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Regulation and targets of Mal-D during border cell migration in *Drosophila melanogaster* oogenesis

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Summary

Cell migration is an important process in the life of many organisms. In multicellular organisms it is tightly regulated by the action of cell signaling pathways and their transcriptional outputs. Although cell signaling and transcriptional changes that lead to the induction of migratory behavior are relatively well studied, transcriptional changes that occur during the migratory behavior and the signaling pathways that get activated in response to mechanical interactions between cell and substrate are largely unknown.

Border cells, a group of specialized follicle cells that commit collective migration during the oogenesis of *Drosophila,* constitute a useful migration model. Previous work in our laboratory by Kalman Somogyi identified Mal-D, a transcriptional co-activator of DSRF, is important for border cell migration. *mal-D* mutation causes decrease of F-Actin levels and loss of cellular integrity in border cells. Moreover Mal-D was found to accumulate in the nucleus of some border cells while the cluster is migrating and only if the cluster is migrating. A suggested mechanism was that the border cells receive a migration related signal, such as an increase of cellular tension and send Mal-D to the nucleus.

The first part of my project was to understand how Mal-D is regulated by the migration. In order to visualize subcellular distribution of Mal-D I generated a tagged version of the endogenous protein by using homologous recombination. Analysis of subcellular distribution of Mal-D with this tool showed that the increase in nuclear levels of Mal-D in migrating cells is the result of an overall increase in the level of Mal-D protein and not redistribution of a fixed amount of protein. Furthermore I identified that mutations in Profilin or DSRF affect the nuclear levels of Mal-D.

In the second part of my project I focused on the targets of Mal-D. I isolated border cell mutant for Mal-D or wild-type, and I compared their gene expression profiles by using microarrays. This analysis identified 171 genes down-regulated more than two fold in *mal-D* mutant border cells reproducibly in all three biological repeats. I analyzed three genes that could be relevant for the observed phenotype of *mal-D* mutants, namely *CG30440, CG1344* and *if* further. Preliminary data suggests that *CG30440* and *CG1344* may play role in mal-D phenotype in border cell migration.

Zusammenfassung

Zellmigration ist ein wichtiger Schritt im Lebenzyklus vieler Organismen. In mehrzelligen Organismen wird die Zellmigration über Signaltransduktion und die Auswirkungen dieser Signale auf die Transkription kontrolliert. Die Signalkaskaden und Transkriptionsereignisse, welche die Induktion der Zellmigration regulieren, sind vielfach untersucht worden. Im Kontrast dazu sind sowohl die durch mechanische Interaktionen aktivierten Signalkaskaden, als auch die Veränderungen der transkriptionellen Aktivität migrierender Zellen zum Zeitpunkt der Migration vielfach noch unbekannt.

Ein geeingentes Modelsystem für die Analyse von migrierenden Zellen sind die sogenannten *Border Cells*, eine Gruppe von speziallisierten Follikelzellen die in der Oogenese von *D.melanogaster* als Zellcluster migrieren. In vorangehenden Experimenten, welche von Kalman Somogyi durchgeführt wurden, hat unser Labor Mal-D als einen transktioptionellen Co-Aktivator von D-SRF identifiziert, mit einer wichtigen Funktion in der Migration von *Border Cells*. Die Mutation des *mal-D* Gens führt zur Reduktion von filamentösem Aktin und dadurch zum Verlust der zellulären Integrität der *Border Cells.* Darüber hinaus akkumuliert das Mal-D Protein im Nucleus einiger migrierender Zellen des Zellclusters. Es wurde hypothesiert, dass die Akkumulation von Mal-D durch ein migrationsvermitteltes Signal, wie beispielsweise eine mögliche Zunahme der Zellspannung, ausgelöst wird.

Der erste Teil meiner Doktorarbeit behandelt die Regulation von Mal-D in Abhängigkeit von der Zellmigration. Um die intrazelluläre Lokalisation von Mal-D zu untersuchen, habe ich eine getaggte Variante des endogenen Proteins durch homologe Rekombination hergestellt. Die Analyse der intrazellulären Lokalisation anhand dieser Methode zeigte, dass die Akkumulation von Mal-D im Nukleus migrierender Zellen auf eine Stimulation der Mal-D Pruduktion und nicht auf eine Relokalisation einer konstanten Menge an Mal-D Protein zurückzuführen ist. Weiterhin konnte ich zeigen, dass Mutationen von Profilin oder DSRF die Menge an nukleär lokalisiertem Mal-D beinflussen.

Der zweite Teil meiner Doktorarbeit beschäftigt sich mit den Zielgenen, welche in Abhängigkeit von Mal-D reguliert werden. Ich konnte eine Mal-D Mutante isolieren und mit dem korrespondierenden Wildtyp in Bezug auf Veränderungen der Transkriptionsaktivität der *Border Cells* mittels DNA-Microarrays charakterisieren. Durch diese Analyse konnten 171 Gene identifiziert werden, die reproduzierbar um mindestens um einen Faktor von 2 unterschiedlich in Mutante und Wildtyp exprimiert wurden. Drei dieser Gene, welche für den primären Phänotyp der *mal-D* Mutanten relevant sein könnten, nämlich *GC30440*, *GC1344* und *if* wurden von mir näher untersucht. Vorläufige Daten zeigen, dass *GC30440* und *GC1344* eine Rolle bei der Regulation der Migration von *Border Cells* durch Mal-D spielen könnten.

List of Abbreviations

ADF	Actin Depolymerizing Factor
ADP	Adenosine Di-Phosphate
Arp	Actin related protein
ATP	Adenosine Tri-Phosphate
BB	Blocking Buffer
bs	Blistered
BSA	Bovine Serum Albumine
cDNA	Complementary Deoxyribonucleic acid
C/EBP	CCAAT/ Enhancer Binding Protein
chic	Chickadee
C-Terminal	Carboxy-Terminal
DEPC	Diethylpyrocarbonate
Df	Deficiency
dia	Diaphanous
Dig	Digoxygenin
DN	Dominant negative
DSRF	Drosohila Serum Response Factor
E-Cadherin	Epithelial Cadherin
EGF	Epithelial Growth Factor
FACS	Fluorescent-Activated Cell Sorter
FRT	Flippase Recognition Site
GAP	GTPase Activator Protein
Gbe	Grainyhead binding element
GDI	Guanine Dissociation inhibitor
GEF	Guanine nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
HA	Hemagglutinin
hsFLP	heat-Shock Flippase
italics	gene names
JAK	Janus Kinase
LIMK	LIM domain Kinase
MAPK	Mitogen Activated Protein Kinase
MeOH	Methanol
Mhc	Myosin Heavy Chain
Mlc	Myosin Light Chain
MRTF	Myocardin Related Transcription Factor
mRNA	Messenger Ribonucleic acid
MTOC	Microtubule Organizing Center
N-Cadherin	Neural Cadherin
N-Terminal	Amino-terminal
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor/ Vascular Endothelial

	Growth Factor
PFA	Paraformaldehyde
PI3K	Phosphatidyl-Inositol-3Kinase
ROCK	Rho Kinase
RTK	Receptor Tyrosine Kinase
Slbo	Slow border cells
TCF	Ternary Complex Factor
SRF	Serum Response Factor
STAT	Signal Transducers and Activators of Transcription
UAS	Upstream Activating Sequence
Upd	Unpaired
UTR	Untranslated Region
WB	Washing buffer
WT	Wild-type
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
β Gal	β Galactosidase

1 Introduction

1.1 The overview of cell migration

Many biological systems ranging from prokaryotes to multicellular organisms show cell migration behavior. Cell migration is an essential process both in the life of unicellular and multicellular organisms. Unicellular organisms use cell migration to move towards the light or towards better food supplies in a process called taxis. Thus the ability of migration is very important for the fitness of the unicellular organism and for giving the organism a means to respond to changing environment.
In multicellular organisms, migration is important both during development and in adulthood. Migratory behavior in multicellular organisms can be divided in two general categories; migration of constitutively migratory cells or induced migration of stationary cells. Leukocytes are a good example of constitutively migratory cells. For them, migration is not just a phase of their life that they have to pass in order to function in their final destination but it is part of the function that they should perform in order to be effective in protecting the organism against pathogens.

Multiple different cell types on the other hand commit induced cell migration. During development many different cell types are born in places different from the places where they are needed. Those cells need to actively migrate in order to reach their final destination and fulfill their function. Induced cell migration is important in adult life too such as in the case of epithelial wound healing. The cells in the opposite sides of the wound need to migrate towards each other in order to constrict over the wound tissue and close it.

Induced cell migration is tightly regulated. Both the failure and excess of migration cause great problems. Unwanted cell migration is tightly associated with a pathogenic process of metastasis of cancer tissue, where gaining the ability to migrate helps a benign tumor to become malignant. In the cases of failure of migration, the organism faces problems like congenital nervous system defects, problems in morphology.

There are many fundamental issues about cell migration that have been addressed by scientists over the years. The main issues are 1) How do cells become migratory? 2) What is the mechanism of cell migration? 3) How do cells decide where to go?
Different cell signaling pathways have been shown to be important for all those different aspects of cell migration. This thesis is focused mainly on the second question and on a particular question that has in large extend not been addressed so far: What is the response of the cell to the migration process, what is the signaling mechanism that is activated during the migration event and what are the transcriptional changes that occur during the migration?

1.2 The Mechanism of Migration

Most of the knowledge about the motility of the cells and the mechanism of cell migration comes from the analysis of single cells crawling on a substratum. Observation of single cultured cells indicated some underlying principles for cell migration. Many cell types are able to crawl towards a source of an attractant molecule. They do so by polarizing towards the ligand source. They start to show a leading edge and a trailing edge. This polarization is in turn reflected in initiating exploratory membrane protrusions in the leading edge. The initial membrane protrusions are stabilized by forming strong adhesions that generate traction force to pull the cell body forward. Meanwhile the cell lowers the adhesion strength in the back, and contracts the uropod to move forward. (Figure 1.1) This complex sequence of events occurs by coupled action of membrane protrusions, cytoskeletal dynamics and cell adhesion (Ananthakrishnan and Ehrlicher, 2007). Although there are subtle differences in the speed of migration such as fibroblasts being slow and keratocytes being fast migrators, and there are other migration modes such as the axon growth cone where the cell body does not move, still the underlying principle of regulated formation and breakage of adhesions holds.
In the following sections I will briefly mention the regulation of Actin cytoskeleton, cell adhesion and generation of traction force.

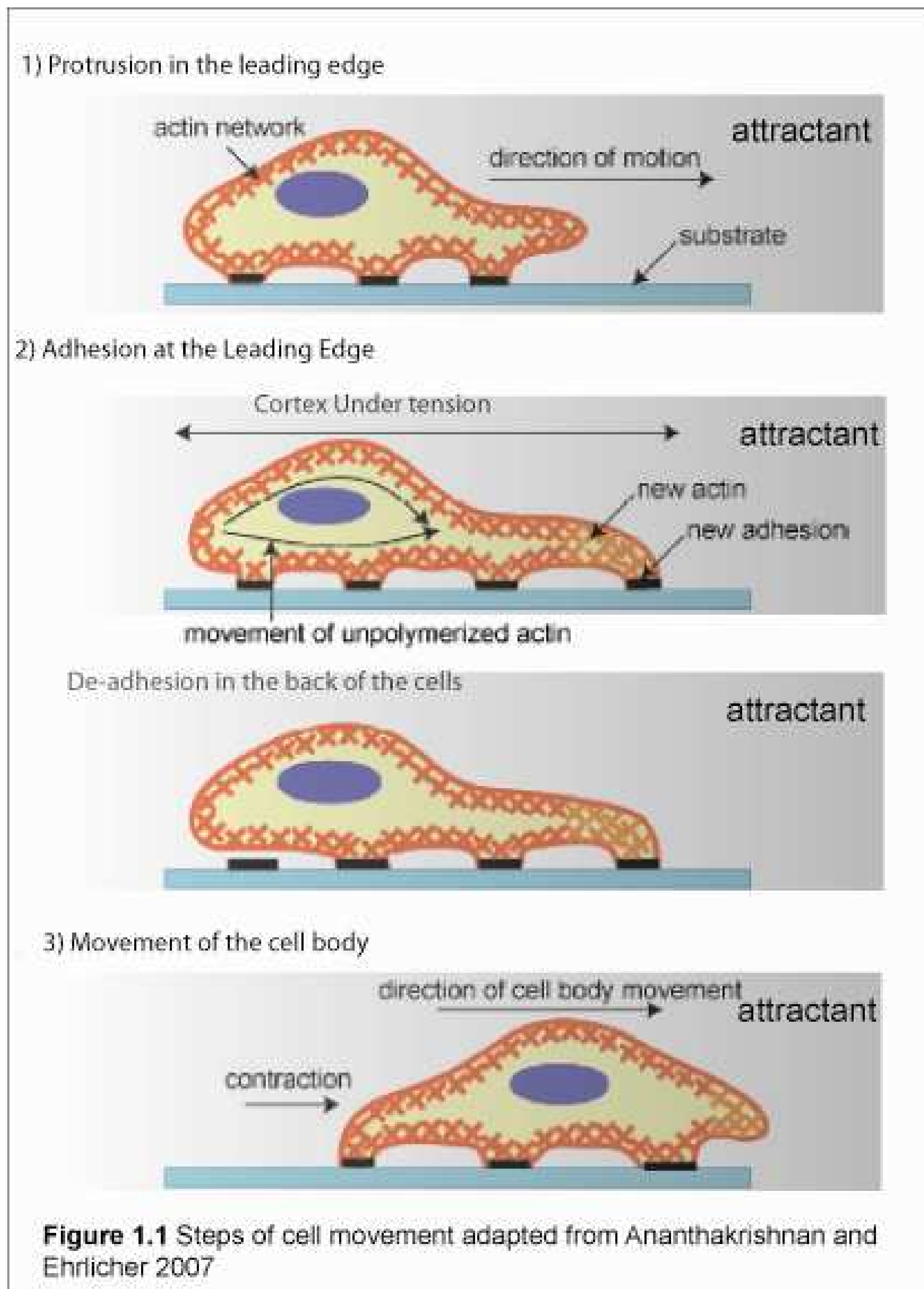

Figure 1.1 Steps of cell movement adapted from Ananthakrishnan and Ehrlicher 2007

1.2.1 The Actin cytoskeleton

1.2.1.1 The General organization of the Actin cytoskeleton

The Actin cytoskeleton is an intertwined network of Actin filaments. It is a very important cell component for the structural organization and morphology of the cell. Actin filaments need to be nucleated from Actin monomers called G-Actin. G-Actin forms filamentous Actin (F-Actin) by polymerizing in a head-to-tail fashion. This gives the Actin filaments an inherent polarity. Actin monomers bound to ATP are added preferentially to the plus end of the filament. Upon addition to the filament after a short delay ATP is hydrolyzed to ADP. Actin monomers bound to ADP are released from the minus end of the filament.

In a cell Actin is found in form of a mix population of G-Actin and F-Actin. Most of the G-Actin is bound to Actin binding proteins such as Profilin and a small protein called β Thymosin 4 that keep the G-Actin levels high while preventing spontaneous, uncontrolled polymerization (Kaiser et al., 1999).

In a cell plus end of the filament, called barbed end , due to their shape observed in electron microscopy of myosin decorated filaments, faces the membrane whereas the minus end of the filament is called pointed end is distal to the membrane (Pollard and Borisy, 2003). The dynamic addition of subunits in the barbed end and subtraction of subunits from pointed end makes the filament intrinsically mobile but the rate of this motility in vitro is too low to account for the motility of the cells. In vivo the processes of nucleation, polymerization and depolimerization are temporally and spatially controlled by the activity of Actin regulators.

1.2.1.2 The regulation of the Actin cytoskeleton in migration

Actin cytoskeleton plays role in several of the steps of the migratory behavior. It is important for the formation of protrusions, stabilization and strengthening of the adhesion in the front.

The main types of protrusions that are sent are large and wide membrane ruffles named lamellipodia and rod like structures called filopodia (Mitchison and Cramer, 1996). Both of those structures are Actin rich structures. The organization of Actin filaments in lamellipodia and filopodia are different. Whereas in lamellipodia Actin filaments form a branched, intertwined mesh like structure, in filopodia Actin filaments are bundled by the action of multiple Actin bundling and cross-linking proteins (Figure 1.2)(Mitchison and Cramer, 1996).

The cell has a variety of Actin regulators in order to regulate the Actin polymerization rate and position and orchestrate the assembly of Actin filaments into defined structures. Those structures in turn push the surrounding membrane forward to generate the protrusions.

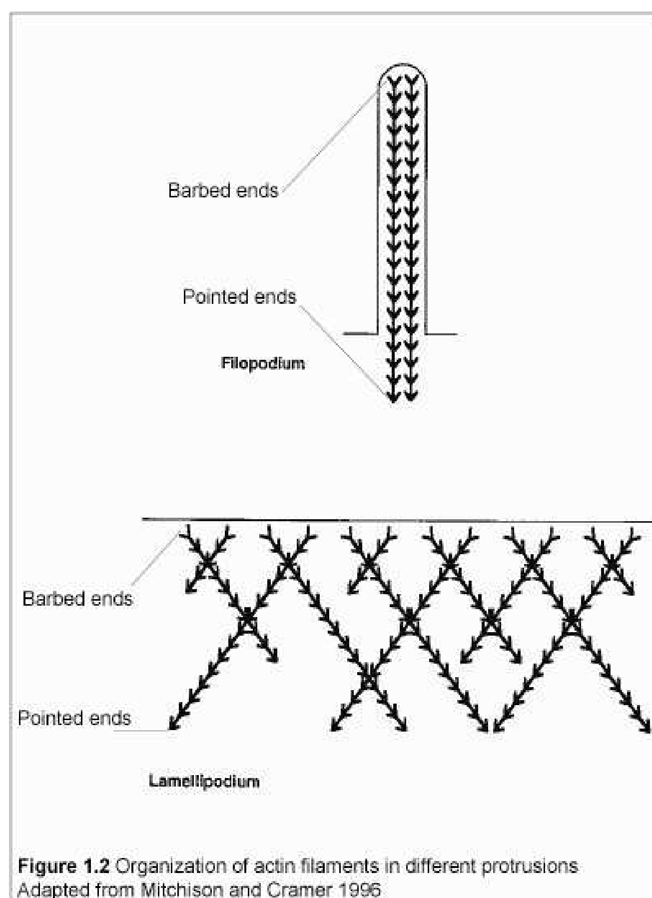

Figure 1.2 Organization of actin filaments in different protrusions Adapted from Mitchison and Cramer 1996

A system that is used as a model for Actin polymerization organization for generating protrusions is the rocketing movement of Listeria and Shigella to move in the living cells that they infect. Those bacteria can hijack the Actin machinery of the host

cell in order to generate Actin comets that they use as a propulsion force. This force generation by the Actin polymerization does not require myosin (Loisel et al., 1999). It has been shown that localized polymerization of Actin can generate enough force to move those bacteria. Although there are over 60 different Actin regulator classes, the minimal requirements for assembling Actin to generate movement of bacterial particles can be reconstituted in vitro by using purified proteins. (Loisel et al., 1999) The minimal components are G-Actin, Arp2/3 (which is activated in this system by bacterial factors that mimic the activity of WASP/SCAR family of Arp2/3 activators), Actin Depolymerizing Factor (cofilin/ADF), and capping protein (Loisel et al., 1999). Adding Profilin and VASP further increased Actin polymerization based motility of the bacteria. I will introduce those factors and how they act in the following paragraphs.

Actin filaments can be nucleated by Arp2/3 family Actin nucleators as a branch of an existing filament. Arp2/3 is a complex of 7 subunits that contain the Actin related proteins 2 and 3, which mimic an Actin dimer and 5 more subunits that keep them stabilized in the inactive state, ARPC1-5 (Pollard, 2007). The activity of Arp2/3 factors is regulated by WASp family of proteins or SCAR/WAVE family of proteins (Figure 1.3). WASp family is consisting of Wiscott Aldritch Syndrome Protein (WASP) and SCAR.

Although most of the nucleation in a lamellipodium goes through Arp2/3, it is not the only Actin nucleator in the cells and there are other classes of Actin nucleating factors that may be important for Actin regulation. Formins for instance are a class of Actin nucleators that can form Actin filaments from soluble G-Actin pool without the requirement for a prior Actin filament (Pollard, 2007). There are multiple members of the Formin family which can be identified by three regions of homology called Formin Homology 1,2 and 3 (FH1, FH2, FH3) domain (Goode and Eck, 2007). FH1-FH2 domains can form dimers and bind to Actin dimers and stabilize this thermodynamically unstable nucleation intermediate which helps to continue the nucleation in vitro (Pring et al., 2003; Pruyne et al., 2002). Moreover when nucleation occurs and filament elongation starts FH1-FH2 dimer remains associated to the barbed end of the filament and prevents the binding of capping proteins meanwhile allowing addition of more Actin subunits (Zigmond et al., 2003). This way of action is called processive capping or leaky capping

and results in unbranched Actin filament elongation (Figure 1.3). FH1 domain which is situated next to FH2 domain can bind to Profilin and incorporate the G-Actin bound to Profilin to the growing F-Actin (Romero et al., 2004).

One of the formins that have been implicated to have a role in cell migration is Diaphanous (mDia) (Watanabe et al., 1999; Watanabe et al., 1997). mDia localizes to the leading edge of a migrating cell (Watanabe et al., 1997) and has role in the formation of unbranched Actin filaments that form filopodia. Other than that mDia is important for the formation of stress fibers in response to Rho GTPase (Watanabe et al., 1999). Diaphanous can be divided into two parts: C terminal part which harbors FH1 and FH2 domains which activate the nucleation and elongation of F-Actin, and Diaphanous Auto-inhibitory Domain (DAD); and N terminal part which is the regulatory part, consisting of Diaphanous Inhibitor Domain and GTPase Binding Domain (GBD). In default state diaphanous is auto-inhibited due to binding of its N terminal region to its C-Terminal region (Watanabe et al., 1999). When active Rho GTPase binds to GBD it relieves the auto-inhibition and activates the protein (Jaffe and Hall, 2005; Watanabe et al., 1999; Watanabe et al., 1997). In fact Diaphanous which lack auto-inhibitory domain is constitutively active. Another factor that nucleates Actin filaments from G-Actin is Spire, which binds to four Actin monomers and aligns them, forming the start of a new filament backbone(Quinlan et al., 2005).

Profilin is a small protein that directly binds to G-Actin. Moreover it facilitates both the addition of ATP bound G-Actin to F-Actin plus end, and it exchanges ADP to ATP in ADP bound G-Actin, thus activating it. Profilin is recruited to the plus end of the filaments by the interaction with Formin proteins too.

Capping protein binds to the plus end of F-Actin and prevents further actin polymerization in that filament (Cooper et al., 1984; Isenberg et al., 1980). Actin polymer growth occurs as a competition between elongation and capping. There are proteins such as Formins and Ena/VASP which actively compete with the capping protein in order to continue the elongation without capping in regions where elongation is favored. (Bear et al., 2002; Zigmond et al., 2003)

Cofilin is an Actin severing factor. It binds to F-Actin and twists it, which causes the tension in the filament to increase and at the end leads to the breakage of the filament. The activity of cofilin is inhibited by direct phosphorylation of the protein by a protein called LIMK (Yang et al., 1998). LIMK in turn is activated by the action of Rho family small GTPases. Inactive, phosphorylated cofilin is reactivated by dephosphorylation by the action of cofilin phosphatase (Nishita et al., 2005; Niwa et al., 2002). Cofilin's role in Actin based force generation is two fold. One is that it severs Actin filaments that are bound to capping protein thus that cannot grow any more, and generates free barbed ends that can be used for further Actin polymerization. Two is that it replenishes G-Actin pool that eventually would get depleted if all the F-Actin generated would stay stable. It is believed that the aged filament would get chopped by the activity of cofilin in order to replenish G-Actin pool.

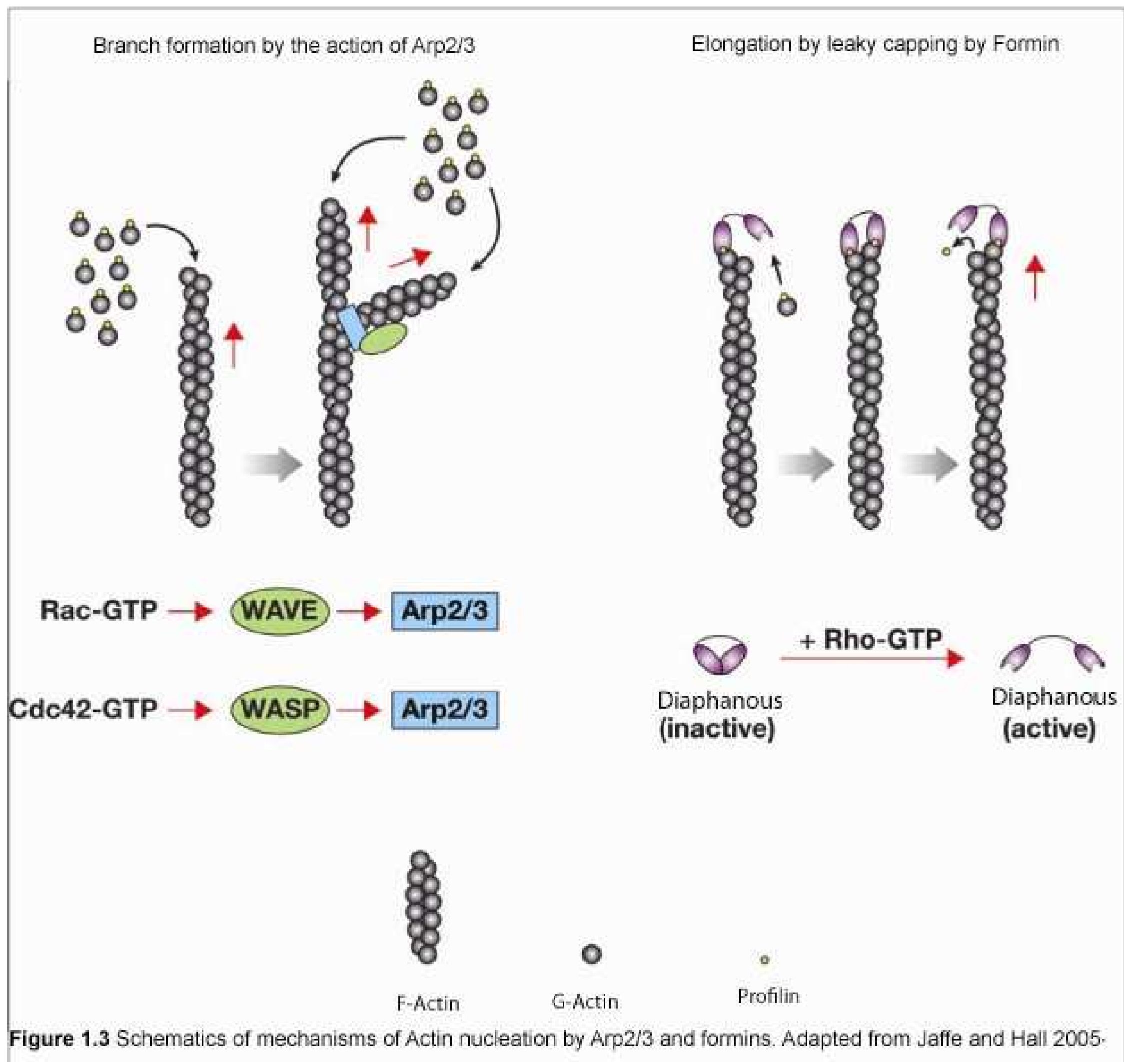

Figure 1.3 Schematics of mechanisms of Actin nucleation by Arp2/3 and formins. Adapted from Jaffe and Hall 2005.

