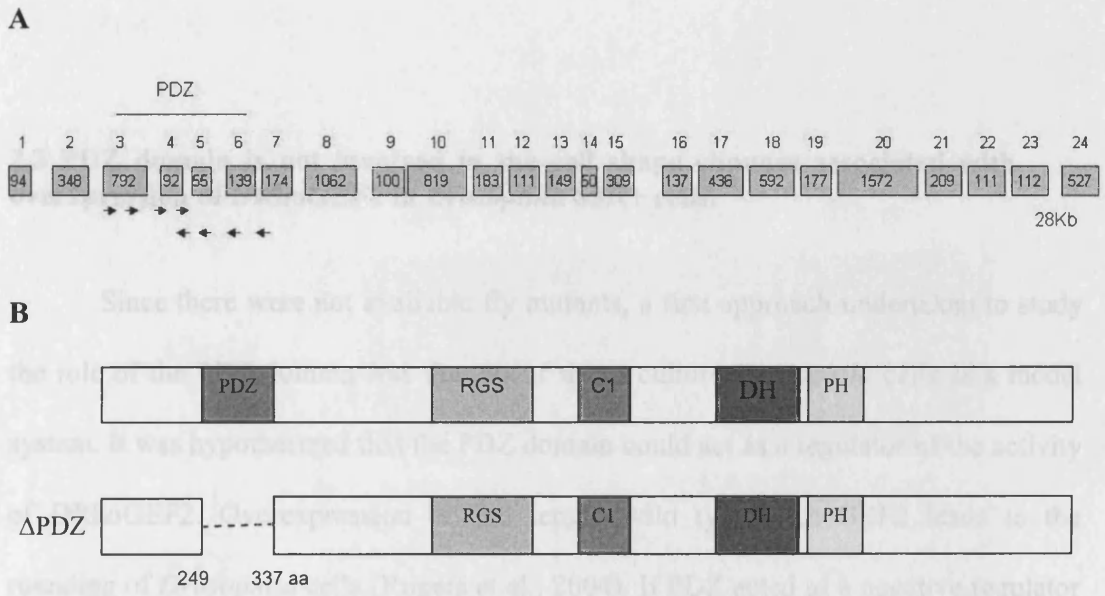


Figure 3.1: A) Schematic representation of DRhoGEF2 gene: bands indicate the exons, the number inside the band indicates the length of the exon in base pairs, the number on top of the bands indicates the number of exon, and the arrows indicate the primer pairs used for the sequencing of PDZ locus (encoded by exons 3-6). B) Schematic representation of the DRhoGEF2ΔPDZ construct.

Due to the lack of available fly mutants for the region of interest whose phenotypic analysis might have helped in the attribution of a functional role, a construct of DRhoGEF2 lacking the PDZ domain was generated (DRhoGEF2ΔPDZ) (S. Rahman, unpublished) (Fig. 3.1B). The construct DRhoGEF2ΔPDZ was generated by

excision of the entire PDZ domain from DRhoGEF2 cDNA and then subcloned into a UAS vector. In order to study the role of the PDZ domain for the function of DRhoGEF2, this construct was used for expression under the control of the GAL4/UAS expression system (Brand and Perrimon, 1993) in *Drosophila* cell lines as well as for the generation of transgenic flies.

2.2 PDZ domain is not involved in the cell shape changes associated with overxpression of DRhoGEF2 in *Drosophila* S2R+ cells.

Since there were not available fly mutants, a first approach undertaken to study the role of the PDZ domain was the use of tissue culture *Drosophila* cells as a model system. It was hypothesized that the PDZ domain could act as a regulator of the activity of DRhoGEF2. Overexpression of full length wild type DRhoGEF2 leads to the rounding of *Drosophila* cells (Rogers et al., 2004). If PDZ acted as a negative regulator of DRhoGEF2, overexpression of the DRhoGEF2ΔPDZ construct in S2R+ cells should cause enhanced cell rounding. On the contrary, if it acted as a positive regulator overexpression of DRhoGEF2ΔPDZ should not cause the observed phenotype. For this experiment, a set of S2R+ cells was transfected with expression vector for full length wild type DRhoGEF2 and another set with expression vector for the DRhoGEF2ΔPDZ construct, under the control of GAL4/UAS system. Cells for the two sets were plated at the same density (10^6 cells/cm^2), that is confluent upon plating. The cultures were seeded for 2 days. The cell density should not have been modified during the course of the study as these cells have been observed to divide every 2 days. The transfected cells

were identified with an antibody raised against DRhoGEF2. The actin cytoskeleton was visualized using TRITC-phalloidin. It was observed that wild type S2R+ cells spread well on the substrate and take up a flatten morphology upon plating on a plastic surface (Fig. 3.2A-C); in contrast cells overexpressing DRhoGEF2 were rounded (Fig. 3.2D-F). The same morphology was observed in cells overexpressing the DRhoGEF2ΔPDZ construct (Fig.3.2G-I).

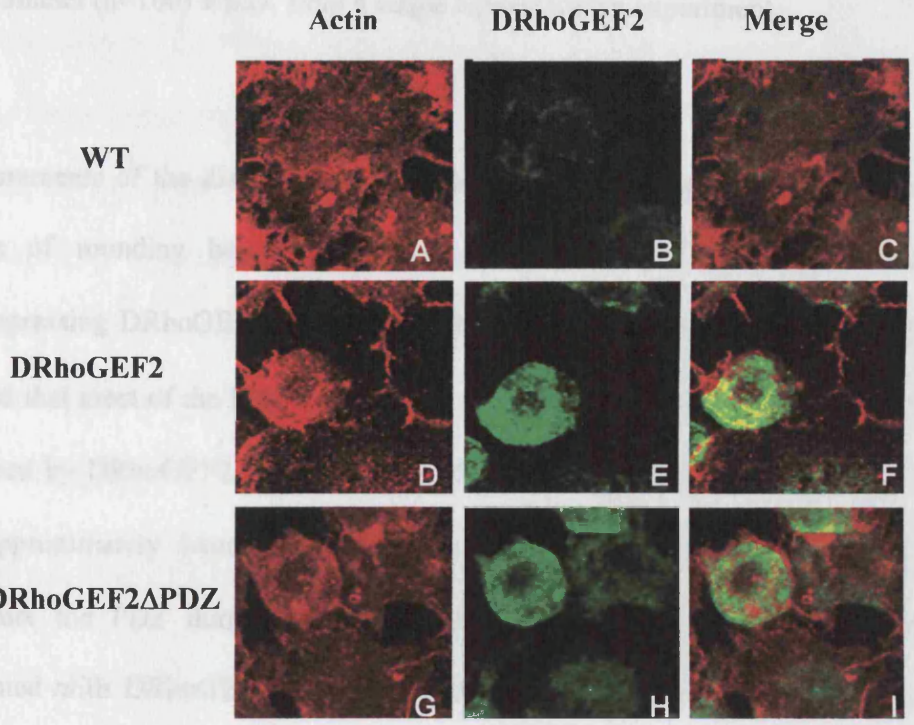


Figure 3.2: Overexpression of DRhoGEF2 and DRhoGEF2ΔPDZ in S2R+ cells. Panels A, D, G show the actin cytoskeleton (red) stained with TRITC-phalloidin. Panels B, E, H show the transfected cells (green) as stained with an antibody against DRhoGEF2. Panels C, F, I are a merge of the phalloidin with α-DRhoGEF2. Overexpression of DRhoGEF2 and DRhoGEF2ΔPDZ causes the cells to round up.

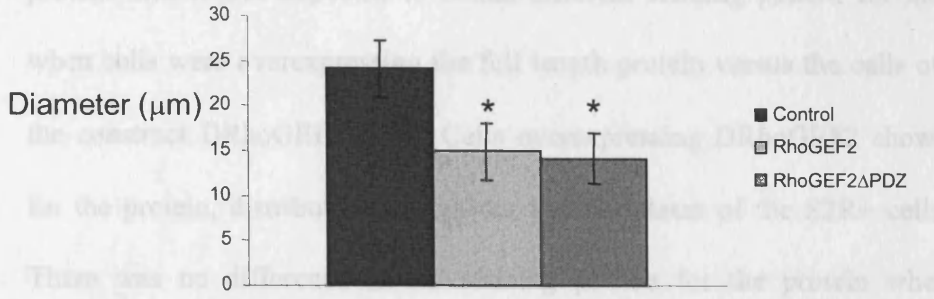


Figure 3.3: Quantification of cells with round shape after overexpression with DRhoGEF2 or DRhoGEF2ΔPDZ. Statistical significance was determined for the difference in cell diameter for cells overexpressing DRhoGEF2 or DRhoGEF2ΔPDZ compared to control by Student's *t* test where $P < 0.005$. The results show the average cell diameter (n=100) ± S.D. from a single representative experiment.

Measurements of the diameter of the transfected cells indicated no difference in the degree of rounding between the cells overexpressing DRhoGEF2 versus cells overexpressing DRhoGEF2ΔPDZ. Quantification of the observed phenotype (Fig.3.3) showed that most of the DRhoGEF2ΔPDZ expressing cells duplicated the morphology produced by DRhoGEF2 overexpression. The average cell diameter for both samples was approximately 14μm instead for the control cells was approximately 24μm. Therefore the PDZ domain does not enhance nor inhibit the unusual phenotype associated with DRhoGEF2 overexpression. The results show that the PDZ domain does not act as a regulator of the activity of DRhoGEF2 in this specific system used here.

It was then hypothesized that the PDZ domain is involved in the placing of DRhoGEF2 to the plasma membrane where it can interact with other partners to induce the cell shape changes. If the PDZ domain was important for the localization of the

protein then it was expected to obtain different staining pattern for the DRhoGEF2 when cells were overexpressing the full length protein versus the cells overexpressing the construct DRhoGEF2ΔPDZ. Cells overexpressing DRhoGEF2 showed a staining for the protein, distributed through out the cytoplasm of the S2R+ cells (Fig. 3.2E). There was no difference in the staining pattern for the protein when cells were transfected with DRhoGEF2ΔPDZ (Fig. 3.2H). Since overexpressed DRhoGEF2 is found abundantly in the cytoplasm it is not possible to assess what happened to the protein localization with the DRhoGEF2ΔPDZ. With the system used here it cannot be concluded whether the PDZ domain is involved or not in the localization of the protein. The question regarding the functional role of the PDZ domain might be better addressed if the Rho1 pathway is activated and the other interacting partners of DRhoGEF2 are expressed at the appropriate levels in the used system. Consequently, it is necessary to try various conditions in order to conclude whether the PDZ domain has a functional or structural role.

2.3 The role of the PDZ domain in different *Drosophila* tissues

To address the role of the PDZ domain as a positive or negative regulator for the activity of DRhoGEF2 the transgenic approach was subsequently used in order to study that in the fly as the tissue culture cells is a simple system and components of the Rho signaling pathway might be missing or not expressed at the appropriate levels. Transgenic flies that expressed wild type DRhoGEF2 (EMBL) and transgenic flies that expressed DRhoGEF2ΔPDZ (EMBL) under the control of the GAL4-UAS expression

system (Brand and Perrimon, 1993) were generated. The aim was to study the effects of the DRhoGEF2ΔPDZ construct in the fly using different drivers and compare these effects with the effects of the wild type transgene.

Firstly, the effects of overexpression of three different lines of wild type DRhoGEF2 transgenes (denoted as RG2-1a, RG2-6a, and RG2-6b) were tested using three different GAL4 drivers because different drivers cause different expression levels of the transgene. To drive expression of wild type DRhoGEF2 the eyeless-GAL4 (ey-GAL4) was used which targets expression to the eye. In addition, the MS1096-GAL4 line was used which directs expression in the wing imaginal disc, and the VP16-V32-GAL4 which is a ubiquitously expressing driver. Crosses were carried out at 25^0C and 18^0C to account for different expression levels due to temperature (Duffy, 2002). Overexpression of wild type DRhoGEF2 with any of the three aforementioned drivers caused a high lethality levels in all transgenic lines at both temperatures (Table 3.1, 3.2 and Fig. 3.4., 3.5). These results suggested that leaky expression for eyeless and MS1096 promoters in vital tissues blocked development. The high lethality levels with the VP16-V32 driver might be due to the fact that this driver targets expression early during oogenesis. The surviving adult flies overexpressing wild type DRhoGEF2 displayed rough eyes with the eyeless driver (Fig. 3.6) and crumpled wings with the MS1096 driver (Fig. 3.7). With the VP16-V32 driver there was not any visible defect of the surviving adult flies. It might be that the effects of the overexpression of the transgene by this driver are subtle or in internal organs.

Table 3.1

Driver	Transgenes	Expected % of flies with appropriate Genotype	Observed % of flies with appropriate genotype	% Lethality	% Phenotype
Ey-GAL4	RG2-1a	50	14 (81/576)	72	15 (12/81)
Ey-GAL4	RG2-6a	50	15 (60/400)	70	50 (30/60)
Ey-GAL4	RG2-6b	50	8 (43/537)	84	28 (12/43)
MS-1096	RG2-1a	50	29 (80/273)	42	89 (71/80)
MS-1096	RG2-6a	50	19 (65/342)	62	40 (26/65)
MS-1096	RG2-6b	50	20 (44/219)	60	55 (24/44)
VP16-V32	RG2-1a	50	10 (52/521)	80	N/D
VP16-V32	RG2-6a	50	9 (59/653)	82	N/D
VP16-V32	RG2-6b	50	15 (64/428)	70	N/D

Table 3.1: Phenotypes of wild type transgenes of RhoGEF2 expressed with three different drivers at 25^0C. The percent of lethality and the corresponding to the driver phenotype for the genotype of interest are indicated in the table. In parenthesis is indicated the sample number. N/D: Not defined.

Table 3.2

Driver	Transgenes	Expected % of flies with appropriate Genotype	Observed % of flies with appropriate genotype	% Lethality	% Phenotype
Ey-GAL4	RG2-1a	50	10 (64/638)	80	20 (13/64)
Ey-GAL4	RG2-6a	50	13 (44/336)	74	41 (18/44)
Ey-GAL4	RG2-6b	50	7 (35/535)	86	14 (5/35)
MS-1096	RG2-1a	50	28 (105/376)	44	84 (88/105)
MS-1096	RG2-6a	50	22 (63/285)	56	38 (24/63)
MS-1096	RG2-6b	50	25 (69/275)	50	64 (44/69)
VP16-V32	RG2-1a	50	6 (35/584)	88	N/D
VP16-V32	RG2-6a	50	10 (42/422)	80	N/D
VP16-V32	RG2-6b	50	11 (52/472)	78	N/D

Table 3.2: Phenotypes of wild type transgenes of RhoGEF2 expressed with three different drivers at 18^0C. The percent of lethality and the corresponding to the driver phenotype for the genotype of interest are indicated in the table. In parenthesis is indicated the sample number. N/D: Not defined.

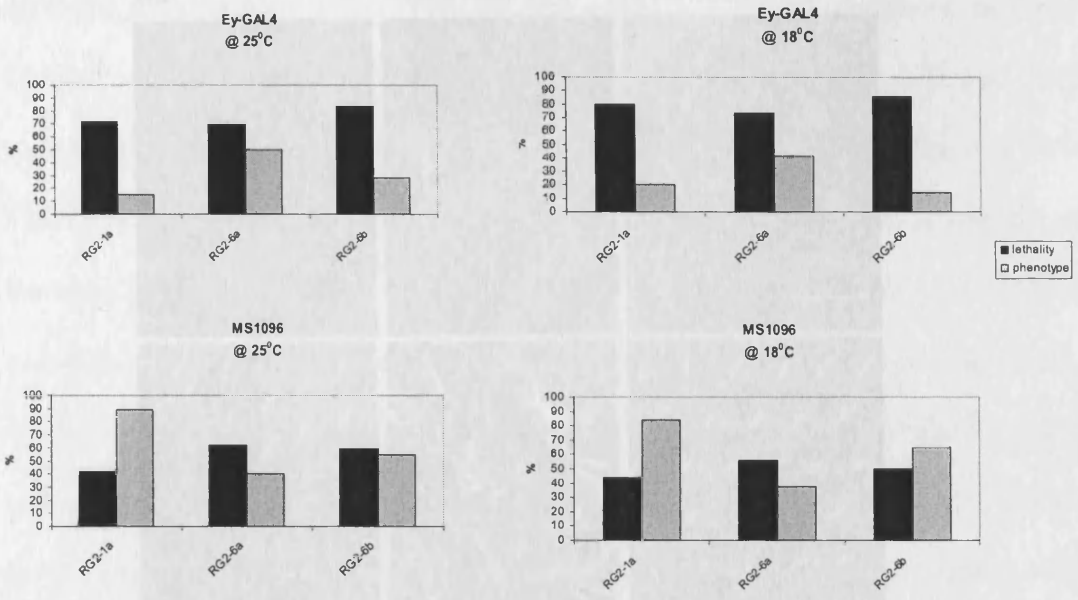


Figure 3.4: Percentages of lethality and phenotypes obtained after overexpression of DRhoGEF2 wild type transgenes with ey-Gal4 and MS1096 at the two temperatures tested.

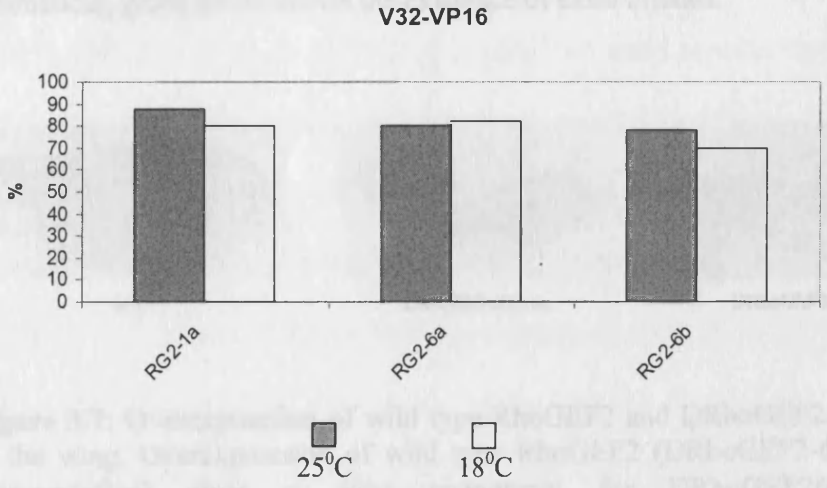


Figure 3.5: Lethality percentage of DRhoGEF2 wild type transgenes expressed with V32-VP16 driver at the two temperatures tested.

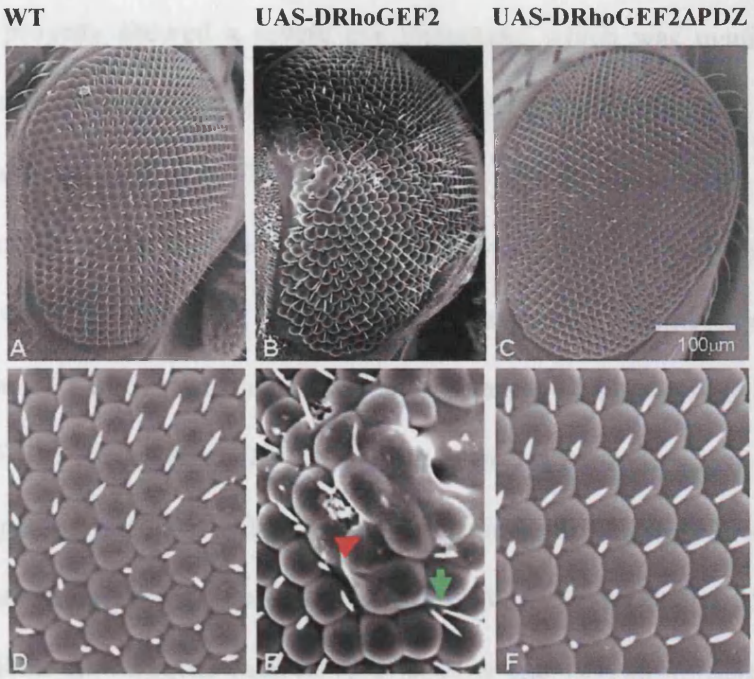


Figure 3.6: Overexpression of DRhoGEF2 wild type by ey-GAL4 disrupts normal eye development. Representative SEMs of Drosophila compound eyes. The genotypes are as follows: (A) OregonR (WT), (B) DRhoGEF2 transgene (line 6b used as representative sample), (C) DRhoGEF2ΔPDZ transgene (line 4a used as representative sample). Magnifications 250x (A-C) and 500x (D-F). Red arrow shows fused ommatidia, green arrow shows the existence of extra bristles.

Figure 3.7: Overexpression of wild type RhoGEF2 and DRhoGEF2ΔPDZ transgenes in the wing. Overexpression of wild type RhoGEF2 (DRhoGEF2-6a line used as a representative) gives a wing phenotype; for DRhoGEF2ΔPDZ transgene (DRhoGEF2ΔPDZ^{2b} used as a representative) no wing phenotype is observed.

The results are summarized in Table 3.1 and 3.2 and in Figures 3.4 and 3.5. In more detail, transgenic line RG2-1a at 25^{0}C showed 72% lethality and 15% (n=12) of the

surviving progeny showed a severe eye phenotype which was manifested as fused ommatidia or of irregular shape, with occasionally extra bristles (Fig. 3.6B and 3.6E compare to 3.6A and 3.6D). When the offspring was raised at 18^0C, there was 80% lethality and 20% (n=13) of the same eye phenotype. Driving expression of this line of transgene with MS1096 driver gave a moderate percentage of lethality of 42% at 25^0C, and 44% at 18^0C; however a much higher population of the surviving progeny (89%, n=71 at 25^0C; 84%, n= 88 at 18^0C) showed a severe wing phenotype in which the wing was crumpled and folded (Fig. 3.7). Transgenic line RG2-1a showed the highest lethality percentages when expressed with VP16-V32 driver (80% at 25^0C versus 88% at 18^0C). RG2-6a transgene showed 70% lethality and 10% (n=6) phenotype with the eyeless driver at 25^0C and a similar lethality percentage for the cross at 18^0C (74%), instead the phenotype percentage was much higher (41%, n=18). Driving expression of RG2-6a transgene with MS1096 driver caused a moderate percentage of lethality as seen for line RG2-1a (62% at 25^0C; 56% at 18^0C). In contrast with what observed for line RG2-1a regarding the phenotype percentages, much less of the surviving progeny from line RG2-6a showed the same severe wing phenotype (40%, n=26 at 25^0C; 38%, n= 24 at 18^0C). The highest percentage of lethality was seen again when expression was driven by VP16-V32-GAL4 driver at both temperatures (80% at 25^0C; 82% at 18^0C). On the contrary, line RG2-6b showed the highest lethality percentage when expressed with the eyeless-GAL4 driver (84% at 25^0C; and 86% at 18^0C) but a low percentage of phenotype (28%, n=12 at 25^0C; 14%, n= 5 at 18^0C). The lethality percentage was 60% at 25^0C and 50% at 18^0C, when RG2-6b transgene was expressed with the wing driver MS1096. However, a much high population of the surviving progeny showed a severe

wing phenotype (55%, n=24 at 25^0C versus 64%, n=44 at 18^0C). Expression with VP16-V32 driver caused the death of 78% at 25^0C, and 70% at 18^0C of the progeny with the appropriate genotype.

From the results obtained here it is observed that overexpression of the transgenic line DRhoGEF2-6a with the ey-GAL4 causes a change in the eye at a higher percentage compared to the other two lines. Instead, overexpression of the transgenic line DRhoGEF2-1a with the MS1096-GAL4 causes a change in the wing at a higher percentage compared to the other two lines. These results suggest that the insertion site of the transgene in combination with the transcriptional activator of the driver influence the expression levels of the transgene.

Furthermore, the above results in accordance with the results obtained from the tissue culture studies indicate that overexpression of DRhoGEF2 causes morphological changes. This suggests that a controlled expression of DRhoGEF2 is necessary for a normal development. High levels of DRhoGEF2 might be causing overactivation of Rho1 which leads to these morphological aberrations.

The relevance of the PDZ domain was analyzed by overexpressing the PDZ mutants [i.e. overexpressing DRhoGEF2 lacking the PDZ domain, (denoted DRhoGEF2ΔPDZ)] in transgenic flies using the Gal4-UAS system (Brand and Perrimon, 1993). To drive DRhoGEF2ΔPDZ expression the ey-GAL4, MS1096-GAL4, and VP16-V32-GAL4 drivers were used. As described above it was observed that overexpression of wild type DRhoGEF2 transgene causes malformation of the eye and wing depending on the driver used. These morphological changes were used as parameters to assess whether the PDZ domain acted as a regulator of the activity of

DRhoGEF2. If the PDZ domain was a positive regulator, then transgenic flies overexpressing DRhoGEF2ΔPDZ should have shown less severe morphological changes or no changes at all. On the other hand, if it acted as a negative regulator then these changes should have been more severe. Therefore the same experiment described before for the wild type DRhoGEF2, was carried out at the same time with five different DRhoGEF2ΔPDZ transgenic lines. All of the DRhoGEF2ΔPDZ transgenic lines tested showed a high percentage of lethality when expressed with the ey-GAL4 driver at 25^0C. Viability did not increase when the crosses were carried out at 18^0C except for line DRhoGEF2ΔPDZ^{4a} for which the lethality percentage from 50% at 25^0C dropped to 10% at 18^0C. When these lines were expressed with MS1096-GAL4 and VP16-V32-GAL4 moderate lethality percentages were observed and viability increased for all lines when the same crosses were carried out at 18^0C. The results are summarized in Tables 3.3 and 3.4 and in Figure 3.8. DRhoGEF2ΔPDZ^{2b} expressed at 25^0C with ey-Gal4 showed 60% lethality; the lethality percentages dropped when the offspring was raised at 18^0C, giving 56% lethality. Driving expression of this line of transgene with MS1096 driver there was a low percentage of lethality of 12% at 25^0C, and 6% at 18^0C. RhoGEF2ΔPDZ^{2b} gave 16% lethality when expressed with VP16-V32 driver at 25^0C and 14% at 18^0C. RhoGEF2ΔPDZ^{3a} transgene showed 46% of lethality at 25^0C and 32% at18^0C with the eyeless driver. Driving expression of RhoGEF2ΔPDZ^{3a} transgene with MS1096 driver there was a moderate percentage of lethality (20% at 25^0C; 18% at 18^0C). The viability was much higher for this line of transgene when expressed with VP16-V32-GAL4 driver at both temperatures (4% of lethality at 25^0C and 18^0C).

Table 3.3

Driver	Transgenes	Expected % of flies with appropriate Genotype	Observed % of flies with appropriate genotype	% Lethality
Ey-GAL4	ΔPDZ^{2b}	50	20 (84/417)	60
Ey-GAL4	ΔPDZ^{3a}	50	27 (116/436)	46
Ey-GAL4	ΔPDZ^{4a}	50	25 (96/378)	50
Ey-GAL4	ΔPDZ^{5c}	50	28 (86/312)	44
Ey-GAL4	ΔPDZ^{6a}	50	17 (51/295)	66
MS-1096	ΔPDZ^{2b}	50	44 (158/364)	12
MS-1096	ΔPDZ^{3a}	50	40 (137/344)	20
MS-1096	ΔPDZ^{4a}	50	46 (121/264)	8
MS-1096	ΔPDZ^{5c}	50	46 (158/344)	8
MS-1096	ΔPDZ^{6a}	50	47 (168/357)	6
VP16-V32	ΔPDZ^{2b}	50	42 (71/169)	16
VP16-V32	ΔPDZ^{3a}	50	48 (145/302)	4
VP16-V32	ΔPDZ^{4a}	50	47 (103/220)	6
VP16-V32	ΔPDZ^{5c}	50	46 (116/253)	8
VP16-V32	ΔPDZ^{6a}	50	40 (95/238)	20

Table 3.3: Percentages of lethality for DRhoGEF2ΔPDZ transgenes (indicated on the second column of the table just as ΔPDZ with a subscript of an arbitrary line number) expressed with three different drivers at 25^0C. In a parenthesis is indicated the sample number.

RhoGEF2ΔPDZ^{4a} showed a high lethality percentage when expressed with the eyeless-GAL4 driver at 25^0C but when the same cross was carried out at 18^0C the lethality percentage dropped to 10%. Similarly to the other lines, RhoGEF2ΔPDZ^{4a} gave low percentages of lethality with MS1096-GAL4 and VP16-V32-GAL4 at both temperatures (8% at 25^0C; 6% at 18^0C with MS-1096 driver and 6% 25^0C; 2% at 18^0C with VP16-V32 driver).

Table 3.4

Driver	Transgenes	Expected % of flies with appropriate Genotype	Observed % of flies with appropriate genotype	% Lethality
Ey-GAL4	ΔPDZ^{2b}	50	22 (62/280)	56
Ey-GAL4	ΔPDZ^{3a}	50	34 (90/264)	32
Ey-GAL4	ΔPDZ^{4a}	50	45 (156/348)	10
Ey-GAL4	ΔPDZ^{5c}	50	32 (113/354)	36
Ey-GAL4	ΔPDZ^{6a}	50	37 (100/270)	26
MS-1096	ΔPDZ^{2b}	50	47 (109/232)	6
MS-1096	ΔPDZ^{3a}	50	41 (88/215)	18
MS-1096	ΔPDZ^{4a}	50	47 (95/202)	6
MS-1096	ΔPDZ^{5c}	50	49 (103/210)	2
MS-1096	ΔPDZ^{6a}	50	48 (98/205)	4
VP16-V32	ΔPDZ^{2b}	50	43 (93/217)	14
VP16-V32	ΔPDZ^{3a}	50	48 (112/234)	4
VP16-V32	ΔPDZ^{4a}	50	49 (82/168)	2
VP16-V32	ΔPDZ^{5c}	50	48 (84/174)	4
VP16-V32	ΔPDZ^{6a}	50	43 (81/188)	14

Table 3.4: Percentages of lethality for DRhoGEF2ΔPDZ transgenes (indicated on the second column of the table just as ΔPDZ with a subscript of an arbitrary line number) expressed with three different drivers at 18^0C. In parenthesis is indicated the sample number.

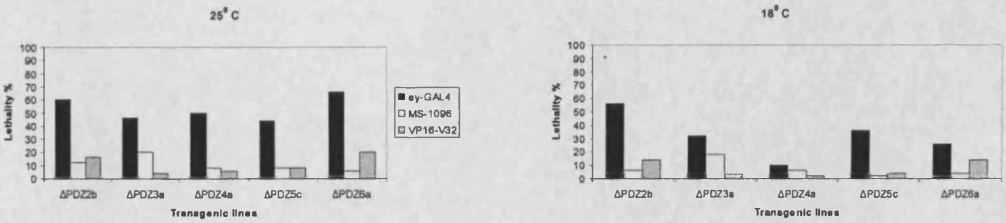


Figure 3.8: Lethality percentage for five different lines of DRhoGEF2ΔPDZ transgene expressed with three different drivers at 25^0C and 18^0C. The transgenic lines are indicated in the graph just with the line number.

DRhoGEF2ΔPDZ^{5c} transgene showed 44% of lethality at 25^{0}C and 36% at18^{0}C with the eyeless driver. The lethality percentage was quite low when DRhoGEF2ΔPDZ^{5c} transgene was expressed with the wing driver MS1096, (8% at 25^{0}C; 2% at 18^{0}C) and with VP16-V32 driver (8% at 25^{0}C, and 4% at 18^{0}C). DRhoGEF2ΔPDZ^{6a} transgene showed the highest lethality percentage of all lines when expressed with ey-GAL4 at 25^{0}C (66%). However this percentage dropped to 26% when the cross was carried out at 18^{0}C. High viability was observed when this transgene was expressed with MS1096 driver (6% at 25^{0}C; 4% at 18^{0}C). On the contrary there was moderate lethality when DRhoGEF2ΔPDZ^{6a} transgene was expressed with VP16-V32 driver (20% at 25^{0}C compared to14% at 18^{0}C). To visualize any eventual defects in the eyes of DRhoGEF2ΔPDZ flies in greater detail, scanning electron micrographs (SEMs) of eyes from wild type, DRhoGEF2 wild type transgenes and from DRhoGEF2ΔPDZ transgenes were compared (Fig. 3.6). Flies overexpressing wild-type DRhoGEF2, using a UAS-DRhoGEF2 transgene and an eyeless-GAL4 driver had rough eyes (Fig. 3.6B and 3.6E). In marked contrast the UAS-DRhoGEF2ΔPDZ transgenic flies had eyes that were indistinguishable in appearance from those of wild-type flies (Fig. 3.6C and 3.6F compared to 3.6A and 3.6D) with each ommatidium having the regular hexagonal shape surrounded by the right number of bristles. In a similar way, flies overexpressing wild-type DRhoGEF2, using a UAS-DRhoGEF2 transgene and an MS-1096-GAL4 driver had defective wings (Fig. 3.7). On the contrary, the UAS-DRhoGEF2ΔPDZ transgenic flies had wings that were indistinguishable in appearance from those of wild-type flies (Fig 3.7).

The fact that lethality is observed with the tissue specific driver indicates that the driver is leaky. The expression levels of the transgene seem overall to be comparable between the lines tested (compare the different lines with the same driver). Carrying out the crosses at 18^0C does not seem to affect significantly the expression levels compared to 25^0C.

To summarize the DRhoGEF2ΔPDZ expressing transgenes do not show any morphological changes associated with the overexpression as it is observed for the wild type transgenes. The results of the overexpression of wild type DRhoGEF2 taken together with the results of the overexpression of DRhoGEF2ΔPDZ could suggest that the PDZ domain acts as a positive regulator for the function of DRhoGEF2. Therefore when PDZ domain is absent the activity of DRhoGEF2 is moderated and kept at low levels.

2.4 Expression levels of DRhoGEF2 required for development

To further understand the role of the PDZ domain for the function of DRhoGEF2, it was aimed to use the DRhoGEF2ΔPDZ transgenic flies in combination with alleles of DRhoGEF2 which cause a phenotypic change and study whether the DRhoGEF2ΔPDZ construct could rescue this phenotype. As a control the flies expressing the wild type DRhoGEF2 transgene were tested as to whether they could rescue the phenotypic change caused by the DRhoGEF2 alleles.

Therefore, the wild type transgene was used with transallelic combinations of DRhoGEF2. The alleles used were: $DRhoGEF2^{1.1}$, $DRhoGEF2^{4.1}$ and $DRhoGEF2^{6.1}$. The EMS allele $DRhoGEF2^{1.1}$ has a point mutation generating a stop codon before the

RGS domain (Fig. 3.9) and it was considered to be a null allele (Barrett et al., 1997). The DRhoGEF2$^{4.1}$ was also an EMS allele (Barrett et al, 1997). Since the DNA rearrangement in DRhoGEF2$^{4.1}$ occurs upstream of the RGS, C1 and PH domains (Fig. 3.10), it is expected that this is a functionally null allele also. The allele DRhoGEF2$^{6.1}$ had a DNA fragment derived from a P-element remaining in the intron upstream of the coding sequence (Fig 3.10). Therefore this is probably a hypomorphic allele giving reduced expression levels of the protein. Alleles DRhoGEF2$^{4.1}$ and DRhoGEF2$^{1.1}$ give identical phenotypes and are completely penetrant (Barrett et al., 1997). The hypomorphic allele DRhoGEF2$^{6.1}$ in combination with the null allele DRhoGEF2$^{4.1}$ gives 49% viability and the surviving adult flies have crumpled wings and rough eyes. The null allele DRhoGEF2$^{1.1}$ in combination with the null allele RhoGEF2$^{4.1}$gives 0% adult viability.

Thus this genetic background of null/hypomorph and null/null alleles of DRhoGEF2 was used as a sensitized system to assess whether wild type transgenes could rescue the lethality, wing and eye phenotypes. Since RhoGEF2$^{1.1}$/RhoGEF2$^{4.1}$ gives a much more severe phenotype (0% viability) in comparison to RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$ (49% viability) the two different allelic combinations of DRhoGEF2 were used for the rescue experiment in order to assess the strength of the DRhoGEF2 wild type transgene.

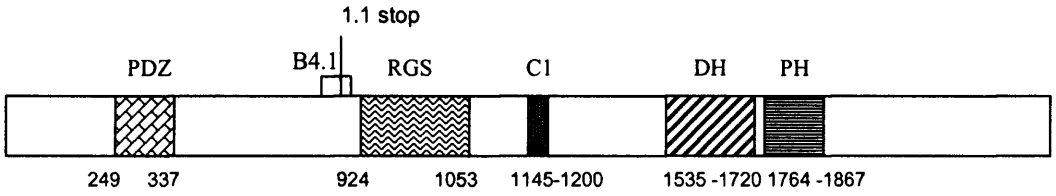


Figure 3.9: Schematic representation of the DRhoGEF2 cDNA. The boundaries of the breakpoint in RhoGEF2$^{4.1}$ allele is indicated by B4.1.RhoGEF2$^{1.1}$ null allele generated by a stop codon is represented as a shorter RhoGEF2 containing only the PDZ domain.

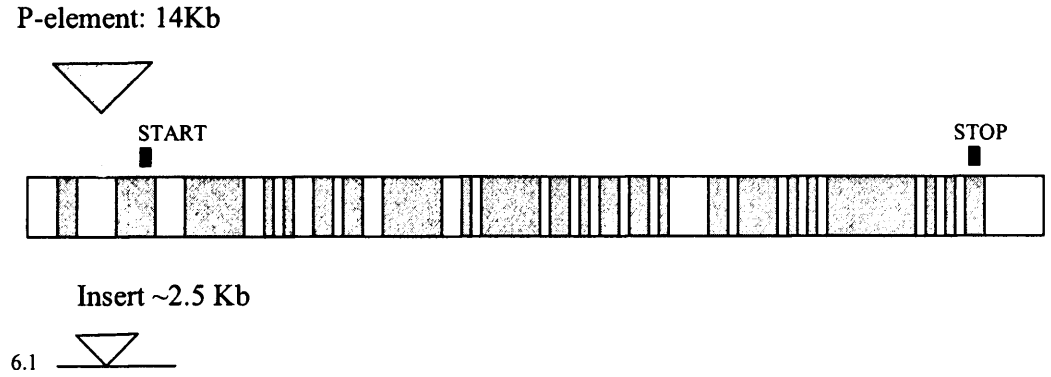


Figure 3.10: Exon-intron map of DRhoGEF2 with the insertion of P-element (grey boxes indicating exons). Map of the insertion and deletion of the P-element imprecise excision allele of DRhoGEF2, cDNA: 8435bp, genomic sequence: 17412bp.

Since DRhoGEF2 is ubiquitously expressed the drivers tubulin-Gal4 (tub-Gal4) and daughterless-Gal4 (da-Gal4) were chosen to drive also ubiquitously the expression of the wild type transgene. For the rescue experiment four lines of wild type transgenes (UAS-DRhoGEF2-2a denoted RG2-2a, UAS-DRhoGEF2-4a denoted RG2-4a, UAS-DRhoGEF2-4b denoted RG2-4b, and UAS-DRhoGEF2-5 denoted RG2-5) chosen randomly, were first tested whether they survive through embryogenesis so that they

can be used in this study. Therefore, to determine whether there are embryogenesis problems associated with the overexpression of the transgenic lines the development of the flies was followed (Table 3.5 and Fig. 3.11). When transgene RG2-2a was not expressed 96% of the fertilized eggs made it through embryogenesis. Instead when the expression of this transgene was driven with either tub-Gal4 or da-Gal4, 28% and 66% respectively survived throughout embryogenesis. Similar results were obtained with transgene RG2-4a: when this transgene was not expressed 97% of the fertilized eggs made it through embryogenesis but when it was overexpressed with tub-Gal4 only 38% survived and with da-Gal4 63% did. Transgene RG2-4b gave 92% viability of the fertilized eggs when not expressed and when overexpressed the viability with tub-Gal4 dropped to 36% and with da-Gal4 to 62%. Finally transgene RG2-5 when not expressed 95% of the fertilized eggs survived embryogenesis; in contrast when overexpressed with tub-Gal4 of the fertilized eggs only 22% survived and when overexpressed with da-Gal4 75% made it through embryogenesis.

From the results above, it is concluded that the overexpression of the transgene had a heavy toll on the embryogenesis of the flies which was much more severe when any of the transgenic lines tested was expressed with tub-Gal4. Instead, overexpression of the transgenic lines with da-Gal4 gave moderate viability levels at early stages. These data indicate that tub-Gal4 drives expression of the protein at higher levels than da-Gal4 (Fig. 3.11).

Table 3.5

transgene	driver	chorion %	Dead %
RG2-2a	no driver	96%	4%
RG2-2a	tub	28%	72%
RG2-2a	da	66%	34%
RG2-4a	no driver	97%	3%
RG2-4a	tub	38%	62%
RG2-4a	da	63%	37%
RG2-4b	no driver	92%	8.%
RG2-4b	tub	36%	64%
RG2-4b	da	62%	37%
RG2-5	no driver	95%	5%
RG2-5	tub	22%	78%
RG2-5	da	75%	25%

Table 3.5: Percentage of wild type transgenes expressed with tubulin-Gal4 or daughterless-Gal4 that make it through embryogenesis.

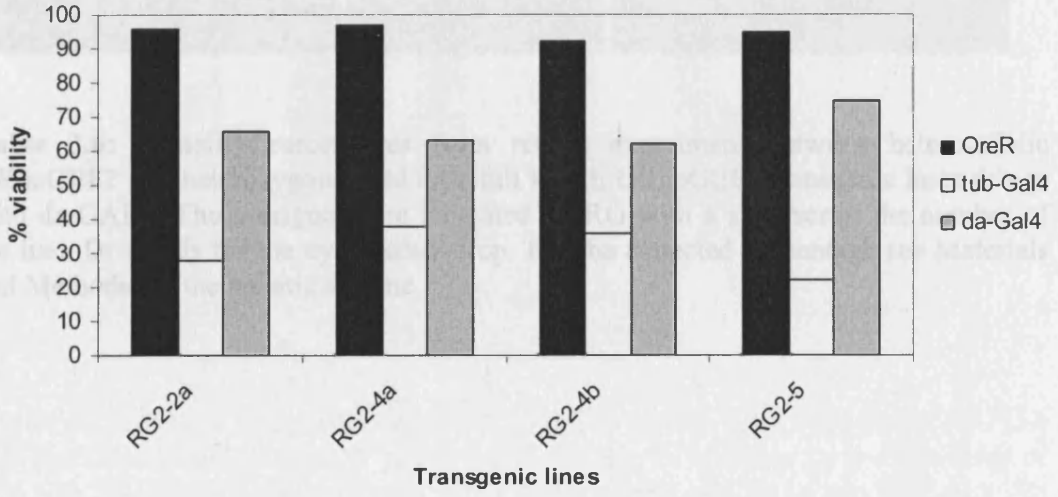


Figure 3.11: Viability percentages during embryogenesis of wild type transgenic DRhoGEF2 flies. The graph shows the percentage of DRhoGEF2 wild type transgenes (denoted RG2-2a, RG2-4a, RG2-4b, RG2-5) that survive embryogenesis.

It was then tested whether reducing the protein levels would allow the flies to survive. Reduction of the protein levels was achieved by combining DRhoGEF2 wild type transgene with hypomorph/null allelic combination DRhoGEF2$^{6.1}$/DRhoGEF2$^{4.1}$.

Table 3.6

Genotype of interest	Expected %	Observed %	% Lethality
RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$	25	13 (n=100)	49
RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$; Dr/RG^{2a}	17	7 (n=102)	59
RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$; da-GAL4/RG^{2a}	17	0	100
RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$; Dr/RG^{4a}	17	10 (n=111)	41
RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$; da-GAL4/RG^{4a}	17	1	94
RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$; Dr/RG^{4b}	17	12 (n=112)	29
RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$; da-GAL4/RG^{4b}	17	1	94
RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$; Dr/RG^{5}	17	9 (n=154)	47
RhoGEF2$^{6.1}$/RhoGEF2$^{4.1}$; da-GAL4/RG^{5}	17	0	100

Table 3.6: Lethality percentages from rescue experiment between hetero-allelic DRhoGEF2 and heterozygous wild type full length DRhoGEF2 transgenic lines driven with da-GAL4. The transgenes are indicated by RG with a superscript the number of the line. Dr stands for the eye marker drop. For the expected percentage see Materials and Methods for the genetic scheme.

Table 3.7

Genotype of interest	Expected %	Observed %	% Lethality
$RhoGEF2^{6.1}/RhoGEF2^{4.1}$	25	13 (n=100)	49
$RhoGEF2^{6.1}/RhoGEF2^{4.1}$; Dr/RG^{2a}	17	10 (n=118)	41
$RhoGEF2^{6.1}/RhoGEF2^{4.1}$; tub-GAL4/$RG^{2a}$	17	2	88
$RhoGEF2^{6.1}/RhoGEF2^{4.1}$; Dr/RG^{4a}	17	12 (n=122)	30
$RhoGEF2^{6.1}/RhoGEF2^{4.1}$; tub-GAL4/$RG^{4a}$	17	0	100
$RhoGEF2^{6.1}/RhoGEF2^{4.1}$; Dr/RG^{4b}	17	10 (n=110)	41
$RhoGEF2^{6.1}/RhoGEF2^{4.1}$; tub-GAL4/$RG^{4b}$	17	0	100
$RhoGEF2^{6.1}/RhoGEF2^{4.1}$; Dr/RG^{5}	17	8 (n=100)	53
$RhoGEF2^{6.1}/RhoGEF2^{4.1}$; tub-GAL4/$RG^{5}$	17	3	82

Table 3.7: Lethality percentages from rescue experiment between hetero-allelic RhoGEF2 and heterozygous wild type full length RhoGEF2 transgenic lines driven with tub-GAL4. The transgenes are indicated by RG with a superscript of the number of the line. Dr stands for the eye marker drop. For the expected percentage see Materials and Methods for the genetic scheme.

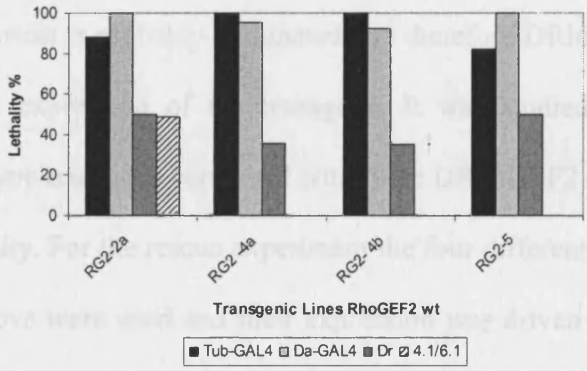


Figure 3.12: Rescue experiment of the DRhoGEF2 mutant phenotype by DRhoGEF2 wild type transgenes. The graph shows the percentage of lethality for the four lines of wild type DRhoGEF2 overexpressed with the two drivers tub-GAL4 and da-GAL4. All transgenic lines were heterozygous combinations with the hetero-allelic mutants $DRhoGEF2^{6.1}/DRhoGEF2^{4.1}$ on the second chromosome. Dr stands for the drop marker. The percentage of the inert transgenes is an average of the percentages obtained in the two independent set of crosses.

Flies with overexpressed DRhoGEF2 wild type driven either by tub-GAL4 or da- GAL4 in combination with DRhoGEF2$^{6.1}$/DRhoGEF2$^{4.1}$ were not recovered or presented very low viability percentages. There was 100% lethality for all lines except for RG2-4a and RG2-4b that both showed 94% lethality when expressed with da-Gal4 driver (Fig. 3.12). Instead with tub-GAL4 there was 100% lethality except for lines RG2-2a that showed 88% lethality and line RG2-5 with 82% lethality (Fig. 3.12). For the surviving progeny, the wild type transgene was not able to rescue the phenotype either thus the surviving adult flies had a wing or eye phenotype. The DRhoGEF2$^{6.1}$/DRhoGEF2$^{4.1}$; Dr/RG2^{TG} progeny (RG2^{TG} indicating any transgenic line) showed low viability as expected from the previous results obtained for the alleles DRhoGEF2$^{6.1}$/DRhoGEF2$^{4.1}$ alone. The results are summarized in Table 3.6 and 3.7 and Fig. 3.12.