Arp2/3, WASP, Profilin, Capping Protein, Cofilin function in the formation of lamellipodia as well,(Reviewed in Pollard and Borisy 2003). (Figure1.4)

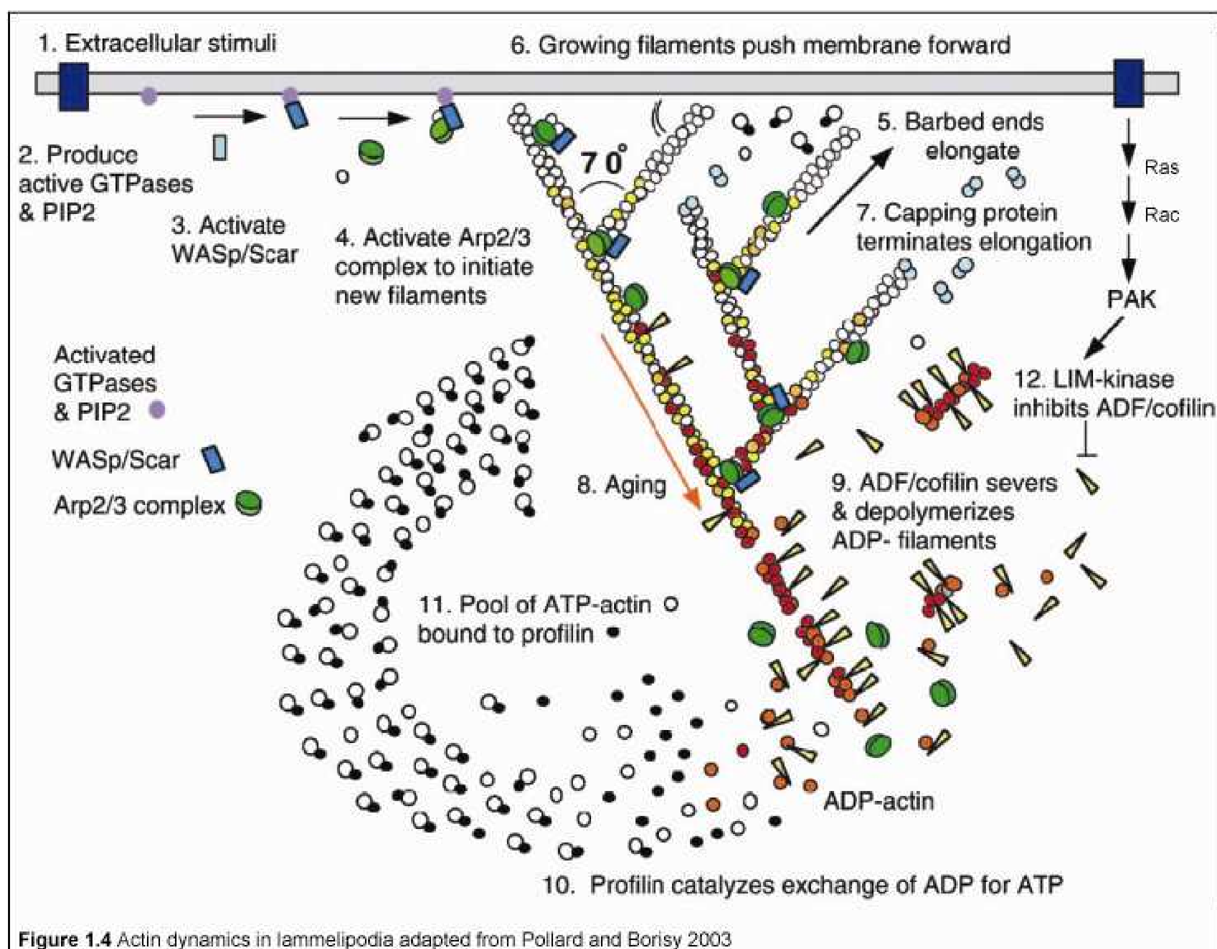

Figure 1.4 Actin dynamics in lammelipodia adapted from Pollard and Borisy 2003

In addition to those proteins that regulate Actin polymerization, branching, depolymerization and capping, there are other Actin ultrastructural organizers that cross link the Actin cytoskeleton. For example cross linking of Actin filaments in *Dictyostelium* by myosin II has been shown to be important for cortical integrity of the cell migrating under differing concentrations of agar, directly affecting how much the cell can deform the surrounding while maintaining its cortical integrity (Laevsky and Knecht, 2003). Interestingly the motor activity of myosin is not required for this organizational role since myosin light chain mutants can deform the substrate as much as the wild type cells (Laevsky and Knecht, 2003).

Although Actin role in lamellipodium formation is well established, the involvement of microtubules is more complex. Through disruption of microtubules and observing whether the migration still occurs it has been shown that microtubules are important for

the migration of big and complex cells such as fibroblasts or epithelial cell layers in response to wounding but not required for the migration of specialized migrating cells such as keratocytes and leukocytes (Waterman-Storer and Salmon, 1999). During the polarization of the cell, Microtubule Organizing Center (MTOC) gets repositioned between the leading edge and the nucleus of the cell (Gomes et al., 2005). Some views on the role of microtubules in cell migration include the involvement of microtubules in disrupting focal adhesions and tail retraction. (Ballestrem et al., 2000)

1.2.1.3 Rho family of small GTPases in Actin regulation

Small GTPases are proteins that are in general considered as molecular switches. They bind to Guanosine triphosphat (GTP). GTP bound GTPases are active and they activate diverse downstream effectors to engage diverse processes such as cell cycle progression, phagocytosis, cell morphology and Actin cytoskeleton dynamics (Etienne-Manneville and Hall, 2002). GTPases have an intrinsic GTP hydrolysis activity that is slow, that convert bound GTP to GDP, rendering it inactive again. There are multiple regulators of this cycle of activation and inhibition (Figure 1.5A) (Luo, 2000; Raftopoulou and Hall, 2004).

GTP hydrolysis activity of a GTPase can be boosted by the action of GTPase Activating Proteins (GAP)s. GAPs therefore promote turning off of GTPases. There is a second family of proteins called GTP Exchange Factors (GEF)s that promotes the exchange of GDP to GTP on an inactive GTPase, thus activating it. A third family of GTPase regulators are called GDP Dissociation Inhibitors (GDI)s that inhibit the release of GDP from a GDP bound inactive GTPase, thus keeping it inactive for longer time (Figure 1.6A)(Etienne-Manneville and Hall, 2002).

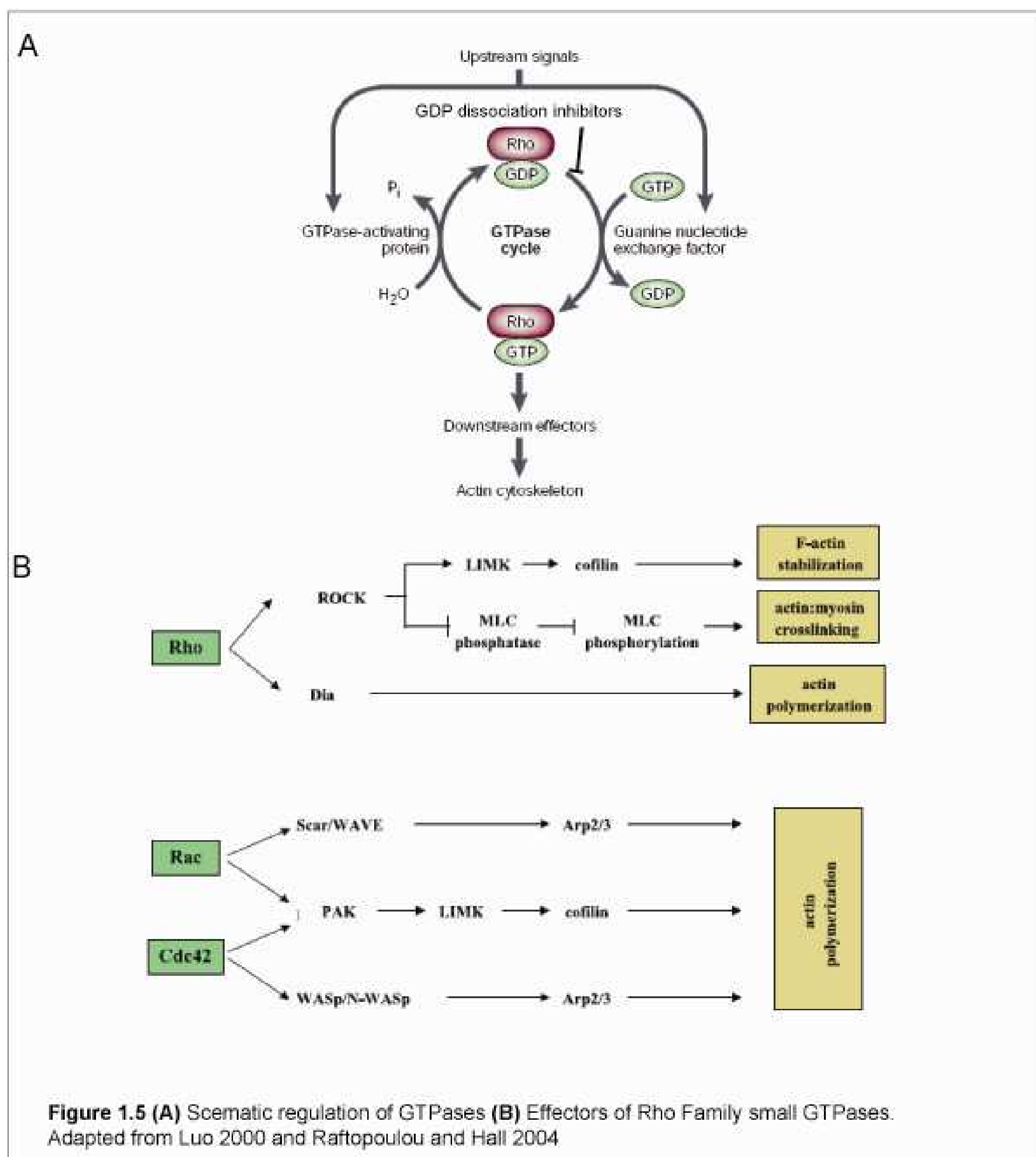

Figure 1.5 (A) Scematic regulation of GTPases **(B)** Effectors of Rho Family small GTPases. Adapted from Luo 2000 and Raftopoulou and Hall 2004

Several processes that are triggered by the activity of GTPases and Actin cytoskeleton dynamics is one of them. Members of the Rho family of small GTPases are implicated in multiple Actin driven processes to coordinate the activity of key effectors such as Formins and Arp2/3 through the activation of WASP as discussed below (Figure 1.5 B).

There are three main members Rho GTPases: Rho, Cdc42 and Rac. Rho has been implicated to be important for the formation of stress fibers, filaments of Actin that are

localized in the adhesion sites and are thought to be important for cell rigidity. Moreover one of the effectors of Rho GTPase called ROCK phosphorylates Myosin light chain phosphatase (MLCP) and causes increase in myosin II phosphorylation, thus activates myosin II (Amano et al., 1996; Essler et al., 1998; Matsui et al., 1996). Another effector of Rho GTPase is Diaphanous (Watanabe et al., 1999). Rho GTPase has been shown to be important in diverse cell types to sense the extracellular matrix and the forces that the cell is submitted to.

Cdc42 is important for induction of filopodia, thus probing the environment. Cdc42 has been shown to activate WASP that activates Arp2/3 family of nucleators. Rac was proposed to be important for the formation of lamellipodia. Its effectors PAK and LIMK are important for inhibiting cofilin (Arber et al., 1998) and to activate SCAR, which in turn activates the Arp2/3 complex. (Ng and Luo, 2004)

1.2.2 The Regulation of the Cell Adhesion

Migrating cells need to stabilize their protrusions in the front of the cell in order to generate traction force to pull the cell body forward. This is accomplished by assembling new adhesion complexes in the front of the cell. Concomitantly cells need to release their adhesion in the back of the cell. This is achieved through internalization of adhesion molecules or dissociation of adhesion complexes at the back of the cell. Many cells use Integrins to bind to extracellular matrix and use it as a substrate on which they migrate. Integrins are formed by heterodimerization of two single-pass transmembrane subunits called α and β subunits (Brown et al., 2000). There are several α and β subunits and the association of different α and β subunits causes the binding to different ligands in the extracellular matrix. The binding specificity is regulated by the large extracellular domains of integrin subunits. These subunits have a short intracellular domain important for association with the Actin cytoskeleton and for regulation of adhesion by different kinds of regulators such as kinases, phosphatases and adaptor molecules. Upon binding to their ligands, integrins recruit multiple cytoplasmic proteins, forming focal complexes (Hynes, 2002). Those focal complexes either maturate into large supra molecular assemblies called focal adhesions or disappear (Laukaitis et al., 2001). Focal adhesions bind the adhesive complex to the Actin cytoskeleton and strengthen it. Furthermore, they

are involved in the subsequent maturation steps of the adhesion. In many migrating cells it has been shown that the leading edge has more integrins than the trailing edge does. This is in part established by the endocytosis and recycling of the integrin complexes (Caswell and Norman, 2006). Different integrin heterodimers are internalized and recycled by using short or long endosomal recycling pathways and blocking recycling routes of transmembrane molecules causes the asymmetrical distribution of integrins to disappear and the cells to slow down. (Strachan and Condic 2004; Caswell and Norman 2006). There are other regulators of integrin signaling including Rho GTPases, different kinases and phosphatases that either act directly on focal adhesion constituents or their regulators.

Integrins are not the only adhesion molecules that are used in cells for migration. There are other classes of adhesion molecules that are used by different kinds of cells for their migration. For example neurons use Neural Cell Adhesion Molecule for their migration on rostral migratory stream and border cells use cadherin in their migration (Niewiadomska et al., 1999). The process and the mechanism of formation of the cell adhesion in the leading edge and dissociation of it in the rear of the cell is still the underlying mechanism required for migration.

1.2.3 Pulling the cell body by contractile forces

The contractile forces are generally generated by the activity of myosin II in the migrating cells. Myosin II activity is spatially regulated during migration. The protein is activated in the back and on the sides of the cell but not in the leading edge (Xu et al., 2003). Myosin II is a hexamer that is formed by two heavy chains and four light chains. They assemble to form a long tail and two large heads that bind to Actin filaments(Bresnick, 1999). Myosin hydrolyzes ATP to generate a cyclic movement that causes a stroke on the Actin filament. First the head region binds the filament, then pulls it and releases the filament which makes a new round of the cycle possible. In the cell, the activity of Myosin II is regulated mainly by the activity of ROCK (Rho kinase) that phosphorylates and activates myosin II, and the activity of myosin light chain phosphatase (MLCP) which dephosphorylates and inactivates myosin II (Bresnick, 1999). ROCK phosphorylates myosin phosphatase and inactivates it which stabilizes the

activation of myosin. ROCK in turn is activated by Rho. In migrating cells Rho is activated in places other then the leading edge which may account for the localized activation of Myosin II. (Explained in more detail in the following section)

1.2.4 Sensing directionality

The initial directionality of the migration and cell polarity is in most cases a direct consequence of extracellular signaling molecules. Cells are very successful in sensing even very shallow gradients of attractors and are able to polarize and move towards them. The initial small change of concentration of attractant over the length of the cell is first sensed and then amplified intracellularly in order to give a robust migration trajectory. A migration system that was studied in this regard is *Dictyostelium discodeum.* cAMP is a potent chemoattractant for this organism and Dictyostelium cells are very sensitive in determining the gradient. Indeed they can sense a change as little as 2% of cAMP concentration over their cell bodies. The mechanism that allows them to be that sensitive can be summarized as localized activation and global inhibition. (Figure1.6) (Jin and Hereld 2006; Willard and Devreotes 2006)

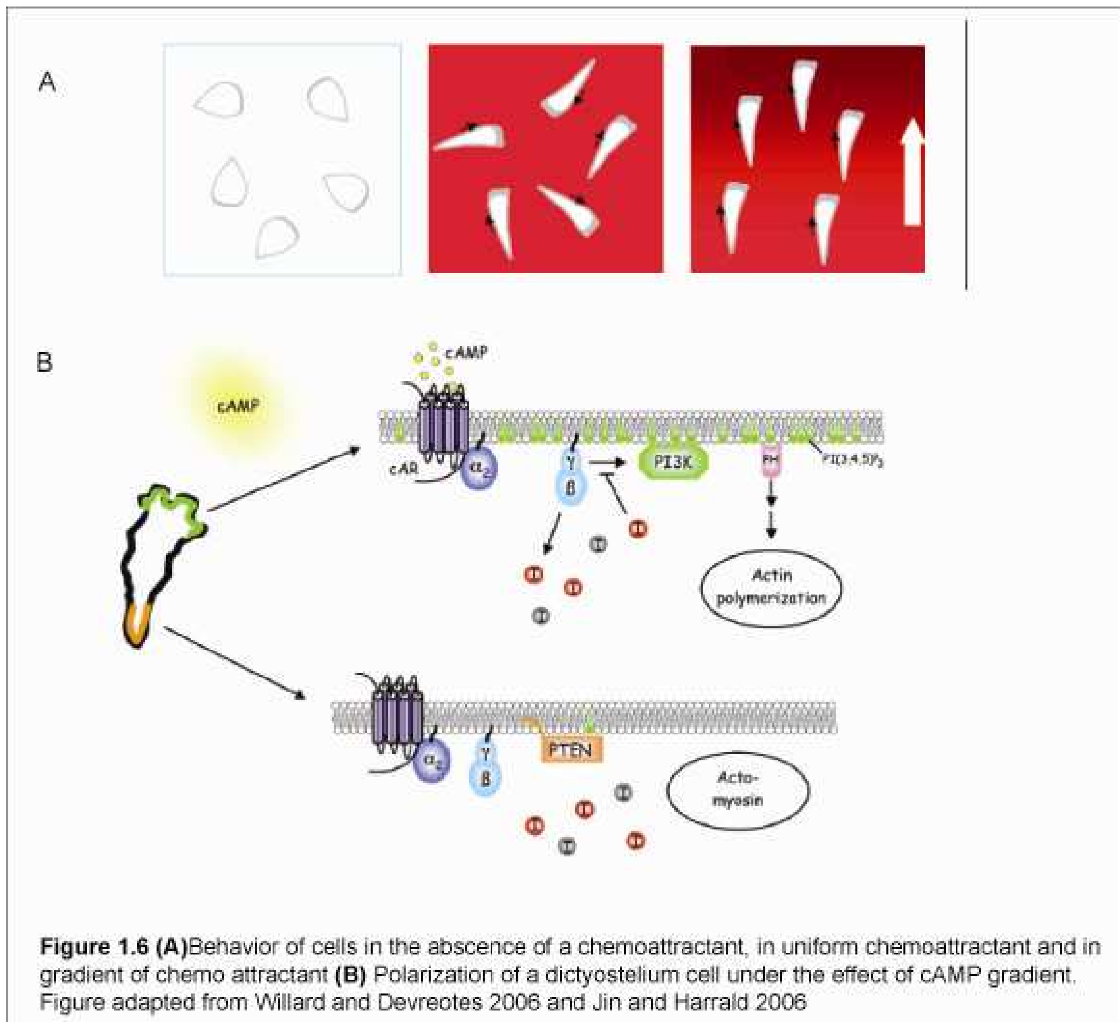

Figure 1.6 (A) Behavior of cells in the abscence of a chemoattractant, in uniform chemoattractant and in gradient of chemo attractant **(B)** Polarization of a dictyostelium cell under the effect of cAMP gradient. Figure adapted from Willard and Devreotes 2006 and Jin and Harrald 2006

In this model cAMP binds to a G Protein Coupled Receptor (GPCR). This causes dissociation of Trimeric G protein into Gα and Gβγ subunits. Gβγ, through activation of Ras causes Phosphatidylinositide 3-Kinase (PI3K) to get activated on the site of receptor activation. PI3K is an enzyme that phosphorylates Phophatidylinositol (PtdIns) to form PtdIns(3,4,5)3 Phosphate (PIP3). The activation of PI3K causes polarization of the cell into a clear leading edge and a rounded trailing edge by the accumulation of PIP3 locally, which in turn recruit proteins with Pleckstrin homology domain, PH domain, PX domains and FYVE domains, towards the leading edge. In the rest of the cell surface the ectopic action of PI3K is counteracted by a phosphatase called PTEN (Funamoto et al., 2002). The initial activity of the PI3K in the leading edge and the removal of PTEN from the leading edge in turn activates a relay of events, amplifying the initial signal thus making the response more robust.

Another migration system that uses localized PIP3 as a polarization means is neutrophils. The initial polarization of PIP3 leads to the activation of Rac GTPase specifically in the leading edge (Xu et al., 2003). That in turn causes increase of Actin polymerization in the leading edge. Increased Actin polymerization causes further increase of PIP3 in the leading edge by means that are not fully understood but may be caused by aggregation of membrane micro domains. On the other hand at the back of the cell Rho GTPase and ROCK cause myosin contractility (Xu et al., 2003). Rho activity and Rac activity are mutually exclusive which causes a robust polarization of the cell, limiting the myosin contractility in the back and Actin polymerization in the front. (Xu et al., 2003). Moreover Cdc42 has been shown to be activated in the leading edge. This goes through recruitment of a complex of PIX (A GEF for Cdc42), and PAK1 (an effector of Cdc42) by Gβγ in the leading edge. Interestingly in this situation PAK1 which is an effector of Cdc42 acts as an activator of it as well. (Li et al. 2003). There are several negative and positive feedback loops that are suggested to make the initial polarity more robust. For example transport of exogenous PIP3 is able to increase the activity of endogenous PIP3 generating machinery and polarize PIP3. This behavior was shown to require PI3K, Rac activity and was shown to depend on Actin cytoskeleton dynamics and it is important to make migration in one direction persistent.(Wang et al., 2002; Weiner et al., 2002)

Border cells migration is another system in which guidance is studied in detail. Border cells differentiate in the anteerior pole of the developing egg chamber and migrate posteriorly towards the oocyte (discussed in detail in the following chapters). Border cell migration is guided by the activity of two receptor tyrosine kinases (RTKs), PVR and EGFR (Duchek and Rorth 2001; Duchek et al. 2001) (Figure 1.7). PVR is the *Drosophila* single orthologue for two separate growth factors in mammals Platelet Derived Growth Factor Receptor/Vascular Endothelial Growth Factor receptor. EGFR is the *Drosophila* orthologue of EGF receptor. In the part of the migration which starts at the anterior pole where the border cells specify, and it ends at the border between nurse cells and the oocyte PVR and EGFR behave in a redundant way. Over expression of either of the ligands within the cluster abrogates the migration (Duchek et al. 2001). Moreover ectopic

over expression if the ligand on the sides of the egg chamber can misguide the border cell cluster to the ectopic expression location (McDonald et al., 2003)
The second part of the migration, dorsal migration towards the oocyte nucleus which happens after reaching the border between oocyte and nurse cells, EGFR has been shown to provide guidance (Figure 1.7 B) (Duchek and Rorth, 2001). The ligand for EGFR, Gurken, is expressed in the anterior dorsal side of the oocyte and forms a gradient that attracts border cells.

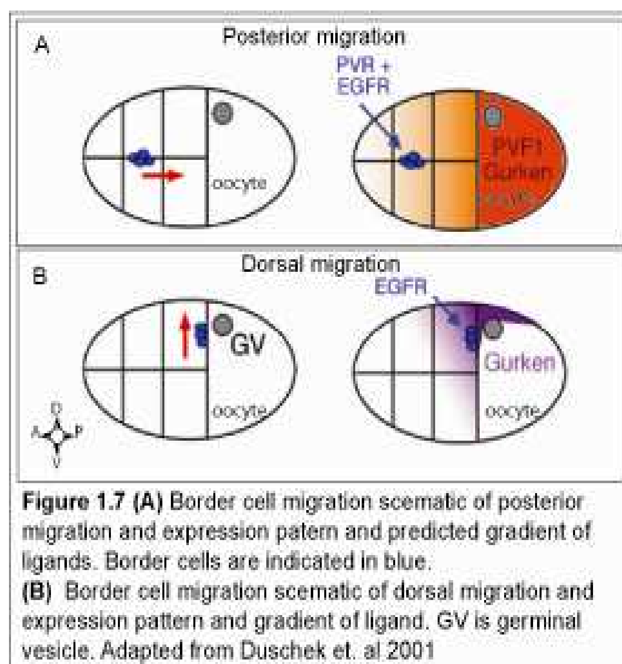

Figure 1.7 (A) Border cell migration scematic of posterior migration and expression patern and predicted gradient of ligands. Border cells are indicated in blue.
(B) Border cell migration scematic of dorsal migration and expression pattern and gradient of ligand. GV is germinal vesicle. Adapted from Duschek et. al 2001

Polarization of the signal transmitted by those RTKs has been shown to be important for the regulation of guidance of the border cells, and there are some genes that are acting to keep the activity of those RTKs polarized (Jekely et al., 2005). Activated RTKs in turn activate diverse downstream effectors, such as Rac GTPase that induces Actin cytoskeletal changes, and signaling components such as MAP kinase signaling pathway, PI3K, PLCγ (Duchek et al. 2001; Bianco et al. 2007).

1.2.5 The Role of Transcription and Cell signaling in migration

The roles of transcriptional changes in cell migration are mostly associated with the induction of cell migration. Many transcription factors have been implicated to be important for making the cell migratory. In the following paragraphs I will give some examples.

Transcription factors Twist and Snail have been shown to be important inducing factors in the mesoderm to start invaginating during gastrulation (Leptin and Grunewald, 1990).

The direct hierarchical action of diverse transcription factors is responsible for the migratory fate decision. Interestingly Twist has been shown to be important for induction of metastasis in diverse mammalian cancer types (Yang et al., 2004). Moreover ectopic expression of Twist in MDCK cells, which are normally not migratory in response to serum, renders them migratory (Yang et al., 2004). This establishes Twist as a potent inducer of migratory behavior.
Snail on the other hand was the first factor to be shown to be an important factor for the migration of Neural Crest Cells and has been shown to be important for the induction of Eptihelial Mesenchymal Transition (EMT) in all the EMT systems where it has been analyzed (Reviewed in (Barrallo-Gimeno and Nieto, 2005).. EMT process is a sequence of events that overall leads the epithelial cells to loose their epithelial morphology and become more loosely shaped like a fibroblast. Epithelial cells break their apical basal polarity, they loosen the cell-cell contacts, decrease the expression of epithelial components (Such as E-cadherin, α and γ catenin) express mesenchymal components (such as vimentin, N-Cadherin, smooth muscle Actin and fibronectin), rearrange their cytoskeleton and become migratory at the onset of EMT (Thiery, 2002). EMT is a recurrent theme in the development of the organism, from gastrulation to organogenesis (Hay, 2005) One of the hallmarks of many EMT events is the repression of E-cadherin expression, albeit it is not enough per se for EMT, and the cells should still start to express mesenchymal components (Yang et al., 2004). Snail performs its role in EMT at least partly by directly repressing the transcription of E-Cadherin (Cano et al., 2000). In addition to Snail two more transcription factors were shown to be important for NCC migration, Sox9 and FoxD3 (Cheung et al., 2005). Sox9 is important for making the cell competent to become NCC, and to promote survival, whereas FoxD3 is mostly important for the down-regulation of N cadherin and expression of integrin (Cheung et al., 2005).

In tissue culture cells TGF β was shown to be an important regulator of EMT in epithelial cells of diverse origins. Interestingly in this context TGF β causes induction of transcriptional repressor Hey1 directly and this has been shown to be required for EMT onset (Zavadil et al., 2004). TGF β signaling induces the actication of Mitogen Activated Protein Kinase (MAPK) signaling as well (Zavadil et al., 2001). Moreover TGF β

signaling induces indirectly Notch signaling on a longer timescale and this induction is required for EMT process as well (Zavadil et al., 2004).

An analysis of mutations in Elongator complex which is suggested to be important for elongation step of transcription, showed that mutation or RNA interference (RNAi) mediated knock-down of a key factor of this complex, IKAP/hELP1 in fibroblasts decreases transcript levels of multiple cell motility related genes and decreases migratory behavior of mutated fibroblasts (Close et al., 2006).

Work on *Caenoharbiditis elegans* anchor cell migration showed that FOS-1 transcription factor is essential for the invasive migration of anchor cells during the development by providing the means of breaking the basal lamina through which those cells migrate (Sherwood et al., 2005).

Janus Kinase/ Signal Transducer and Activator of Transcription (JAK/STAT) pathway was shown to be another signaling/transcription factor couple important for multiple cell migration systems. It was first implicated to have a role in induction of migration in the Border Cell migration system (Discussed more in detail later) (Beccari et al., 2002; Silver and Montell, 2001). Border cells differentiate among the anterior follicle cells with the action of two specialized follicle cells called polar cells. Polar cells induce border cell fate in the cells surrounding them by activating JAK/STAT pathway. Polar cells express the ligand Unpaired and border cells express the receptor Domeless (Beccari et al., 2002; Silver and Montell, 2001). In response to signals from polar cells, border cells start to express *slbo*, the *Drosophila* CAAT Enhancer Binding Protein (C/EBP) transcription factor homologue in *Drosophila* (Montell et al., 1992). Slbo in turn activates transcription of many genes that are important for migratory behavior. Slbo is absolutely essential for border cell migration, since border cells mutant for *slbo* are not even motile and are stuck in the anterior pole of the oocyte (Montell et al., 1992; Rorth et al., 2000). Overall, border cell fate is gained by transcriptional activation of multiple genes including transcription factors, cytoskeletal regulators and muscle specific genes (Borghese et al. 2006; Wang et al. 2006). Interestingly temperature sensitive alleles of STAT showed that if STAT function is impaired after border cell specification, border cells still have migration

delays which suggests that JAK/STAT pathway is important not only for the specification of the border cells but also during the border cell migration (Silver et al., 2005).
JAK/STAT signaling is important for the induction of migration of Primordial Germ Cells in *Drosophila* as well at the end of germband retraction (Kunwar et al., 2006).

1.4 Differences between cell migration in cell culture and in vivo cell migration

Cell culture migration systems give clear advantages in terms of amenability to manipulations and the ease of imaging. Although the key cellular mechanism for motility are most likely the same between migrating cultured cells and in vivo migration systems, there are clear differences. First of all the migration substrate of in cell culture migration systems is two dimensional whereas for the in vivo migration the substrate most of the time is three dimensional. This causes significant limitations for the membrane movements compared to the cell culture situation where the cell membrane is not hindered from one side.
A further difference is that the in vivo, both the start and the end of migration should be tightly regulated. Cells need to stop moving when they reach their target.
An important difference arises for collective migration. Some of the migration systems, such as the one I am interested in, undergo collective migration, meaning that the cells actively migrate together while they are part of a cluster or tissue. In cell culture migration models generally focus on the movement of a single cell in response to a motility cue. The difference between the collective migration and single cell migration is even more important while thinking about forces that are applied on a migrating cell in vivo during collective migration. Both pulling and pushing within the cluster of cells generate intra cluster forces. A nice example is the dorsal closure in the *Drosophila* embryo. This is a migration system where an entire epithelial sheet migrates collectively to close over the dorsal hole that is created over amnioserosa, the extra embryonic epithelial tissue covering the dorsal side of developing embryo, at the end of germ band retraction in embryogenesis (Jacinto et al., 2002). Elegant experiments with laser ablations showed that those cells are pulling each other and are thus under tension(Kiehart et al., 2000). If one makes a laser cut in the epithelium, cells retract,

reminiscent of a string under tension that is cut. Surrounding cells find a new equilibrium and then continue to migrate (Kiehart et al., 2000). Myosin II contractility is the driving force of this closure. Myosin II is localized specifically in the leading edge of the migrating epithelium along with a strong, organized, Actin cable (Franke et al., 2005). The driving force comes from both the constriction of this actomyosin cable in the leading edge and the constriction of the amnioserosa (Kiehart et al., 2000).

In some collective migration models guidance cues are sensed by leader cells and the action of leader cells organize the rest of the cluster to follow. One tissue where it is analyzed is the tracheal migration in *Drosophila*. Tracheal branches form by budding of an epithelium and migration of the cells forming the epithelium as a group of cells (Ghabrial et al., 2003). They are guided through the activity of an RTK where ligand is expressed in the surrounding tissue. In this system the cell that receives the most FGF signal becomes the leading cell and directs the follower cells in the migrating group (Ghabrial and Krasnow, 2006). It sends a secondary signal to the follower cells to make them differentiate into tubes. It has been shown that the presence of the receptor only in the leading cell is enough to direct the migration (Ghabrial and Krasnow, 2006). Another system with this kind of cluster dynamics is the lateral line migration in zebrafish. In this system, a large cluster of cells migrate along the dorsal side of the developing fish to drop lumps of cells that will form the mechanosensors of the fish. The guidance has been shown to be established by SDF1 and its receptor CXCR4 (a GPCR). In this system elegant mosaic analysis showed that a whole cluster that is mutant for the receptor, thus unable to get the guidance cue, can be rescued by the presence of a few cell with the receptor (Haas and Gilmour, 2006).

One suggested mechanism for the signaling from the leader cell to the follower cells is mechanical signaling. In this model, the leading cell pulls the follower cells and this pulling force is perceived by the follower cells and makes them know they are followers. This type of signaling does not need to be unidirectional and the follower cells can cause stretching and mechanical tension on the leader cells as well.

1.5 Border cell migration

1.5.1 Overview of border cell migration

Border cell migration is a collective migration of about 8 cells that happens during the stage 9 to stage 10 of oogenesis of *Drosophila melanogaster*. Developing drosophila egg chamber consists of 15 germline derived giant cells called nurse cells, an oocyte, and about 1000 somatic cells that cover them called follicle cells (Figure 1.8).

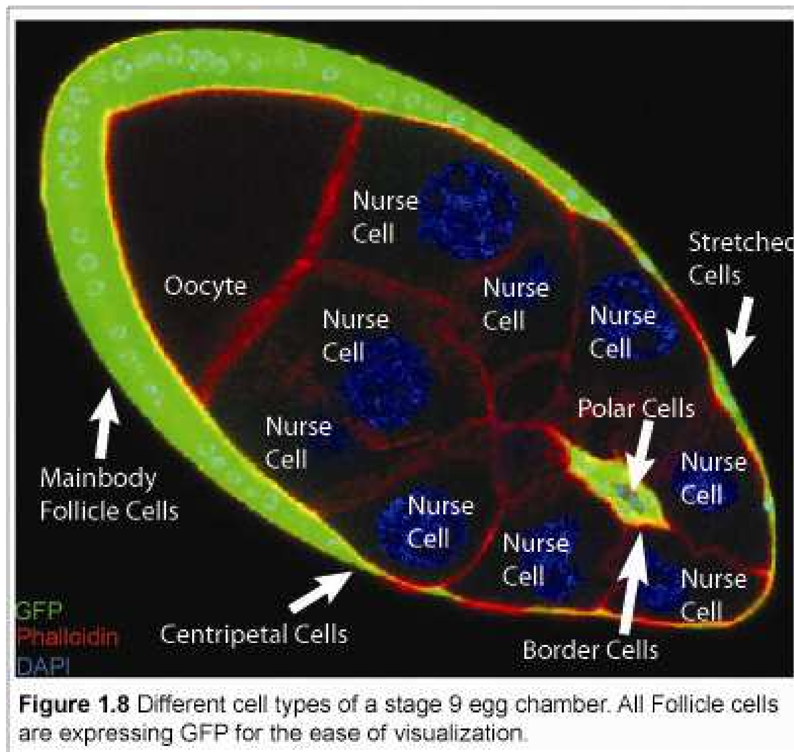

Figure 1.8 Different cell types of a stage 9 egg chamber. All Follicle cells are expressing GFP for the ease of visualization.

After getting specified at the anterior pole of the egg chamber, border cells form a cluster that surrounds the polar cells at the anterior pole, send a long cellular extension (Fulga and Rorth, 2002), and start their migration process at stage 9 of oogenesis (Figure 1.9). At this stage of oogenesis follicle cells start to undergo a morphogenetic movement as well. Most of the follicle cells move towards the oocyte and form a columnar epithelium covering the oocyte, leaving a group of about 50 extremely flattened cells that cover the nurse cells, called stretched cells (Horne-Badovinac and Bilder, 2005)(Figure 1.8).

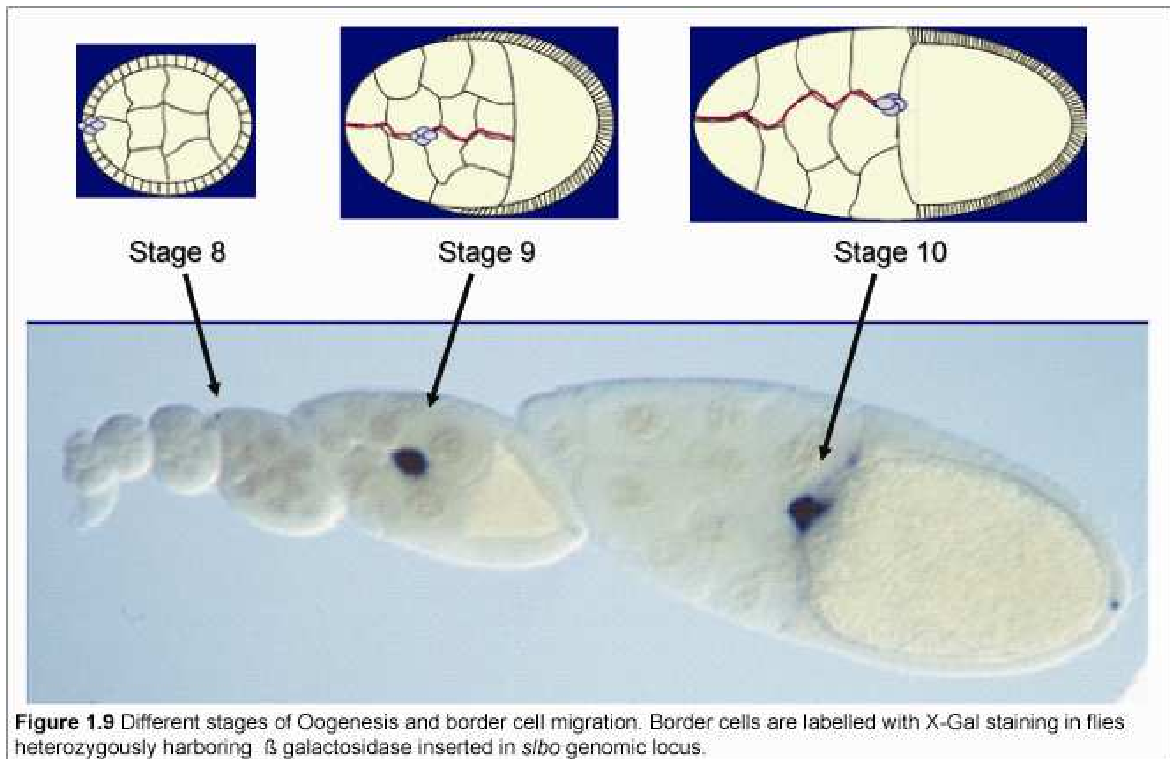

Figure 1.9 Different stages of Oogenesis and border cell migration. Border cells are labelled with X-Gal staining in flies heterozygously harboring ß galactosidase inserted in *slbo* genomic locus.

Border cell migration is a stereotypical migration. At a given time one can predict how much they should have migrated. Accordingly one can assess whether they are delayed or not delayed. One can score border cell migration by looking at their position relative to retracting centripetal cells (Figure 1.8). In wild type situation they are seen in the same distance to the border between oocyte and nurse cells.

Border cell migration is an invasive migration since border cells invade in between nurse cells. It has been shown that border cells migrate on nurse cells by using DE-cadherin, a well established cell-cell adhesion molecule (Niewiadomska et al., 1999). If border cells or nurse cells are mutant for DE-cadherin, border cells cannot migrate. How adhesion in border cells is regulated is not fully understood however the link between the Actin cytoskeleton and DE-cadherin is required but not regulated in the level of DE-Cadherin – α catenin, and constitutively binding DE-cadherin with α-catenin can replace the function of the endogenous protein. (Pacquelet and Rorth, 2005) A possible mechanism of adhesion regulation is the turnover of adhesion complexes by endocytosis.

Throughout the migration process border cells remain attached to each other. Indeed, if one generates a border cell cluster consisting of wild-type cells and cells mutant for an essential factor like *slbo*, mutant cells are pulled into the migrating cluster (Rorth et al., 2000). Mutant cells always trail behind and the more cells are mutant in the cluster the more delayed the cluster is. This suggests that those cells are not contributing to migration and are pulled by the wild-type cells that attempt to migrate. The identity of the adhesion molecule that binds them is still not known but it is known that it is not only DE-cadherin since border cell clusters composed of DE-Cadherin mutant cells and wild-type cells keep their cluster morphology (Niewiadomska et al., 1999).

Both laser ablation of border cells and genetic manipulation of border cells to stop their migration caused defective morphology of the sperm channel. At the end of the migration, border cells differentiate and form the pore leading to micropyle, the sperm channel that is crucial for the fertilization of the oocyte (Montell et al., 1992). Another role of border cell migration is the induction of the gene torso-like in the oocyte which is important for patterning of the resulting embryo after fertilization (Savant-Bhonsale and Montell, 1993). Thus, a mutation that completely blocks border cell migration causes sterility of the females.

1.5.2 Role of Mal-D and DSRF in border cell migration

Mal-D has been identified by the work of Kalman Somogyi in our laboratory because of impaired border cell migration and decrease in F-Actin levels in cells mutant for this gene (Somogyi and Rorth 2004). Sequence analysis identified the gene as the only Drosophila member of MRTF family of SRF coactivator. Before going into detail about the phenotypes of Mal-D I would like to introduce Mal-D and DSRF and the knowledge that we have from their analysis in different systems.

1.6 SRF and MAL

Serum Response Factor (SRF) has been studied in mammalian cell culture system for a long time. In mammalian cell culture system, it was identified as the transcription factor crucial for the expression of immediate early genes (Norman et al., 1988; Treisman,

1987). Immediate early genes are a group of genes that are activated if serum is added after serum starvation. Their expression level increases within 30 minutes of serum addition and this increase does not require prior protein synthesis. This list of genes includes cell proliferation and survival factors such as c-Fos, c-Myc and c-Jun. The sequence motif that renders SRF responsiveness has been identified. The motif is $CC(A/T)_6GG$ (Treisman, 1985).

SRF is a member of the MADS (MCM1, Agamous, Deficiens, SRF) family of transcription factors. Those transcription factors share homology in a 57 amino acid region called the MADS box (Shore and Sharrocks, 1995). Although there are many members of MADS box proteins in plants the only members of the family in animals are Mef2 (myocyte enhancer factor 2) subfamily that has role in muscle differentiation and SRF. The conserved MADS box contains sequences important for homodimerization and DNA binding of those proteins. SRF has an extension of this motif that can bind to its transcriptional coactivators. MADS box is highly conserved in SRF from different species and it is 93% identical from Drosophila to human SRF (Affolter et al., 1994).

Analysis of SRF activity showed that by itself SRF is a poor activator of transcription. The activity of SRF depends on binding to different transcription coactivators. There are two major classes of coactivators that activate the transcription of two separate groups of targets (Gineitis and Treisman, 2001) (Figure 1.10). The first group of targets has been shown to be responsive to growth factor signaling and they are inhibited by using MAP kinase pathway inhibitors. The transcription coactivator family responsible for the activation of this class of targets is Ternary Complex Family (TCF). This family is composed of Sap1, Elk-1 and Net. They are phosphorylated directly by MAPK signaling and bind to SRF and a consensus sequence on the DNA next to the SRE and activate a group of SRF targets (Gineitis and Treisman, 2001).

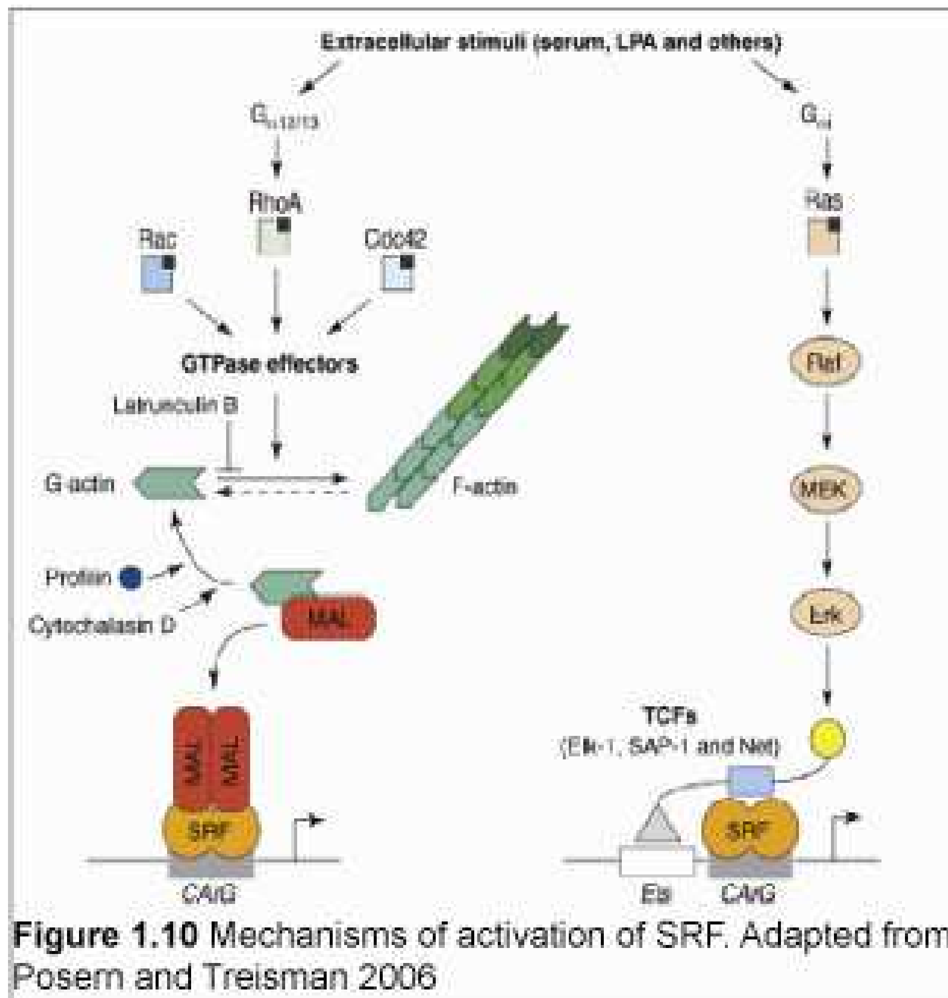

Figure 1.10 Mechanisms of activation of SRF. Adapted from Posern and Treisman 2006

(Posern and Treisman, 2006)

In addition to its regulation by MAPK signaling it has been known that serum induction of a different subset of SRF target genes was blocked by blocking Rho small GTPase using C3 transferase or by inhibiting Actin polymerization (Hill et al., 1995). Using drugs that bind to G-Actin such as cytochalasin D or swinholide A on the other hand activates the transcription of those targets in NIH3T3 cells. (Hill and Treisman, 1995; Mack et al., 2001; Sotiropoulos et al., 1999).

The link between Actin polymerization and transcriptional activation remained elusive until the identification of MAL as potent transcription coactivators of SRF. I will mention the other members of the MRTF family and how they are regulated in the following sections. MAL is cytoplasmic when NIH3T3 cells are serum starved, and shifts to the nucleus in a rapid manner in response to serum (Miralles et al., 2003). Moreover this

shifting to the nucleus can be inhibited by either blocking Rho or Actin polymerization (Miralles et al., 2003).

MAL has three Actin binding motifs called RPEL motifs in its N terminus. Deletion or point mutation of those motifs causes Mal to accumulate constitutively in the nucleus without the requirement to serum activation (Miralles et al., 2003). This led to the hypothesis that MAL is kept cytoplasmic by the action of G-Actin. Growth factors in serum activate Rho, which in turn causes G-Actin to form F-Actin with the action of Rho effector Diaphanous. This accumulation of F-Actin causes G-Actin depletion in the cells, thus rendering MAL free of cytoplasmic retention. This causes MAL to go to the nucleus where it binds to SRF and causes the upregulation of Actin, Vinculin and other factors that increase the F-Actin levels. (Miralles et al., 2003; Morita et al., 2007). On the other hand the regulation of MAL by Actin cytoskeleton may be more complex. An Actin point mutant that binds strongly to MAL was shown to drive MAL into nucleus showing that Actin may have a more active role in MAL regulation rather than cytoplasmic retention (Posern et al., 2004). A recent study indicated that in cells that are not stimulated with serum MAL continuously rapidly shuttles between nucleus and cytoplasm since blocking nuclear export causes rapid accumulation of MAL in the nucleus and photo activation of a photoactivatable GFP fused to MAL in the nucleus shows dispersal of the signal in the cytoplasm (Vartiainen et al., 2007). Suggested mechanism is that in cells that are serum starved the nuclear export of MAL is so rapid that MAL can only be seen in the cytoplasm, (Vartiainen et al., 2007). 5 minutes after blocking nuclear export MAL was accumulated in the nucleus, which is a rate that is faster than serum induced nuclear accumulation of MAL indicating that basal shuttling rate of MAL is higher than induced nuclear transport meaning that the effect of serum activation goes through at least partly by blocking nuclear export (Vartiainen et al., 2007). Continuous shuttling in the serum starved cells is dependent on cytoplasmic Actin dynamics, and nuclear Actin pool, since treating the cells with Actin sequestering drugs or Rho inhibitors prior to inhibiting nuclear export abrogates nuclear accumulation in response to nuclear export inhibition (Vartiainen et al., 2007). Actin regulates MAL by binding to it in the cytoplasm and inhibiting nuclear import, binding to MAL in the nucleus and leading to its nuclear export

and binding to MAL in the nucleus to prevent it to stimulate SRF target genes. (Vartiainen et al., 2007)

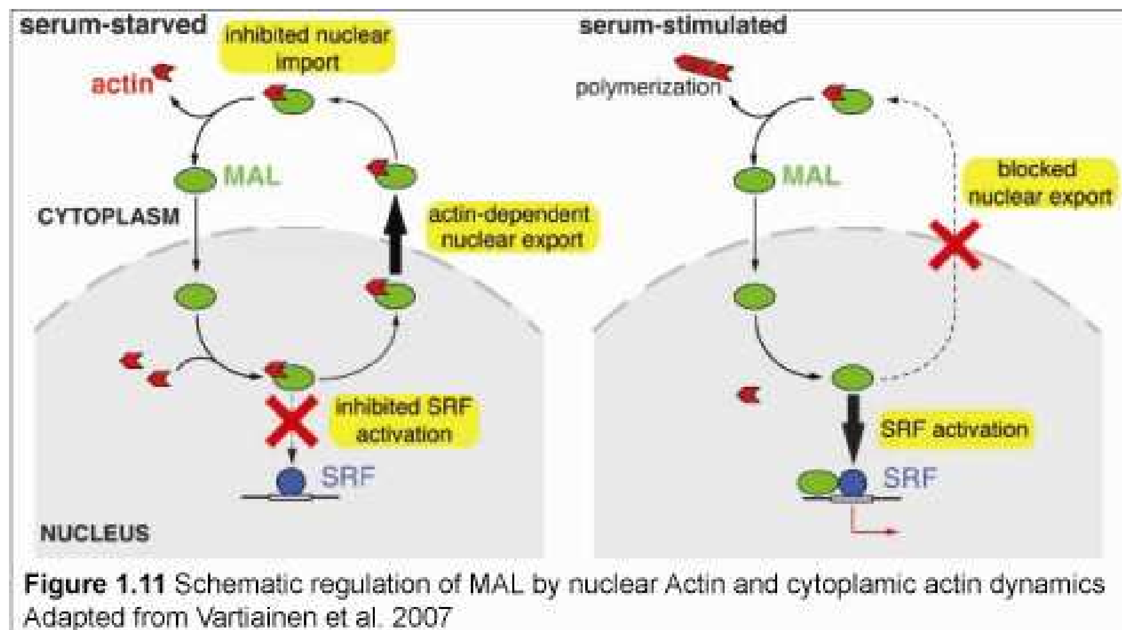

Figure 1.11 Schematic regulation of MAL by nuclear Actin and cytoplamic actin dynamics Adapted from Vartiainen et al. 2007

1.6.1 SRF and MRTFs in vivo

SRF has been shown to be important in many processes in the mouse development and adult life. Homozygous mutation in SRF causes the embryos to die as early as gastrulation, due to a defect in mesoderm specification (Arsenian et al., 1998). Conditional disruption of SRF in different tissues indicated roles of SRF in cardiac development (Niu et al., 2005), skeletal muscle development (Li et al., 2005), postnatal skeletal muscle growth and regeneration(Charvet, Houbron et al. 2006), neural circuit assembly (Knoll, Kretz et al. 2006), learning (Etkin et al., 2006; Lindecke et al., 2006) and lymphocyte development (Fleige et al., 2007) in mouse. Moreover in mouse SRF has been implicated to be important for the migration of new born neurons from the subventricular zone to the olfactory bulb, along rostral migratory stream. (Alberti et al., 2005). Additionally SRF mutant Embryonic Stem cells (ES) have less Actin, lamellipodia and focal adhesions. (Schratt et al., 2002)

There are three members of the MRTF family of transcription factors. The founding member, Myocardin, has been identified because of its restricted expression domain.

Myocardin is specifically expressed in smooth muscle, and cardiac muscle. Ectopic expression of Myocardin is sufficient to express smooth muscle and cardiac muscle markers in non muscle cells in *Xenopus* (Small et al., 2005). Moreover mice mutant for myocardin dies during embryonic development due to problems with vasculature, showing again the importance of myocardin in smooth muscle differentiation (Li et al., 2003). In contrast to other members of the family myocardin is constitutively nuclear in the cell types it has been analyzed (Wang et al., 2001).

In contrast to tight tissue specific expression of myocardin, MAL and MRTF-B are ubiquitously expressed in mouse. A knock out of MAL is viable and fertile, but shows a phenotype that is specific for lactating females. The mothers that are homozygous mutant for MAL fail to feed their pups.(Li et al., 2006; Sun et al., 2006b) There was a problem with embryonic heart development as well but it was not fully penetrant meaning MAL is not essential for embryonic heart development but may have roles according to environmental stress. (Sun et al. 2006) Knock-out of MRTF-B on the other hand causes problems in development of neural crest derived smooth muscle cells in branchial arteries and causes embryonic lethality in mid gestation. (Oh et al., 2005) Although MAL and MRTF-B are similar and are expressed ubiquitously they do not act in a fully redundant manner meaning they may have diverged in roles. Other possibility is that the proteins are redundant and removing either MAL or MRTF-B in the affected cells causes the total level of SRF dependent transcription of targets to go down. The gene causing the phenotype may be the one higher expressed in that tissue that can compensate the mutation in the other family member. Generation of double mutant mice would clarify this issue.

Members of MRTF family of transcriptional coactivators have similar domain structure. They contain RPEL motifs in their N-Terminus, 2 in the case of Myocardin and 3 for MAL and MRTF-B that is essential for the regulation of MAL and MRTF-B. They all contain a SAP domain, a leucine zipper and a very potent C terminal transcription activator domain (Figure 1.12)(Wang et al., 2001). SAP domain is important for binding to SRF and is important for the activity of the protein. MAL and myocardin form homodimers and this dimerization is important for the activity of the proteins (Miralles et

al., 2003). Myocardin related transcription factors share homology in their SAP domain as well. SAP motif is a motif of 35 amino acids, named after SAF-A/B, Acinus and PIAS. SAP motif is rich in positive residues, which may work for binding the backbone of the DNA. In different proteins that have SAP domain it has been suggested to conduct diverse roles such as chromosome organization, nuclear breakdown and apoptotic DNA fragmentation. (Aravind and Koonin, 2000; Pipes et al., 2006) The role of SAP domain of MRTF family is vague since its deletion affects the expression of some of the target genes and not others, suggesting that there might be locus specific interactions between MRTF family of transcriptional co-activators SAP motif and target DNA sequences (Wang et al., 2001).