In addition, for this rescue experiment the allelic combination null/null DRhoGEF2$^{1.1}$/DRhoGEF2$^{4.1}$ was used which gives 0% viability; with this combination the endogenous protein is probably eliminated and therefore DRhoGEF2 present in the flies derives from expression of the transgene. It was studied whether the UAS-DRhoGEF2 wild type transgene combined with these DRhoGEF2 mutants could rescue the observed lethality. For the rescue experiment the four different wild type transgenic lines described above were used and their expression was driven ubiquitously by tub-GAL4 and da-GAL4. DRhoGEF2$^{1.1}$ /DRhoGEF2$^{4.1}$; MKRS/RG2^{TG} progeny (RG2^{TG} indicating any transgenic line; MKRS is a marker) was not recovered as expected in accordance with the previous results obtained showing that DRhoGEF2$^{1.1}$ /DRhoGEF2$^{4.1}$ is 100% lethal.

Two transgenic lines of DRhoGEF2 out the four tested were able to rescue to a certain degree the lethality observed with the heterozygous DRhoGEF2$^{1.1}$/DRhoGEF2$^{4.1}$. Line RG2-2a showed 59% lethality and line RG2-5 showed 53% lethality (Table 3.8, Fig 3.13). On the contrary the other two lines gave 100% lethality. These results indicate that the insertion site of the transgene must play a role in its expression levels.

The results of the rescue experiment of the wild type transgene in combination with the hypomorph/null alleles and null/null alleles of DRhoGEF2 suggest that an appropriate amount of the protein is necessary for the normal development of the fly (Fig.3.14).

Table 3.8

Genotype of interest	Expected %	Observed %	% Lethality
RhoGEF2$^{1.1}$/RhoGEF2$^{4.1}$; MKRS/RG^{2a}	17	0	100
RhoGEF2$^{1.1}$/RhoGEF2$^{4.1}$; da-GAL4/RG^{2a}	17	7 (n=33)	59
RhoGEF2$^{1.1}$/RhoGEF2$^{4.1}$; MKRS/RG^{4a}	17	0	100
RhoGEF2$^{1.1}$/RhoGEF2$^{4.1}$; da-GAL4/RG^{4a}	17	0	100
RhoGEF2$^{1.1}$/RhoGEF2$^{4.1}$; MKRS/RG^{4b}	17	0	100
RhoGEF2$^{1.1}$/RhoGEF2$^{4.1}$; da-GAL4/RG^{4b}	17	0	100
RhoGEF2$^{1.1}$/RhoGEF2$^{4.1}$; MKRS/RG^{5}	17	0	100
RhoGEF2$^{1.1}$/RhoGEF2$^{4.1}$; da-GAL4/RG^{5}	17	8 (n=25)	53

Table 3.8: Lethality percentages from rescue experiment between hetero-allelic RhoGEF2 and heterozygous wild type full length RhoGEF2 transgenic lines.

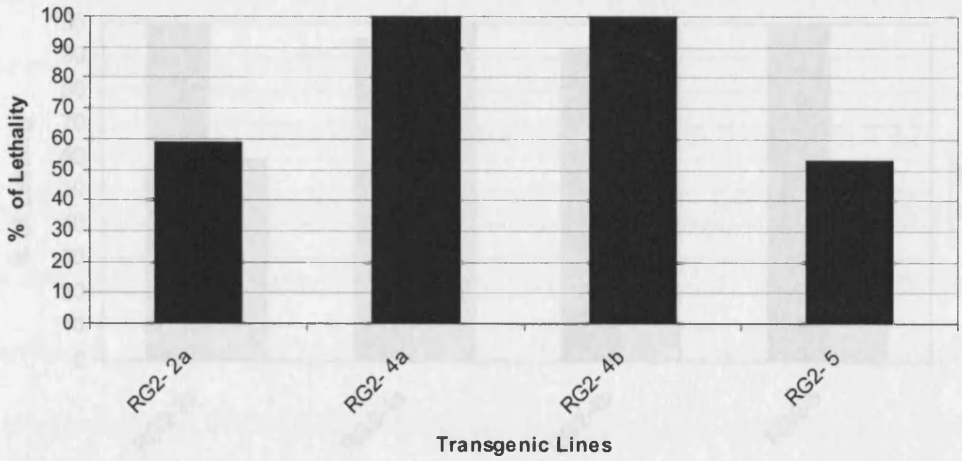


Figure 3.13: Rescue of the DRhoGEF2 mutant phenotype by DRhoGEF2 wild type transgenes. The graph shows the lethality percentage of the four lines of wild type DRhoGEF2 overexpressed with the da-GAL4. All transgenic lines were heterozygous combinations with the hetero-allelic mutants DRhoGEF2$^{1.1}$/DRhoGEF2$^{4.1}$on the second chromosome.

When the wild type transgene is combined with the null/hypomorph (DRhoGEF2$^{6.1}$/DRhoGEF2$^{4.1}$) there is an uncontrollably high expression of the protein that is lethal for the fly. Reducing the protein levels by combining the transgene with a null/null (DRhoGEF2$^{1.1}$/DRhoGEF2$^{4.1}$), allows some rescuing of the lethality.

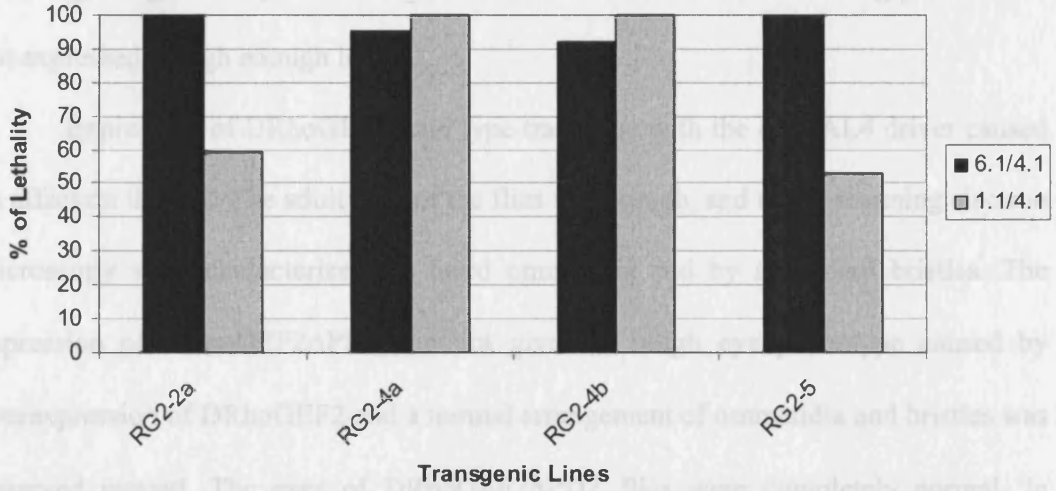


Figure 3.14: Rescue experiment of the DRhoGEF2 mutant phenotype by DRhoGEF2 wild type transgenes. The graph shows a comparison between the lethality percentages of DRhoGEF2 wild type transgene driven with da-Gal4 with combinations of DRhoGEF2 mutants. All transgenic lines were heterozygous combinations with the hetero-allelic mutants $DRhoGEF2^{6.1}$/$DRhoGEF2^{4.1}$ (denoted on the graph as 6.1/4.1) and $DRhoGEF2^{1.1}$/$DRhoGEF2^{4.1}$ (denoted on the graph as 1.1/4.1) on the second chromosome and the four lines of wild type DRhoGEF2 (denoted on this graph by: RG2-2a, RG2-4a, RG2-4b, RG2-5).

3. CONCLUSIONS

In order to understand the role of the PDZ domain for the function of DRhoGEF2 it was examined how overexpression of DRhoGEF2ΔPDZ construct affected the morphology of S2R+cells. Wild type DRhoGEF2 induced cell contraction. The same morphology was observed when DRhoGEF2ΔPDZ was expressed in the cells. Thus, under the conditions used in S2R+ cells the PDZ domain did not have an effect for the function of DRhoGEF2 that is it did not act as regulator for the activity of the protein. The localization of the protein could not be assessed as overexpression of the protein was found diffuse in the cytoplasm. This might be due to the fact that the

Rho1 signaling pathway has to be prior activated or that other interacting partners are not expressed at high enough levels.

Expression of DRhoGEF2 wild type transgene with the ey-GAL4 driver caused an effect on the eye. The adult eyes of the flies were rough, and under scanning electron microscopy were characterized by fused ommatidia and by additional bristles. The expression of DRhoGEF2ΔPDZ did not give the rough eye phenotype caused by overexpression of DRhoGEF2 and a normal arrangement of ommatidia and bristles was observed instead. The eyes of DRhoGEF2ΔPDZ flies were completely normal. In addition, overexpression of wild type DRhoGEF2 by MS1096-GAL4 caused a malformation of the wings. On the contrary, overexpression of the DRhoGEF2ΔPDZ did not change the shape of the wings. These preliminary results suggest that the PDZ domain could act as a positive regulator for the activity of DRhoGEF2.

Finally, from the rescue experiment it is observed that appropriate expression levels of DRhoGEF2 are necessary for correct development. Expression of the transgene in combination with the trans-allelic combination of DRhoGEF2 null/null is able to rescue the lethality; on the contrary the expression of the transgene in combination with the trans-allelic combination of DRhoGEF2 hypomorph/null is not able to rescue the lethality. Thus moderate expression levels of DRhoGEF2 are required for the development of the fly. It would be interesting to study whether expression of the transgene DRhoGEF2ΔPDZ is also able to rescue the lethality of null/null alleles of DRhoGEF2. This experiment will give a better insight into the role of the PDZ domain for the function of DRhoGEF2.

IV. RESULTS CHAPTER 4

Chapter 4: CHARACTERISATION OF DMEC2

1. INTRODUCTION

In order to learn more about the DRhoGEF2's function and its positioning in the Rho1 signaling pathway, it was sought to identify other interacting partners for this multidomain protein. In particular, one candidate domain that could provide the basis for additional communications not characterised as of yet is its PDZ domain. A yeast two-hybrid system was elected to screen for interacting proteins for the PDZ domain of DRhoGEF2 (Barrett K., unpublished data). In this screen, the PDZ encoding sequence was fused to Gal4 activation domain as the bait fusion; a *Drosophila* cDNA library was fused with the Gal4 DNA-binding domain. Three "PDZ-specific" proteins were identified over the course of a single yeast-two hybrid screen, one of which was the novel gene CG7635. The other two candidates were the tumor suppressor gene called MCC and the predicted gene CG9795. All three of these proteins have at their carboxy terminus the signature motif that could be a target for the PDZ domain. These three candidate interacting partners could be involved in an association with DRhoGEF2 at different times or in different tissues therefore contributing to the selection of outcome of Rho1 activation by DRhoGEF2. In this thesis the focus was on the interaction of CG7635 with the PDZ domain of DRhoGEF2; analysis of the other candidate PDZ binding factors are described elsewhere.

In this chapter CG7635 is characterized. Subsequently it is studied whether it can affect the actin cytoskeleton and the microtubule network. To approach these questions, the functional role of CG7635 in an actin cytoskeleton context is studied in

tissue culture cells, as well as in various fruit fly tissues. Furthermore, since its orthologue, the *C. elegans* Mec2 is involved in mechanosensation, it was aimed to study whether DMec2 can also be established as a molecule involved in the transduction of mechanical cues.

2. RESULTS

2.1 General Domain Organization and Structural Features

The predicted gene CG7635, that was named *Dmec2* to follow the nomenclature of the *C. elegans* protein, is located on the X chromosome at position 18A6 of the cytogenetic map. The gene is predicted to be encoded by 4 exons of relatively small length ranging from 165 bp to 513 bp (Fig. 4.1).

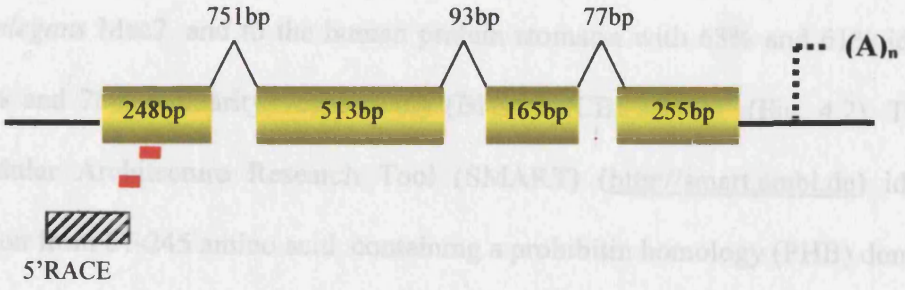


Figure 4.1: *Dmec2* Gene structure. The positions of the 4 Exons (yellow boxes), the poly(A) tail (dashed line) the gene-specific primers used for 5'RACE (red bar) and sequence obtained by 5'RACE (hatched box) are indicated.

In order to confirm that the available clone of DMec2, derived from a *Drosophila* cDNA library (Du, et al., 1996) used for the yeast two-hybrid assay, was

full length, primer extension experiments were carried out to identify the 5' end. Sequences obtained by 5'RACE (Smart Race, Clonetech) were verified by comparison to corresponding genomic DNA sequence. The sequencing of the produced DNA showed that the available cDNA was indeed the full length. The first ATG is found 49 nucleotides after the beginning of the mRNA at the first exon, and in the close proximity there are three more start sites (45bp, 81bp, and 90 bp apart from the first one). These start sites do not have around them sequences that match important consensus ones, with the exception of the fourth start site which has a Kozak sequence (CCAC upstream of the AUG) and a G at +4 (Cavener, 1987).

The *Drosophila* Mec2 encodes for a 350 amino acid protein with a predicted molecular mass of approximately 38KDa. In order to check whether the gene is redundant a BLAST search of the *Drosophila* database (http://flybase.bio.indiana.edu) with DMec2 sequence revealed that the Drosophila genome does not have a related gene. In addition, the BLAST searches showed that the fly Mec-2 is homologous to the *C. elegans* Mec2, and to the human protein stomatin with 63% and 61% identity and 82% and 78% similarity, respectively (Blast , NCBI server) (Fig. 4.2). The Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de) identified a region from 87-245 amino acid containing a prohibitin homology (PHB) domain found also in stomatin and in many other proteins associated with lipid rafts (Morrow and Parton et al., 2005).

It was shown by proteolytic digestion of intact human erythrocytes that stomatin is located solely at the cytoplasmic surface of the erythrocyte membrane and it does not have an extracellular portion (Hiebl-Dirschmied et al., 1991). By inference, DMec2 is

assumed to have also a monotopic structure with both N- and C- terminal portions of the molecule exposed at the cytoplasmic face of the lipid bilayer (Fig. 4.3A). In addition, there is some evidence to support this hypothesis as the hydrophilicity plot by Kyte and Doolittle (Fig. 4.3B) shows a single 33-aminoacid hydrophobic (and presumably membrane-spanning) segment close to the N-terminus (amino-acid residues 67-100) preceded by a hydrophilic 66-aminoacid N-terminal region and followed by the C-terminal part containing most of the 350aminoacids. A database search of recognized amino acid sequence motifs showed potential N-glycosylation sites at two positions (193 and 298), but because these are all in the inferred cytoplasmic region it is unlikely that these are so modified. The amino acid sequence has also three potential Protein Kinase C (PKC) phosphorylation sites (176, 184, 199) and three potential Casein K II (CK2) phosphorylations sites (57, 199, 211). Furthermore, it has an Alanine rich stretch (240-271) and five predicted threonine phosphorylated sites (56,184,199,211,311). It will be shown later that DMec2 interacts with the PDZ domain of DRhoGEF2. It is noted here that regulation of the PDZ-peptide interaction can occur by phosphorylation of residues near the C terminus. In fact the -2 residue (counting from the end of the protein and setting the last amino acid as residue 0) of the PDZ-binding C-terminal peptides is frequently an amino acid that can be phosphorylated such as threonine, serine, or tyrosine. For example, the -2 serine of inward rectifier K^+ channel Kir2.3 falls within a consensus sequence for Protein Kinase A (PKA); phosphorylation of this site by PKA abolishes Kir2.3 interaction with PSD-95's PDZ domains (Cohen et al., 1996). Another example is the phosphorylation of -2 serine of the β2-adrenergic receptor by G-protein-coupled receptor kinase GRK5 that disrupts receptor binding to the PDZ

domain of NHERF (Cao et 1999; Hall et al., 1998). Residues that can be phosphorylated need not be at -2 position to affect PDZ binding. For instance -3 serine of the AMPA receptor subunit GluR2 C- terminus can be phosphorylated by PKC, and this modification prevents GluR2 binding to the PDZ domain protein GRIP (Matsuda et al., 1999). On the contrary, for DMec2 threonine at position 348 arguably the most critical residue for PDZ recognition, is not predicted to be phosphorylated and the next phosphorylatable residue is found quite far from the C-terminus, at position 311. In any event, all these sites mentioned above might represent regulatory sites; however, these are only speculations as there are not available data for such regulation of the homologous proteins. There are a number of cysteine residues at the N-terminus that could be palmitoylated as it has been shown to be in stomatin (Snyers et al., 1999) and could be contributing to the anchoring of the protein onto the inner leaflet of the plasma membrane. Palmitoylation confers greater membrane affinity, but can also affect a protein functionally or influence its interaction with other proteins and specific membrane domains such as lipid rafts, which are membrane microdomains associated with protein complexes, cholesterol, and sphingolipids.

Although the central portion of the amino acid sequences of the *C. elegans* Mec2, human stomatin and DMec2 display homology, these proteins show also important differences that might imply modified or different functions. The most salient difference between these three proteins is a signature motif at the C-terminus, recognizable by PDZ domain Class I, present only in DMec2 (Fig. 4.3C). This interaction will be discussed in Chapter 5.

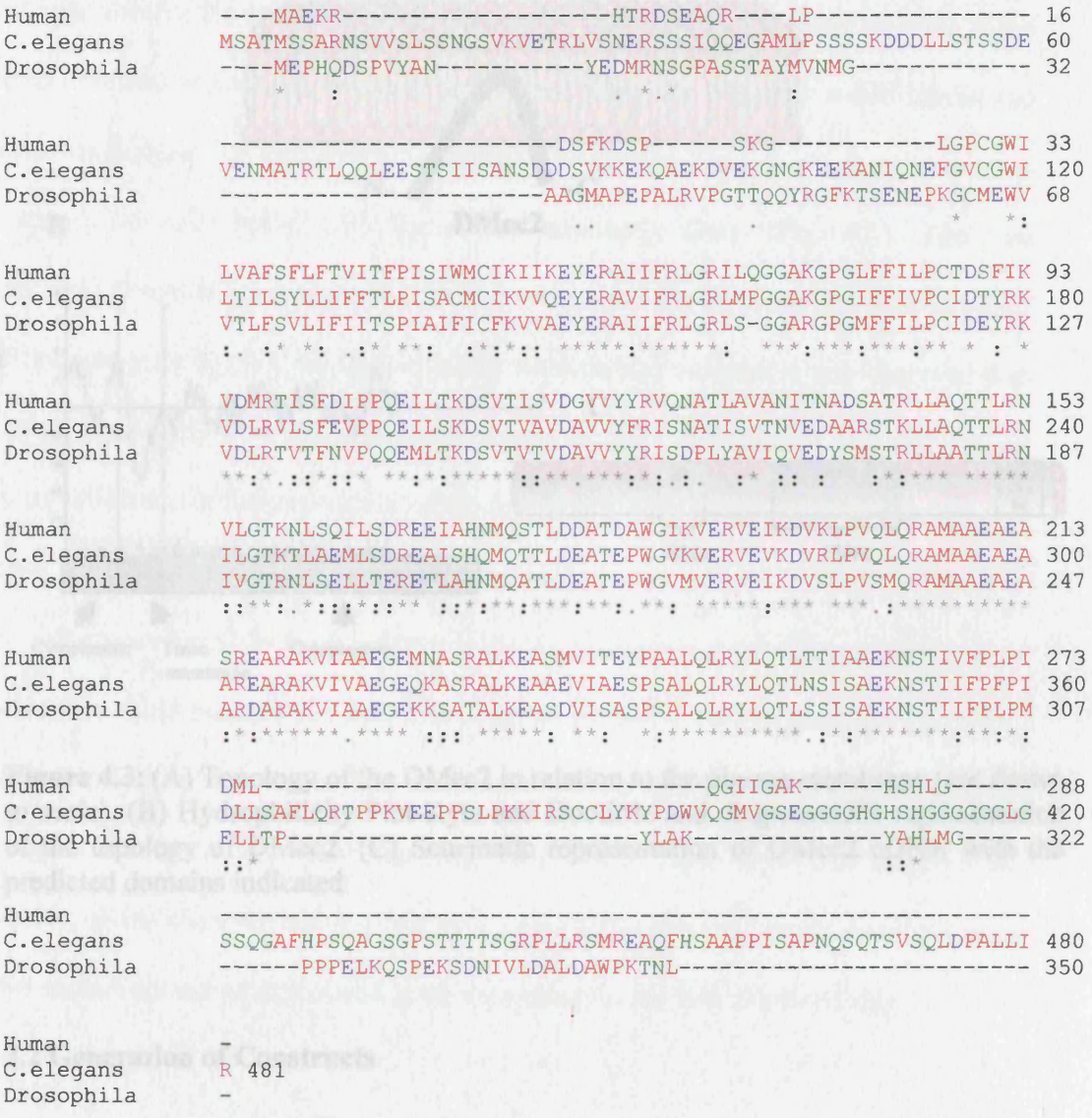


Figure 4.2: Sequence alignment of DMec2, human stomatin, and *C. elegans* Mec2. The sequences were aligned using the ClustalW program, which highlights matching amino acids with the same colour. Under the sequences the identical amino acids are indicated by an asterisk, amino acids of the same nature by a double dot, instead when all three or two out of the three aligned amino acids are of the same nature they are indicated by a single dot.

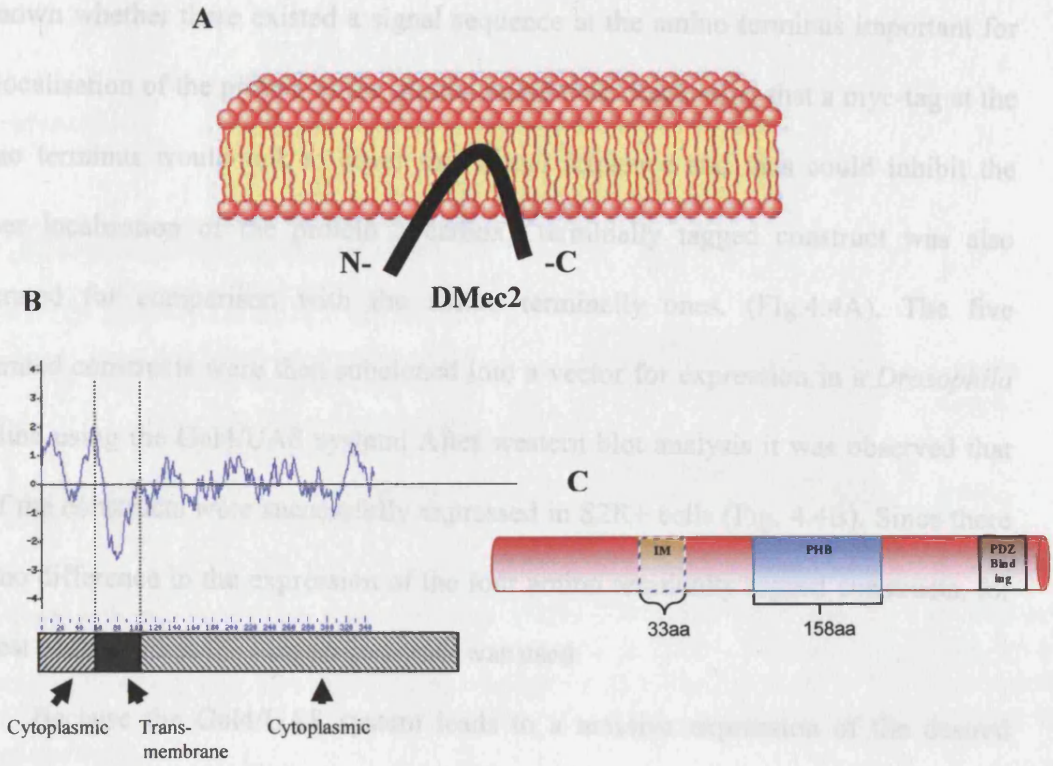


Figure 4.3: (A) Topology of the DMec2 in relation to the plasma membrane (not drawn to scale). (B) Hydrophilicity Plot-Kyte and Doolittle and diagrammatic representation of the topology of DMec2. (C) Schematic representation of DMec2 cDNA with the predicted domains indicated.

2.2 Generation of Constructs

In order to study the function of this novel protein *in vivo,* constructs encoding DMec-2_{myc} were generated by PCR using the *mec-2* cDNA as a template and different primers (Table in Materials and Methods) containing other than the myc tag, a Kozak sequence as well to increase the translation efficiency. Since the exact start site for DMec-2 protein was not known four different amino terminally tagged constructs were generated for the four possible start sites mentioned above (Fig. 4.4A). It was also

unknown whether there existed a signal sequence at the amino terminus important for the localisation of the protein to the plasma membrane. Reckoning that a myc-tag at the amino terminus would risk to cover that signal sequence and thus could inhibit the proper localisation of the protein a carboxy terminally tagged construct was also generated for comparison with the amino terminally ones. (Fig.4.4A). The five generated constructs were then subcloned into a vector for expression in a *Drosophila* cell line using the Gal4/UAS system. After western blot analysis it was observed that all of the constructs were successfully expressed in S2R+ cells (Fig. 4.4B). Since there was no difference in the expression of the four amino terminally tagged constructs, for the rest of the studies the longest construct was used.

Because the Gal4/UAS system leads to a massive expression of the desired protein that could saturate the cell, it was aimed to find a way to moderate its levels. One way employed to achieve that was to carry out a time course experiment during which the cell lysate was harvested after one, two or three days after transfection. However, it was observed that expression follows a "hit and run" mode starting 3 days post-transfection and no expression at all happening on the first or second day.

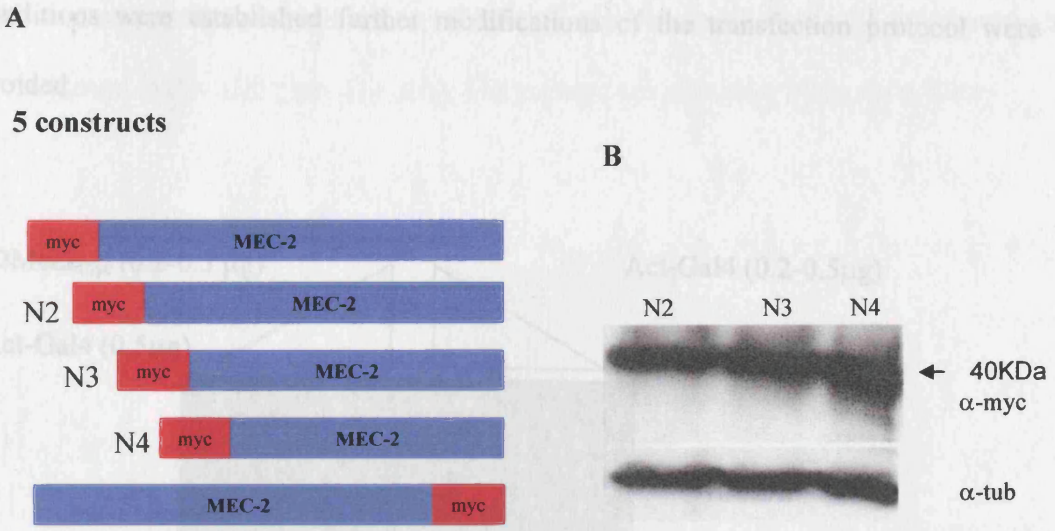


Figure 4.4: A. Schematic representation of the five contructs made. B. The shorter constructs indicated as N2, N3, N4 are expressed in S2R+ cells as it is shown by a western blot analysis. The anti-tubulin blot serves as a loading control. The expression of N1 construct is shown later; the expression of carboxy-terminally tagged construct is not shown.

Therefore moderating the expression levels of the protein by harvesting the cells early after transfection was not possible. A second method applied to fine tune the expression levels of the protein was the use of a gradient of administered DNA for identifying the best working concentration (Fig. 4.5). The same cells were also transfected with UAS-GFP in order to be able to calculate the transfection efficiency. After ensuring by looking under the microscope for GFP expression that these sets of transfections had the same percentage of success, it was concluded that Gal4 and the UAS construct had to be 1:1 mass ratio to get protein production. Thus moderating the expression levels by transfecting with less DNA was not possible to achieve. Successful transfections depended also on the transfection agent and the amount used. Once these

conditions were established further modifications of the transfection protocol were avoided.

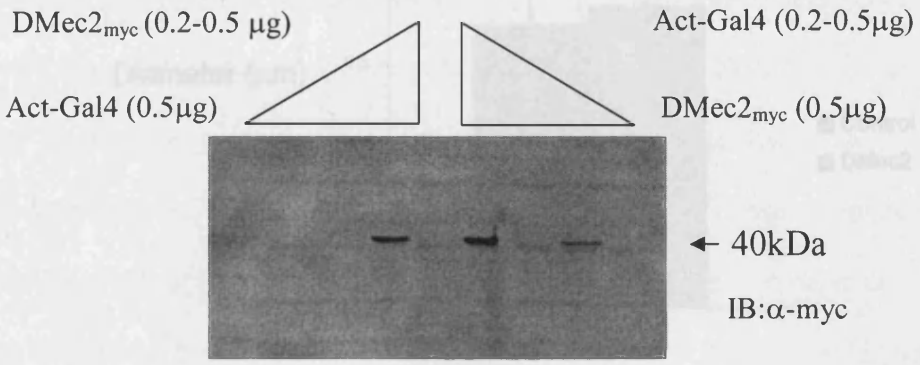


Figure 4.5: Expression levels of UAS-DMec2$_{myc}$ using a gradient of transfected DNA. S2R+ cells were transfected with increasing amounts of UAS-DMec2$_{myc}$ (0.2-0.5µg) and a constant amount of Actin5c-Gal4 (0.5µg) or increasing amounts of Actin5c-Gal4 (0.2-0.5µg) and a constant amount of UAS-DMec2$_{myc}$ (0.5µg). Three days post-transfection the cells were harvested, subjected to SDS-PAGE (10%) and analysed by western blot using an anti-myc antibody.

2.3 DMec2 does not alter the morphology of Drosophila cells

Subsequently, it was studied whether DMec2 could cause cell shape changes. Whether DMec2 could affect the actin cytoskeleton was studied by overexpressing it in *Drosophila* tissue culture cells. For that reason, S2R+ cells were transfected with the longest amino-terminally tagged DMec-2$_{myc}$. Three days post-transfection the cells were fixed and double stained with anti-myc and the actin binding compound phalloidin labeled with TRITC. S2R+ wild type cells upon plating spread well on the surface and are flat. Quantification using Image J and measuring the spreading by

taking as a parameter the diameter of the cells showed that the cells did not change size compared to the wild type (Fig. 4.6). The average cell diameter of the cells where

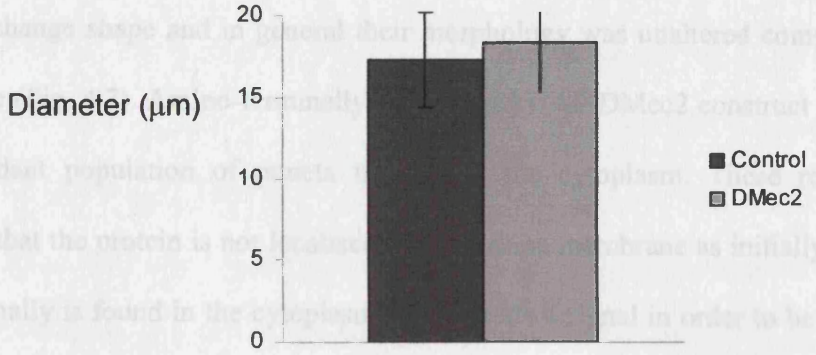


Figure 4.6: Quantification of cell spreading after overexpression of $DMec2_{myc}$ construct. Statistical significance was determined for the difference in cell diameter for cells overexpressing DMec2 compared to control by Student's *t* test where $P < 0.005$. The results show the average cell diameter (n=150) ± S.D. from a single representative experiment.

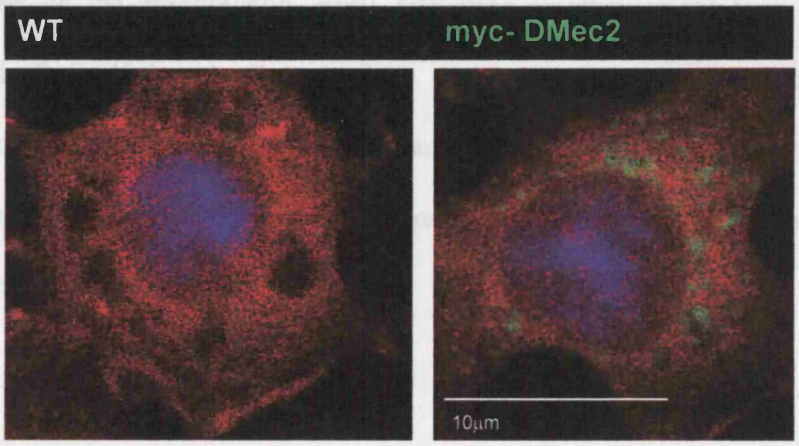


Figure 4.7: DMec2 expression in S2R+ cells. S2R+ cells were transfected with Actin5c-GAL4 and expression vector for the amino terminally tagged UAS-$DMec2_{myc}$. Three days after transfection the cells were fixed and stained with an antibody to myc to show localization of expressed protein. The experiment was analysed by confocal microscopy. This is a representative figure where the expressed protein is seen as green dots. DAPI staining shows the nucleus and TRITC-phalloidin stains the actin cytoskeleton. DMec2 is found in the cytoplasm in a punctate form.

DMec2 was overxpressed was 18μm compared to that of the wild type cells that was 17 μm. From the same experiment it was also observed that overexpression of amino terminally-tagged $DMec2_{myc}$ did not have an effect on the actin cytoskeleton, the cells did not change shape and in general their morphology was unaltered compared to the wild type (Fig. 4.7). Amino-terminally myc-tagged UAS-DMec2 construct was seen as an abundant population of puncta throughout the cytoplasm. These results could indicate that the protein is not localised to the plasma membrane as initially thought or that normally is found in the cytoplasm and it needs a signal in order to be transported to the plasma membrane. Therefore, the possibility that DMec2 could indeed have an effect on the actin cytoskeleton cannot be excluded. The lack of morphological differences, after overexpression of DMec2, contrary to what was expected, might be explained by the fact that the protein is not found in the right conditions to have an effect on the cytoskeleton. Rho1 signalling pathway might have to be activated or DMec2 itself has to be somehow activated in order to induce changes in the morphology of the cell. Another reason for failing to see an effect might be due to the fact that other interacting partners are absent or in very low expression levels in the cells used.

2.4 DMec2 and vesicle localisation

The punctate staining of DMec2 obtained from the immunofluorescence studies is reminiscent of an inclusion of the protein in vesicles. That suggestion prompted the study of vesicular localisation for $DMec2_{myc}$. In order to test the possibility that DMec2 is localised in vesicles, S2R+ cells were transfected with Actin5c-GAL4 and expression vector UAS-$DMec2_{myc}$. Three days after transfection the cells were fixed and double stained with an antibody to myc to show localisation of expressed protein and with an antibody to clathrin as a vesicle marker. Clathrin was used because it mediates transfer of vesicles that bud from the *trans*-Golgi (Molecular Cell Biology, 4th edition). Using that marker could indicate whether DMec2 might be following the Golgi pathway for attachment to the plasma membrane. As it is suggested by figure 4.8, there is colocalisation between DMec2 and vesicles in the region around the nucleus. However, it cannot be concluded that DMec2 is included in the vesicles; it might be just in the same area with the vesicles. Moreover, it is observed in figure 4.8 that there is also a massive population of DMec2 further away from the nucleus not colocalising with the vesicles marked by the anti-clathrin antibody. This could indicate that the protein is not included in the vesicles or that it is included in another kind of vesicular structures that could have been visualised using another marker.

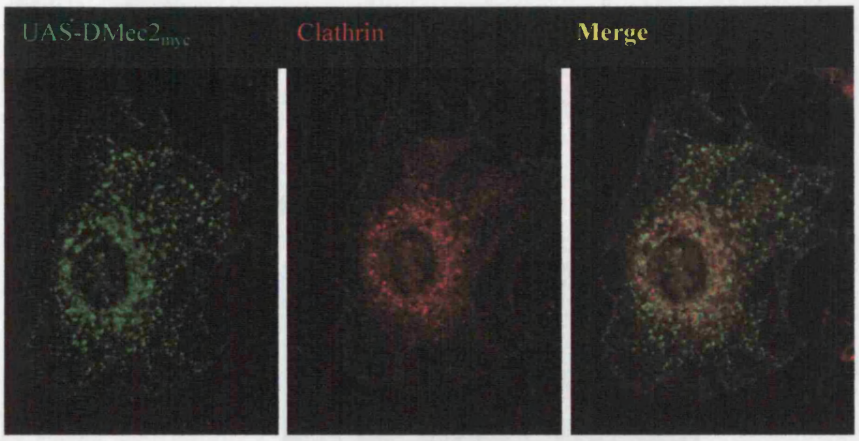


Figure 4.8: $DMec2_{myc}$ expression in S2R+cells and vesicles localisation. The expression of the protein is massive with a substantial population of $DMec2_{myc}$ found in vesicles around the nucleus and a conspicuous amount scattered in the cytoplasm or other type of vesicles.

Vesicles that bud from the *trans*-Golgi network then can fuse with late endosomes; subsequently vesicles which bud from the late endosomes can be sorted to lysosomes. Therefore, the population of DMec2 further away from the vesicles coated with clathrin might represent inclusion of the protein in lysosomes on its way to be degraded.

2.5 Loss of DMec2 does not affect the actin cytoskeleton nor the microtubule network of *Drosophila* cells

In previous section was shown that overexpression of amino terminally-tagged $DMec2_{myc}$ does not have an effect on the morphology of *Drosophila* cells in tissue culture conditions. In addition, it was studied whether loss of DMec2 has any effect on the cell shape of S2R+ cells.

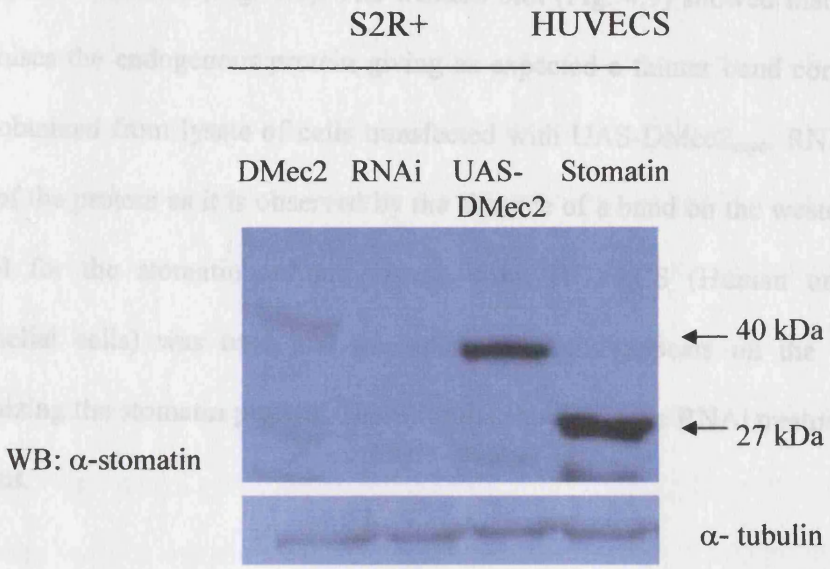


Figure 4.9: Western Blot analysis for RNAi experiment, using an antibody to DMec2 orthologue, stomatin. For the western blot analysis cell lysate was used from non transfected S2R+ cells, S2R+ cells expressing UAS-DMec2, and cells treated with dsRNA to DMec2 for six days. As a control for the anti-stomatin antibody cell lysate was used from HUVECS expressing stomatin. Control for loading is shown as an immunoblot with anti-alpha-tubulin antibody. RNAi has eliminated most of the endogenous DMec2 present in S2R+ cells.

RNA interference (RNAi) is a hitherto well established method to do functional analysis of genes. RNAi in *Drosophila* cells is efficient, reducing or eliminating target-gene expression to elicit partial to complete loss-of-function phenotypes upon the simple addition of double stranded RNA (ds RNA) to the culture medium (Clemens et al., 2000). In order to test in detail the effects of DMec2 on the cell morphology, S2R+ cells were treated with dsRNA against DMec2 for six days. Subsequently they were fixed and stained for immunofluorescence studies. Some of the treated cells were harvested and the cell lysate was analysed by western blotting using an antibody against stomatin, in order to confirm that the protein was eliminated by the introduction of

gene-specific dsRNA (Fig. 4.9). The western blot (Fig. 4.9) showed that the antibody recognises the endogenous protein giving as expected a fainter band compared to the band obtained from lysate of cells transfected with UAS-DMec2$_{myc}$. RNAi eliminates most of the protein as it is observed by the absence of a band on the western blot. As a control for the stomatin antibody lysate from HUVECS (Human umbilical vein endothelial cells) was used and as expected a band appears on the western blot recognizing the stomatin protein. These results show that the RNAi treatment was very efficient.

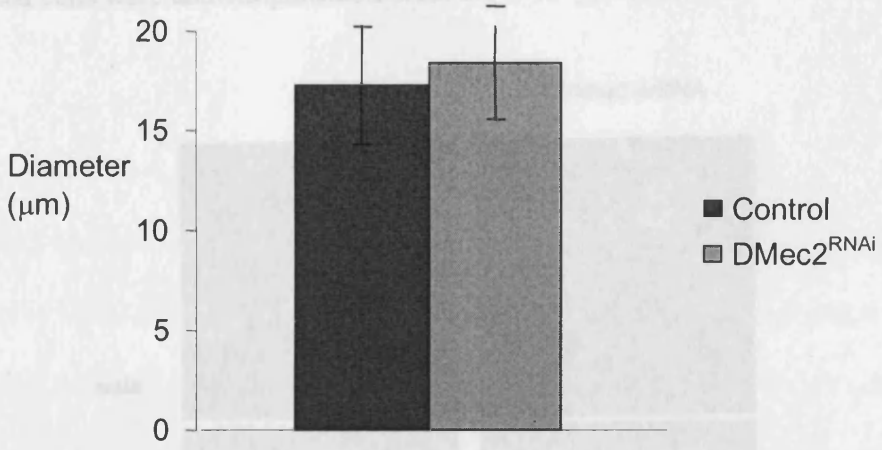


Figure 4.10: Quantification of cell spreading of control cells and cells treated with double stranded RNA against DMec2 for six days. Statistical significance was determined for the difference in cell diameter for cells where DMec2 has been eliminated compared to control by Student's *t* test where $P < 0.005$. The results show the average cell diameter (n=120) ± S.D. from a single representative experiment.

Control S2R+ cells spread well and are flat when they are plated on plastic surface, as previously described. The same spreading and flattening upon plating was observed with the cells treated with DMec2 double stranded RNA. Quantification of the spreading of the cells by taking as a parameter the diameter of the cells (Fig. 4.10) showed that there was no significant difference between the untreated and the treated with RNAi cells and the cell size for both cell categories was approximately 20μm.

In order to see the effects of DMec2 elimination on the actin cytoskeleton, the cells were stained with phalloidin labelled with TRITC. As seen in Figure 4.11, no change in form was observed after treatment of cells with RNAi targeting DMec2; the treated cells were undistinguishable from the wild type cells.

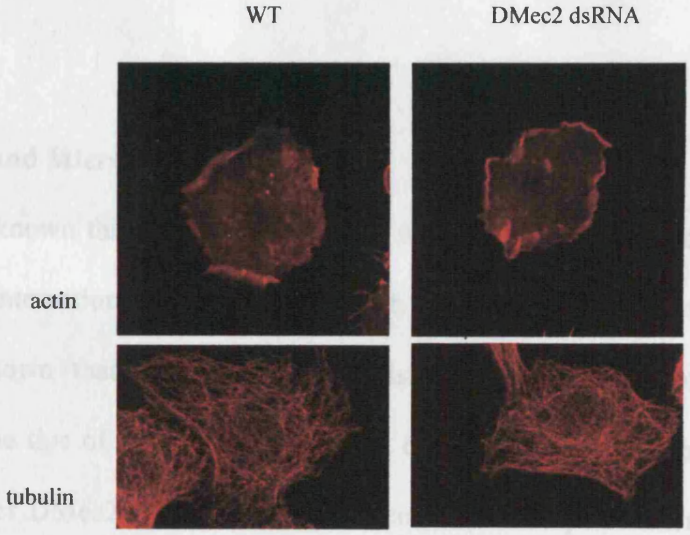


Figure 4.11: RNAi experiment. F-actin was visualized with TRITC-labeled phalloidin 6 days after the addition of DMec2 specific dsRNA. Tubulin was visualized with anti-alpha tubulin antibody 6 days after the addition of DMec2 specific dsRNA. Elimination of DMec2 does not have an effect on the actin nor microtubule network.

Furthermore, the cells were stained with anti-α tubulin to visualise the microtubule network. In the wild type cells a radial microtubule array was observed. Treatment of cells with RNAi did not perturb the microtubule network. The microtubules were still seen as a meshwork of long struts diverging from the centre of the cell.

In conclusion, depletion by RNAi of DMec2 has no effect on the actin or microtubule network. Taken together the results from the overexpression and loss of DMec2 experiments, it is shown that with the assays used the protein under study does not have an effect on the actin filaments or microtubule network in *Drosophila* cells and general cell shape of the S2R+ cells.

2.6 DMec2 and Microtubule Network

It is known that microtubules play a major role in defining cell shape through the specific interaction of their plus ends with proteins at the cell cortex. In addition, it has been shown that DRhoGEF2 colocalises with EB1-a microtubule associated protein- at the tips of microtubules (Rogers, et al., 2004). Therefore, it was aimed to check whether DMec2 could be found associated at the tips of microtubules. To this end, S2R+ cells were transfected with $DMec2_{myc}$ and its localisation was visualised after stabilisation of the mictotubule network. Microtubules were visualised with anti β-tubulin, and exhibited the usual radial array. As it is seen in figure 4.12 DMec2 is not associated with the tips of the microtubules under the conditions tested; instead the protein is found in the perinuclear area.

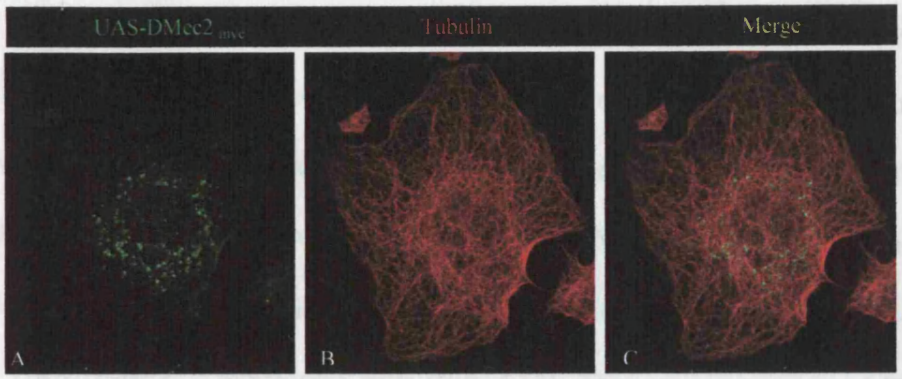


Figure 4.12: DMec2 and tips of Microtubules. S2R+ cells were transfected with Actin5c-GAL4 and expression vector UAS-DMec2$_{myc}$. Three days after transfection the cells were fixed with a special protocol to preserve the microtubule network and stained with an antibody to myc to show localization of expressed protein and with an antibody to β-tubulin to visualise the microtubule network. This is a representative figure where the expressed protein is seen as green dots and the microtubule network is seen in red.