All members of the MRTF family of transcription factors contain a basic region that resembles structurally to the B box of TCF family of SRF coactivators. Indeed replacement of basic regions with the B box of Ets protein, a member of TCF family, does not perturb the activity of Myocardin (Wang et al., 2004). Thus MRTF family of transcriptional coactivators competes for the same region on SRF for binding and activating the protein. This kind of competition causes the formation of a binary switch in cell fate decision. Binding of SRF to activated Elk-1 causes SRF to activate growth associated targets whereas association with myocardin causes the execution of muscle differentiation program (Wang et al., 2004). A further role for the basic domains is for MAL nuclear transport in response to serum induction. (Miralles et al., 2003)

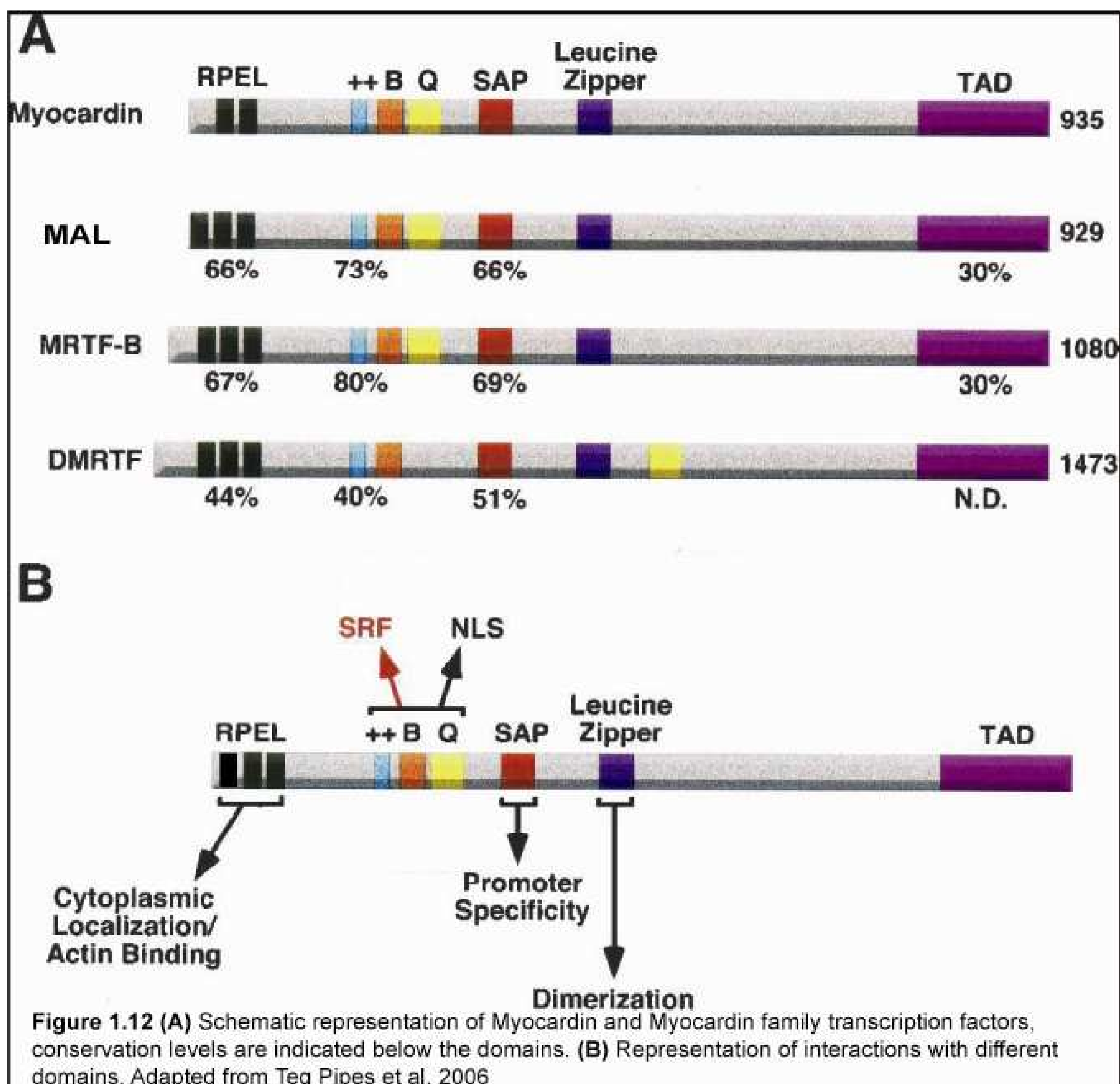

Figure 1.12 (A) Schematic representation of Myocardin and Myocardin family transcription factors, conservation levels are indicated below the domains. **(B)** Representation of interactions with different domains. Adapted from Teg Pipes et al. 2006

The C terminus of members of MRTF family of transcription factors harbors the transcription activator domain. Members of MRTF family of transcription factors are not well conserved in this transcription activator domain and replacing this domain by an exogenous transcription activator domain (TAD) such as VP16 TAD does not cause problems in the activity of the proteins (Wang et al., 2001). Moreover the domain can be fused to Gal4 DNA binding domain and boost transcription in Gal4 responsive sites. If one removes the C-Terminal TAD from MRTF family of transcription factors one generates a dominant negative factor which presumably binds and sequesters SRF (Wang et al., 2001) (Figure (Pipes et al., 2006)).

Proteins of MRTF family of transcriptional coactivators do not bind directly to DNA. This differential behavior of different coactivator families of SRF is thought to give target specificity to the activity SRF in response to TCF family and MRTF family.

1.6.2 DSRF and Mal-D

In *Drosophila* there is one homologue of SRF, namely DSRF encoded by the gene *blistered*. It is an essential gene and many mutants have been identified. DSRF was shown to be important for the terminal branching of the trachea, the outgrowth of the terminal branches (Guillemin et al., 1996), and the differentiation of inter-vein cells in the wing (Fristrom et al., 1994). In the absence of intervein cell differentiation dorsal and ventral sides of the wing do not adhere strongly and wings show blisters, hence the gene is called blistered in flies. The targets that are up-regulated by DSRF activity are not known, and coactivators of DSRF were not known, specifically there is no TCF gene in the sequenced fly genome. Mal-D is the only identified DSRF coactivator in *Drosophila melanogaster* (Figure 1.13). Mal-D is the only orthologue of MRTF family of transcription factors in flies and it shares the highest homology to MAL.

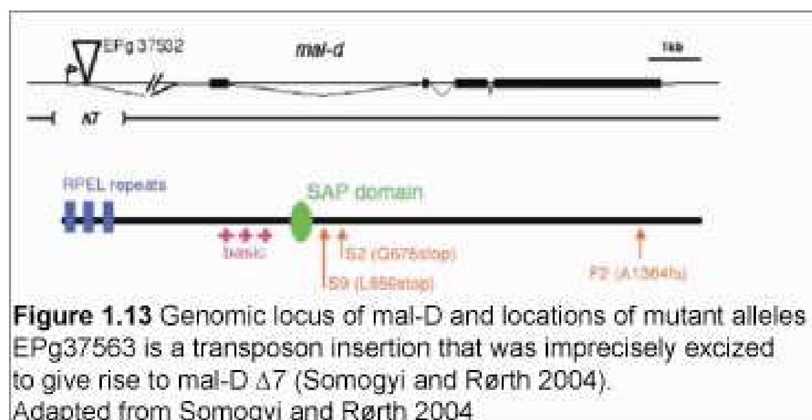

Figure 1.13 Genomic locus of mal-D and locations of mutant alleles EPg37563 is a transposon insertion that was imprecisely excized to give rise to mal-D Δ7 (Somogyi and Rørth 2004). Adapted from Somogyi and Rørth 2004

Mal-D is an essential gene in *Drosophila* (Han et al., 2004; Somogyi and Rorth, 2004). Hypomorphic allelic combinations of Mal-D showed kinked bristles on the notum of the mutant flies (Somogyi and Rorth, 2004). Bristles are actin based structures and many actin regulators have been found to cause kinked bristle phenotype in *Drosophila*. RNAi mediated Mal-D knock-down showed problems with tracheal out branching similar to DSRF loss of function (Han et al., 2004). Moreover over expression of a dominant

negative form of Mal-D that lacks the C terminal TAD (Mal-D ΔC) in wings causes blisters in the wing epithelium the same way as DSRF does. When overexpressed in the developing mesoderm Mal-D ΔC causes the ventrodorsal migration of muscle cells and subsequent organization of the heart tube. (Han et al., 2004) However neither the wing phenotype nor the migration problems in the mesoderm are observed in the mutant embryos meaning that over expression of a dominant negative form meaning the dominant negative Mal-D may generate phenotypes unrelated to the loss of Mal-D (Kalman Somogyi Personal communication).

1.6.3 Phenotype of Mal-D loss of function in the border cell migration

Mal-D mutant border cells have severely delayed migration. Most of the Mal-D mutant border cells migrate very poorly (Figure 1.14 B). DSRF mutant border cells show the same phenotype as well. (Figure 1.14 B)

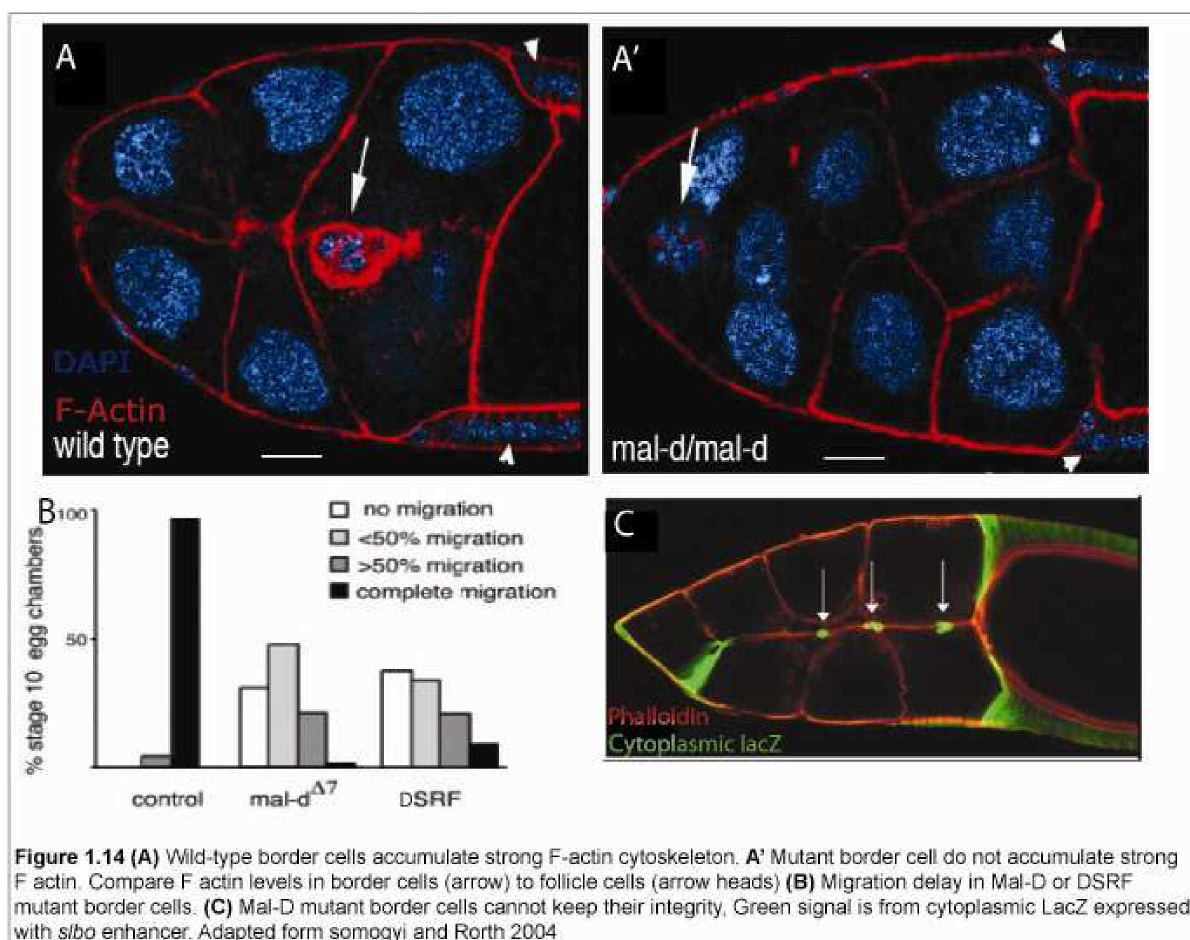

Figure 1.14 (A) Wild-type border cells accumulate strong F-actin cytoskeleton. **A'** Mutant border cell do not accumulate strong F actin. Compare F actin levels in border cells (arrow) to follicle cells (arrow heads) **(B)** Migration delay in Mal-D or DSRF mutant border cells. **(C)** Mal-D mutant border cells cannot keep their integrity. Green signal is from cytoplasmic LacZ expressed with *slbo* enhancer. Adapted form somogyi and Rorth 2004

Moreover during the onset of migration F-Actin levels increase in the wild-type migrating border cell clusters, compared to the follicle cells, prior neighbors of border cells. In Mal-D mutant border cell clusters this increase is not seen (Somogyi and Rorth, 2004) (Figure 1.14A-A'). Another phenotype associated with Mal-D loss of function is that the border cells mutant for Mal-D cannot keep their cellular integrity, and they tend to shed blobs of cytoplasm that continue their migration, separated from the main cell body (Somogyi and Rorth, 2004)(Figure 1.14C). This breaking apart phenotype is specific to border cells that undergo an invasive migration. Although there is a decrease in the level of F-Actin in follicle cells as well *mal-D* mutant follicle cells do not break (Somogyi and Rorth, 2004). This result indicates that the border cell guidance and migration mechanics can go in a transcription independent way with the local activity of proteins that are in the leading edge. This kind of migratory behavior was previously identified in pieces of leukocytes that can generate fragments of cytoplasm that do not contain the nucleus, centrosomes, microtubules and majority of organelles, but continue to migrate in response to chemo attractants. (Keller and Bessis, 1975)

1.6.4 What is known about the regulation of Mal-D in border cell migration?

An antibody raised against Mal-D shows that Mal-D can be observed nuclear in some of the cells of the migrating border cell cluster (Somogyi and Rorth, 2004). The level of nuclear accumulation is variable in the border cell clusters from cell to cell. There are some cells that show nuclear Mal-D signal whereas other border cells of the same cluster do not show the nuclear accumulation (Somogyi and Rorth, 2004). The probability of a cell showing nuclear Mal-D, to be in the front positions in the migrating cluster is the same as it being in the back positions of the cluster, thus there is no prototype of nuclear accumulation of Mal-D in the border cells. A border cell cluster that is stretched has more chance of having some border cells with nuclear Mal-D than a rounded up border cell cluster (Somogyi and Rorth, 2004).

The nuclear accumulation of Mal-D has been shown to be regulated by migration of the border cells. If one generates a border cell cluster consisting only of border cells mutant for *slbo* the cluster (Full clone) does not move and stays in the anterior pole of the egg

chamber. In this situation Mal-D is not seen to accumulate in the nucleus (Somogyi and Rorth, 2004). On the other hand if one generates border cell clusters consisting of wild-type cells and cells mutant for *slbo* (Partial clone), wild-type cells attempt to migrate and as they are bound to the mutant cells, they pull the mutant cells in the migrating cluster (Rorth et al., 2000). In this situation the mutant border cells can accumulate nuclear Mal-D (Somogyi and Rorth, 2004). The mutant cells that are part of a full clone cluster or a partial clone cluster are genetically identical and the difference mainly arises from the fact that mutant cells that are part of a full mutant clone are not incorporated into a migrating cluster whereas mutant cells that are part of a partial clone are pulled into the migrating cluster by the action of wildtype cells. This suggests that there is a migration-related signal that promotes nuclear accumulation of Mal-D (Somogyi and Rorth, 2004). This migration-related signal may be the pulling force of the other cells, or stretching of the cells in response to this pulling force, or the increase of cell tension. An attractive scenario is that the cells sense the migration-related signal, they accumulate Mal-D in the nucleus where it binds to DSRF, and transcribes factors that are needed for the increase F-Actin levels of the cells, thus increasing the robustness of the cell to counteracting the tension. In the absence of Mal-D the cells cannot increase their F-Actin levels and loose their integrity, as they cannot counteract the forces related to migration.

2. The Aim of the Project

Mal-D is an interesting factor that has an important role of making the cells more robust for the migration. Moreover it is regulated by the migration event. Understanding how Mal-D is regulated by the migration event will provide information about how, and what the cells perceive during the migration. The first aim of my project is to find out which factors are required for the regulation of Mal-D during the border cell migration. This way I plan to understand the nature of the signal perceived by the migrating cell that leads to transcriptional output form Mal-D/DSRF complex.

On the other hand Mal-D gives a peculiar phenotype which is the breaking of the cells. The other aim is to identify targets of Mal-D/DSRF by using whole genome expression arrays on border cells mutant for Mal-D or wild-type. Unraveling the targets of Mal-D that lead to the phenotype can help to understand what the cells are missing in the absence of Mal-D and would be telling about what the cells need to become more robust in order to counteract the hardship of migrating and invading through another tissue.

3 Results

Part I MAL-D Regulation

3.1 Tools for Visualizing Mal-D Subcellular Localization

Previous antibody staining results in fixed samples showed that Mal-D can be seen nuclear in some border cells of the migrating border cell cluster (Somogyi and Rorth, 2004). This nuclear accumulation depends on the migration process and if the border cells are rendered non migratory by mutating them, nuclear accumulation of Mal-D is lost. Number of cells with nuclear Mal-D as well as the position of those cells within the cluster varies form eggchamber to eggchamber, suggesting that there is a dynamic regulation of subcellular localization of Mal-D. For understanding this regulation it is important to have a means of observing localization of Mal-D.

I needed to generate new tools to visualize nuclear localization of Mal-D since the previous antibody staining was not robust, and had a high background and since the affinity purified antibody ran out and further attempt to do affinity purification failed.

3.1.1 Transgenic approaches

One common way to visualize the subcellular localization of a protein in vivo is to tag that protein with Green Fluorescent Protein (GFP) and express it exogenously. It gives further advantage of possibility of doing live imaging, which was one of my plans but was not pursued later on. In the case of Mal-D when the protein was over expressed protein accumulated in the cytoplasm (Somogyi and Rorth, 2004), thus it was very difficult to observe any nuclear accumulation. I needed to find a means of expressing the protein in amounts low enough to not to accumulate in the cytoplasm, but high enough to be detectable. For addressing this technical difficulty I cloned 3 GFPs in tandem at the C

terminus of Mal-D (Mal-D-3XGFP). This way I was planning to have higher signal for every overexpressed molecule of Mal-D, thus increase my detection with lower over expression levels. I cloned this construct in *Drosophila* transgenesis vectors with different promoters. I used Tubulin promoter (a ubiquitous promoter), Armadillo promoter (weaker ubiquitous promoter), UAS promoter (weak basal activity that can be very much enhanced by expression of GAL4 enhancer) and heat shock promoter (weak basal activity that can be increased with heat shock (See materials and methods)). I tested multiple transgenic fly lines that carry those constructs, since the site of insertion of the transgenic construct on the genome greatly affects its expression levels.

While expressing a tagged protein one should make sure that the modified protein is still functional, and regulated in a similar way to the endogenous protein. This transgene driven by different promoters were crossed to *mal-D* lethal allelic background mal-D ^{s2}/ mal-D ^{f2} and it could rescue lethality of those mutant alleles. Border cells could migrate normally in the flies overexpressing this transgene. Over expressing constitutive active diaphanous which lacks its auto inhibition domains in the C terminus, along with Mal-D-3XGFP induced strong nuclear accumulation of Mal-D-3XGFP in follicle cells and border cells (Figure 3.1 A and B), the same way that it causes wild-type Mal-D to accumulate in the nucleus. (Somogyi and Rorth, 2004)

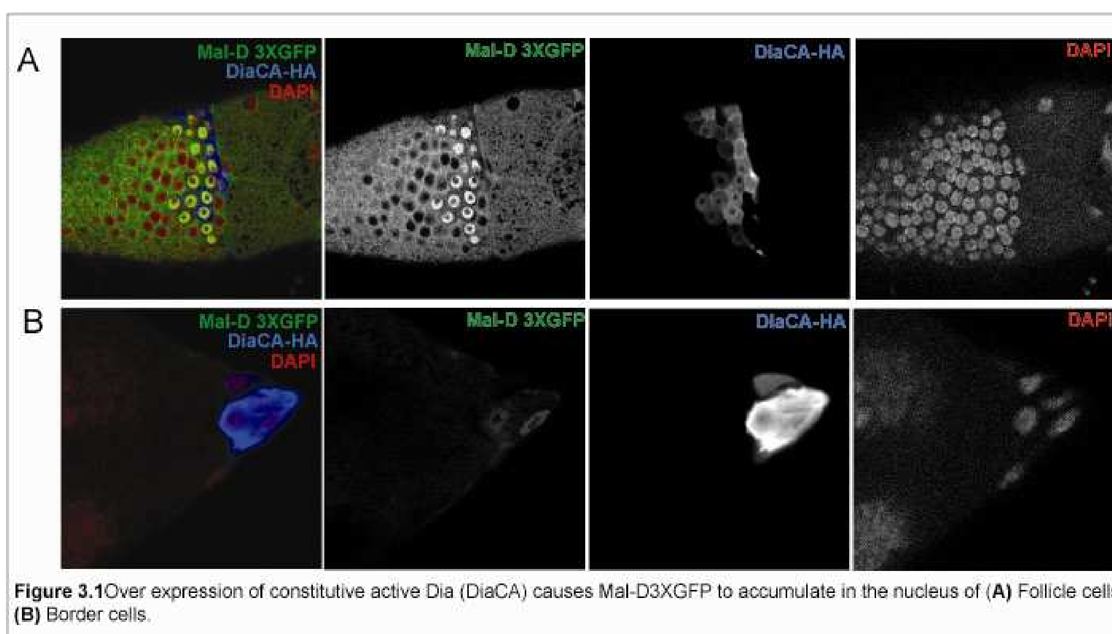

Figure 3.1 Over expression of constitutive active Dia (DiaCA) causes Mal-D3XGFP to accumulate in the nucleus of **(A)** Follicle cells **(B)** Border cells.

Only problem with this approach is that I could not find a level of expression low enough for the overexpressed protein not to accumulate in the cytoplasm (Figure 3.2). This made it hard to see nuclear accumulation of Mal-D.

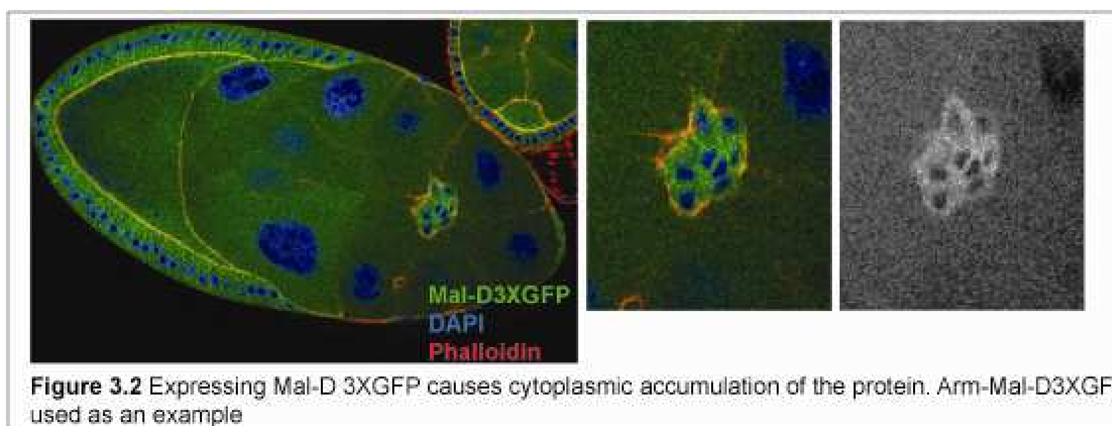

Figure 3.2 Expressing Mal-D 3XGFP causes cytoplasmic accumulation of the protein. Arm-Mal-D3XGFP used as an example

In all combinations of driver and transgenic lines, cytoplasmic accumulation was a problem. One important conclusion with this approach is that the protein is tolerant to modifications on its C-terminus, as fusing a large tag such as 3XGFP does not completely perturb the functionality.

3.1.2 Knock-in approach

3.1.2.1 Construction of Mal-D9HA

Low levels of staining on the endogenous protein with different antibodies show that the protein is not highly expressed. Over expression increases the detection but disturb the subcellular distribution of the protein. To circumvent both of these problems I knocked-in 9 Hemagglutinin (HA) tags in the endogenous *mal-D* locus by using homologous recombination technique (Gong and Golic, 2003). This technique makes it possible to modify genomic sequences specifically by using homologous recombination. This would give me 9 copies of a good epitope fused directly to the endogenous protein, thus expressed in the endogenous levels. I targeted the C terminus of the protein because the results of transgenic approach showed that C terminus of the protein was tolerant to modifications. I added a fragment that contains 9 HA tags to the 5' homology region leaving the rest gene mostly unmodified (Figure 3.3 A). One feature of the technique is

the insertion of an eye color marker (*white*) in the chromosome that underwent homologous recombination. Although having the eye color marker was helpful in order to select the initial recombination event it might cause problems in the expression levels since it was situated between coding region of the gene and the 3'UTR of the gene. This marker was flanked by LoxP sites which can be removed by using site specific recombinase Cre. For minimizing the amount of modification made to the endogenous locus I removed the *White* marker by using hs-Cre. After removing selection marker, the sole difference to the endogenous gene is an addition of a LoxP foot of 22 nucleotides in the 3' untranslated region of the gene, and the presence of 9 HA Tags (Figure 3.3 A). The correct insertion of the construct was confirmed by PCR (Figure 3.3 B,C,D and refer to Materials and Methods 4.2.4) and sequencing.

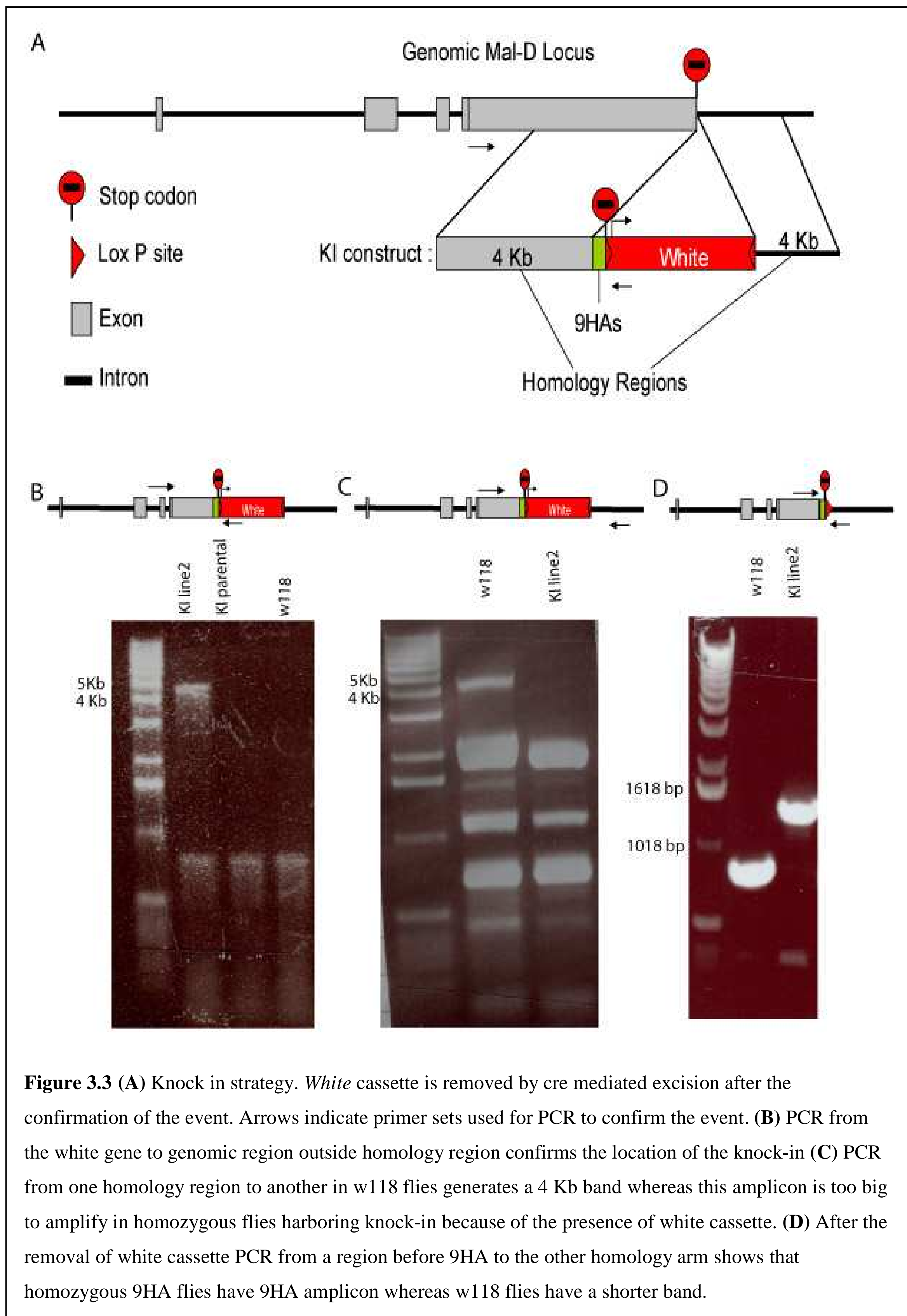

Figure 3.3 (A) Knock in strategy. *White* cassette is removed by cre mediated excision after the confirmation of the event. Arrows indicate primer sets used for PCR to confirm the event. **(B)** PCR from the white gene to genomic region outside homology region confirms the location of the knock-in **(C)** PCR from one homology region to another in w118 flies generates a 4 Kb band whereas this amplicon is too big to amplify in homozygous flies harboring knock-in because of the presence of white cassette. **(D)** After the removal of white cassette PCR from a region before 9HA to the other homology arm shows that homozygous 9HA flies have 9HA amplicon whereas w118 flies have a shorter band.