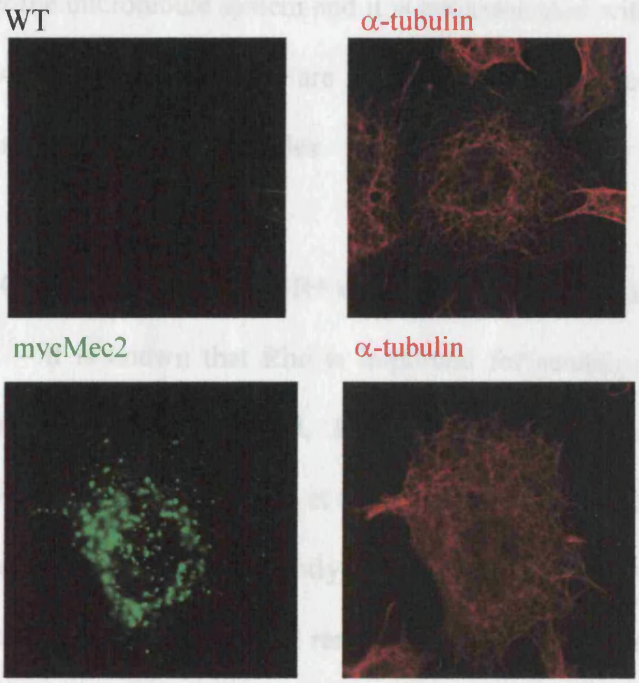


Figure 4.13: DMec2 and microtubule network. S2R+ cells were transfected with Actin5c-GAL4 and expression vector UAS-DMec2$_{myc}$. Three days after transfection the cells were fixed and stained with an antibody to myc to show localization of expressed protein and with an antibody to β-tubulin to visualize the microtubule network. The experiment was analysed by confocal microscopy. This is a representative figure where the expressed protein is seen as green dots and the microtubule network is seen in red. Overexpression of DMec2 does not affect the microtubule network.

To further study the relationship of DMec2 with the microtubule network it was analysed whether DMec2 could have an effect by overexpressing it. For that reason, S2R+ cells were transfected with the expression vector UAS-DMec2$_{myc}$. Three days post-transfection the cells were fixed and stained. Microtubules were visualised with anti β-tubulin; a dense, radial microtubule array was observed in both wild-type cells and UAS-DMec2$_{myc}$ expressing cells (Fig. 4.13). Thus overexpression of DMec2 did not seem to perturb the microtubule network under the conditions tested. In the previous section was shown that loss of DMec2 did not have an effect on the microtubule cytoskeleton. Taken together these results suggest that DMec2 does not alter the microtubule system and it is not associated with the tips of it at the cell cortex. It is possible that the cells are not found in the right environment so that DMec2 can interact with the microtubules.

2.7 Characterisation of S2R+ cell line for shear stress experiments

It is known that Rho is important for sensing mechanical stress (Geiger and Bershadsky, 2001). Indeed, Rho GTPases have been implicated in endothelial responses to shear stress (Li et al., 1999; Tzima et al., 2001), a frictional force exerted by laminar flow. It was recently shown that Rho1 is activated during the early stages of endothelial actin cytoskeletal remodelling induced by shear stress and that it is required for initial cell contraction and depolarisation (Wojciak- Stothard and Ridley, 2003). This suggests that Rho is mediating cytoskeletal changes in response to a form of physical force such as shear stress. In addition, one of the effectors of Rho is the mechano-enzyme, Myosin II. From this evidence it was reasoned that components

upstream of Rho1 might also be responding to mechanical cues other than chemical ones. To explore a possible involvement of DRhoGEF2 and DMec2 in this process it was aimed to establish DMec2 as a relay molecule acting between two mechanosensors and demonstrating that it plays a key role in actin remodelling and reinforcement in response to mechanical forces. To this end, it was first tested whether the Drosophila cell line S2R+ (embryonic epithelial derivative) is amenable to such experiments, due to the lack of Drosophila endothelial cell line. A time course experiment was initially carried out during which S2R+ cells were subjected to shear stress caused by fluid running on top of them at 3dyn/cm^2 (Wojciak-Stothard and Ridley, 2003). Human umbilical vein endothelial cells (HUVECs) within 5 min of stimulation with shear stress show a rapid increase in the number of actin stress fibers (Wojciak-Stothard and Ridley, 2003). This was taken as the hallmark event of the manifestation of a cytoskeletal response to shear stress. Therefore the cells were fixed after 5, 10, 15, 20 minutes of fluid force application and stained with phalloidin labelled with TRITC to visualise F-actin. The shear stress applied seems not to cause the formation of stress fibers during the time intervals and the magnitude of force used (Fig. 4.14).

Nevertheless, it has to be pointed out that there are other kinds of forces that a cell can respond to other than shear stress. Different types of cells are best suited to respond to different types of stimuli.
In order to study the involvement of DMec2 and DRhoGEF2 in a cytoskeletal remodelling due to shear stress another cell line is necessary to be used as a model system.

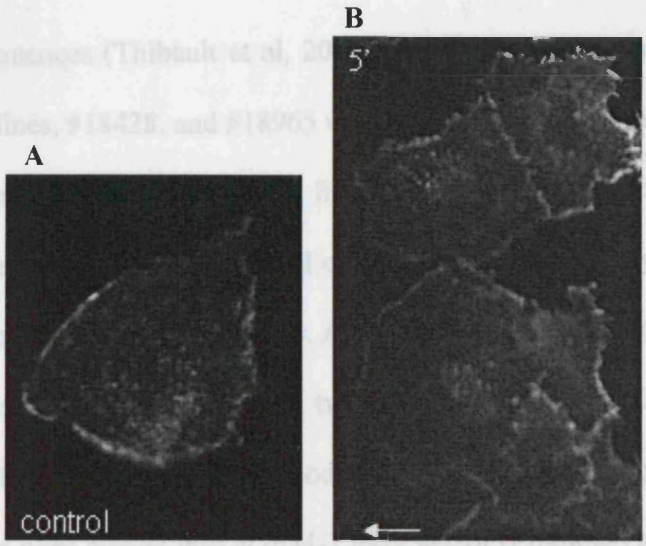


Figure 4.14: Shear stress experiment. Both pictures show F-actin staining in S2R+ cells. The direction of flow is indicated by an arrow. A shows a cell in control (static) conditions, whereas B shows cells stimulated with shear stress 3 dyn/cm^2 for 5 min.

2.8 Function of DMec2 in vivo

To characterise the role of DMec2 during the development of *Drosophila*, it was sought to find mutants for this gene. Two *piggyBac* transposon insertions in the DMec2 locus have been generated by Exelixis and have been defined molecularly by recovery of flanking genomic sequences. The transposon called *piggyBac* is structurally related to Class II inverted repeat elements, it is 2.5 kb long, possesses 13-bp inverted terminal repeats and a 2.1-kb ORF, and demonstrates specificity for the tetranucleotide target sequence TTAA, which it duplicates upon insertion and precisely regenerates upon excision (Lobo et al., 1999). *piggyBac* excisions from the germ line are nearly always precise, it does not share chromosomal hotspots associated with P element and is more effective at gene disruption because it lacks the P element bias for insertion in 5'

regulatory sequences (Thibault et al, 2004). The limits of the *piggyBac* insertions for both DMec2 lines, #18428, and #18965 were confirmed by Inverse PCR.

Genomic DNA was harvested from flies with the transposon inserted into the CG7635 gene and digested by Hin P1I or Sau3AI for the recovery of the 3' and 5' end respectively of the *piggyBac* sequence. After digestion, the fragments were ligated. The ligated genomic DNA was used for two rounds of inverse PCR using two sets of primers (Table in Materials and Methods) for each end of the piggyBac sequence. The PCR products were cloned into pGEM-T easy vector (Promega) and sequenced (MWG Biotech). The flanking genomic sequences recovered coincided with those obtained by Exelixis. Line # 18428, has the transposon inserted in the first exon (position 132918bp of the genomic sequence, accession number AE003511) and for line # 18965 the transposon is found in the fourth exon (position 134584bp of the genomic sequence, accession number AE003511), (Fig. 4.15).

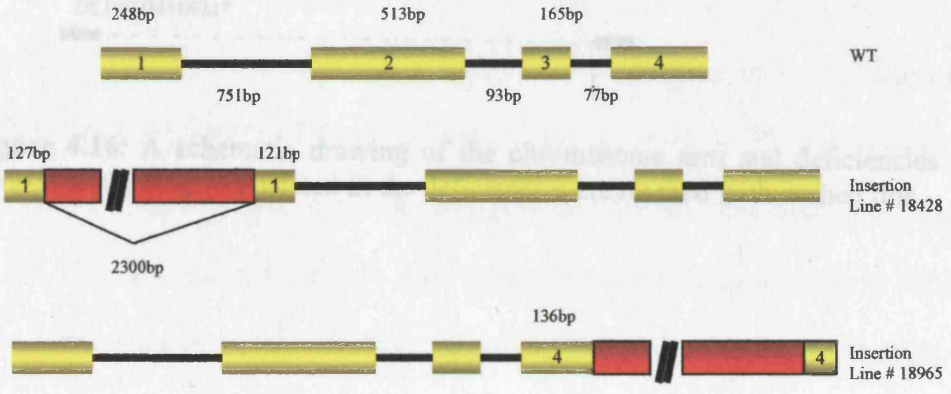


Figure 4.15: Schematic representation of piggyBac insertion into *Dmec2*

The flies with the *piggyBac* insertion were homozygous viable with no visible phenotype. A complementation test using two different deficiencies, Df(1)JA27 and

Df(1)ED7441 ranging [18A5-18D] and [18A3-18C2] respectively (both deleting approximately 40 predicted genes) (Fig. 4.16) were crossed with each *piggyBac* line. The results of these crosses are summarised in the table below (Table 4.1). Given the Mendelian expectation of 34% for the viable genotypes (hemizygous deficiency on Y chromosome is lethal), offspring from these crosses showed no reduced viability. In addition, there was no a visible phenotype.

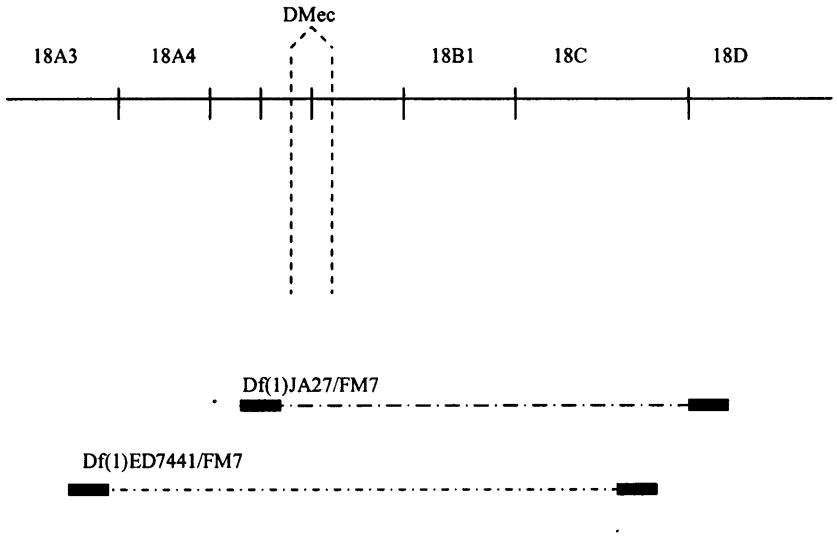


Figure 4.16: A schematic drawing of the chromosome arm and deficiencies around DMec2 locus. Regions deleted in the deficiencies are marked with dashed line.

Table 4.1.

Genotype	Expected % of flies with appropriate genotype	Observed % of flies with appropriate genotype	% Lethality
Df(1)JA27/18428	34	39 (n=168)	0
Df(1)ED7441/18428	34	43 (n=112)	0
Df(1)JA27/18965	34	39 (n=194)	0
Df(1)ED7441/18965	34	33 (n=138)	0

Table 4.1: Results from the crosses of the Deficiencies with the *piggyBac* lines. In parenthesis is indicated the sample number.

2.8.1 Overexpression of DMec2 does not alter the morphology of different tissues

Not having any lethal alleles of DMec2 that might have allowed to assess the function of the gene *in vivo* the transgenic approach was undertaken. Thus, in order to study whether DMec2 could affect the actin cytoskeleton and induce morphological changes, transgenic UAS-DMec2$_{myc}$ flies were generated (EMBL) for overexpression of the gene in different tissues. Five transgenic lines were chosen at random and tested for the effects of overexpression using three different drivers at 18^{0}C and 25^{0}C as the temperature dependence of GAL4 activity in Drosophila is well established (Duffy, 2002). The GAL4-driven DMec2$_{myc}$ protein was analysed by Western blot to ensure that the DMec2$_{myc}$ fusion protein was being produced in the transgenic flies (Fig. 4.17).

The three different drivers used were: i) the eyeless-Gal4 driver which allows gene expression to be specifically targeted to the eye, ii) the MS1096-Gal4 which drives expression in the wing and iii) VP16-V32-GAL4 which drives expression ubiquitously. Overexpression of DMec2 with VP16-V32-GAL4 did not cause high lethality (Table 4.2) and no observable effect. Overexpression of the gene using the ey-GAL4 and MS1096-GAL4 drivers did not cause any defects of the tissues under study;

in other words there were no flies recovered which had an

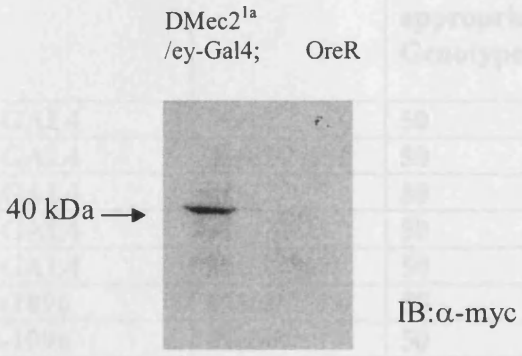


Figure 4.17: Western Blot analysis of protein extracts from adult fly eyes to check for expression of the DMec2 protein in the transgenic flies. The proteins were separated by SDS-PAGE (12%) and transferred to nitrocellulose. DMec2 was identified with anti-myc antibody.

effect in the shape of the eye, or an effect on the shape, hairs, vein network or campaniform sensilla sensory organs of the wing or any other effect. The eyes from adult flies carrying one copy of DMec2 transgene were study in more detail for subtle defects using scanning electron microscopy (SEM) and sectioning of the eye tissue. Scanning electron micrographs of the adult eyes revealed normal hexagonal shape ommatidia and normal number of bristles (Fig. 4.18). The sectioning of the adult eyes of the flies revealed no subtle defects of the different UAS-DMec2 lines. The photoreceptors were characterized by normal shape (Fig.4.19). To sum it up, the eye of the transgenic flies were indistinguishable from those of the wild type. There was no difference in the results between the two temperatures used to carry out the crosses.

Table 4.2:

Driver	Transgenes	Expected % of flies with appropriate Genotype	Observed % of flies with appropriate genotype	% Lethality
Ey-GAL4	$Mec2^{1a}$	50	49 (n=192)	2
Ey-GAL4	$Mec2^{1b}$	50	45 (n=120)	9
Ey-GAL4	$Mec2^{5}$	50	57 (n=146)	0
Ey-GAL4	$Mec2^{10}$	50	49 (n=158)	2
Ey-GAL4	$Mec2^{11}$	50	50 (n=115)	0
MS-1096	$Mec2^{1a}$	50	55 (n=220)	0
MS-1096	$Mec2^{1b}$	50	49 (n=180)	2
MS-1096	$Mec2^{5}$	50	56 (n=239)	0
MS-1096	$Mec2^{10}$	50	48 (n=239)	4
MS-1096	$Mec2^{11}$	50	54 (n=271)	0
VP16-V32	$Mec2^{1a}$	50	50 (n=344)	0
VP16-V32	$Mec2^{1b}$	50	52 (n=338)	0
VP16-V32	$Mec2^{5}$	50	50 (n=128)	0
VP16-V32	$Mec2^{10}$	50	51 (n=367)	0
VP16-V32	$Mec2^{11}$	50	52 (n=246)	0

Table 4.2: UAS-DMec2 expression in different tissues. Crosses were carried out at 25^0 C. In parenthesis is indicated the sample number.

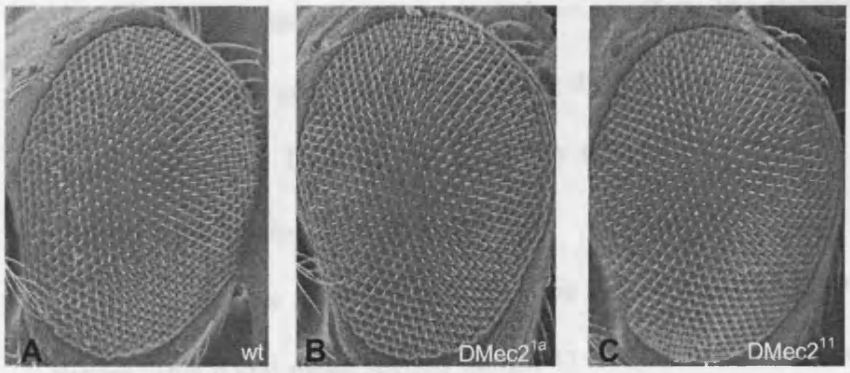


Figure 4.18: Characterisation of the DMec2 eye effect (A-C).Scanning electron micrographs of adult eyes of (A) OreR (wild type, +), and (B-C) two of the transgenic lines of UAS-DMec2 (UAS-DMec2/ey-Gal4). Overexpression of DMec2 does not have an effect on overall eye size, ommatidial shape or bristle number.

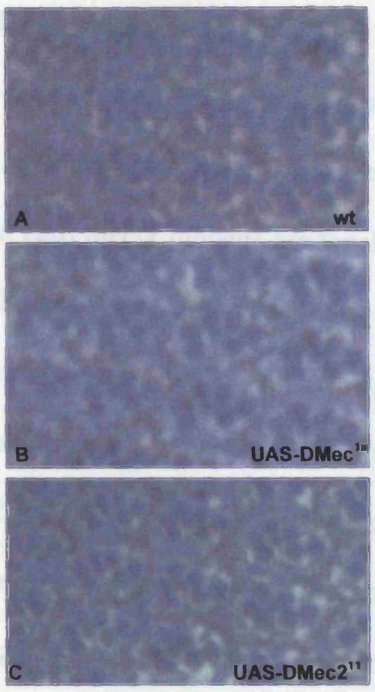


Figure 4.19: (A-C) Toluidine blue-stained transverse retinal section of eyes of wild type (A) and two different DMec2 transgenic lines (B-C) showing normal eye development. Eye section stained with toluidine blue to to visualize the shape of the ommatidia.

The absence of defects observed after overexpression of DMec2 might be due to the fact that still the protein is not expressed at high enough levels. Therefore it was tested whether higher expression would have an effect. The expression levels of DMec2 were increased by generating flies having three copies of DMec2 and expression was driven in the eye. This did not yield any observable effects in the eye nor reduced viability with VP16-V32-GAL4.

The results show no effect of the overexpression of DMec2 in the studied tissues. This could mean several things: 1) DMec2 is not essential for embryogenesis, or eye or wing formation or 2) there might be a compensation for its overexpression, or 3) DMec2 has a completely different function that could not be assessed with the methods used here. For instance it can be a behavioural gene.

2.9 Behavioural test for touch insensitivity

Since there were no observable effects after overexpression of DMec2 in cells and transgenic flies, it was then tested whether this is a behavioural gene. The *C. elegans mec-2* mutants are touch insensitive. Assuming that the *piggyBac* lines have a disrupted gene affecting its normal function, larvae from *piggyback Dmec2* homozygous females and hemizygous males were studied for touch insensitivity. As a paradigm for the behavioural test the assay described by Kernan et.al., (Neuron 12.,1994) was used. Every larval segment has various external and internal mechanosensory organs, including sensory hairs, campaniform sensilla, chordotonal organs and multidendritic neurons, which could be involved in both sensing the stimulus and eliciting the response. A larvae shows a stereotypical response after being stroked by a mechanical stimulus which is a series of multiple waves of the thoracic segments and retreat away from the stimulus. In the screen, larvae moving forward were stroked with the tip of an eyelash across one side of the thoracic segments and the response of the larvae was observed. To quantify the responsiveness of a larva, scores of 0-4 were assigned to the following behaviours: no response (0), hesitation (1), anterior withdrawal/turn (2), single reverse contractile wave (3), multiple waves/retreat (4). One hundred and fifty first instar larvae were tested for touch sensation and all of them fell in the fourth category which is the wild type one; that is after the stimulus the larvae showed multiple waves of the thoracic segments and retreated. These results might suggest that the gene is not disrupted by the *piggyBac* insertion or that the gene is not involved in mechanotransduction of external stimuli in *Drosophila*. UAS-RNAi

DMec2 flies (Joshua Ainsley, personal communication) also did not show any defects in sensing mechanical cues or defects in locomotion.

3. CONCLUSIONS AND DISCUSSION

In this chapter it was tested whether DMec2 can induce cell shape changes. From the results herewith presented, the functional role of DMec2 *per se* is not understood. Overexpression of DMec2 does not seem to affect the cell morphology of quiescent *Drosophila* S2R+ cells. It is noted here the evidence that exists about the DMec2 orthologue, the human stomatin, expressed in mammalian cells and its effect on the actin cytoskeleton. Treatment of UAC (Human amniotic) cells with IL-6 and dexamethasone upregulated stomatin five to six-fold and this treatment was accompanied by a slight morphological change (the cells became bigger and intracellular contacts were more visible) suggesting some modification of the cytoskeleton and/or the plasma membrane. The same treatment had no effect on HeLa (cervix carcinoma) and HMEC (endothelial) cells or any other cell line investigated (Snyers et al., 1997). In the same study it was also shown that stomatin colocalized with actin to some extent in induced UAC cells and that there was a specific association of stomatin with cortical actin microfilament system. The presence of high order oligomers of stomatin on the cytoplasmic side of plasma membrane, its partial association with the cytoskeleton as well as its co-localization with cortical filaments suggest a structural role and indicate that stomatin can play a role in the cortical morphogenesis in UAC cells and perhaps other cells (Snyers et al., 1997). However, it

cannot be excluded that DMec2 may actually have an effect on the actin cytoskeleton as from experiments designed to assess the subcellular distribution of DMec2 in *Drosophila* cells there is some indication that the protein may be retained in vesicles and for that reason might not be reaching the right place for it to act. It cannot be ruled out that the punctate staining corresponds to other organelles, e.g lysosomes, or that the protein aggregates in the cytoplasm because of a certain instability. Using another cell line or another expression vector might have been able to shed more light into the role of DMec2. Endogenous stomatin in UAC cells after upregulation with interleukin-6 and dexamethasone appears divided into two separate pools: one in the plasma membrane and one perinuclear (Snyers et al., 1997). Interestingly, stomatin fused to the myc-epitope at the N-terminus does not reach the plasma membrane but is blocked in the Golgi apparatus and/or the endoplasmic reticulum which might represent polypeptides en route to the plasma membrane (Snyers et al., 1998). On the contrary, C-terminal tagged stomatin displays a fluorescence concentrated in fine plasma membrane folds and extensions and also in the intra-cytoplasmic pool within the Golgi region, staining pattern identical to endogenous stomatin in UAC cells (Snyers et al., 1998). The problem of the intracellular retention of the protein in perinuclear aggregates might have been solved by introducing an internal tag proximal to the C terminus. For example it has been reported that small tags at the N or C terminus of flotillin caused a perturbation of the protein trafficking and resulted in its retention in perinuclear aggresomes. Instead when the tag was introduced internally close to the C terminus flotillin was efficiently transported to the membrane as the wild type protein (Morrow et al., 2002). Alternatively, this perinuclear pattern might be a fixation artifact and

having used another permeabilisation agent a different staining pattern might have been obtained. For example experiments in MDCK cells showed that endogenous RhoB was found in cytoplasmic vesicles when the cells were permeabilised with Triton X-100; instead when they were permeabilised with saponin the protein was found in a juxtanuclear structure (Michaelson et al., 2001).

In another set of experiments it was examined whether small interfering RNA (siRNA) against DMec2 affected cell shape. Depletion of DMec2 protein seems also not to alter the cell shape of the cells examined. In conclusion, with the assays used, DMec2 had no visible effect on S2R+ cells' actin organization, microtubule network, or morphology in general. These results might be due to the fact that the protein is not found in the right conditions in order to act. One possibility could be that DMec2 is part of a complex of proteins one or several of which interact with the cytoskeleton and which interacting partners are missing from the used system. Another possibility is that oligomerisation with itself is prevented, or that the right stimulation was not put forward in order to have an effect on the cell shape in response to the overexpression or elimination of the protein. Alternatively, an association with the actin cytoskeleton could have been better checked with sedimentation experiments and a gel overlay assay.