Flies carrying the modification in the endogenous locus are viable and fertile. Moreover they do not have any kinked bristles which is a phenotype seen even very mild Mal-D allelic combinations. Homologous recombination may cause complex genomic rearrangements that may result in duplications of the endogenous locus. For testing whether Mal-D 9HA was the only *mal-D* locus, and whether the insertion event caused a duplication, I used different PCR primers in homozygous flies. First I showed that a 4kb amplicon that could be amplified in the wild-type flies was disrupted when the knock in construct with *white* cassette is homozygous. (Figure 3.3 C). In the presence of this construct the amplicon is more than 7 Kb which was too long to be amplified in those conditions. Second I showed that after the removal of the *white* cassette, the only amplicon that could be amplified by using a set of primers located near 3'end of 5'homology region and 5'end of 3' homology region (reverse), was shifted equal to the size of 9HA fragments in knock-in flies compared to wild-type flies (Figure 3.3 D). Those PCR results along with sequencing results showed that the Mal-D 9HA insertion did not cause a complex genomic reorganization.

3.1.2.2 The phenotype of Mal-D 9HA

In flies homozygous for Mal-D 9HA there was an unexpected border cell migration phenotype. At stage 9 the border cells started their migration later than their wild-type counterparts (Figure 3.4 A). At stage 10 although more than 70% of the border cell clusters reached their destination there was about a population of 20% of border cells that migrated only half way (Figure 3.4 B). This phenotype could have different reasons. One possible reason is that the new construct was a hypomorphic allele of *mal-D*. The function of the protein might be compromised in a partial way so that the protein was functional enough to cause only a mild phenotype. I tested whether the border cell delay phenotype I had with homozygous Mal-D 9HA flies would get worse when Mal-D 9HA was trans-heterozygous with different alleles of Mal-D. I used mal-D ^{s9}, ^{s2}, ^{s5} and $^{\Delta7}$

alleles. ^{S} alleles are EMS mutants that cause stop codons in the coding region of *mal-D* that generate lethal mutations in the gene, and mal-D $^{\Delta7}$ removes regulatory regions, that result in viable flies with no detectable Mal-D protein in ovaries with Western Blot (Material and Methods 4.2.2, (Somogyi and Rorth, 2004)). Mal-D 9HA or *mal-D* alleles were crossed to Oregon R wild-type stock as a control. The migration delay was quantified in stage 9 and stage 10 of oogenesis (Figure 3.4 A and B). For ^{s2} and ^{s9} alleles heterozygous *mal-d* mutant alleles gave the same migration delay as trans-heterozygous *mal-D* mutant alleles over Mal-D 9HA. For mal-D $^{\Delta7}$ and mal-D ^{s5} heterozygous *mal-D* mutant border cells migrated better then the trans-heterozygous mutant alleles over Mal-D 9HA.

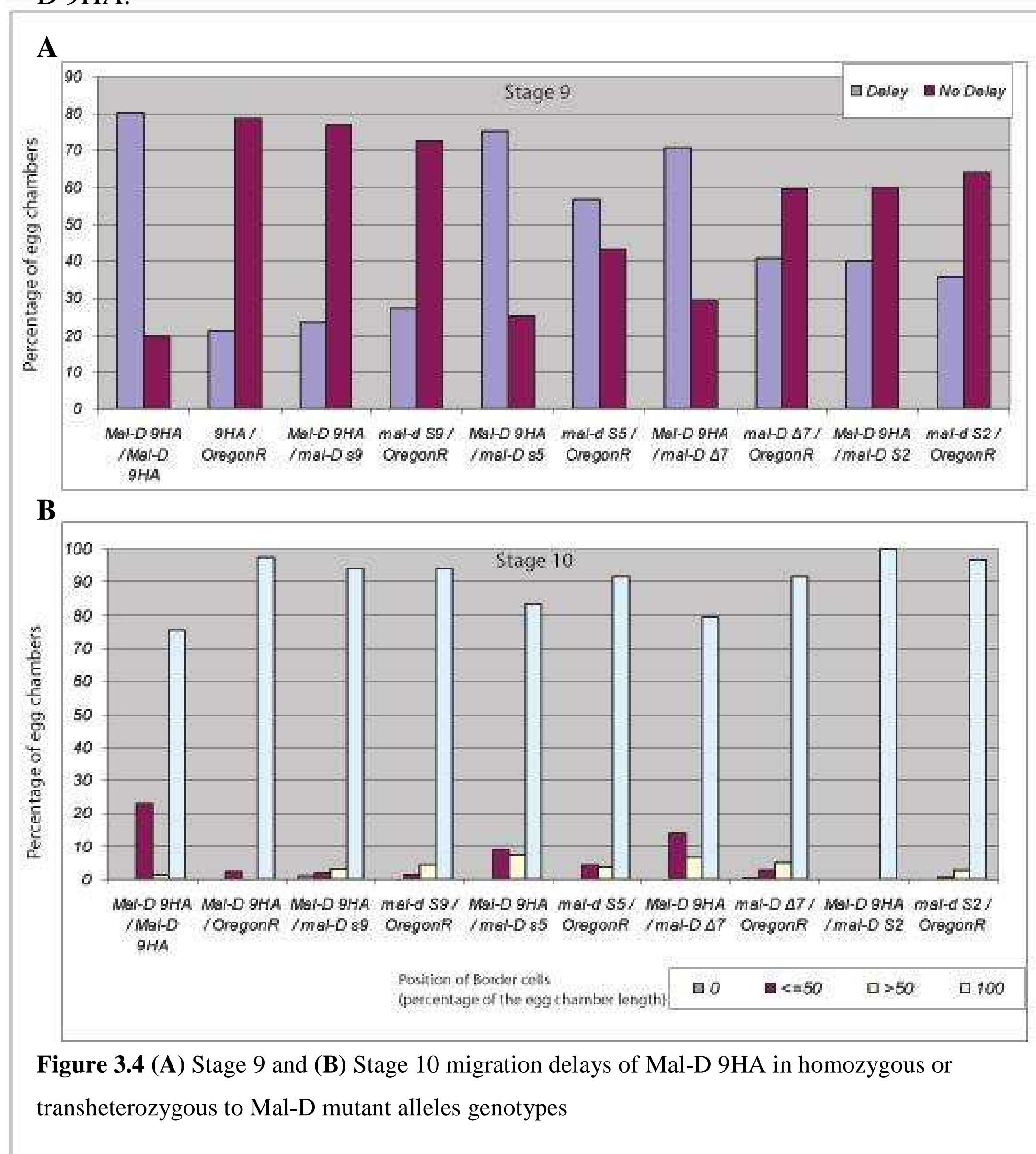

Figure 3.4 (A) Stage 9 and **(B)** Stage 10 migration delays of Mal-D 9HA in homozygous or transheterozygous to Mal-D mutant alleles genotypes

The migration delay caused by homozygous mutant border cells for the mentioned alleles is equal, meaning that for border cell migration all the alleles are equally penetrant (Somogyi and Rorth, 2004).

A different approach that I pursued was to rescue the migration phenotype of Mal-D 9HA homozygous flies by over expressing wild-type Mal-D exogenously with a transgene that rescues the phenotypes of null allelic combinations of *mal-D* (Somogyi and Rorth, 2004). This did not rescue the border cell migration delay in stage 9 of the migration (Figure 3.5).

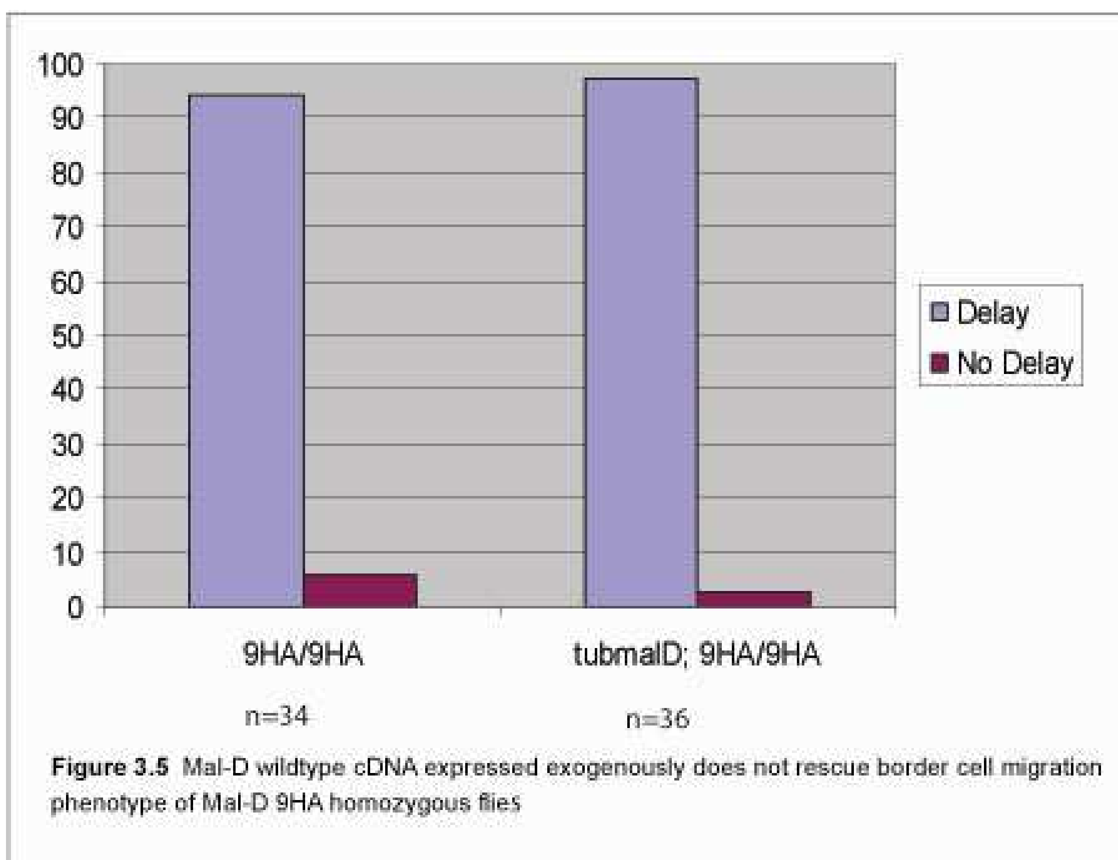

Figure 3.5 Mal-D wildtype cDNA expressed exogenously does not rescue border cell migration phenotype of Mal-D 9HA homozygous flies

The reason for the mild migration delay is not clear. Mal-D 9HA does not behave as a clear hypomorph, since in trans-heterozygous situations the phenotype was not worse than homozygous Mal-D 9HA, and since the phenotype that I observed in homozygous Mal-D 9HA flies could not be rescued by over expressing a wild-type transgene. On the other hand the phenotype of Mal-D 9HA over some, but not all mutant alleles of Mal-D gave stronger phenotype than the heterozygous situation of those alleles. Remaining possibilities are that the protein is a neomorph or there is a background mutation,

proximal to knock-in site on the knock-in chromosome. In either case Mal-D 9HA can replace endogenous function of the protein to a large extend, since the migration delay that was observed was mild and at stage 10 of oogenesis most of the border cell homozygous mal-D 9HA or trans-heterozygous with Mal-D 9HA over *mal-D* mutant alleles complete their migration (Figure 3.4 B).

3.1.2.3 Visualizing nuclear Mal-D 9HA by immunofluorescence.

Staining with Anti HA antibody showed the nuclear Mal-D 9HA population in a manner reminiscent of previous results obtained by affinity purified antibody. Mal-D 9HA was seen to be nuclear in a fraction of migrating border cells. (Figure 3.6A) Moreover the nuclear accumulation pattern in border cells was reminiscent to the old antibody staining pattern meaning it was more readily seen during the migration event and was no longer nuclear at stage 10 of oogenesis when border cells finish their posterior migration. The staining results were specific for Mal-D 9HA since staining of w118 flies that did not have Mal-D 9HA did not give such staining patterns (Figure 3.6 B).

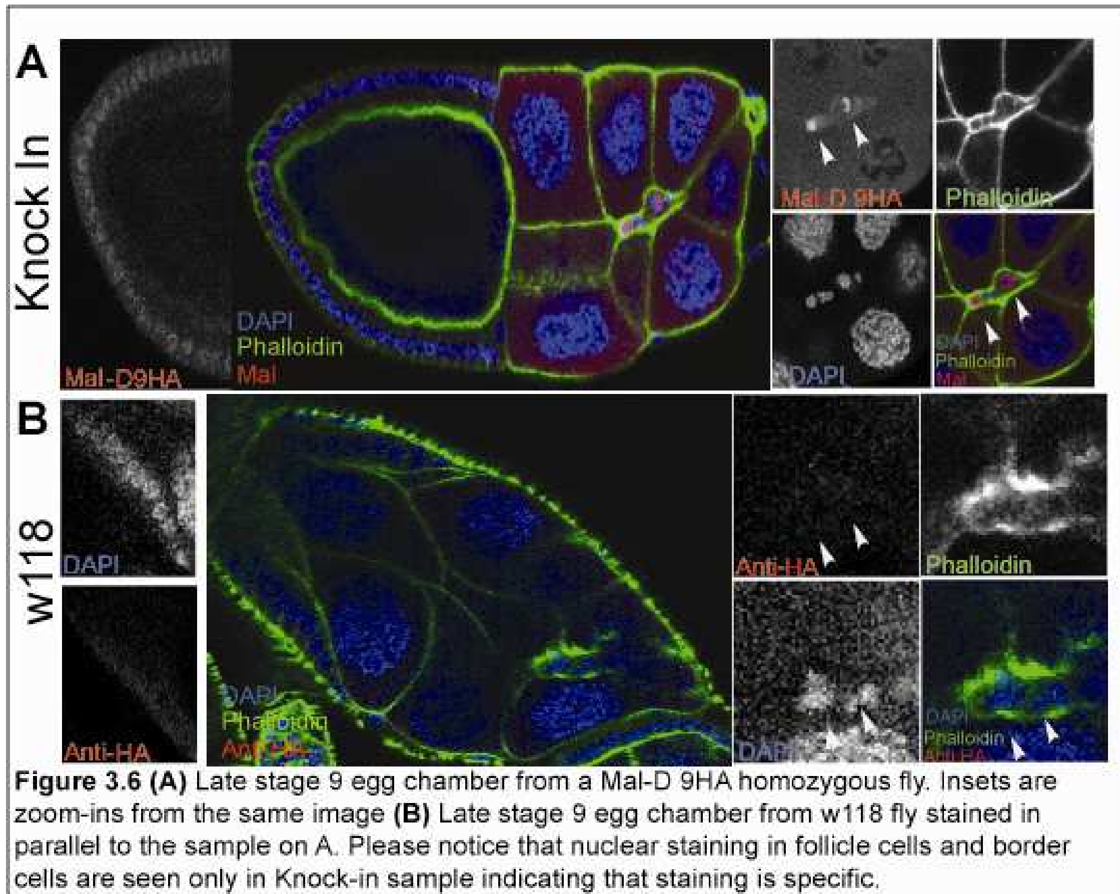

Figure 3.6 (A) Late stage 9 egg chamber from a Mal-D 9HA homozygous fly. Insets are zoom-ins from the same image **(B)** Late stage 9 egg chamber from w118 fly stained in parallel to the sample on A. Please notice that nuclear staining in follicle cells and border cells are seen only in Knock-in sample indicating that staining is specific.

I could observe nuclear Mal-D 9HA in different cells as well I could detect low nuclear Mal-D 9HA signal in follicle cells both at stage 9 and stage 10 of oogenesis as well (Figure 3.6 A inset). There was a specific nuclear Mal-D 9HA signal in stretched cells during the early stage 9 of oogenesis, while the centripedal cells migrate to cover over the oocyte and cause the stretching of the stretched cells (Figure 3.7). This staining disappeared at late stage 9 and at stage 10.

These staining patterns were not observed before with the old antibody, most likely due to the lower detection limit. The follicle cell staining was to be expected because of the fact that there was a decrease of F-Actin phenotype in the follicle cells mutant for Mal-D 9HA in the follicular epithelium, showing that the protein had a function in follicle cells (Somogyi and Rorth, 2004).

These results show that the staining that I have is comparable qualitatively to the old antibody staining, meaning Mal-D 9HA is regulated similar to the endogenous protein.

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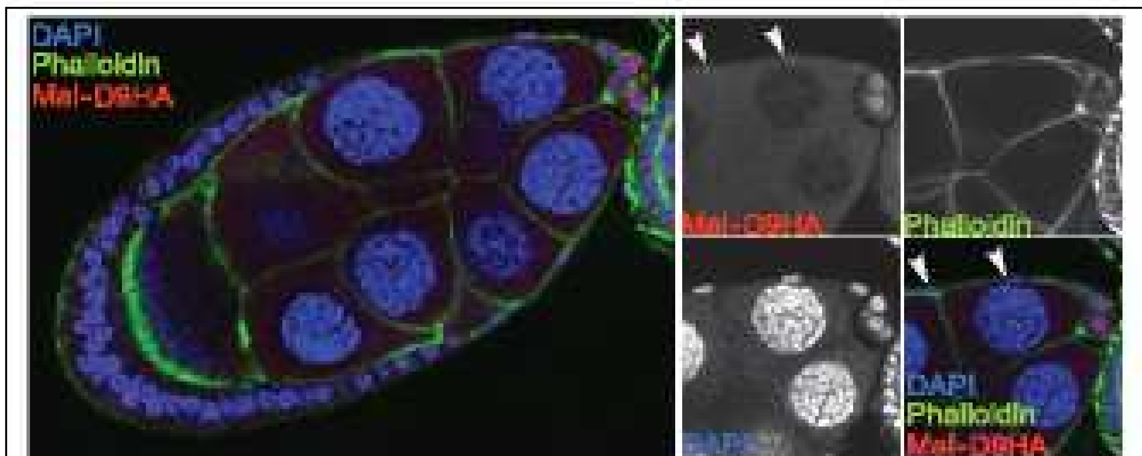
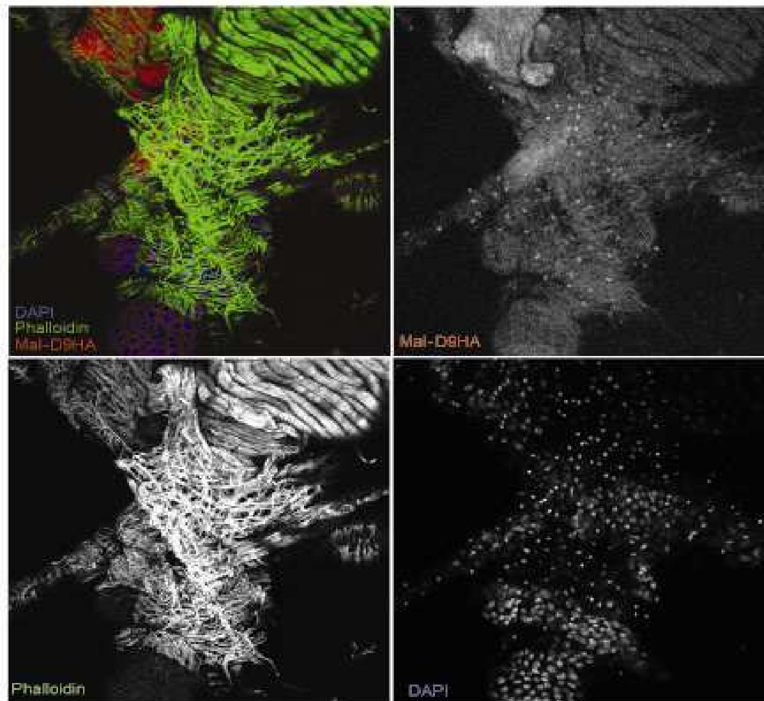


Figure 3.7 Nuclear Mal-D is seen in stretch cells at early stage 9 of oogenesis. Stretched cells are indicated by white arrow heads

Other than the staining in the ovary, I could detect Mal-D 9HA in the nuclei of muscle cells both in the muscle sheet surrounding the ovary and the developing embryo somatic muscles. (Figure 3.8) The significance of Mal-D in the muscle development remains to be addressed. The work of Kalman Somogyi showed that the embryos mutant maternal and zygotic mutant for neither *mal-D* nor *bs* caused a change in gross morphology of the embryonic muscles, but this analysis was not done in great detail and maybe there was a subtle phenotype that we could not observe. Moreover larvae zygotic mutants for *mal-D* cause the larvae to have a sluggish appearance which is reminiscent of a muscle defect phenotype (Kalman Somogyi personal communication). Another possibility is that there may be a differential splicing in the muscle or a redundant protein that can replace for loss of Mal-D. The role of Mal-D in muscle development if there is any, remains to be determined.



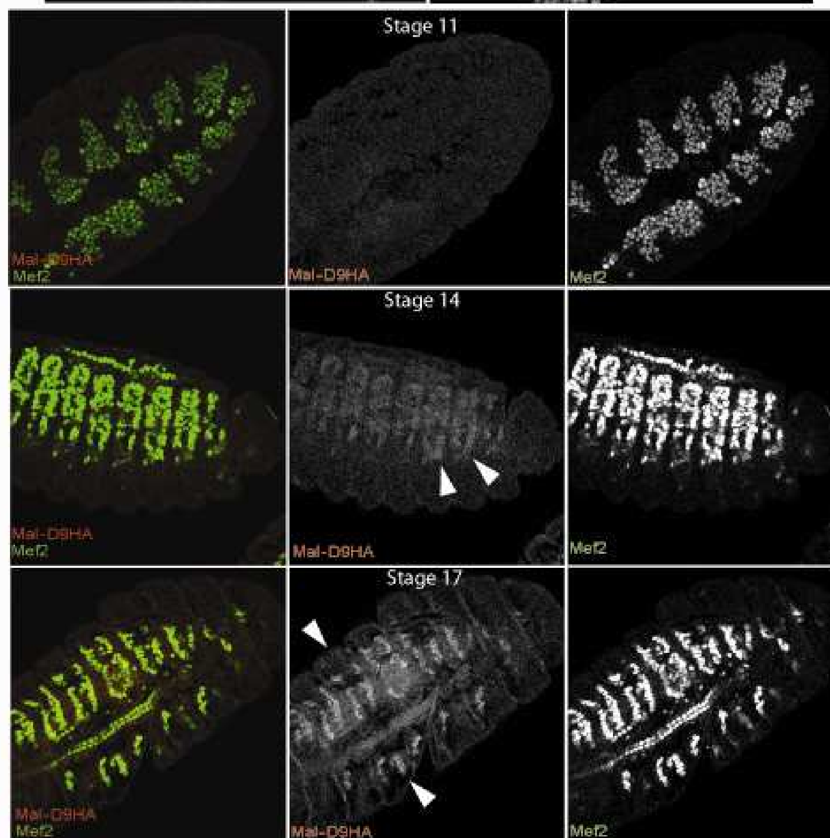


Figure 3.8 **(A)** Nuclear Mal-d 9HA can be observed in the nuclei of muscle sheet surrounding the oocyte **(B)** Different stages of embryonic muscle development. Mef 2 is a nuclear protein that has a role in the muscle development. Note that Mal-D 9HA is nuclear in the differentiated muscle weakly at stage 14 and strongly at stage 17 (white arrow heads).

3.2 Regulation of Mal-D

3.2.1 High levels of nuclear accumulation of Mal-D 9HA is regulated by migration related signal

There seems to be different levels of nuclear accumulation in different cells. In follicle cells there is the low accumulation. In border cells there is sometimes very high accumulation, sometimes higher than follicle cells accumulation and sometimes about the same level as the follicle cell accumulation. By using Photoshop software I quantified the nuclear levels of Mal-D 9HA in border cells and in follicle cells in the same egg chamber, the same picture and quantified the ratio between the signals. The pictures were taken in non saturating conditions. If the ratio of border cell nuclear/follicle cell nuclear signal was between 1 and 1.5 I called it the nuclear Mal-D index (NMI) of 1 (border cells that had about the same nuclear levels as follicle cells), if 1.5 to 2 fold NMI 2 (border cells that had higher nuclear Mal-D 9HA than follicle cells) and more than 2 fold as NMI 3 (The border cells that had very high nuclear Mal-D 9HA accumulation) (Figure 3.9).

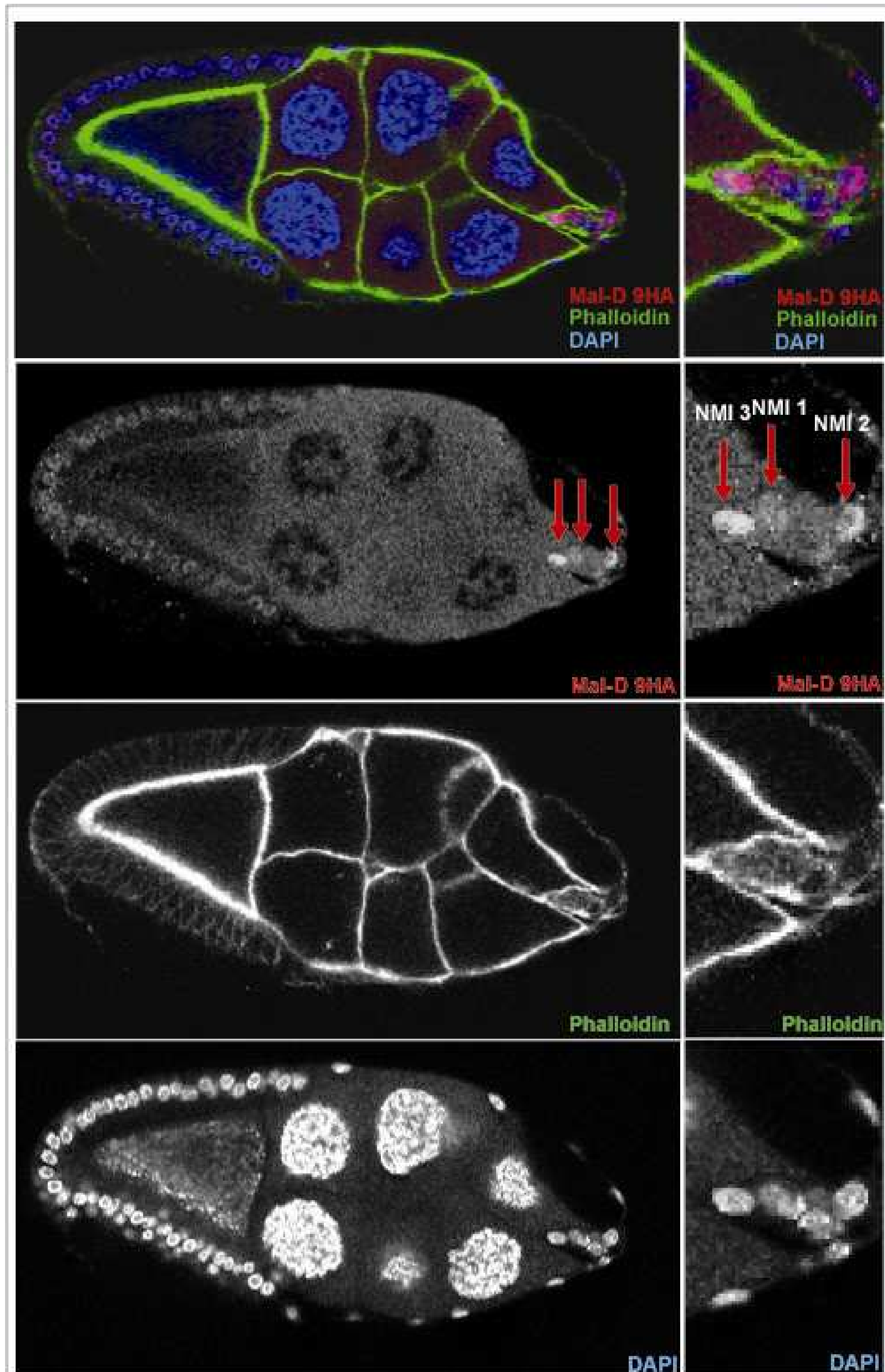


Figure 3.9 A stage 9 Mal-D 9HA homozygous eggchamber stained with HA antibody. Border Cells with different Nuclear Mal-D Indeces are shown with red arrows.