Since the cell culture studies did not give much insight, the transgenic approach was then undertaken to elucidate the role of DMec2. However, even overexpression of DMec2 in a couple of different tissues on the whole fly did not seem to have an effect. Programmed overexpression of DMec2 by the eye-specific driver *eyeless* did not give a distinct eye phenotype. The eyes of DMec2/ey-GAL4 flies had normal size, with bristle

number and hexagonal shaped ommatidia as the wild type OreR flies. To pinpoint the function of DMec2, the effects of increasing the DMec2 activity by generating flies having three copies of DMec2 was examined but again overexpression of DMec2 did not have an effect on the overall eye size. These data suggest that DMec2 overexpression in the fly eye does not disrupt the ordered structure observed in the wild-type eye, both externally and internally. Overexpression of DMec2 was driven also in the wing and in the whole fly; however, no effect was observed in either case.

Two lines of flies with the transposon *piggyBac* inserted in the DMec2 gene were also tested. These lines were crossed with deficiency lines that eliminate Dmec2 gene together with other genes however these yielded viable flies with no phenotype. With the aim of generating a stronger allele, it was taken advantage of the fact that the *piggyBac* is inserted with an FLP site in the same orientation for two lines. In that case the two piggyBac lines were combined to excise the gene between the FLP sites. A heat-shock flipase was used in order to excise the gene. Subsequently the excision of the gene would have been confirmed with PCR. UAS-RNAi flies for DMec2 gene were also viable (Joshua Ainsley, personal communication) with no phenotype and no touch insensitivity. Therefore the excision of the gene was aborted.

It is not known what is the significance of similarity of DMec2 to only the central part of stomatin and Mec2. All three of these proteins contain a central domain, called the prohibitin like domain (PHB). This domain is present in a number of proteins that are associated with lipid rafts which are microdomains in the plasma membrane involved in the clustering of signalling molecules. The PHB domain is evolutionarily conserved and is found in eukaryotic and prokaryotic membrane proteins. Except for

the prokaryotic proteins carrying the PHB domain some eukaryotic ones seem to have similar functions but others do not, therefore it is not clear whether this domain renders these proteins structurally or functionally related. The human stomatin modulates the gating of ion channels but how this is done is not well understood (Price et al., 2004). In addition, there are other mammalian proteins such as flotillins implicated in various cellular processes that possess the conserved central region common to the aforementioned proteins (Liu et al., 2005). The genome of *C. elegans* encodes nine stomatin-related genes, three of which have been studied genetically and are involved in mechanotransduction (MEC-2) (Huang et al., 1995; Tavernarakis and Driscoll., 1997), locomotion (UNC-24) (Barnes et al., 1996) and responsiveness to volatile anaesthetics (UNC-1) (Rajaram et al., 1998). In *C. elegans* Mec2 has been shown also to interact with ion channel subunits and potentiate the current when expressed in *Xenopus* oocytes (Goodman et al., 2002). It has actually been shown that MEC-2 interacts with the MEC-4 degenerin ion channel subunit via its stomatin-like region, which therefore in this case acts as protein binding domain, while its nonstomatin domains regulate channel activity (Zhang et al., 2004). Finally, the *E. coli* plasma-membrane proteins HflK and HflC (high frequency of lysogenisation) have the region of similarity found in the stomatin family and have a role in the switching from lysogenic to lytic cycle during λ-phage infection (Tavernarakis et al., 1999). All these proteins have different functions in the organisms they are part of therefore it is suggested that this central region forms a distinct domain; it is thought that the specificity for the function of these proteins is conferred by their amino and carboxy termini which are not conserved amongst them. On the other hand the conserved

domain may serve a structural role. For example in *C. elegans* and mammalian stomatin part of this region seems to be important for homo-oligomerisation (Snyers et al., 1998). Perhaps the general role of this domain might be in lipid raft association as it is usually observed that the apparently unrelated PHB-containing proteins are associated with these microdomains.

In conclusion, the role of DMec2 remains an enigma. It might be that DMec2 is not an essential gene or that its function is completely different from what it has been tested for here. For example it may be a behavioural gene involved in sensing anaesthetics or pain as some other members of the stomatin family are. Since it is not known where and when it is expressed it is difficult to speculate on its function. More experiments are definitely needed to establish the physiological function of this gene.

V. RESULTS CHAPTER 5

Chapter 5: ANALYSIS OF THE PHYSICAL AND FUNCTIONAL INTERACTION BETWEEN DRhoGEF2 AND DMec2

1. INTRODUCTION

DRhoGEF2 is thought to receive a signal from Fog through Cta, but it has a more severe phenotype in gastrulation than *cta* and *fog* (Barrett et al., 1997). For this reason, it is possible that DRhoGEF2 may be activated in a Fog/Cta-independent pathway. In fact, because *fog* and *cta* have a non-essential function in the mesoderm, it is thought that there is a second pathway commanding cells to undergo shape changes (Costa et al., 1994). One potential interaction with other components of other signaling pathways could occur through the PDZ domain of DRhoGEF2.

Interestingly the typical GLGF motif that is thought to comprise the ligand binding pocket of PDZ domains (Doyle et al., 1996) is changed to GYGM in DRhoGEF2. The GLGF binding loop has been shown specifically to interact with a (S/T)-X-(V/I/L) (X denoting any amino acid) motif found at the carboxyl terminus of various proteins (Songyang et al., 1997). Consequently, the DRhoGEF2-PDZ domain having a slightly different binding motif might have a different binding specificity.

The novel protein DMec2 was initially identified by a yeast two-hybrid screen (K. Barrett, unpublished data) as a candidate interacting partner of the PDZ domain of DRhoGEF2. DMec2 has a cytoplasmic region with type I C-terminus PDZ-binding motif (T-N-L) conforming to the consensus sequence (S/T)-X-(V/I/L) (Songyang et al., 1997). Therefore, the yeast-two-hybrid assay indicates that DMec2 might actually be a

target for the binding loop of DRhoGEF2-PDZ domain despite the fact that the latter is different from the archetypal PDZ domain. Because DMec2 was initially detected as the prey of PDZ in a yeast-two-hybrid assay, it was sought to confirm the interaction by co-immunoprecipitation. Therefore, the physical interaction between these two proteins is described and further characterized. In addition, two separate approaches were taken to elucidate the functional relevance of this interaction: first the interaction was tested using *Drosophila* cells as a model system to study possible effects on the actin cytoskeleton. In the second approach, the interaction was tested genetically using transgenic flies and alleles of the two genes.

2. RESULTS

2.1 Physical Interaction between the PDZ domain of DRhoGEF2 and DMec2

2.1.1 The Carboxy terminus of DMec2 interacts with the PDZ domain of DRhoGEF2

In order to test whether the PDZ domain interacts with the carboxy terminus of DMec2 co-immunoprecipitation experiments were carried out. S2R+ cells were transfected with an expression vector for the N-terminal myc-epitope-tagged DMec2 (described in Chapter 4). The PDZ domain of DRhoGEF2 was digested from the full length cDNA and subcloned in frame with the T7 tag present in a vector (pETc, Novagen) for expression in *E.coli* cells (BL.21, Invitrogen) after IPTG induction. The cell lysate containing DMec2 was mixed with the purified recombinant T7-PDZ and

immunoprecipitated with an anti-T7 antibody on protein G-Sepharose beads and analyzed by Western Blot with anti-myc. As shown in figure 5.1, the PDZ domain precipitates the myc-tagged DMec2 from transfected cells as seen by a band at 40kDa mark.

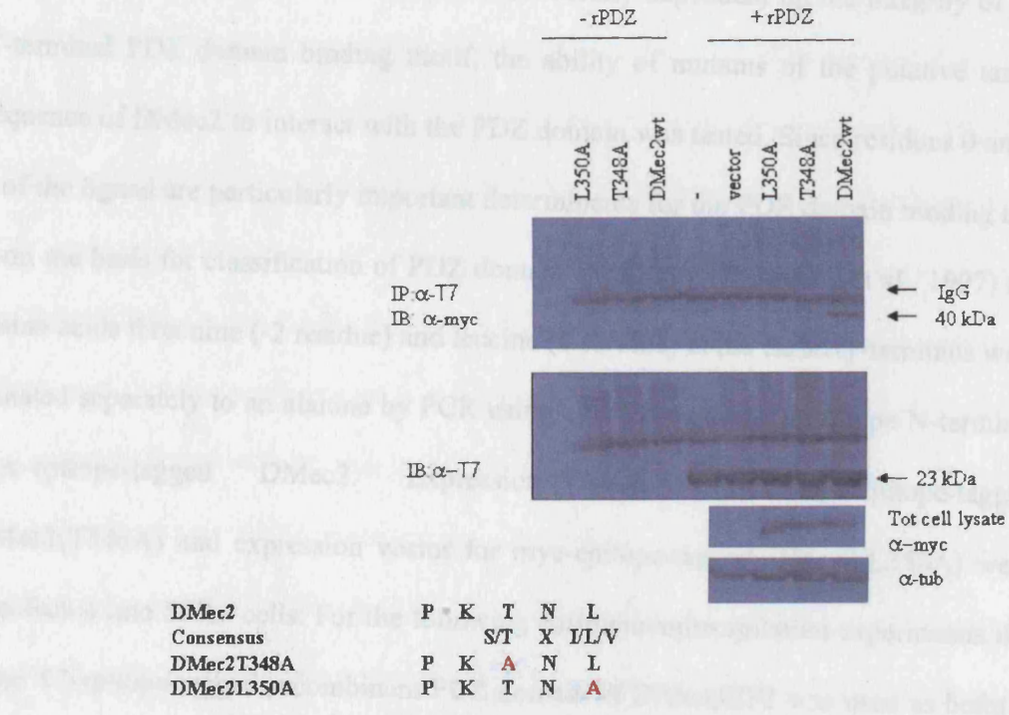


Figure 5.1: Interaction between DMec2 and the PDZ domain of DRhoGEF2. Cells were transfected with amino terminally myc-tagged DMec2 constructs: wild type (DMec2wt), mutated DMec2 where Threonine 348 was changed to Alanine (T348A), and mutated DMec2 where Leucine 350 was changed to an Alanine (L350A). The whole cell lysate was mixed with recombinant amino terminally T7 tagged PDZ domain. The T7-PDZ was immunoprecipitated with the anti-T7 antibody coupled to G-Sepharose coated beads. Shown is the western blot of immunoprecipitated DMec2 probed with anti-myc. The expression of the DMec2 protein was determined by Western blot analysis of the whole cell lysate with the anti-myc antibody. The presence of the recombinant PDZ domain was analysed with the anti-T7 antibody by reprobing the co-immnoprecipitation blot. The anti-tubulin blot serves as a loading control.

Confirmation of the presence and absence of the recombinant protein is shown by the same blot below, after stripping and reprobing it for α-T7, revealing the T7-tagged recombinant PDZ migrating at ~ 23 kDa. Control for loading is shown as an immunoblot with anti α-tubulin.

To check whether this interaction is critically dependent on the integrity of the C-terminal PDZ domain binding motif, the ability of mutants of the putative target sequence of DMec2 to interact with the PDZ domain was tested. Since residues 0 and -2 of the ligand are particularly important determinants for the PDZ domain binding and form the basis for classification of PDZ domain specificity (Songyang et al., 1997) the amino acids threonine (-2 residue) and leucine (0 residue) at the carboxy-terminus were mutated separately to an alanine by PCR using as a template the wild-type N-terminal myc-epitope-tagged DMec2. Expression vector for myc-epitope-tagged DMec2(T348A) and expression vector for myc-epitope-tagged DMec2(L350A) were transfected into S2R+ cells. For the following co-immunoprecipitation experiments the same T7-epitope-tagged recombinant PDZ domain of DRhoGEF2 was used as before. As expected, these mutants failed to immunoprecipitate with the PDZ domain (Fig. 5.1). These results indicate that the interaction occurs through the consensus sequence motif at the carboxy-terminus of DMec2. In addition, they indicate that the amino-acids at position 0 and -2 are necessary for the interaction to take place since mutation of these putative amino-acids is able abolish the binding of the target to the PDZ domain.

2.1.2 Specificity of the Interaction between PDZ domain and DMec2

To test whether the interaction between DMec2 and the PDZ domain of DRhoGEF2 is specific, a mutated form of the PDZ domain was generated by site directed mutagenesis (K. Barrett, unpublished). To generate this mutated form the corresponding residues on the PDZ domain of DRhoGEF2 involved in the binding were deduced by alignment of the primary sequences with the third PDZ domain of PSD-95 (PSD-95-3) and labeled according to their positions in the crystal structures of PSD-95-3. Consequently, in the PDZ mutant two amino acids in the carboxylate-binding groove were mutated. More precisely, tyrosine a hydrophobic amino-acid with a bulky aromatic side chain was substituted with a leucine that is also a non-polar, hydrophobic amino-acid. For the same mutant a methionine, a non-polar and hydrophobic amino-acid was substituted with phenylalanine also a hydrophobic amino-acid but with much bulkier side chain, an aromatic group -the construct presented here was called PDZ(YLMF). In other words the GYGM motif was changed to GLGF. Therefore with these two mutations the carboxylate-binding loop of the PDZ domain of DRhoGEF2 ends up being identical to the binding loop of the third PDZ domain of PSD-95. The aim of these substitutions was to modify the interaction and not to abolish it. It is reported that variations in the size and geometry of the hydrophobic pocket presumably account for the differential preference of various PDZ domains for valine, leucine, isoleucine, phenylalanine, or alanine at the very end of peptide ligands (Songyang et al., 1997).

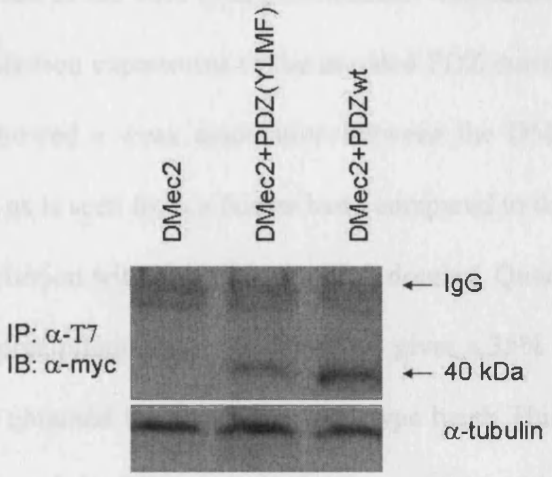


Figure 5.2: Specificity of the Interaction between C-terminal of DMec2 and the PDZ domain of DRhoGEF2. Cells were transfected with amino-terminally myc-tagged DMec2 construct and mixed with recombinant amino-terminally T7 tagged PDZ domain of DRhoGEF2 wild type (PDZwt) or a mutated form of it [PDZ(YLMF)]. The T7-PDZ domains were immunoprecipitated with the anti-T7 antibody coupled to G-Sepharose coated beads. Shown is the western blot of immunoprecipitated DMec2 protein probed for anti-myc antibody. The anti-tubulin blot serves as a loading control.

The mutated form of the PDZ domain of DRhoGEF2 [designated hereafter PDZ(YLMF)] was digested from the full length cDNA and subcloned in frame with the T7 tag present in the vector (pETc, Novagen) for expression in *E.coli* cells (BL.21, Invitrogen) after IPTG induction. N-terminal myc-epitope-tagged DMec2 was obtained after transfection of S2R+cells. The cell lysate containing DMec2 was mixed with the purified recombinant T7-PDZ(YLMF) and immunoprecipitated with an anti-T7

antibody on protein G-Sepharose beads and analyzed by Western Blot with anti-myc. Co-immunoprecipitation of the wild type PDZ domain was carried out in parallel with the co-immunoprecipitation experiment of the mutated PDZ domain. The Western blot analysis (Fig. 5.2) showed a weak association between the DMec2 and the mutated form of PDZ domain as is seen from a fainter band compared to the band obtained from the co-immunoprecipitation with the wild type PDZ domain. Quantification of the band from the co-immunoprecipitation with PDZ(YLMF) gives a 35% decrease in the signal relative to the signal obtained from the PDZ wild type band. This result could suggest that the mutated form of the PDZ domain forms a pocket with a modified size and shape that causes a less efficient interaction with the Carboxy-terminus of DMec2. The experiment described here indicates that the interaction of DMec2 with the mutated form of PDZ domain was less strong therefore it indicates a specific interaction between DMec2 and the PDZ domain of DRhoGEF2.

2.2 Functional Role of the Interaction of DMec2 with PDZ domain

2.2.1 Interaction between DRhoGEF2 and DMec2 in Drosophila tissue culture cells

After having shown *in vitro* that DRhoGEF2 interacts with the carboxy-terminus of DMec2 via its PDZ domain, the physiological relevance of this interaction was studied. In order to study the functional significance of this interaction that is whether the two proteins can induce cell shape changes, *Drosophila* S2 cells were used as a model system. A set of S2 cells was co-transfected with an expression vector for

N-terminal myc-epitope-tagged DMec2 and full length DRhoGEF2. As controls were used a set of S2 cells transfected with the expression vector for N-terminal myc-epitope-tagged DMec2 alone and another set with expression vector for DRhoGEF2 alone. Expression of the proteins was under the control of the (Actin-5c driver)Gal4/UAS system as was previously described. For all experiments cells were plated at the same density 10^6 cells/cm^2 that is confluent upon plating. The S2 cells upon plating attach well and spread on a plastic substrate (Fig. 5.3).

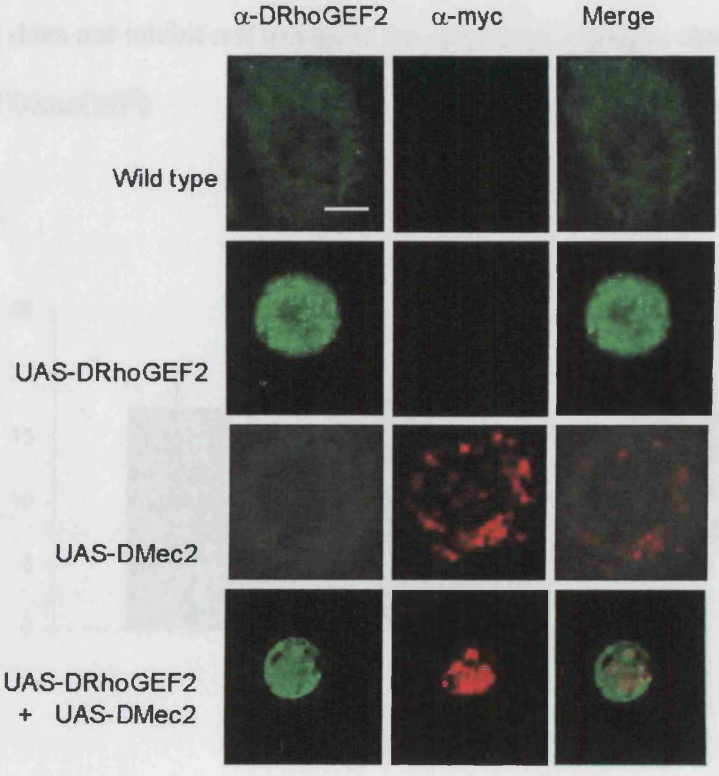


Figure 5.3: Overexpression of DMec2 together with DRhoGEF2 does not enhance nor inhibits the cell rounding that it is observed after overexpression of DRhoGEF2 alone. S2 cells were transfected with (Act5c-Gal4) UAS-DRhoGEF2 and UAS-DMec2$_{myc}$. DRhoGEF2 is visualised in green with an antibody against DRhoGEF2. DMec2 is visualised in red with an antibody against the myc tag. Scale bar 10μm.

When transfected with DRhoGEF2 the cells rounded up as was described in Chapter 3. Instead transfection of S2 cells with DMec2 alone does not seem to cause a change in cell shape. It was aimed to study whether DMec2 could make the cell shape change obtained after overexpression of DRhoGEF2, more or less severe. Co-expression of DRhoGEF2 with DMec2 did not modify the observed phenotype; the cells still rounded to the same degree (Fig. 5.3) giving a size within the range observed when the cells were transfected with DRhoGEF2 alone. The degree of cell rounding was quantified by taking as a parameter the diameter of the cells (Fig. 5.4). The results obtained here show that DMec2 does not inhibit nor enhances the phenotypic changes observed after overexpression of DRhoGEF2.

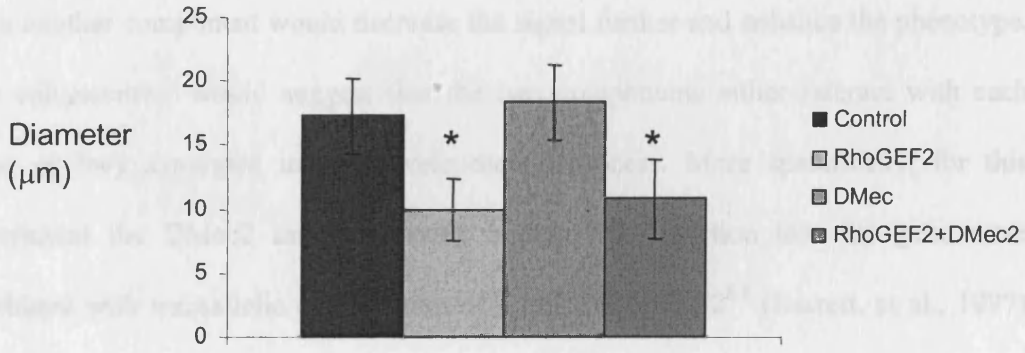


Figure 5.4: Quantification of cell rounding by taking as a parameter the diameter of the cells after transfection of S2 cells with DRhoGEF2 and DMec2. Statistical significance was determined for the difference in cell diameter for cells overexpressing DRhoGEF2, DMec2 or both compared to control by Student's *t* test where $P < 0.005$. The results show the average cell diameter (n=100) ± S.D. from a single representative experiment.

2.2.2 Interaction between DRhoGEF2 and DMec2 in flies

Since the tissue culture studies were not very informative regarding the functional significance of the interaction between DMec2 and DRhoGEF2, it was tested whether the two interacted genetically. Two approaches were used to study the genetic interaction between DMec2 and DRhoGEF2. In the first approach, the interaction between the two genes was studied using a hetero-allelic combination for DRhoGEF2 that causes an easily scored phenotypic change and a mutant copy of DMec2. More precisely, the genetic background of DRhoGEF2 null/hypomorph has an adult wing phenotype that was used as a sensitised system to assess the genetic interaction of DRhoGEF2 with DMec2. The addition of a heterozygous mutation of another molecule presumably participating in the same signaling pathway was expected to cause a more severe phenotype. Since there is still some signal through DRhoGEF2, a reduced signal from another component would decrease the signal further and enhance the phenotype. The enhancement would suggest that the two components either interact with each other or they synergise in the developmental process. More specifically, for this experiment the DMec2 mutants having a piggyBac insertion into the gene were combined with transallelic combination of a null DRhoGEF2$^{4.1}$ (Barrett, et al., 1997) (described in Chapter 3) over a hypomorphic DRhoGEF2$^{6.1}$ (Barrett et al., 1997) allele (described in Chapter 3), and their phenotypes and viability percentages were compared. Both piggyBac lines have 100% viability. Hetero-allelic combinations of DRhoGEF2$^{4.1}$/DRhoGEF2$^{6.1}$ (DRhoGEF2$^{4.1/6.1}$) were approximately 60% viable (Table 5.1 and Fig.5.5). Of the surviving adults, approximately 55% had crumpled wings (Fig.5.6). When the heteroallelic combination of DRhoGEF2 null/hypomorph was

Table 5.1

	RhoGEF2$^{4.1/6.1}$	**18428/+;RhoGEF2$^{4.1/6.1}$**	**18965/+;RhoGEF2$^{4.1/6.1}$**
Viability	60%	63%	57%
Wing phenotype	55%	54%	52%

Table 5.1: Genetic interactions between hetero-allelic DRhoGEF2 (DRhoGEF2$^{4.1}$/DRhoGEF2$^{6.1}$) and heterozygous piggyBac lines (18428 and 18965) of DMec2. In the table the wing phenotype and viability percentages are reported. The piggyBac lines are 100% viable.