I quantified this index for stage 9 egg chambers of 10 independent staining days of Mal-D 9HA homozygous ovaries so that I have a general idea about the behavior of wild-type Mal-D 9HA border cells. (Figure 3.10)

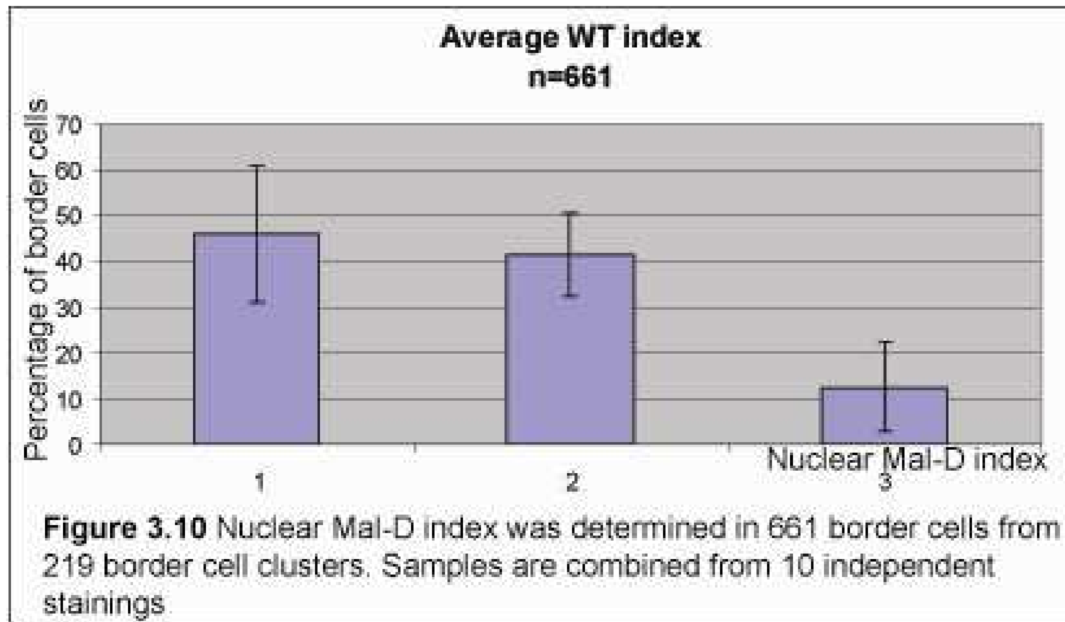


Figure 3.10 Nuclear Mal-D index was determined in 661 border cells from 219 border cell clusters. Samples are combined from 10 independent stainings.

In order to define whether the nuclear staining levels that I observed in NMI 2 and 3 correspond to the migration induced nuclear accumulation I generated *slbo* or *shg* (gene encoding DE-Cadherin) mutant border cell clusters by inducing mitotic clones with slbo ^{8ex2} or shg ^{R69} alleles (Materials and methods 4.2.3). Both of them are deletion of most of the coding regions of the genes and are loss of function alleles. If all of the cells that form the cluster were mutant for either of those factors (Full mutant clone), border cells did not move at all and remained in the anterior pole of the egg chamber. In this situation all the border cells that were quantified for NMI showed NMI 1 (Figure 3.11, figure 3.12). If the border cell clusters were consisting of a mixed population of wild-type and homozygous mutant border cells (partial mutant clone), wild-type border cells attempted to migrate and pulled the mutant border cells along to the migrating cluster. In this situation mutant cells that were pulled into the migrating cluster, thus received the migration related signal, accumulated NMI 2 and 3 nuclear Mal-D levels (Figure 3.12). This indicated that NMI 2 and 3 levels of nuclear accumulation of Mal-D corresponded to migration induced levels or Mal-D.

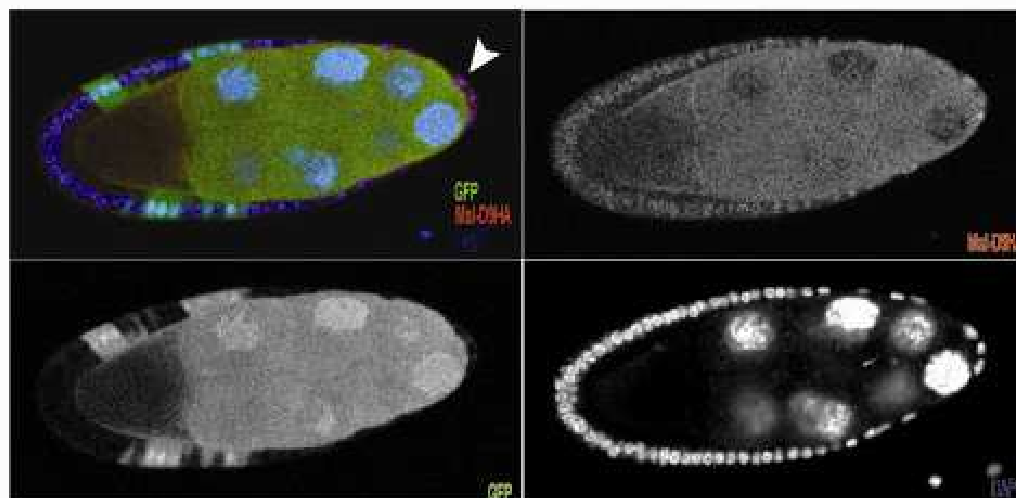


Figure 3.11 Border cell clusters completely mutant for slbo accumulate the same level of nuclear Mal-D as follicle cells. GFP negative cells are homozygous mutant. White arrow indicates border cells

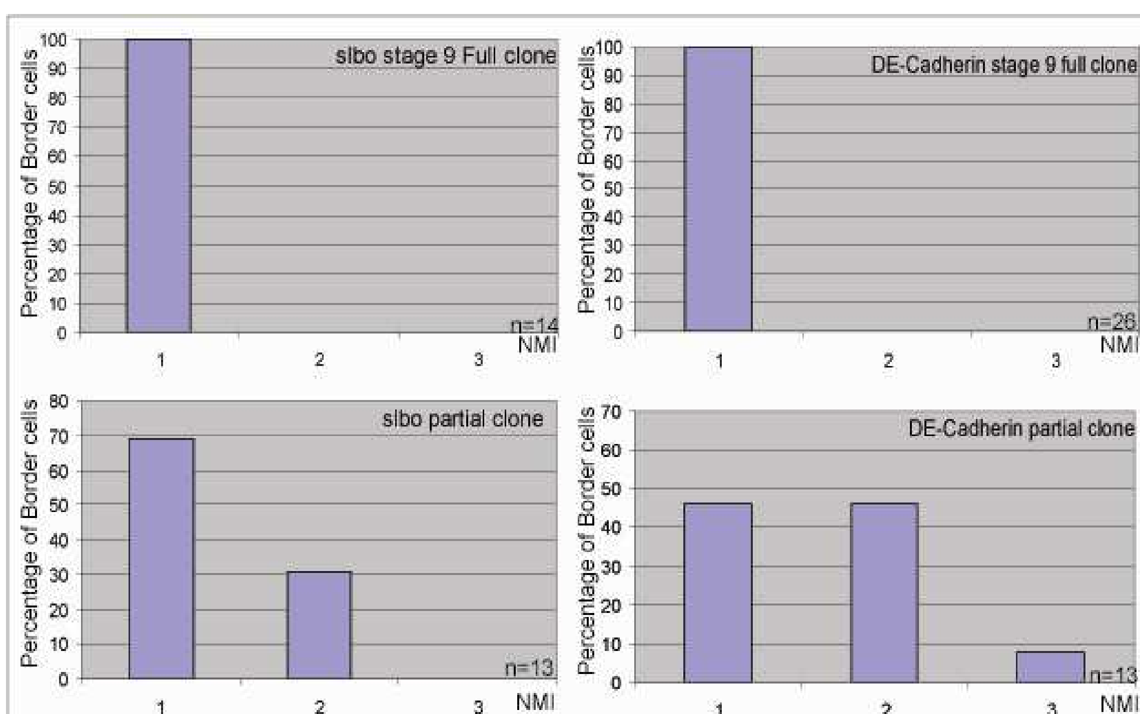


Figure 3.12 Percentages of border cells with given Nuclear Mal-D Index (NMI) for border cells from clusters where all cells are mutant for slbo or DE-Cadherin (full clone), or in clusters consistning of wildtype and mutant cells (Partial clone). Number of border cells are written in the corner of each graph. Please compare with the distribution in Figure 3.11

These results indicated that Mal-D 9HA gives the same staining pattern as the old antibody and that Mal-D 9HA behaves similar to the endogenous protein. The differences that could be observed such as different nuclear accumulation levels and follicle and stretched cell nuclear accumulations result from the increased sensitivity of Mal-D 9HA detection. The nuclear staining that we could observe with the old antibody corresponded to the NMI 2 and 3 with the new antibody. NMI 1 was blending to the background noise with the old antibody and was not detected over the background. In the following parts I will mention Mal-D and Mal-D 9HA interchangeably.

With the increased detection it was possible to detect nuclear and cytoplasmic signal. In order to understand subcellular distribution of Mal-D I quantified this ratio for the border cells and follicle cells from a set of samples stained on the same day (Figure 3.13 A). I stained egg chambers of w118 females in parallel in order to determine the background signal. I then wanted to determine if nuclear accumulation of Mal-D results from a difference in subcellular distribution of a constant level of the protein or higher nuclear levels results from overall increase in the cellular level of the protein. For determining this correlation I plotted average nuclear/ cytoplasmic ratio of the cells to their NMIs (Figure 3.13 B).

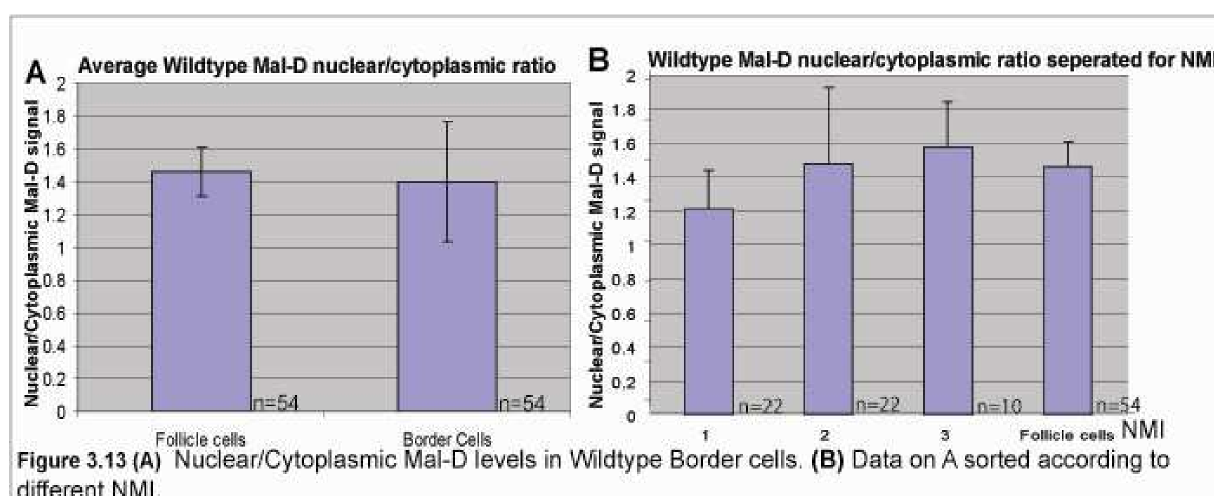


Figure 3.13 (A) Nuclear/Cytoplasmic Mal-D levels in Wildtype Border cells. **(B)** Data on A sorted according to different NMI.

Average nuclear/cytoplasmic ratios of border cells and follicle cells seem to be very close in the wild-type samples (Figure 3.13 A). On the other hand the cytoplasmic Mal-D signals in follicle cells are very close to staining background in the w118 follicle cells,

which makes it hard to quantify it reliably. The average nuclear/ cytoplasmic levels of Mal-D in border cells with different NMIs show that although there is a mild increase of average nuclear/cytoplasmic ratio according to increasing NMI this is not statistically significant. (Figure 3.13 B) This may indicate that the thing that changes in the border cells accumulating high levels of Mal-D is the level of Mal-D in total and not the distribution. In other words nuclear levels increase in border cells because the overall levels increase.

3.2.2 Strategy to identify genes important for Mal-D regulation

The strategy that I used for assessing whether a candidate gene is important for Mal-D regulation is summarized in figure 3.14.

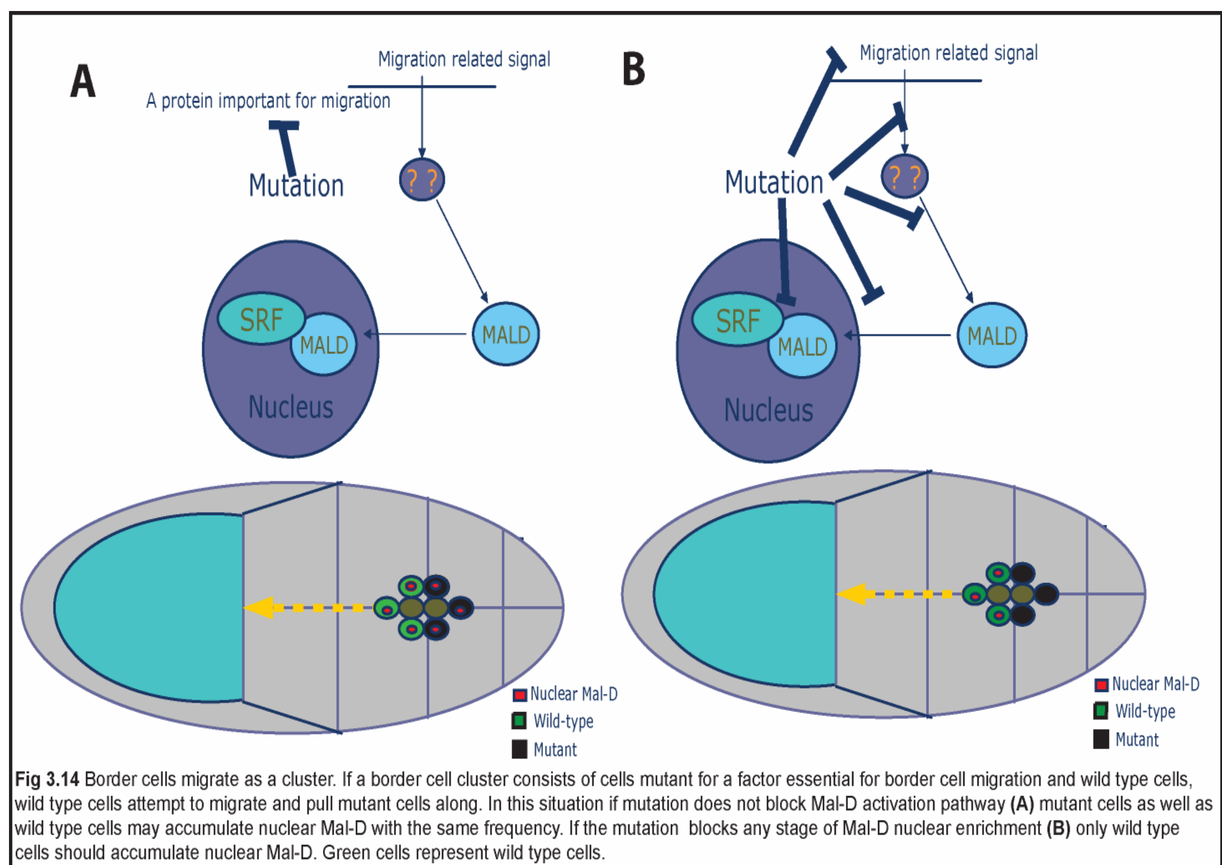


Fig 3.14 Border cells migrate as a cluster. If a border cell cluster consists of cells mutant for a factor essential for border cell migration and wild type cells, wild type cells attempt to migrate and pull mutant cells along. In this situation if mutation does not block Mal-D activation pathway **(A)** mutant cells as well as wild type cells may accumulate nuclear Mal-D with the same frequency. If the mutation blocks any stage of Mal-D nuclear enrichment **(B)** only wild type cells should accumulate nuclear Mal-D. Green cells represent wild type cells.

This strategy gave me a possibility to discriminate candidate genes that I wanted to test according to either being important for perceiving migration related signal and increasing nuclear Mal-D levels or not being related to Mal-D regulation.

3.2.3 Rho and Diaphanous are not essential for the nuclear accumulation of Mal-D in border cell or follicle cell nuclei

Rho and diaphanous are important factors for the migration of border cells. (Bastock and Strutt, 2007; Beccari, 2003). Moreover those factors were previously implicated for the regulation of Mal in mammalian system. I tested whether they had a role in the regulation of Mal-D in Drosophila. I used rho ^{72O} and dia ^{5} alleles, which are null alleles. In partial clones of either rho ^{72O} or dia ^{5}, Mal-D can accumulate strongly nuclear (Figure 3.15 A and 3.16 A) and in the same frequency as strong nuclear Mal-D accumulation of wild-type clusters (Figure 3.17). This shows that Rho and Diaphanous are not essential for strong nuclear accumulation of Mal-D in migrating border cells.

For determining if Rho or Diaphanous are important for the nuclear accumulation of Mal-D in follicle cell and stretched cells I analyzed mutant clones in those cell types. Follicle cells mutant for *rho* or *dia* could accumulate Mal-D the same level as their wild-type counterparts, meaning that Rho and Diaphanous were dispensable for nuclear accumulation of Mal-D in follicle cells (Figure 3.15 C and 3.16 C). In stretched cells homozygous for rho ^{72O} I observed a decrease in nuclear Mal-D levels (Figure 3.15 B) in 10 different egg chambers where I could find a mutant stretch cell clone together with a wild-type counter part. This decrease was not seen in the dia ^{5} mutant stretched cells suggesting that a different effector of Rho had a role in this nuclear accumulation (Figure 3.16 B). One surprising finding from this analysis is that the nuclear accumulation of Mal-D in those related cell types goes through genetically dissectible pathways.

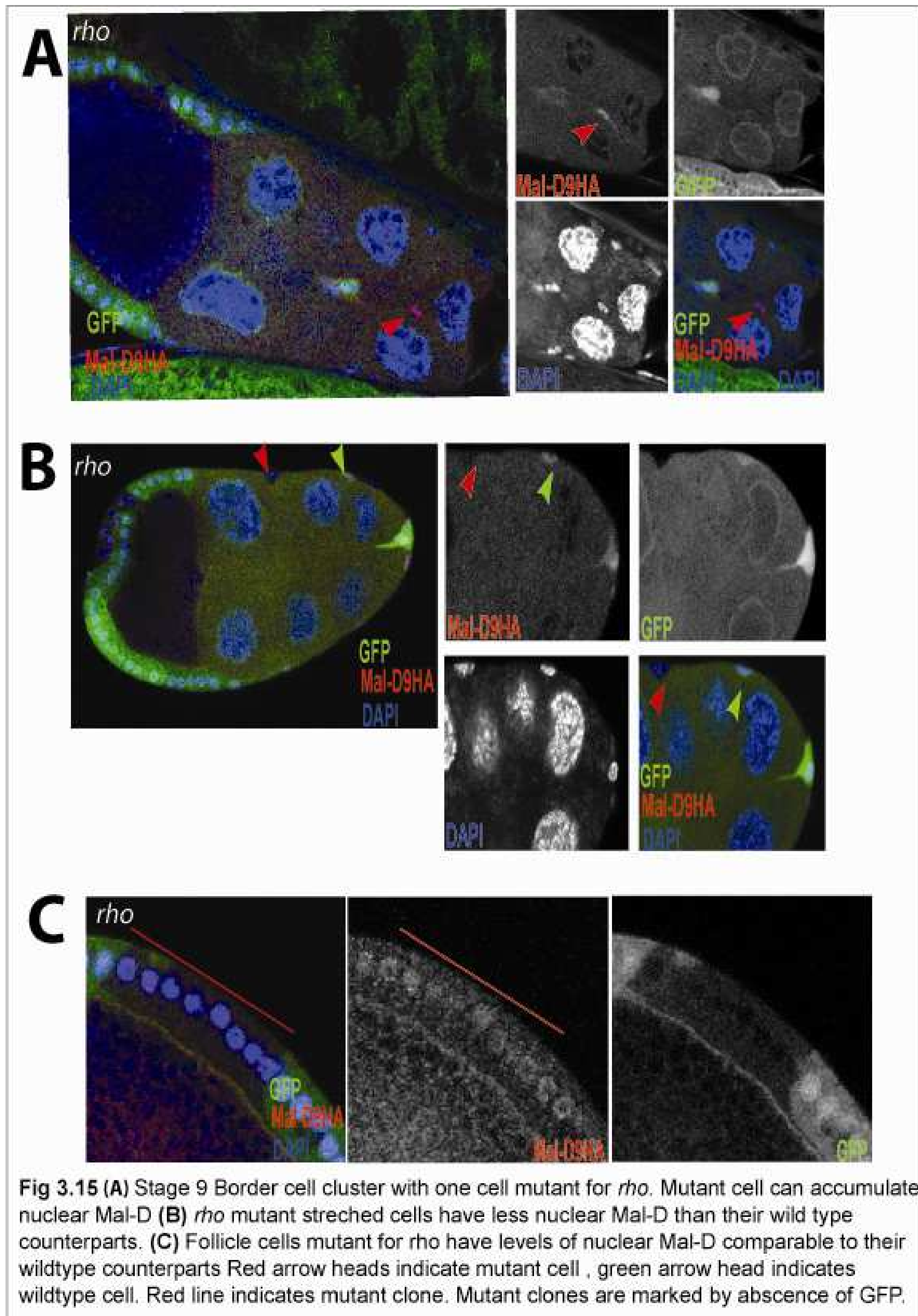


Fig 3.15 (A) Stage 9 Border cell cluster with one cell mutant for *rho*. Mutant cell can accumulate nuclear Mal-D **(B)** *rho* mutant streched cells have less nuclear Mal-D than their wild type counterparts. **(C)** Follicle cells mutant for rho have levels of nuclear Mal-D comparable to their wildtype counterparts Red arrow heads indicate mutant cell , green arrow head indicates wildtype cell. Red line indicates mutant clone. Mutant clones are marked by abscence of GFP.

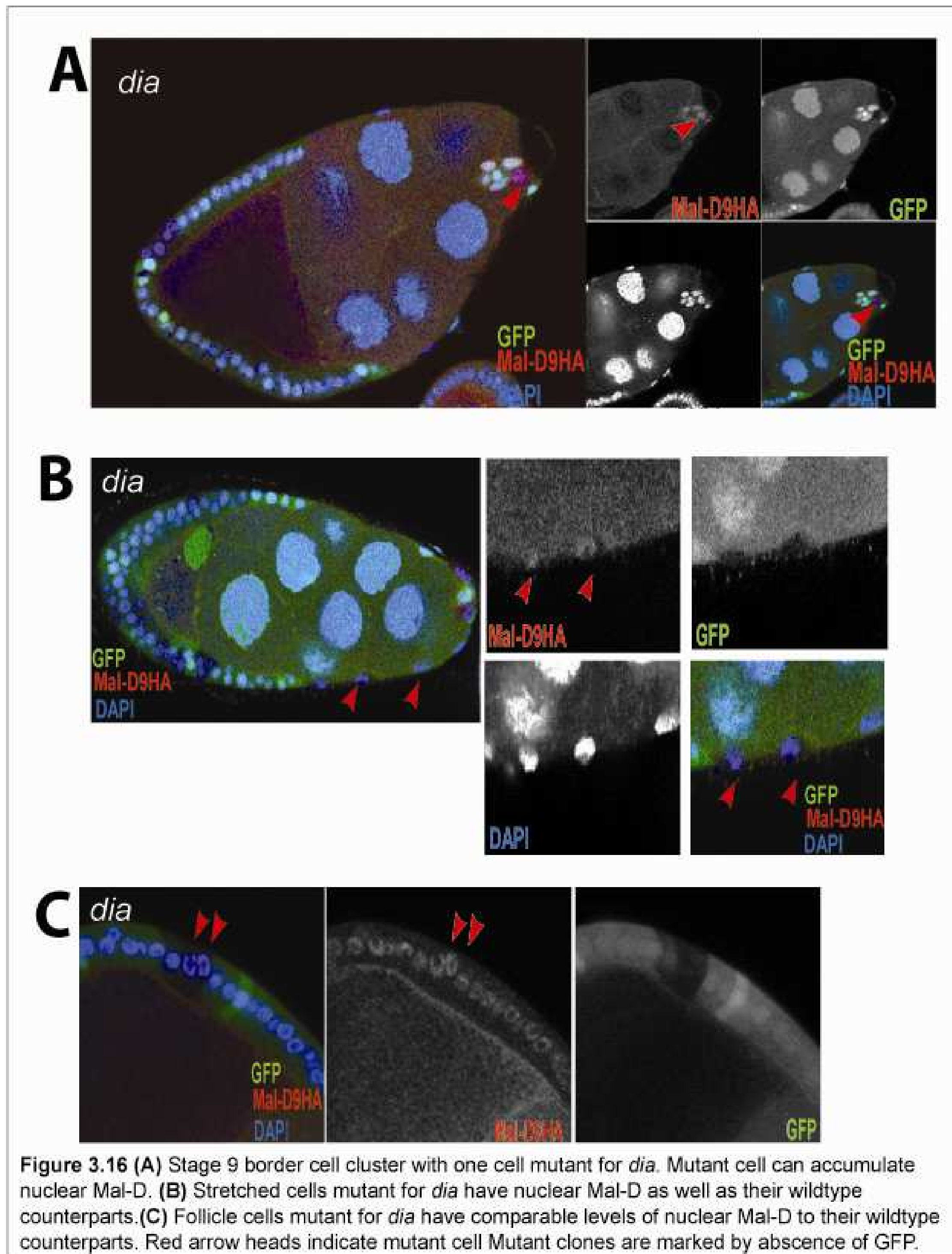


Figure 3.16 (A) Stage 9 border cell cluster with one cell mutant for *dia*. Mutant cell can accumulate nuclear Mal-D. **(B)** Stretched cells mutant for *dia* have nuclear Mal-D as well as their wildtype counterparts.**(C)** Follicle cells mutant for *dia* have comparable levels of nuclear Mal-D to their wildtype counterparts. Red arrow heads indicate mutant cell Mutant clones are marked by abscence of GFP.

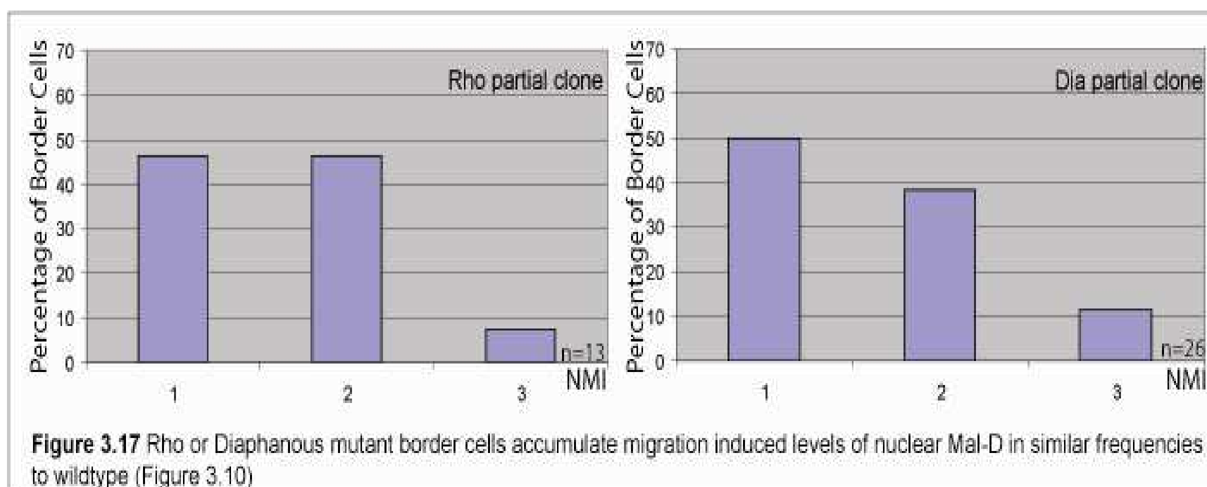


Figure 3.17 Rho or Diaphanous mutant border cells accumulate migration induced levels of nuclear Mal-D in similar frequencies to wildtype (Figure 3.10)

3.2.4 Profilin is important for nuclear localization of Mal-D in border cells, follicle cells and stretched cells.

I then generated *chic* (the gene encoding Profilin) mutant clones in order to investigate the relation between Mal-D and Actin cytoskeleton. Profilin is a protein that binds to monomeric Actin and presents it to the growing tip of Actin filaments. Its function has been analyzed in border cell migration and in oogenesis. (Geisbrecht and Montell, 2004; Verheyen and Cooley, 1994) It is an important factor for border cell migration, and in follicle cells, cell mutant for Profilin have been shown to have lower F-Actin levels. Moreover most of the free G-Actin in the cells are bound to Profilin in order to prevent spontaneous polymerization (Kaiser et al., 1999).

Chickadee is an essential gene in *Drosophila*. I used a loss of function allele, chic ^{221}.In chic ^{221} partial clones mutant cells did not accumulate strong nuclear Mal-D levels (Figure 3.18A , Figure 3.19). Even the heterozygous cells in the same border cell clusters accumulated nuclear Mal-D poorly. Moreover in 14 out of 32 cases Mal-D could be seen excluded from nucleus and accumulated in cytoplasm of the mutant cells (Figure 3.18A). The cytoplasmic accumulation of Mal-D was not observed in wild-type border cells.

In chic ^{221} mutant follicle cells, mutant cells did not have nuclear Mal-D and Mal-D could be seen more cytoplasmic than in the wild-type cells (Figure 3.18B). Moreover cells

heterozygous for profilin mutation have higher nuclear Mal-D than mutant cells but lower nuclear Mal-D levels than the twinspot wild-type cells (For details See material and methods)(Figure 3.18 B). This suggests that *chic* has a phenotype on Mal-D localization even in the heterozygous situation. Stretched cells mutant for Profilin do not accumulate nuclear Mal-D either (Figure 3.18 C).

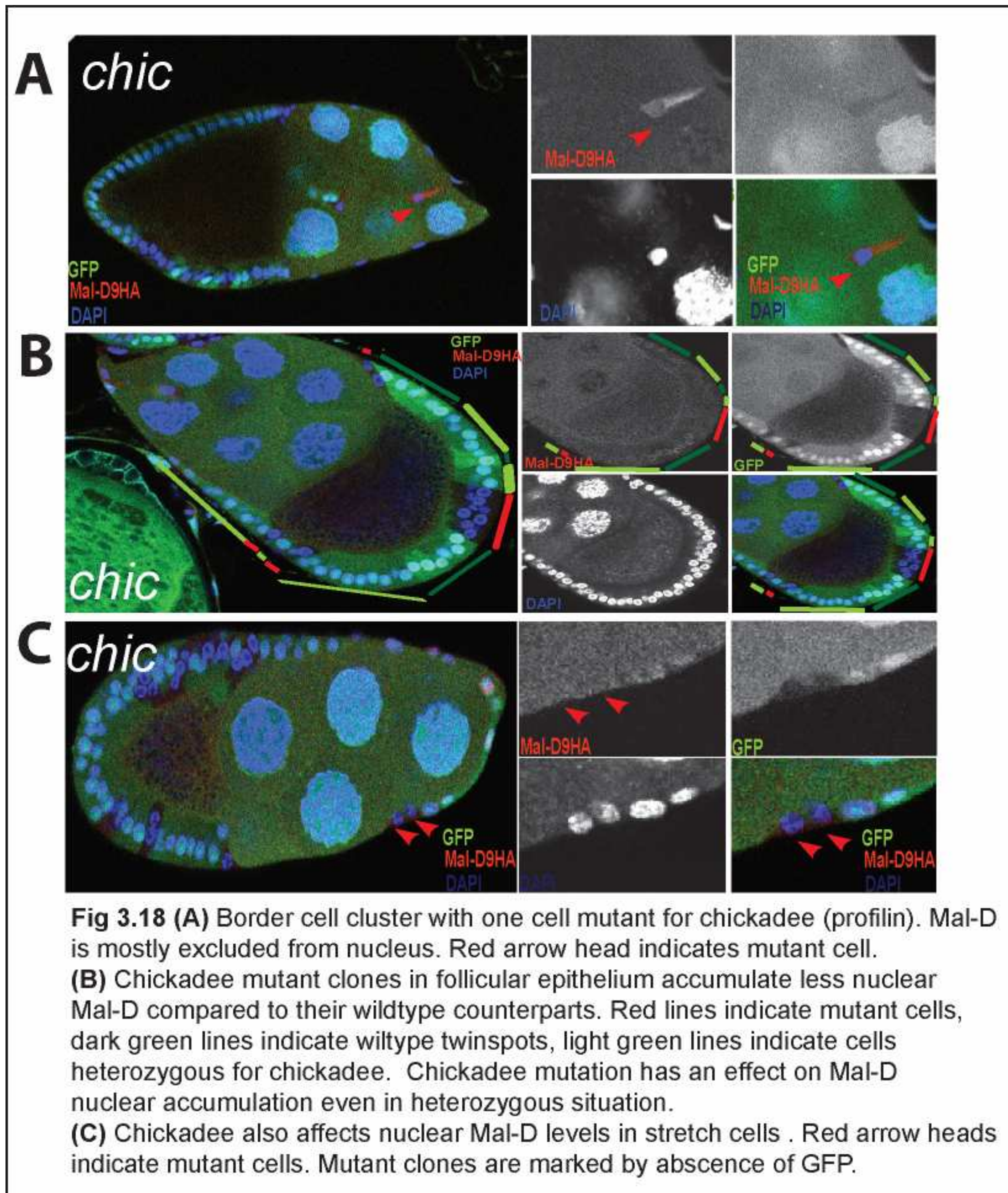


Fig 3.18 (A) Border cell cluster with one cell mutant for chickadee (profilin). Mal-D is mostly excluded from nucleus. Red arrow head indicates mutant cell.
(B) Chickadee mutant clones in follicular epithelium accumulate less nuclear Mal-D compared to their wildtype counterparts. Red lines indicate mutant cells, dark green lines indicate wiltype twinspots, light green lines indicate cells heterozygous for chickadee. Chickadee mutation has an effect on Mal-D nuclear accumulation even in heterozygous situation.
(C) Chickadee also affects nuclear Mal-D levels in stretch cells . Red arrow heads indicate mutant cells. Mutant clones are marked by abscence of GFP.

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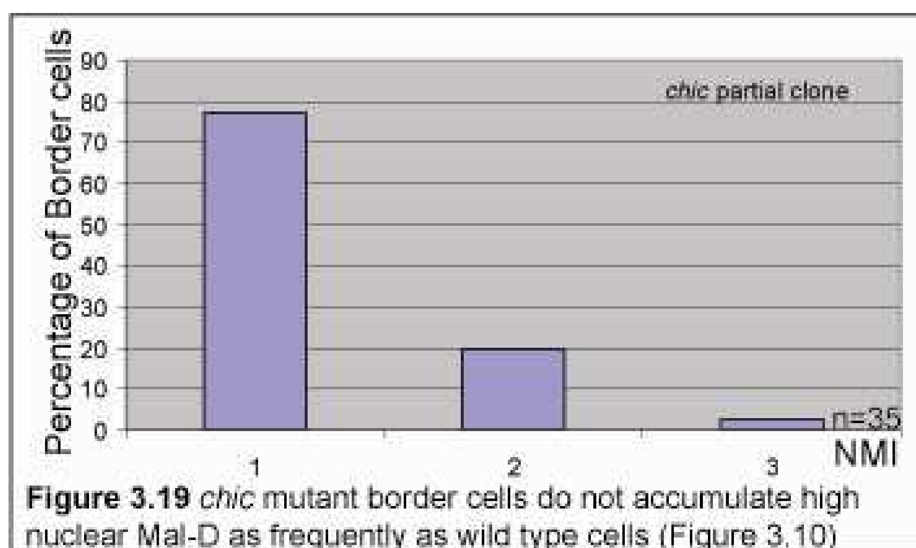


Figure 3.19 *chic* mutant border cells do not accumulate high nuclear Mal-D as frequently as wild type cells (Figure 3.10)

In order to understand the altered subcellular distribution of Mal-D in *chic* mutant border cells I quantified nuclear/cytoplasmic ratio for *chic* mutant border cells and follicle cells. (Figure 3.20)

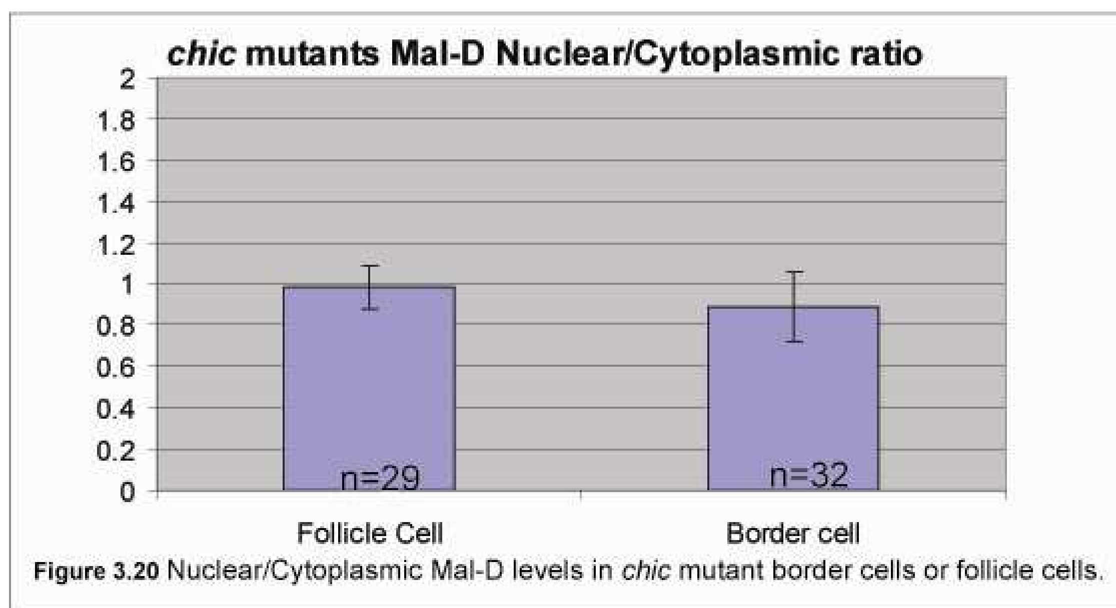


Figure 3.20 Nuclear/Cytoplasmic Mal-D levels in *chic* mutant border cells or follicle cells.

In chic mutant border cells and follicle cells nuclear/cytoplamic levels of Mal-D decrease meaning that the protein is redistributed to the cytoplasm (Figure 3.20)

3.2.5 DSRF mutation causes Mal-D to accumulate in the nuclei of border cells, but not in follicle cells or stretched cells

DSRF is the partner of Mal-D in transcription. It is the factor that binds to DNA and regulates the transcription of the target genes. I used a loss of function allele bs^{14} which causes appearance of an early stop codon that results in the formation of a truncated protein that end before the MADS domain. In order to analyze the behavior of Mal-D in the abscence of its partner I generated border cell clusters partial mutant for bs^{14} and quantified NMI.

In bs ^{14} partial clones, border cells mutant for bs ^{14} accumulated high levels of nuclear Mal-D more frequently than the wild-type border cells (Figure 3.22 A). NMI in bs ^{14} mutant border cells was more skewed to NMI 2 and 3. (Figure 3.21) Moreover at stage 10 of migration, the stage where border cells reach the oocyte border and finish their migration, border cells mutant for bs ^{14} continued to have higher nuclear Mal-D than their wild-type counterparts in the same cluster. (Figure 3.22 B)

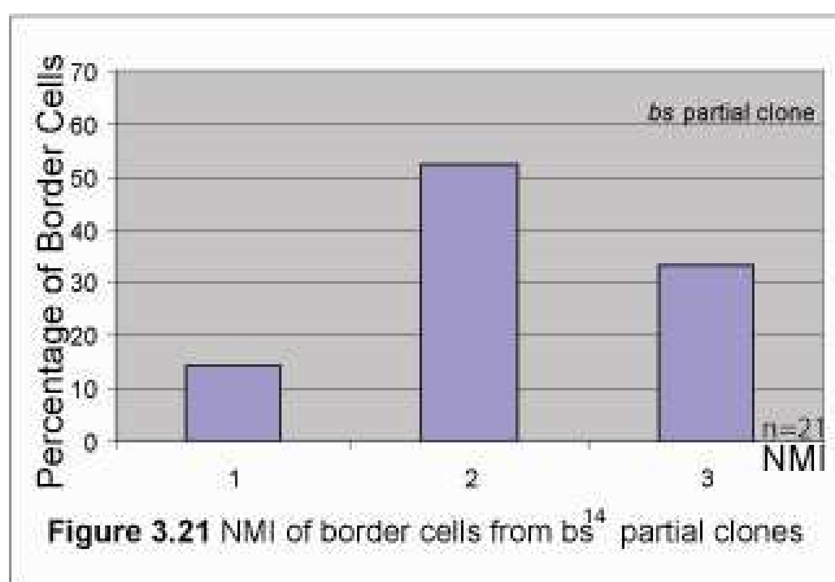


Figure 3.21 NMI of border cells from bs^{14} partial clones

Those phenotypes were seen only in the border cells. Follicle cells accumulated same amount of nuclear Mal-D no matter whether they were bs ^{14} mutant or wild-type (Figure 3.22 C). The persistence of signal at stage 10 did not occur in stretch cells either (Figure 3.22 D). Those results indicate that bs ^{14} causes nuclear accumulation of Mal-D specifically in border cells.

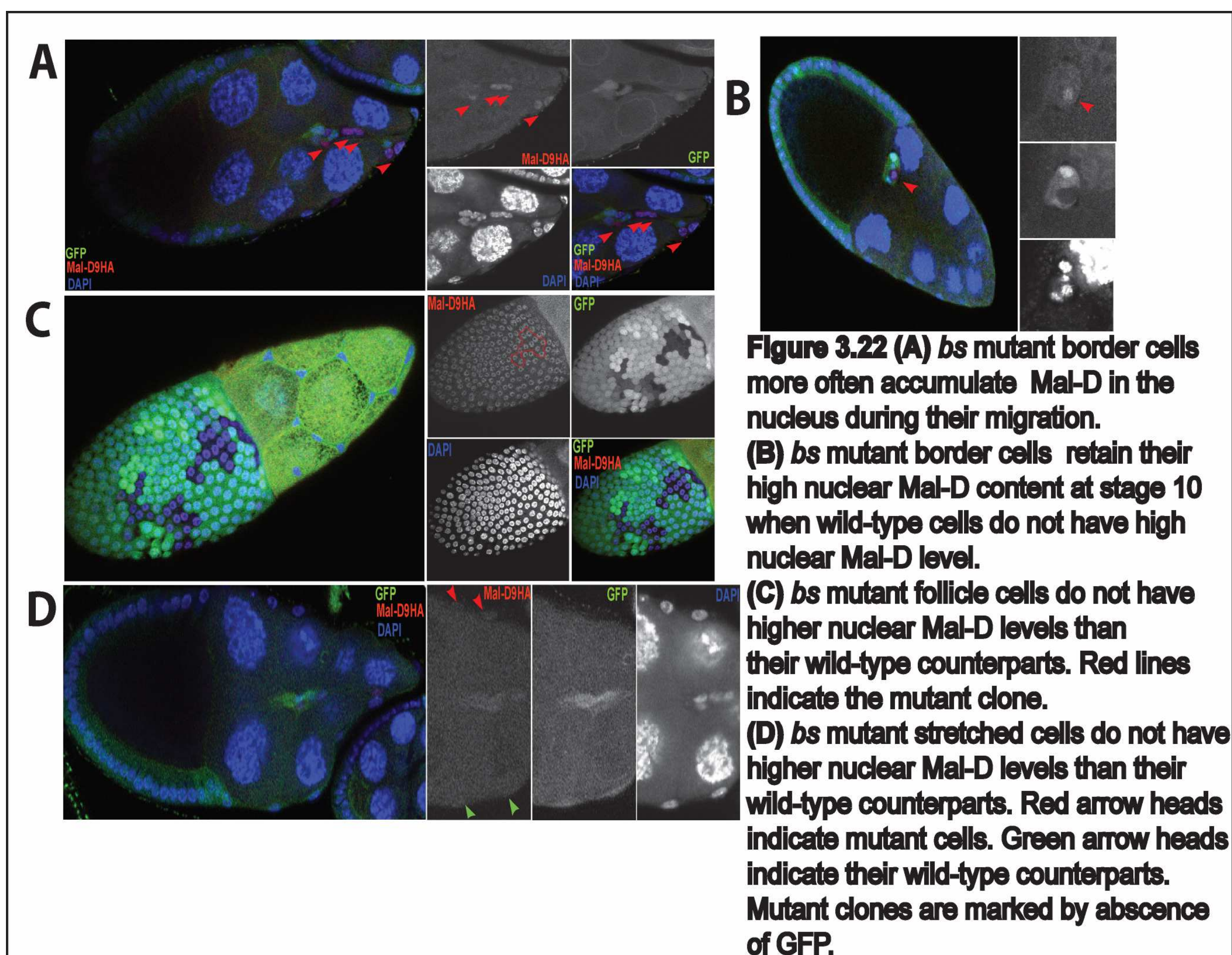


Figure 3.22 (A) *bs* **mutant border cells more often accumulate Mal-D in the nucleus during their migration.**
(B) *bs* **mutant border cells retain their high nuclear Mal-D content at stage 10 when wild-type cells do not have high nuclear Mal-D level.**
(C) *bs* **mutant follicle cells do not have higher nuclear Mal-D levels than their wild-type counterparts. Red lines indicate the mutant clone.**
(D) *bs* **mutant stretched cells do not have higher nuclear Mal-D levels than their wild-type counterparts. Red arrow heads indicate mutant cells. Green arrow heads indicate their wild-type counterparts. Mutant clones are marked by abscence of GFP.**

Part II Function of Mal-D

3.3.1 Transcriptional output of Mal-D and DSRF

3.3.1.1 Designing a reporter in S2 cells

SRF is a factor that has been studied in mammalian cell culture system for a long time. Its binding consensus site and its targets are known. Mammalian SRF binds to a consensus site named CATG boxes or SREs that consist of $CC(A/T)_6GG$. Three copies of this motif upstream a basal promoter and a reporter gene is enough in the mammalian cell culture system to render the reporter gene responsive to the transcriptional activity of SRF (Hill et al., 1993). As *Drosophila* SRF is 90% identical in the DNA binding domain to the mammalian SRF (Affolter et al., 1994) I decided to adapt this reporter approach to fly proteins.

In order to test whether the same site could function in *Drosophila* I cloned a block of 3 SRF binding sites (SREs) cloned in tandem, upstream a basal promoter driving β Gal reporter gene. This construct was transfected to *Drosophila* S2 cell line along with DSRF and either Mal-D full length cDNA or Mal-D ΔN which behaves as a constitutive active form of the protein. (Miralles et al., 2003; Somogyi and Rorth, 2004) As control I transfected the construct alone or with only DSRF. I used the reporter gene with the basal promoter, without SREs, in order to confirm the specificity of activity. The activity of the reporter was measured by doing a β-gal activity test (See materials and Methods). Transfection of the reporter alone or reporter with DSRF did not activate the β gal activity (Figure 3.23 A). Over expressing full length Mal-D which can still be regulated together with DSRF increased the reporter activity and this increase was specific to the presence of SREs since a reporter without those sites was not activated (Figure 3.23 A). Over expressing Mal-D ΔN, constitutive active form of Mal-D together with the reporter increased the activity of the reporter in a SRE dependent manner. This coul be explained by the presence of endogenous DSRF in the S2 cells as could be seen with Western Blot analysis with DSRF antibody with S2 cells either overexpressing DSRF or wild-type

(Figure 3.23 B). The combined activity of DSRF and Mal-D ΔN over expressed together on the other hand was enough to activate the reporter gene more than 100 fold.

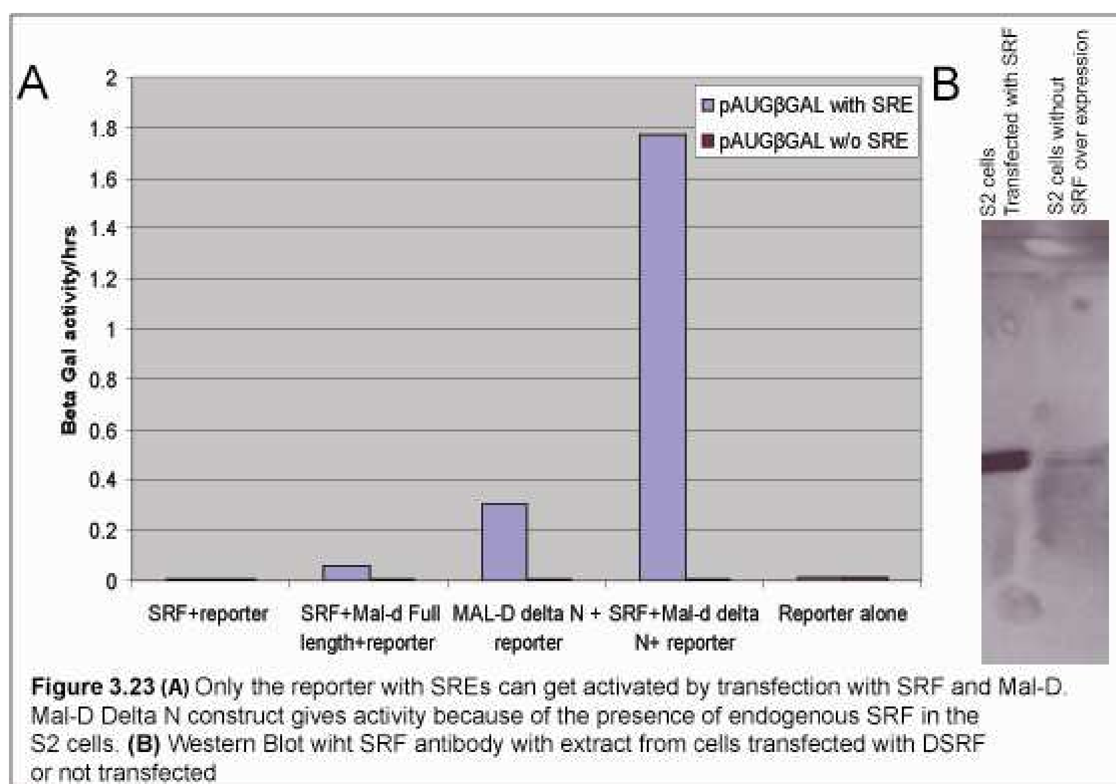


Figure 3.23 (A) Only the reporter with SREs can get activated by transfection with SRF and Mal-D. Mal-D Delta N construct gives activity because of the presence of endogenous SRF in the S2 cells. **(B)** Western Blot wiht SRF antibody with extract from cells transfected with DSRF or not transfected

This result showed that Mal-D and DSRF can cooperate on SREs in our system as well.

3.3.2 Mal-D activity towards Actin in vivo goes through DSRF

Over expression of Mal-D ΔN in follicular epithelium, causes over accumulation of F-Actin (Somogyi and Rorth, 2004). On the other hand DSRF loss of function in a clone of cells causes the F-Actin level to decrease in a cell autonomous manner (Somogyi and Rorth, 2004). I performed an epistasis experiment to understand whether the activity of Mal-D over expression on F-Actin goes through DSRF, thus through transcriptional activation of target genes. I over-expressed constitutive active Mal-D in a field of follicular epithelium, while removing DSRF in clones of cells. I over expressed Mal-D ΔN by using UAS/Gal4 system (See materials and methods). I used slbo Gal4 driver that is expressed in border cells and a subset of follicle cells. I generated bs^{14} homozygous clones in this combination so that I had wild-type cells over-expressing UAS Mal-D ΔN as controls next to bs^{14} mutant cells that over express the same transgene.

I could observe that the F-Actin level in the bs^{14} cells goes down even though they over express Mal-D ΔN (Figure 3.24). If the action of Mal-D went through another factor, I should have observed accumulation of F-Actin no matter whether I have DSRF or not. As over-expressing Mal-D while removing DSRF caused the phenotype of DSRF loss of function, which is decrease of F-Actin, Mal-D ΔN activity on F-Actin levels requires the presence of DSRF, indicating that work together in vivo in the fly ovary.

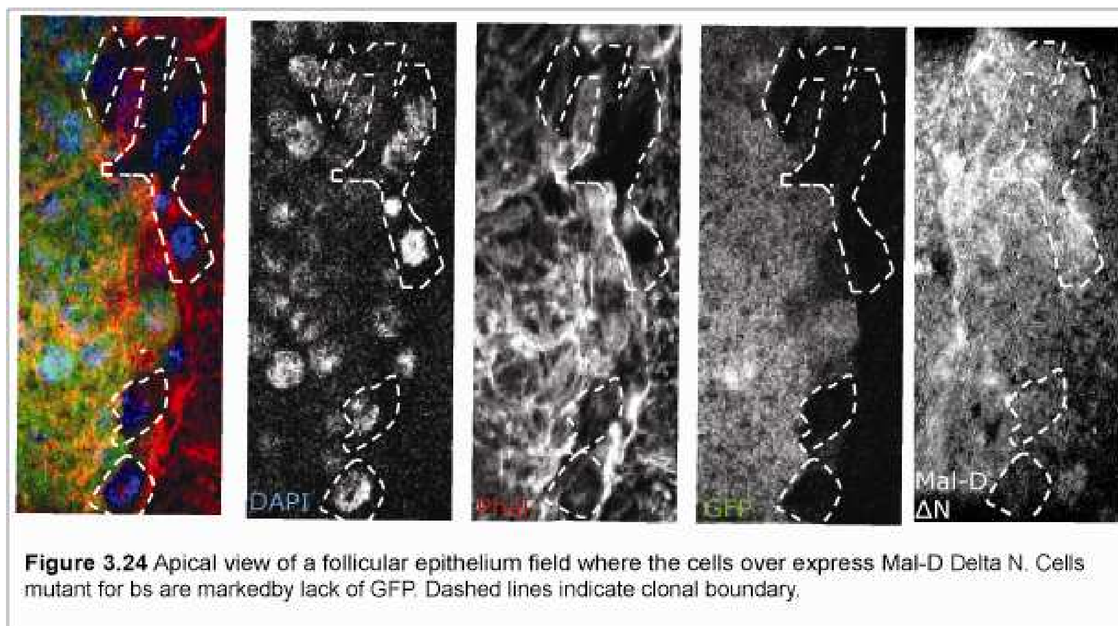


Figure 3.24 Apical view of a follicular epithelium field where the cells over express Mal-D Delta N. Cells mutant for bs are markedby lack of GFP. Dashed lines indicate clonal boundary.