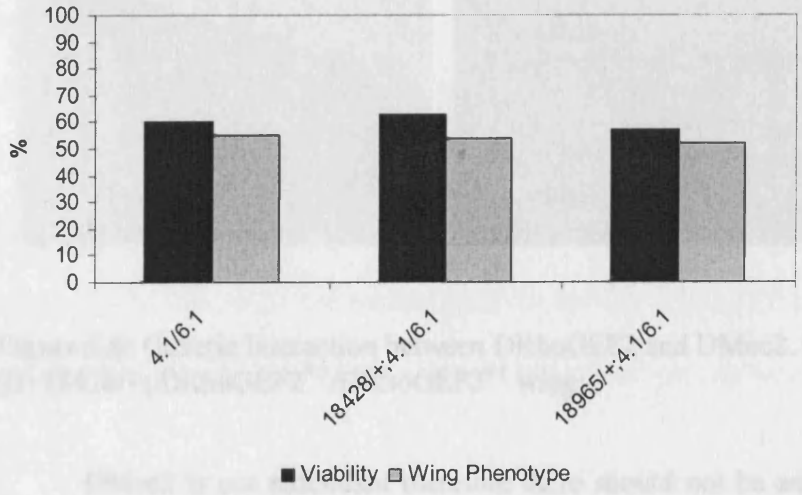


Figure 5.5: Viability and wing phenotype percentages for heteroallelic combination of DRhoGEF2$^{4.1}$/DRhoGEF2$^{6.1}$ (4.1/6.1) with heterozygous *piggyBac* lines18428, and 18965.

present in a trans-heterozygous combination with the piggyBac alleles 18428 and 18965 of DMec2 there was no change in the viability or wing phenotype percentages. DRhoGEF2$^{4.1}$/DRhoGEF2$^{6.1}$ with heterozygous DMec2 allele 18428 showed 63% viability. Of the surviving adults 54% had the crumpled wings. Approximately the same percentages were observed when DRhoGEF2$^{4.1}$/DRhoGEF2$^{6.1}$ was combined with a heterozygous DMec2 allele 18965. Flies with this genotype were 57% viable and of the surviving flies 52% of them had the wing phenotype. From these results is seen that the DMec2 mutants do not have a synergistic effect on the heterozygous combination of DRhoGEF2 alleles (Table 5.1 and Fig.5.5).

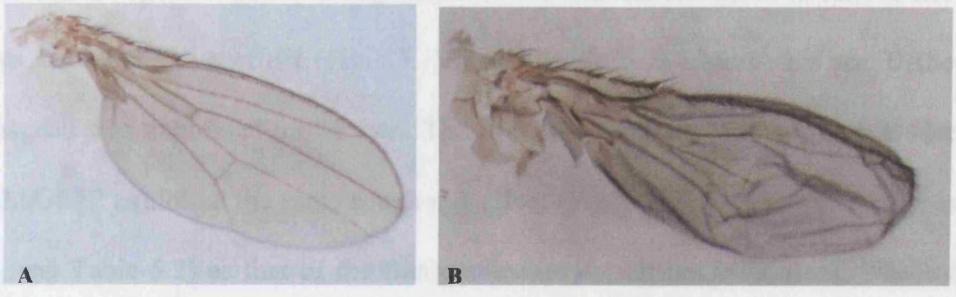


Figure 5.6: Genetic Interaction between DRhoGEF2 and DMec2. (A) Wild type wing, (B) 18428/+; DRhoGEF2$^{4.1}$/DRhoGEF2$^{6.1}$ wing.

DMec2 is not redundant therefore there should not be another gene in the fly genome that could compensate for it. It is difficult to assess whether there is a genetic interaction between DMec2 and DRhoGEF2 using the piggyBac mutants of DMec2 as their nature was not known. It might be that the piggyBac insertion does not disrupt the gene and therefore these are still functional alleles of DMec2.

Therefore in order to test whether DRhoGEF2 and DMec2 interact genetically a second approach was undertaken by using transgenic flies for both genes. Overexpression of Rho1 in the developing fly eye using the synthetic promoter called GMR causes a rough eye phenotype (Hariharan et al., 1997). Thus the *Drosophila* eye was used as a system to study the effects of overexpression of DMec2. Overexpression of DMec2 using the ey-Gal4 did not seem to have an effect on eye development. In contrast, it was shown that excessive activity of DRhoGEF2 perturbed the normal development of the eye. When overexpressed in transgenic lines, wild type forms of DRhoGEF2 disrupted the normal ommatidial structure of the eye and resulted in an externally "rough" effect (Fig. 5.7 and 5.8 and Table 5.2). Overexpression of wild type DRhoGEF2 together with overexpression of DMec2 did not seem to relieve nor make more severe the eye effect (Fig.5.7, 5.8 and Table 5.2) observed when DRhoGEF2 transgene was expressed on its own. The eyes of transgenic flies for both DMec2 and DRhoGEF2 exhibited the same rough eye effect (Fig.5.8) at the same percentages (Fig. 5.7, and Table 5.2) as that of the transgenic flies for DRhoGEF2 alone. These results suggest that DMec2 does not interact genetically with DRhoGEF2. It is noted that the eyeless-GAL4 driver is leaky that is why a high lethality percentage is observed.

Table 5.2

	Ey-Gal4/RG2-6b;Mec2^{9}/+	Ey-Gal4/RG2-6b;+
Viability	14%	12%
Eye effect	18%	10%

Table 5.2: Percentages of viability and eye phenotype obtained from overexpressed wild type DRhoGEF2 transgene and wild type DRhoGEF2 transgene in combination with DMec2 transgene.

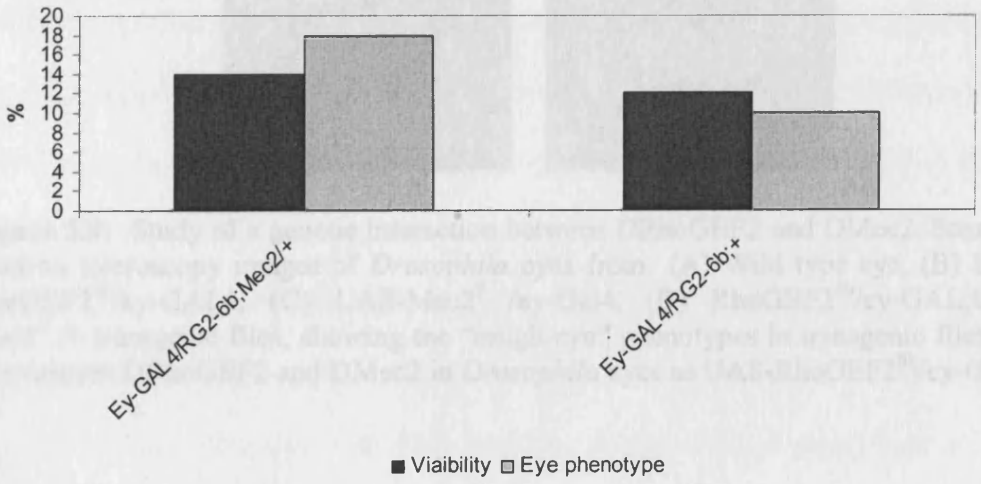


Figure 5.7: Viability and eye effect percentages for heterozygous combination of DRhoGEF2 wild type transgene (line 6b) with DMec2 wild type transgene (line 9) driven by ey-Gal4 (Ey).

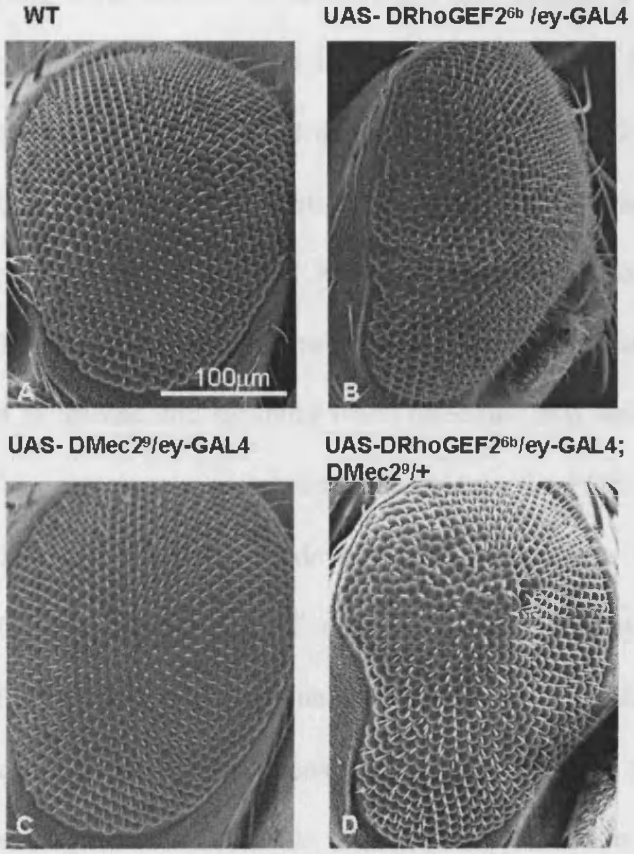


Figure 5.8: Study of a genetic interaction between DRhoGEF2 and DMec2. Scanning electron microscopy images of *Drosophila* eyes from (A) Wild type eye, (B) UAS-RhoGEF2^{6b}/ey-GAL4, (C) UAS-Mec2^{9} /ey-Gal4, (D) RhoGEF2^{6b}/ey-GAL;UAS-Mec2^{9} /+ transgenic flies, showing the "rough-eye" phenotypes in transgenic flies that overexpress DRhoGEF2 and DMec2 in *Drosophila* eyes as UAS-RhoGEF2^{6b}/ey-GAL4 do.

3. CONCLUSIONS AND DISCUSSION

PDZ-containing proteins are often engaged in the formation of supramolecular complexes that carry out localized signaling functions at particular subcellular locations (Harris and Lim, 2001) allowing an efficient signal transduction (Harris and Lim, 2001).

The role of the PDZ domain of DRhoGEF2 in forming a protein-protein complex was addressed. Thus the physical interaction between the PDZ containing nucleotide exchange factor DRhoGEF2 and the novel protein DMec2 was examined. First, it was shown by a co-immunoprecipitation experiment that the interaction occurs through the carboxy terminus of DMec2. Recombinant PDZ domain was able to immunoprecipitate the wild type DMec2. However when the last amino acid of DMec2 leucine was mutated to alanine and similarly when threonine two amino acids before the carboxy end of the protein was mutated to alanine then the PDZ was not able to interact with the constructs and pull them down. These results show that the binding occurs through the C-terminus of DMec2. The lack of interaction between the mutants of DMec2 and the PDZ domain might be due to the fact that the smaller side-chain of alanine could be creating an energetically costly unfilled hole within the hydrophobic cavity rendering the interaction unfavorable. Since the interaction is abolished even when one of the two putative amino acids are mutated it seems that both of them are likely to be necessary for the interaction to occur. This is consistent with the fact that these two amino acids at the C-terminal PDZ domain binding motif are centrally involved in the interaction with PDZ domains. Indeed, PDZ domains bind to short sequences of five to seven residues in their target proteins (Doyle et al., 1996; Songyang et al., 1997). The specificity of these recognition motifs that *per se* are of little importance is typically improved by the requirement that they occur at the C-terminus. The requirement for a C- terminus motif results from a steric rather than an electrostatic mechanism: the peptide-binding pocket is constructed in a way that residues beyond the C-terminus are incompatible with it (Doyle et al., 1996; Harris and

Lim, 2001). In addition, experiments on the effects of salt on the binding reaction suggest that electrostatic contributions are of little consequence (Harris et al., 2003), despite the fact that C-terminal ligands have a negatively charged carboxylate.

The human Na^+/H^+ Exchanger Regulatory Factor (NHERF/EBP50) has a PDZ domain which is similar to the PDZ domain of DRhoGEF2 having a GYGF binding loop. By alignment of the primary sequences of these two PDZ domains, it is seen that they also have a similar amino acid sequence outside the loop. In addition, the target of NHERF/EBP50 has a carboxyl-terminal Leucine. Therefore, the crystal structure of the PDZ1 domain of NHERF/EBP50 could provide insights into the structural basis for carboxyl-terminal Leucine recognition by class I PDZ domains (Karthikeyan et al., 2001). Taking that binding as a paradigm, it could be inferred that the side chain and carboxylate group of DMec2's Leucine could enter into a deep cavity formed by Tyrosine, Glycine, Methionine (of the GYGM loop), Valine (two amino acids outside the loop), and Valine, Isoleucine (in the αB helix). The carboxyl-terminal oxygen atom of Leucine could bind hydrogen directly with the amide nitrogen atoms of Tyrosine and Glycine, and indirectly with the carbonyl oxygen atom of Methionine through a water molecule and through two water molecules with Lysine (Fig. 5.9.). The hydroxyl oxygen of DMec-2's Threonine two amino acids away from the last Leucine residue (Thr-2) could hydrogen bind with the amide nitrogen of Histidine in the αB helix. In addition, there could be hydrogen bonds between the carbonyl oxygen of Thr-2 and the amide nitrogen of Valine as well as between the amide nitrogen of Thr-2 and the

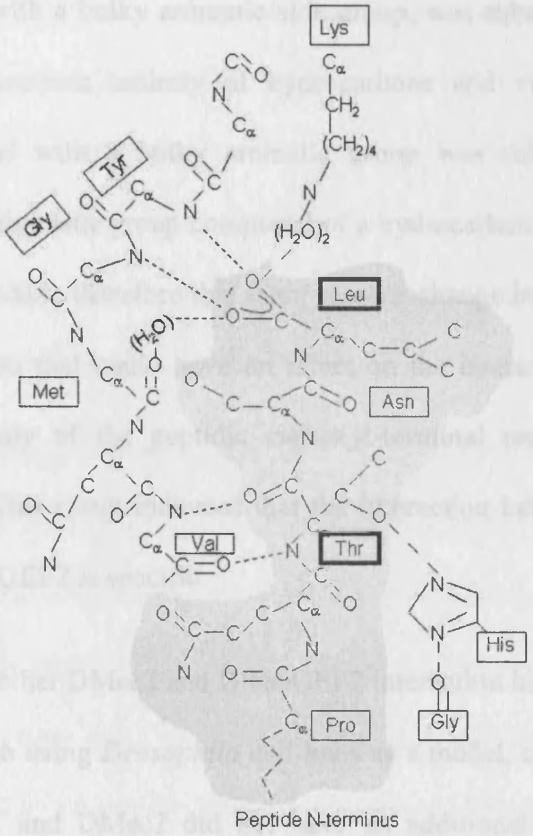


Figure 5.9: Schematic representation of a speculative binding model between the PDZ domain of DRhoGEF2 with its target, the carboxyl terminus of DMec2 (in the shadowed side of the drawing).

carbonyl oxygen of Valine (Val present two amino acids after the binding loop, between βA and βB), (Fig.5.9).

Subsequently, it was studied whether the interaction between DMec2 and the PDZ domain of DRhoGEF2 was specific because that would give an indication of a possible functional relevance of this binding. For this experiment a mutated form of the binding pocket of DRhoGEF2-PDZ domain was used to immunoprecipitate DMec2. The immunoprecipitation of DMec2 by this mutated PDZ domain was less efficient than the one by the wild type PDZ domain. To create the PDZ binding groove mutant

the Tyrosine, an amino acid with a bulky aromatic side group, was substituted with a Leucine whose side chain consists entirely of hydrocarbons and vice versa the Phenylalanine, an amino acid with a bulky aromatic group was substituted with Methionine, an amino acid with a side group composed of a hydrocarbon chain (except for a sulfur atom); it is conceivable therefore that there is some change in the geometry and size of the binding groove that could have an effect on the interaction with the stereochemical complementarity of the peptidic carboxyl-terminal residue and the volume/shape of the cavity. This result indicates that the interaction between DMec2 and the PDZ domain of DRhoGEF2 is specific.

It was then studied whether DMec2 and DRhoGEF2 interaction has a functional relevance. In the first approach using *Drosophila* cell lines as a model, co-transfection of S2 cells with DRhoGEF2 and DMec2 did not have an additional effect to the abnormal phenotype of rounding up due to overexpression of DRhoGEF2 alone. The fact that we do not see any further or less rounding of the cells when DRhoGEF2 is co-expressed with DMec2 compared to what is happening when DRhoGEF2 is overexpressed alone although these two proteins physically interact, might be because this interaction does not have a physiological relevance and actually it does not occur in this cell system or that the conditions of the system are not the right ones to observe a change in the organization and distribution of the actin cytoskeleton. Maybe the two proteins are not in the right stoichiometric proportions, or other interacting partners are absent from the system used or that DMec2 has to be activated. Alternatively, it might be due to the fact that the result of this interaction is uncoupled from effects on the cytoskeleton or that it affects other aspects other than cell rounding. Another

possibility might be that DRhoGEF2 overexpression causes such a severe phenotype that nothing can affect it more. Since DRhoGEF2 is hypothesized to act in a restricted area somehow it has to reach that area or if it is always present to a specific place then somehow it has to be activated locally. It is not clear whether DMec2 physically binds and brings DRhoGEF2 to the place of action or it acts by creating the right architectural milieu for DRhoGEF2 recognition and subsequent activation.

The *Drosophila* wing was previously shown to be a good model system to study the interaction of DRhoGEF2 with other signaling components as these interactions cause phenotypic changes in the wing (K. Nikolaidou unpublished data). Therefore, to test whether there is a physiological relevance of the interaction between DMec2 and DRhoGEF2, DMec2 mutants were put together with a heteroallelic combination of DRhoGEF2 null/hypomorph that causes the wings to become malformed. This study showed that the two genes do not interact. An explanation for not observing a genetic interaction between DMec2 and DRhoGEF2 is the possibility that this not the right system to be looking at. It is possible that the DRhoGEF2 phenotypic change of the wings is not specific to the pathway that DMec2 participates. Since DRhoGEF2 has a lot of phenotypes, maybe DMec2 is in a different pathway. It is also possible that DMec2 acts downstream of DRhoGEF2. For example DMec2 is not used to activate DRhoGEF2 but to become activated by it. Therefore, since DRhoGEF2 in this hetero-allelic combination of null/hypomorph is almost absent DMec2 cannot be activated and so even if it is removed no phenotypic change can be observed.

Studies using transgenic flies for both genes did not reveal a genetic interaction between DMec2 and DRhoGEF2. It is possible that DMec2 is not expressed in high

enough levels therefore increasing its copy number might give an effect. Because DRhoGEF2 overexpression in the eye causes a severe effect it is possible that the effects of overexpressed DMec2 cannot be observed. Therefore future work to test whether these genes interact will have to include a sensitized system without bringing it to its limit, such as the null/hypomorph alleles of DRhoGEF2. Using this genetic background then overexpressed DMec2 can be introduced to study whether there is enhancement or suppression of the null/hypomorph phenotype. More experiments are needed in order to conclude whether DMec2 interacts genetically with DRhoGEF2.

VI. DISCUSSION

Chapter 6 : DISCUSSION

1. Summary

This study showed that DRhoGEF2 causes a rough eye and a crumpled wing effect when overexpressed in the respective tissues. In contrast, overexpression of DRhoGEF2 lacking the PDZ domain did not cause these effects. These preliminary results could suggest that the PDZ domain is acting as a positive regulator for the function of DRhoGEF2. In addition it showed that overexpression or loss of DMec2 does not induce cell shape changes in *Drosophila* tissue culture cells. Furthermore, overexpression of DMec2 in the *Drosophila* eye and wing does not have an effect. DMec2 binds to the PDZ domain of DRhoGEF2. However, overexpression of DMec2 with DRhoGEF2 in cells and in flies does not change the effects caused by overexpression of DRhoGEF2 alone.

2. The function of DMec2

As outlined in the Introduction cell shape changes in morphogenesis are controlled by a sub-family of the Ras family of small GTPases called Rho. The activity of Rho, is controlled by RhoGEFs. The proper function of the Rho pathway requires that the activated Rho is specifically located at the plasma membrane. The mechanism by which Rho is thus located is not yet fully understood. One possibility is that on receipt of a stimulating signal RhoGEF becomes attached to the membrane. Rho then associates with membrane-bound RhoGEF. These stimulating signals can be an

extracellular biochemical one or in particular cases can arise from a mechanical stress coming from outside the cell. Thus for these cases, one could envisage that a protein in the cell membrane has two functions: one to anchor RhoGEF; the other to transduce the mechanical stimuli that control the activity of Rho.

The work in this thesis arose from an observation using the yeast two-hybrid assay that DRhoGEF2 interacted with DMec2. DMec2 belongs to the stomatin family. Stomatin-like proteins are integral membrane proteins with an affinity for lipid raft microdomains (Morrow and Parton, 2005). The central part of these proteins, called the PHB domain, may be the recognition motif for the partitioning of the proteins into lipid rafts. The fact that DMec2 carries this domain, rendered it a very attractive candidate as a binding partner for the PDZ domain of DRhoGEF2 which is thought to act in specific parts of the plasma membrane. It was envisioned that DMec2 could be acting as a chaperone for DRhoGEF2, with the PHB domain detecting the membrane microdomains and then PDZ domain of DRhoGEF2 being responsible for the attachment onto the membrane. Another reason DMec2 was a very appealing candidate was the possibility that DMec2 could be involved in the transduction of mechanical cues. Consequently, DRhoGEF2 could have also been involved in the modification of the actin cytoskeleton due to physical forces. Thus, DMec2 was thought to have a dual role: to help in the localization of DRhoGEF2 as well as to relay information about the physical microenvironment contributing in the spatially restricted activity of DRhoGEF2.

Experiments carried out here to elucidate the function of DMec2 indicate that this protein does not seem to have an effect on the actin or microtubule cytoskeleton.

Based on the tissue culture studies and the experiments using transgenic flies, it seems unlikely that DMec2 participates in the signaling pathway involving DRhoGEF2 leading to cell shape changes. DRhoGEF2 may have other functions for which DMec2 is required. Alternatively, DRhoGEF2 may be influencing any function DMec2 may have. As described above, the interaction between DMec2 and the PDZ domain of DRhoGEF2 might not occur *in vivo*. However, from the co-immunoprecipitation experiments it cannot be excluded that DMec2 could be interacting with another PDZ containing protein.

The mammalian orthologue of DMec2, stomatin, and the *C.elegans* Mec2 do not have a PDZ interaction motif but associate with ion channels via their PHB region. It is therefore possible that DMec2 is interacting via its PHB domain with an ion channel leaving its C-terminus free for interaction with other proteins hence participating in a signaling pathway that has still to be identified.

3. Various domains are involved in the localization of RhoGEFs

Specific subcellular locations are used as hubs of signal transduction pathways. As the starting point of signaling pathways are in defined regions of the plasma membrane, the active proteins initiating them are spatially restricted to a region. Thus, there is a relationship between localization and function of the proteins. The precise subcellular localisation of proteins can depend on certain protein domains and, in some cases, more than one domain is required for proper localisation.

RhoGEFs are one example showing that localisation of the protein is important for activating a signaling pathway. It is thought that the RhoGEFs reside in the cytoplasm and a cue recruits them to the plasma membrane where they can activate Rho. If RhoGEFs reside in the cytoplasm how are they recruited to the plasma membrane? Are they anchored to some cellular structure until a signal comes along to allow their move to the plasma membrane or do they float in the cytoplasm inactive due to a conformational (auto)inhibition?

Here it was hypothesized that the PDZ domain of DRhoGEF2 was important for the targeting of the protein to the membrane. To address this question a DRhoGEF2ΔPDZ construct was used in *Drosophila* cells and its staining pattern was compared with that of the wild type protein. In the absence of activation of the pathway, overexpressed DRhoGEF2 appeared to be distributed throughout the cytoplasm. The same staining was observed for the protein lacking the PDZ domain. Since the wild type protein in quiescent cells was found in the cytoplasm it was impossible to observe a variation in the localisation with the DRhoGEF2ΔPDZ construct. Therefore, it might be that the signaling pathway has to be activated in order to observe the translocation of the protein. Recently this activation has been achieved by transfection with *concertina* (Rogers et al., 2004).

With a substantial cytosolic pool, DRhoGEF2 may be recruited to specific sites by interaction with different proteins. Recruitment of DRhoGEF2 to particular sites may result in the formation of signaling microdomains, where, depending upon the state of activation of its DH/PH domain, DRhoGEF2 could activate Rho1 and hence control local actin filament rearrangements. Rogers et al., (2004) have reported that

DRhoGEF2 associates with the growing ends of microtubules but upon activation it is released from the microtubules and it associates with the plasma membrane.

Is the PDZ domain of DRhoGEF2 the only possible mechanism via which DRhoGEF2 could associate with the plasma membrane? In fact, RhoGEFs can be recruited to the plasma membrane via several different domains; even if they contain a PDZ domain, that is not necessarily used for the shuttling of the protein to the appropriate site of action. For example, Tiam1 is localized to the plasma membrane by virtue of an amino-terminally located PH domain (Michiels et al., 1997). For PDZ-RhoGEF the proline-rich motif next to the DH/PH domain is essential for plasma localization (Togashi et al., 2000). One example that the PDZ domain is used as a means of translocation is the mammalian PDZ containing protein called LARG. LARG is recruited from the cytoplasm to the plasma membrane by plexin-B1 via its PDZ domain (Hirotani et al., 2002; Swiercz et al., 2002).

Other RhoGEFs have PDZ recognition motifs and so are recruited to a subcellular localization by a PDZ containing protein as in the case of βPIX that is recruited to the dendritic spines by the adaptor protein called Shank (Park et al., 2003). Kalirin-7 also requires its PDZ motif for positioning in dendritic spines and mutant Kalirin-7 lacking this motif is diffusely distributed in the cytoplasm (Penzes et al., 2001). There is also the case of RhoGEFs that do not have a PDZ domain or a PDZ binding motif. These RhoGEFs are localized via adaptor proteins as for instance p190 RhoGEF that is probably recruited to subcellular complexes by the adaptor protein JIP-1 (JNK interacting protein-1) (Meyer et al., 1999). One of the homologues of DRhoGEF2, the mammalian p115RhoGEF that does not bear a PDZ domain, is

recruited to the plasma membrane by its RGS and PH domain upon stimulation of the receptor (Bhattacharyya and Wedegaertner, 2003).

Similarly, DRhoGEF2 could be recruited to the plasma membrane via another domain. In fact it has other than the PDZ domain two more domains the PH, and the C1 domain which could be involved in the localization or attachment of proteins to the plasma membrane. The PH domain binds to phosphatidylinositol (Lemmon et al., 1996) and the C1 domain binds to membrane lipids also such as phosphatidylinositol 3-phosphate (Harjes et al., 2006). Thus these domains by binding to lipid molecules could contribute to the membrane association of DRhoGEF2. Some RhoGEFs bind to phospholipids via the PH domain with low affinity and little specificity, which implies that these interactions are insufficient for membrane localization (Snyder, et al., 2001). Therefore it is possible that these domains synergize for the correct positioning of the protein as on their own are not able to provide a strong link to the plasma membrane. One domain might be necessary for the targeting to the plasma membrane and another domain might be necessary for the fine localization and retention to a precise membrane site.

Future work would have to include analysis of a series of epitope-tagged DRhoGEF2 derivatives deleted for the specific domains. This has to be tested in cells transfected with *concertina* as it has been shown that concertina causes the release of DRhoGEF2 from the microtubule tips allowing its association with the plasma membrane (Rogers et al., 2004). Because the protein might be localized in plasma membrane microdomains an immunogold electron microscopy analysis is necessary for more accurate results.

Moreover, PDZ containing proteins are crucial in the generation of signaling complexes at cellular membranes (Fanning and Anderson, 1999). DRhoGEF2, through an interaction with other proteins could recruit additional proteins to these complexes. It is possible therefore that the PDZ domain is not involved in the subcellular localization of DRhoGEF2. Instead it could be important for the localization of an upstream component as for example the receptor. Another possibility is that the PDZ domain could play a role in transporting the target of DRhoGEF2. Activation of certain G protein coupled receptors causes translocation of Rho1 from the cytoplasm to the membrane (Fleming et al., 1996; Kranenburg et al., 2001). Therefore, one critical step in the activation of Rho1 signaling pathway is probably to bring the RhoGEF in close proximity to Rho1 at specific sites of the plasma membrane. It has been recently shown that DRhoGEF2 recruits Rho1 to the cellularization front (Barmchi et al., 2005). It would be very interesting to use DRhoGEF2 mutants for the PDZ domain to test whether DRhoGEF2 is still able to localize Rho1.

4. Role of the PDZ domain for the function of DRhoGEF2

The PDZ domain-mediated interaction between RhoGEF and another molecule may play an important role in the regulation of the GEF activity. For instance the PDZ domains of the mammalian PDZ-RhoGEF and LARG interact with the C-terminus of Plexin-B1 and the insulin-like growth factor (IGF-1) receptor (Taya et al., 2001; Hirotani et al., 2002; Swiercz et al., 2002; Perrot, et al., 2002; Aurandt et al., 2002; Driessens et al., 2002). This interaction is necessary for the activation of Rho1 upon

stimulation of the receptor with the ligand suggesting a role for this PDZ-mediated interaction in the regulation of the GEF activity.

This study also examined whether the PDZ domain acts as a positive or negative regulator for the function of DRhoGEF2. Overexpression of DRhoGEF2 in S2R+ cells caused their rounding. PDZ-RhoGEF when overexpressed in Swiss3T3 and MDCKII cells causes the cell rounding as well (Togashi et al., 2000). Similarly overexpression of p190 RhoGEF (specific activator for Rho1) in N1E-115 cells results in neurite retraction and cell rounding (Gebbink et al., 1997). DRhoGEF2ΔPDZ overexpression did not inhibit nor enhanced the cell rounding observed after overexpression of the wild type construct. This could indicate that the PDZ domain is not involved in the effects of DRhoGEF2 on the actin cytoskeleton. Deletion of the PDZ domain of PDZ-RhoGEF also did not show any demonstrable effects on the ability of PDZ-RhoGEF to induce Rho-dependent pathways (Fukuhara et al., 1999). Similarly, deletion of the PDZ was shown not to affect the biological activities of the Rac1 exchange factor Tiam1 (Michiels et al., 1997).

The role of the PDZ domain for the function of DRhoGEF2 was also studied using transgenic flies. In contrast to the results obtained from the tissue culture studies, experiments using transgenic flies showed that the PDZ domain does have a significant role. Overexpression of DRhoGEF2 in the eye and wings caused a rough eye effect and a malformation of the wings, when its expression was programmed to these specific tissues. These effects might be due to the fact that an increase in cellular concentration of DRhoGEF2 when overexpressed causes an increased activation of Rho1. On the other hand overexpression of the protein lacking the PDZ domain did not have these

effects. These results indicate that the PDZ domain could act as a positive regulator for the function of DRhoGEF2. Since the PDZ domain is essential for the overexpression effects this suggests the PDZ domain is required for Rho1 activation. If that is the case there are two possibilities how that might occur: 1) DRhoGEF2 might be an intermediate between Fog receptor and Rho1 activation, or 2) DRhoGEF2 might be an intermediate between another molecule (from another signaling pathway) and Rho1 activation.

For instance DRhoGEF2 could be interacting with the fog receptor (Fig. 6.1). When DRhoGEF2 is overexpressed more protein can interact with the receptor; therefore the signal is amplified which leads to a Rho1 overactivation. On the other hand if DRhoGEF2 cannot bind to the receptor because it lacks the PDZ domain then the upstream signal cannot be amplified. Furthermore, let's set two assumptions:

a) Concertina antagonizes DRhoGEF2- that is DRhoGEF2 could act as a GAP for Concertina (a $G\alpha_{12}$ homolog). This could happen because DRhoGEF2 bears an RGS like domain and RGS domains have been shown to stimulate GTP hydrolysis of G_α subunits (Berman et al., 1996; Popov et al., 1997). In addition, there is evidence that the mammalian p115RhoGEF acts as a GAP for $G\alpha_{13}$ (Kozasa et al., 1998).

b) DRhoGEF2 interacts with the Fog receptor via the PDZ domain and this interaction is necessary for the Fog-induced Rho1 activation.

Overexpressing only the PDZ domain would turn on the signaling pathway, thus Fog could overactivate Concertina. DRhoGEF2 not being overexpressed could not compensate for the Concertina overactivation because it would not be in sufficient

levels to turn off Concertina in a feedback loop. If no Rho1 activation is occurring that would indicate that DRhoGEF2 is an intermediate between Fog and Rho1 activation.

Alternatively overexpression of DRhoGEF2 entails by itself an amplified signal. When PDZ domain is missing the binding partner cannot transduce the amplified signal downstream in order to influence the activation or the effects of Rho1. Therefore the signal passing through the PDZ domain is necessary for the function of DRhoGEF2.

The activity of DRhoGEF2 has to be tightly regulated in order to achieve the right activation levels for Rho1. The multidomain nature of DRhoGEF2 provides elements for its strict regulation. Each structural element may be assigned with a positive or negative role for the fine tuning of the RhoGEF activation at each stage of the pathway it participates. The closely related to the *Drosophila* DRhoGEF2, PDZ-RhoGEF was shown to interact with the $G\alpha_{12}$ and $G\alpha_{13}$ subunits and that this interaction was mediated by the RGS domain of PDZ-RhoGEF that acts as a negative regulator limiting the extent of activation by the G_{α} subunit (Fukuhara et al., 1999). It is possible that the RGS domain of DRhoGEF2 has the same function when it interacts with Concertina. It is tempting to speculate that the PDZ domain of DRhoGEF2 acts as a positive regulator for the transduction of the signal at the beginning of the pathway instead RGS is needed to damp the intensity of the signal in a subsequent stage (Fig. 6.1).

Future work to explore the biochemical specificity of DRhoGEF2 and the relative contribution of each structural domain needs to include expression plasmids for epitope-tagged forms of wild type and truncated DRhoGEF2 mutants.

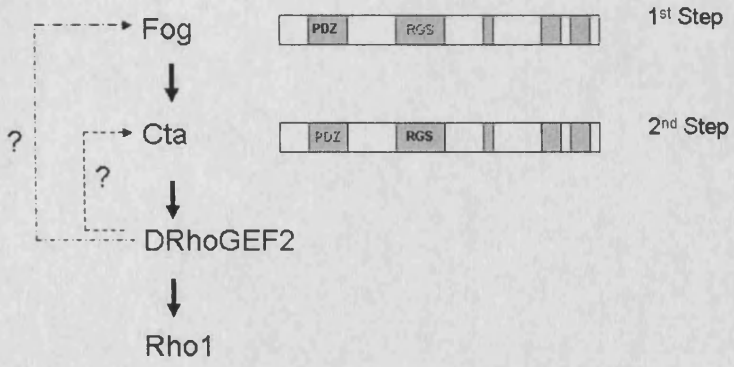


Figure 6.1: Model of the regulation of the Rho1 signaling pathway by DRhoGEF2. In a first step PDZ domain of DRhoGEF2 acts as a positive regulator for the function of DRhoGEF2 possibly by an interaction with the receptor Fog and in a second step the RGS domain through a possible interaction with Concertina acts as a negative one to attenuate the intensity of the signal.

5. PDZ domain of DRhoGEF2: One Versus Multiple targets

Rho GTPases mediate the transduction of extracellular signals that lead to actin rearrangements. However, the mechanism by which they cause cytoskeletal modifications is not completely understood. Regulated reorganization of the actin cytoskeleton is required for precise cell shape changes that occur during morphogenesis (Sullivan and Therkauf, 1995).

DRhoGEF2 functions as a Rho1 specific activator and is an important mediator of the cell shape changes observed during embryogenesis. In addition, there is some evidence suggesting that DRhoGEF2 may regulate specific aspects of Rho1 function (Nikolaidou and Barrett, 2004). However it is not known how the GEF activity of DRhoGEF2 is controlled. DRhoGEF2 has various domains therefore potentially can be

regulated by multiple upstream signals. Evidence supporting control through multiple signaling pathways comes from experiments on *Drosophila* embryogenesis. It has been shown that DRhoGEF2 is required for the well orchestrated cell shape changes during gastrulation (Barrett, 1997; Häcker and Perrimon, 1998). The fact that the phenotypic changes of *fog* or *cta* embryos are not as severe as that of *DRhoGEF2* embryos (Barrett et al., 1997) indicates the requirement for additional signals that work together with DRhoGEF2. One possibility for how these signals are coming into and out of DRhoGEF2 is via its PDZ domain.

The aim of this project was to explore the association of DRhoGEF2 with DMec2 via the PDZ domain and test whether DMec2 could be the transducer of one of these missing signals. As a first approach, it was tested if DMec2 expressed in *Drosophila* cells could associate with the PDZ domain of DRhoGEF2 obtained as a recombinant protein from bacteria. As determined by co-immunoprecipitation the two associated through the carboxy terminal of DMec2. The PDZ domain of DRhoGEF2 is classified as class I, selecting peptides with a hydroxyl amino acid at position -2 (Songyang et al., 1997). The class I PDZ domains interacts preferentially with the C-terminal amino acid sequence (S/T)X(V/I/L) (X representing any amino acid), and bind to the peptides that terminate in a hydrophobic amino acid such as Val, Ile, or Leu. Because the three amino acids of the C terminus of DMec2 are TNL, the finding here is consistent with this prediction. Co-immunoprecipitation experiments using a two amino acid mutated form of the PDZ domain binding loop showed a less efficient association with the DMec2 than with the wild type sequence, indicating a specific interaction between DMec2 and the PDZ domain of DRhoGEF2. Future work to explore better the

binding of DMec2 with the PDZ domain of DRhoGEF2 will have to include full length DRhoGEF2 and the DRhoGEF2ΔPDZ obtained from cell lysates after co-transfection with DMec2. Furthermore, in order to test in greater detail the specificity of the interaction several similar PDZ-containing proteins will have to be included. In addition, to test better the affinity of the interaction, future studies will have to include experiments of isothermal titration calorimetry which is a method used to quantify the binding affinity between two proteins if there is a change in enthalpy upon binding of the two partners. This experiment is carried out by a stepwise injection of one protein in solution into a cell containing the solution of the binding partner. When the two proteins interact heat is released or absorbed in direct proportion of the proteins' molar ratio.

To test whether this binding had a functional consequence, the effect of an interaction between DMec2 and DRhoGEF2 on the actin cytoskeleton of *Drosophila* cells was examined. Overexpression of DRhoGEF2 in S2 cells caused the cells to round. When DRhoGEF2 was co-expressed with DMec2 the phenotype was not enhanced nor inhibited. This might be interpreted as showing that DRhoGEF2-DMec2 interaction is unimportant however much more needs to be known before that conclusion can be accepted. It might be that other factors are needed for the two proteins to cause an effect. The nature of these factors needs further investigation. For instance, it might be that DMec2 has to be activated before the two proteins can come together in the cell. It might be that after the interaction occurs there are other downstream targets in addition that cause an effect on the actin cytoskeleton. Another possibility is that the interaction between DMec2 and DRhoGEF2 does not have an

effect on the actin cytoskeleton. DMec2 might just be creating the right structural environment for the attachment of DRhoGEF2 to the plasma membrane. Another explanation might be that overexpression of DRhoGEF2 causes such a dramatic change in the cell shape that the effects of another protein cannot be observed.

An additional approach was undertaken in order to test if the two genes interact. Transgenic flies overexpressing in the eye or wing wild type DRhoGEF2 showed phenotypic changes in those organs and high lethality levels. Overexpression of DMec2 alone did not seem to have an effect in the eye, wing or lethality levels. If DMec2 had a synergistic or inhibitory effect to the function of DRhoGEF2 then when put together with DRhoGEF2 it should enhance or suppress the phenotype. However, in this study overexpression of DMec2 did not seem to influence the effects of DRhoGEF2 overexpression. This could mean that the two genes do not interact.

The yeast-two-hybrid assay used to find interacting partners for the PDZ domain of DRhoGEF2 revealed two more potential candidates which have not been either confirmed or excluded yet. In addition, there is another possible interaction between the PDZ domain and a protein called T48 whose function is as yet unknown (Maria Leptin, unpublished data). And there could also be the possibility that none of these interactions is really happening *in vivo* and there could be other candidates that are missed out. For instance the PDZ domain could interact directly with the receptor fog.

One question that arises from the indication that there are several potential binding partners is whether it is possible that all of these candidates are actually interacting with the PDZ domain of DRhoGEF2. One possibility is that they could be

interacting at different times and/or tissues or different subcellular locations. An example where PDZ-containing protein binds to several partners is the mammalian RhoGEF called LARG. LARG binds to the carboxy terminus of plexin-B1 receptor (Hirotani et al., 2002), LPA receptor (Yamada et al., 2005), and IGF-1 receptor (Taya et al., 2001). However such alternative binding seems less likely for DRhoGEF2.

DRhoGEF2 is ubiquitously expressed but it seems to have a specialized function. If DRhoGEF2 has such a specialized function it is quite unlikely to have several binding partners for its PDZ domain. One argument in support of this is the conservation of this signaling pathway in processes with similar outcomes even if they happen in different tissues. For example, DRhoGEF2 is involved in processes that require the contraction of actomyosin rings such as epithelial folding occurring in gastrulation (Barrett et al., 1997), and also in imaginal disc development and in salivary gland formation (Nikolaidou and Barrett, 2004). In all these processes Fog and Concertina are used as upstream components of a pathway leading to DRhoGEF2 and Rho1 (Nikolaidou and Barrett, 2004). Since DRhoGEF2 has to control the activation of Rho1 for this purpose alone DRhoGEF2 itself has to be tightly regulated and promiscuous interactions for its PDZ domain could not be afforded. Thus, two of these possible interacting partners have to be eliminated and just one has to be the *bona fide* partner in this particular pathway that leads to the aforementioned changes of the actin organisation. This is not to say that there cannot exist another partner for DRhoGEF2 that might be involved in the activation of Rho1 for other purposes such as regulation of the cytoskeleton during another process (i.e. mitosis).

6. Studying Biomechanics *in vivo*

In the beginning of this study it was hypothesized that a link between mechanotransduction and the DRhoGEF2 signalling pathway could be established (See Introduction). This was based solely on the fact that DRhoGEF2 physically interacted with DMec2, a protein whose homologue in *C. elegans* is known to be involved in the transduction of mechanical signals caused by an external force. Therefore, it was hoped to study the influence of forces on the function of DRhoGEF2 during gastrulation. This hypothesis prompted the study of how to approach this objective. Here, I describe one developmental process-dorsal closure- on which the role of forces has been studied, in order to set the problem, and explain the variables that can be studied. I then draw parallels with gastrulation to explain whether this study is feasible or not.

Several questions arise regarding the study of biomechanics. Is it possible that a developing tissue has mechanical properties and is it possible that such properties might contribute to forces for morphogenesis? In other words, during developmental processes is there a change in the equilibrium of forces that hypothetically define the cell architecture or tissue integrity that can generate a resultant force of enough magnitude that can be harnessed by the cytoskeleton?

One example suggesting that such a possibility could occur in nature comes from studies during dorsal closure in *Drosophila* (Kiehart et al., 2000; Hutson, et al., 2003). During this process the surface of the embryo is under intrinsic tension and multiple forces are contributing to its completion (Kiehart et al., 2000). These forces are generated by a wave of constrictions at the leading edge of the lateral epidermis and by a tension in the amnioserosa (the membrane that covers the hole) (Kiehart et al.,

2000). These forces can contribute to the movement of the leading edge of the lateral epidermis toward the dorsal midline to close the hole. However what is required for the completion of the process (zippering stage) is the opposing force generated by an anisotropic (discontinuous/not uniform within the tissue) tension in the lateral epidermis (Kiehart et al., 2000). Overall, dorsal closure is characterised by a set of cellular processes that generate forces with a contribution to the event.

Is this the only developmental event during which forces have such an explicit role? Is it plausible that *Drosophila* gastrulation could be seen as a biomechanical process? If yes, would that imply that the individual proteins required to carry out this process could be influenced by forces?

Comparably to the dorsal closure, the main event in gastrulation is movement. There are four central movements that make the cell rearrangements during gastrulation: 1) epithelial bending, 2) rearrangements of cells within the plane of epithelia 3) delamination of single cells as well as of whole epithelia for epithelial-mesenchymal transitions, and 4) cell migration of single or group of cells.

During epithelial bending the invaginating cells constrict their apical circumference induced by actomyosin networks (Dawes-Hoang et al., 2005), causing the cells to become wedge-shaped. This cell shape change draws the sheet of cells inward at that point as long as the cells are attached to each other. In theory, this stage could be thought of as driven mainly by contraction forces. In a mechanical model for the morphogenetic folding of embryonic epithelia based on hypothesised mechanical properties of the cellular cytoskeleton, a wave of constrictions is triggered by a single cell at the centre of the future furrow, making the initial constriction (Odell et al.,

1981). Since the cells are thought to be joined to each other the initial constriction causes a propagating contraction which is sufficient to cause invagination of the embryonic epithelia. However, this does not seem to happen in nature. Instead it appears that cells constrict autonomously under the genetic control of fate-determining transcription factors. More precisely, the cell shape changes associated with ventral furrow formation by a small number of constricting cells depend on the transcription factors Snail and Twist, and single wild type cells in a *snail twist* mutant mesoderm are able to undertake the typical shape rearrangements independently of the neighbouring mutant cells that remain unchanged (Leptin and Roth, 1994). Thus during apical constriction there is the generation of intrinsic forces rather than extrinsic ones. In addition DRhoGEF2 function may not be essential for the generation of contractile force, but rather for regulating the temporal and spatial coordination of actomyosin contractility (Barmchi et al., 2005). Therefore it is rather difficult to make a link between DRhoGEF2 and physical forces.

During the subsequent movements such as cell intercalation, epithelial to mesenchymal transition as well as cell migration DRhoGEF2 has not been shown to play a role.

In order to study the contribution of forces during morphogenesis there has to be a tensional force generated in the actin cytoskeleton which is opposed by another tissue or by the extracellular matrix in order to feedback to alter the cell form. The hypothetical mechanotransduction machinery is thought to be composed of two anchors (See Introduction): the extracellular matrix/integrins and the microtubule/actin cytoskeleton. Are these components put in place in the gastrulating *Drosophila*

embryo? The extracellular matrix and its receptors, have been found to play no role in gastrulation movement, as they are expressed only after gastrulation (Leptin, 2005). Ion channels are also components of another model of mechanotranduction machinery. In *C. elegans* Mec2 interacts with members of the DEG/ENaC channels involved in mechanosensation (Goodman et al., 2002). In *Drosophila* one member of this family called ripped pocket (RPK) is expressed in early stage (0-3h) embryos-much before gastrulation- but it is not present in later stages of embryogenesis and it has not been implicated in mechanosensation (Adams et al., 1998). Other members called pickpocket (PPK) are expressed much later in development in the sensory dendrites of a subset of peripheral neurons of late stage embryos and early larvae (Adams et al., 1998).

In conclusion, the system to be used in order to study how tension that arises within a tissue, generates a force which is transduced across and harnessed by the cytoskeleton during a morphogenetic event has to be carefully chosen. For example the sea urchin embryo is an appropriate model to study the mechanics of epithelial invagination because mechanical properties can be attributed to its filamentous cytoskeleton, the cell-cell junctional complexes and adhesion sites between cells and the extracellular matrix with traceable roles in morphogenesis (Davidson et al., 1999).

In order to study forces during a morphogenetic event first it has to be established that there is a relative tension/stiffness between at least two contiguous tissues. Secondly, the cellular structures responsible for this tension can be identified by disrupting their assembly for instance by using cytochalasin D to disrupt the actin, nocodazole to disrupt the microtubule network and glycine extraction to disrupt the

extracellular matrix. Subsequently, the tension can be released for example by photoablation in order to identify from where the endogenous forces emanate.

7. Conclusion

The appropriate response to an extracellular stimulus is dependent on the intensity and duration of the signal. Regulation of the Rho1 signalling occurs at multiple levels, including the receptor, the G protein, the GEF and the effector. In addition the specificity of the signal might be due to the restricted expression of a ligand that initiates the response.

The observations made in this study suggest that the PDZ domain may mediate an interaction between DRhoGEF2 and its partner to play an important role in the regulation of the GEF activity. The mechanism by which this domain regulates the function of the protein is not clear. DRhoGEF2 might be at the crossroad of various pathways integrating different signals or it might be acting in a feedback loop. It still remains an open question an important aspect of the DRhoGEF2 regulation: if and how it is localised to the plasma membrane. Further studies will follow to identify the missing players from the Rho1 signalling pathway.

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