3.3.3 Designing in vivo reporters

Having a reporter gene that reflects the activity of Mal-D and DSRF would be very useful for monitoring the activity of the protein in vivo. Subcellular localization read out for Mal-D is an important read out for Mal-D activation but it only gives an indirect means of monitoring the activity of the Mal-D/DSRF complex. A reporter gene which is activated by Mal-D/DSRF transcriptional activity would give me different and more direct activity readout.

Initial results with reporters in cell culture experiments encouraged me to try this approach in vivo. Previous studies have used this kind of approach successfully to generate in vivo reporter constructs for Notch activity read out (Furriols and Bray, 2001), JAK/STAT signaling activity read out (Gilbert et al., 2005), bicoid dependent

transcription read out (Ochoa-Espinosa et al., 2005), but still the number of such reporters are limited showing that it is not easy to generate a good in vivo reporter.
I generated transgenic flies with the reporter construct in order to get an in vivo activity read out. The way I tested them was by looking at the border cells. In flies mutant for Mal-D the border cells get delays in the beginning of migration, suggesting that the protein activity is required in the early phases of the migration, and the protein is active at those stages in wild-type situation.

With this first reporter construct with 3 SREs I could not get any activity in the flies. In order to increase the sensitivity of the reporter I tried a different approach. I generated reporters with more SRF binding sites, hoping that this would increase the sensitivity of the reporter. 14 Lines were tested by dissecting ovaries with those reporters. Only two lines gave staining in the border cells in late stage 10 of oogenesis. Three independent reporter lines were tested whether they can get activated with constitutive active Mal-D. They all could be activated by over expression of constitutive active Mal-D, meaning that inherently they were responsive to the transcriptional activity of Mal-D and DSRF but they were not sensitive enough to detect the endogenous level of activity in the migrating border cells. The line that was giving better signal was tested in Mal-D mutant background. I generated flies that are mutant for mal-D ^{F2}/ mal-D $^{\Delta 7}$ and that had the reporter construct. mal-D ^{F2} is a strong hypomorphic allele of Mal-D and in this allelic combination there should be very little Mal-D activity. I dissected those flies along with flies that were wild-type and had the reporter only. I stained them in parallel with X-Gal staining. The reporter did not show activity in Mal-D mutant flies (Figure 3.25). This means that the activity of the reporter was Mal-D dependent.

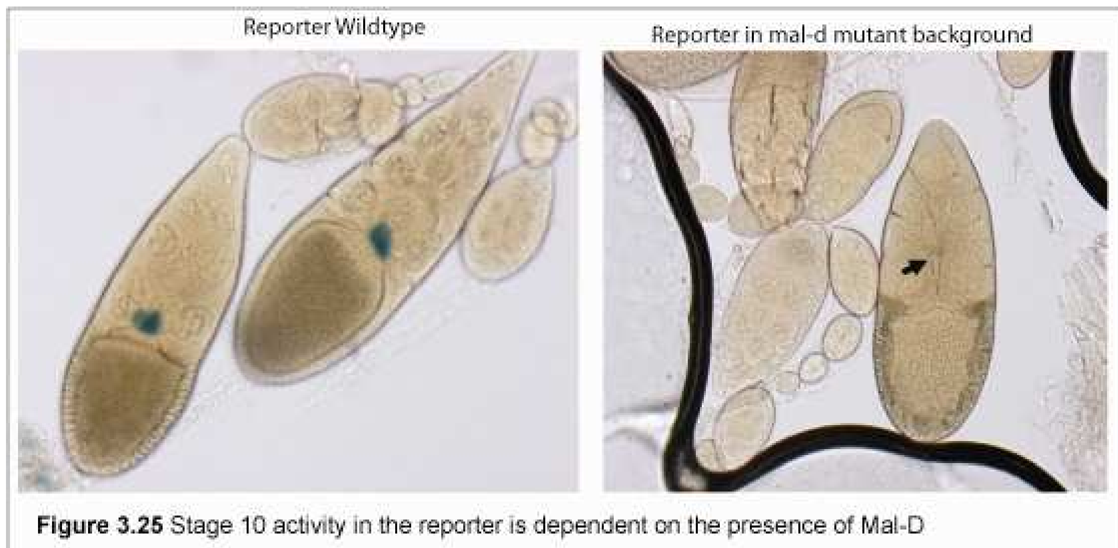
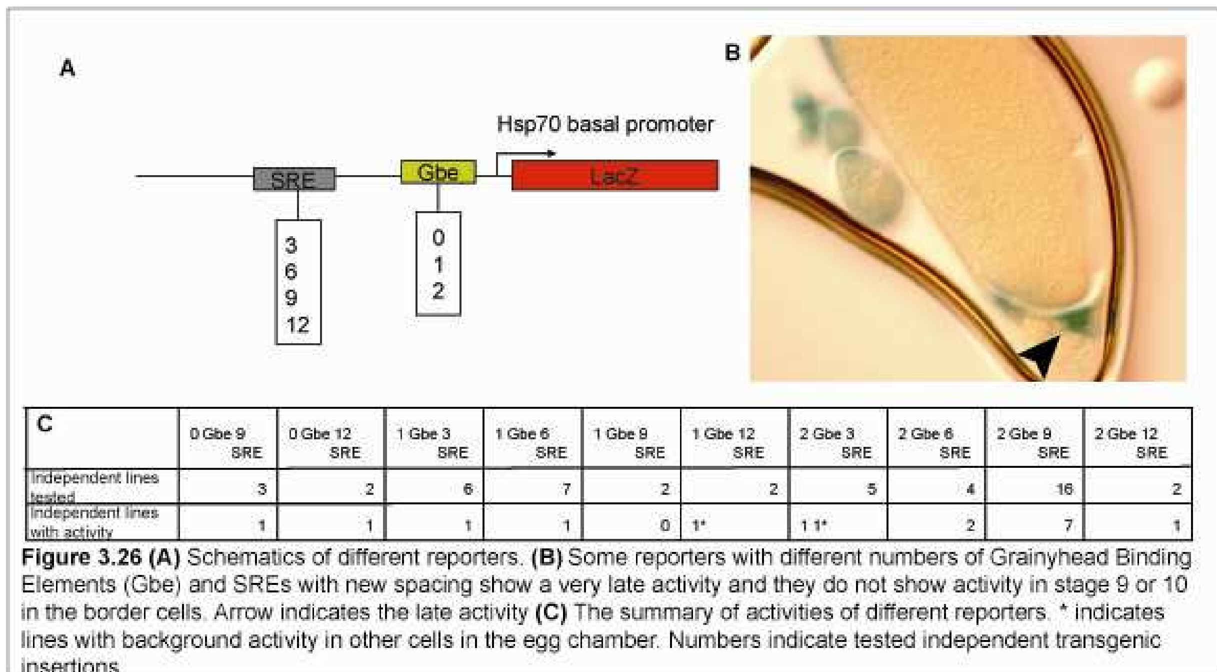


Figure 3.25 Stage 10 activity in the reporter is dependent on the presence of Mal-D

Although the observed specific activity was promising it was still too low to be useful. I needed to have the reporter homozygous to have a decent signal and even in this situation reporter was giving the signal quite later than the actual migration process.

There is a possibility that I might be unwillingly introducing a silencing element with the sequences bridging the SREs. Those sequences were taken directly from the reporter in the mammalian reporter, and not tested in *Drosophila* for any effect they might be causing. Furthermore I decided to boost the basal activity of all reporters by adding a known enhancer site in them, namely Grany head binding element (Gbe). I replaced the bridging sequences, with a different sequence that has been used before to generate a reporter construct for Notch Pathway (Furriols and Bray, 2001). I cloned different reporter vectors with differing number of SREs (3,6,9,12) and differing number of Gbes (0,1,2) and with new spacing. (Figure 3.26A) I tested lines coming from those constructs. Many reporter lines showed a weak activity at late stages of oogenesis (Figure 3.26B and C). None of the reporter lines showed stage 9 activity.



C	0 Gbe 9 SRE	0 Gbe 12 SRE	1 Gbe 3 SRE	1 Gbe 6 SRE	1 Gbe 9 SRE	1 Gbe 12 SRE	2 Gbe 3 SRE	2 Gbe 6 SRE	2 Gbe 9 SRE	2 Gbe 12 SRE
Independent lines tested	3	2	6	7	2	2	5	4	16	2
Independent lines with activity	1	1	1	1	0	1*	1 1*	2	7	1

Figure 3.26 (A) Schematics of different reporters. **(B)** Some reporters with different numbers of Grainyhead Binding Elements (Gbe) and SREs with new spacing show a very late activity and they do not show activity in stage 9 or 10 in the border cells. Arrow indicates the late activity **(C)** The summary of activities of different reporters. * indicates lines with background activity in other cells in the egg chamber. Numbers indicate tested independent transgenic insertions

One last approach that I tried was to use a region in *if* gene (integrin PS α). This gene was seen to be regulated in my expression profiling approach (See below). There is a stretch of highly conserved 3 SREs in one of the introns of *if*. Moreover there is suggestion that *if* is regulated by DSRF in the wing. (Montagne et al., 1996) I cloned this stretch of 1 Kb upstream the basal promoter driving β gal gene. One of the lines that I tested gave me a strong activity at stage 9 of oogenesis in the border cells. It was getting stronger in the further stages of oogenesis. I tested whether that reporter could get activated by ectopic expression of constitutive active Mal-D ΔN. It gave activity in the patches of cells over expressing Mal-D ΔN in the follicular epithelium meaning it could respond to increasing amounts of signal. (Figure 3.27)

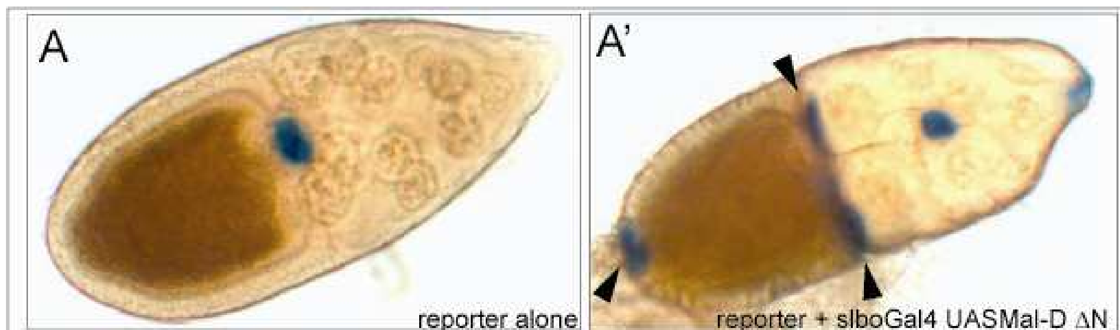


Figure 3.27 Over expression of Constitutive active Mal-D ectopically drives the ectopic expression of the reporter with if enhancer region. Black arrow heads indicate ectopic expression regions.

I tested whether the activity that I was seeing was dependent on DSRF/ Mal-D action. I crossed the reporter construct in the background of flies where I generated clones of cells lacking DSRF. The reporter was still giving activity in the clones of cells lacking DSRF meaning that the staining that I observed was due to an enhancer trap effect and was not due to the activity of DSRF/ Mal-D. This makes that reporter unusable. (Figure 3.28)

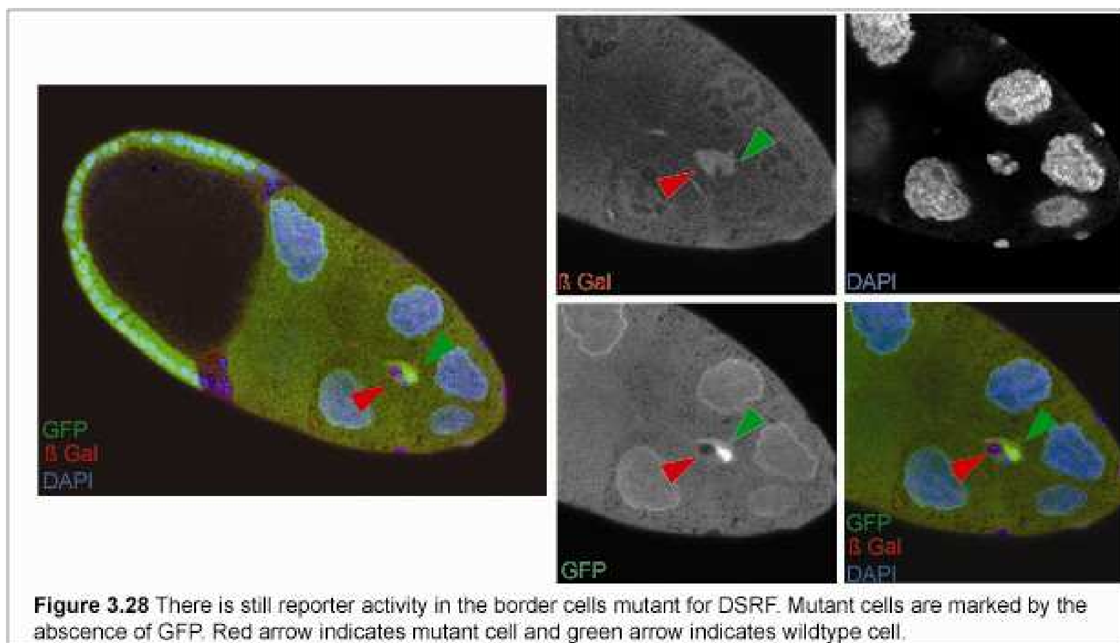


Figure 3.28 There is still reporter activity in the border cells mutant for DSRF. Mutant cells are marked by the absence of GFP. Red arrow indicates mutant cell and green arrow indicates wildtype cell.

3.3.4 Expression profiling with *mal-D* mutant border cells

Finding the transcriptional targets of Mal-D in border cells would be very helpful for understanding the role of the protein and the reasons of the phenotype I observe in the absence of Mal-D. An added bonus would be to find a gene that is transcribed by the

activity of Mal-D, that can be used as an activity readout for Mal-D. For tackling this problem I undertook an expression profiling approach. I isolated border cells mutant for *mal-D* or wild-type by using Fluorescent Activated Cell Sorting (FACS) technique. This method was optimized and used by a previous PhD student in our laboratory, Lodovica Borghese.(Borghese et al., 2006).

3.3.4.1 Isolation of Mutant border cells

Mal-D is an essential protein for *Drosophila*. This creates a challenge since I needed to dissect adult flies in order to collect the border cells. One possibility was to generate homozygous mutant cells in an otherwise heterozygous fly and mark them with GFP by using MARCM system, and sorting those cells with FACS. The problem with this approach was that even one mutant cell in a cluster would incorporate this cluster in my mutant population no matter whether the rest of the cluster is mutant or not. This would contaminate my mutant sample. For this reason I decided to use a different approach. I made use of the UAS/Gal4 system in order to label the border cells specifically with GFP. I expressed UAS GFP Actin with a border cell specific Gal4 driver c522. (Figure 3.29)

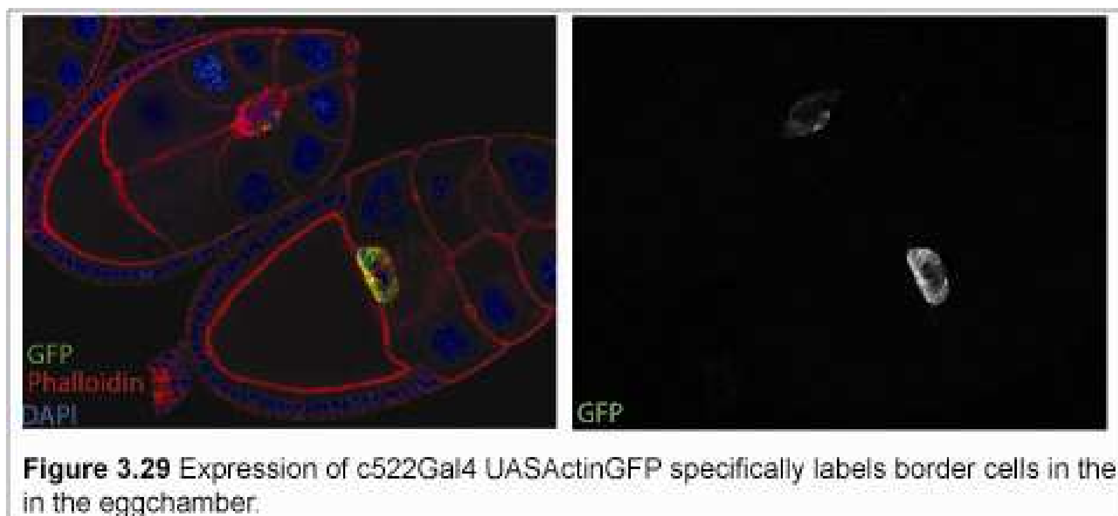


Figure 3.29 Expression of c522Gal4 UASActinGFP specifically labels border cells in the in the eggchamber.

Both of those constructs were recombined to a semi-viable ovary null allele of *mal-D*, namely mal-D $^{\Delta 7}$. Semi viability means that if I set up a cross with heterozygous parents and leave the progeny in the same vial, homozygous mutant progeny does not come in the expected ¼ Mendelian ratio. Presumably the competition with the wild-type siblings

prevents the development of those mutant larvae, since isolating the mutant larvae in a separate vial results in a better viability of the mutant flies. (Kalman Somogyi personal communication) In order to isolate the mutant flies in their early larval stages I used the leakiness of this Gal4 driver in the salivary gland of the larvae. (See Materials and Methods)

I dissected 200 flies in an hour, dissociated the egg chambers by trypsin EDTA and collagenase treatment (Materials and methods) and sorted the resulting cells in FACS sorter (Figure 3.30). I sorted the GFP positive cells in the lysis buffer of the RNA extraction kit and froze them in this solution until I had enough cells to pool and extract RNA from. I pooled the border cells collected in different days both for wild-type and mutant samples in order to have enough material.

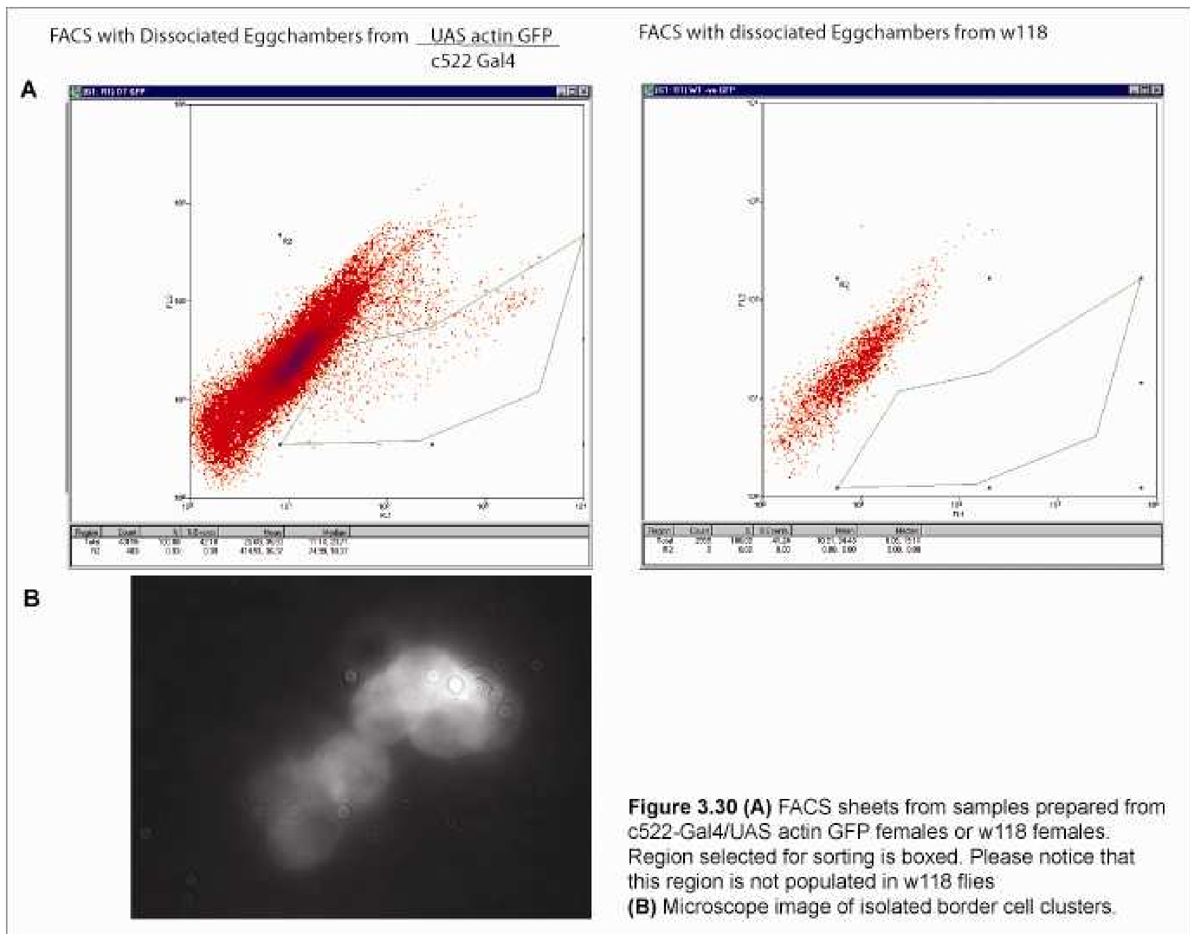


Figure 3.30 (A) FACS sheets from samples prepared from c522-Gal4/UAS actin GFP females or w118 females. Region selected for sorting is boxed. Please notice that this region is not populated in w118 flies
(B) Microscope image of isolated border cell clusters.

3.3.4.2 Isolation and quality control of RNA

I proceeded to RNA extraction when I had 50000 events per sample. I could get an average of 50 ng of total RNA from 50000 events (See Materials and methods). I tested for the quality of RNA by running the RNA on Agilent Bioanalyzer. For conducting good amplification and labeling of RNA it is of critical importance to have intact RNA. Bioanalyzer is sensitive enough to give an estimation of integrity of RNA with using only a few picograms of total RNA. RNA was intact and comparable in quantity for each repeat (Figure 3.31).

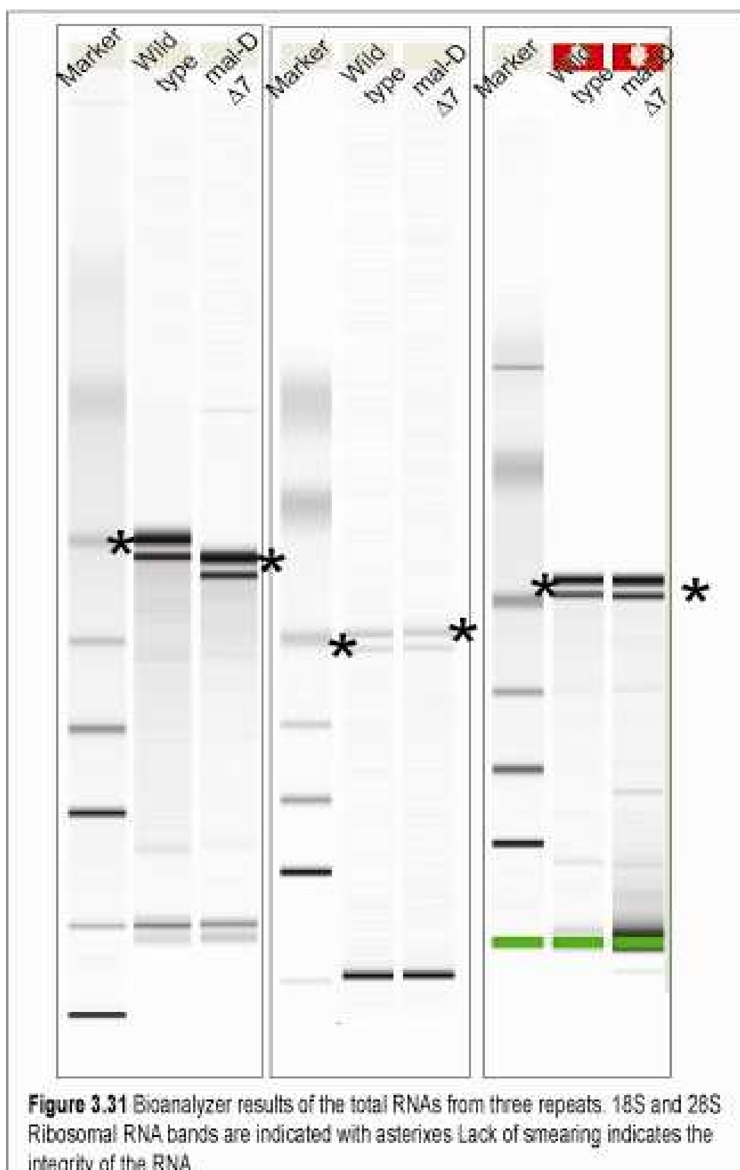


Figure 3.31 Bioanalyzer results of the total RNAs from three repeats. 18S and 28S Ribosomal RNA bands are indicated with asterixes Lack of smearing indicates the integrity of the RNA

Bioanalyzer analysis and further amplification and labeling were conducted by Genecore facility in EMBL by Tomi Ivacevic. The shift in the band sizes in the first repeats RNA sample resulted from an error of the software's analysis mode (Tomi Ivacevic personal communication). The important information from this result was that RNA was intact and there was no smearing. 18S/28S RNAs are the most abundant RNA species in the cells and are used as an indicator of RNA quality. If RNA was degraded one would expect ribosomal RNA bands to be degraded and smeary as well.

3.3.4.3 Linear amplification, labeling and hybridization of arrays

Linear 2 step RNA amplification was conducted by using the Genechip 2 step RNA amplification kit by Tomi Ivacevic. I conducted 3 biological repeats. In order to determine how reproducible different repeats were I determined the correlation of the different WT samples. Unfortunately the correlations that were obtained were not as tight as what Lodovica Borghese obtained using the same method for unknown reasons (Borghese et al., 2006). First and second wild-type samples have a correlation coefficient 0.82, first and third 0.77 and second and third 0.88. Low correlation between different biological repeats made conducting statistics very difficult. I decided to focus on the genes that were at least 2 fold down regulated in mal-D $^{\Delta 7}$ mutant border cells compared to the wild-type border cells in each repeat. There were about 171 genes that were consistently more than two fold down regulated in the mutant border cells. (See Appendix)

3.3.5 Attempt to find direct targets of Mal-D

3.3.5.1 Promoter and enhancer analysis

Some of the genes that were down regulated in mal-D $^{\Delta 7}$ border cells compared to wild-type border cells are expected to be direct transcriptional targets of Mal-D/DSRF complex.

I tested whether there was a bias to have more serum response factor binding sites in the upstream region of those genes. I analyzed upstream 10000 bps of those genes and searched for the presence of serum response elements in those regions. There was no statistically significant overall enrichment for SREs. I proceeded with those genes to test

whether they may be directly regulated by Mal-D. I went on to analyze the genes that have SREs in their promoter/enhancer regions. With the help of Michal Karzynsky I got the sequences orthologous to these upstream regions in different drosophila species in order to determine whether those SREs are conserved. The species that were used were *Drosophila simulans, Drosophila ananassae,Drosophila yakuba, Drosophila mojavensis, Drosophila pseudoobscura* and *Drosophila virilis* (Figure 3.32).

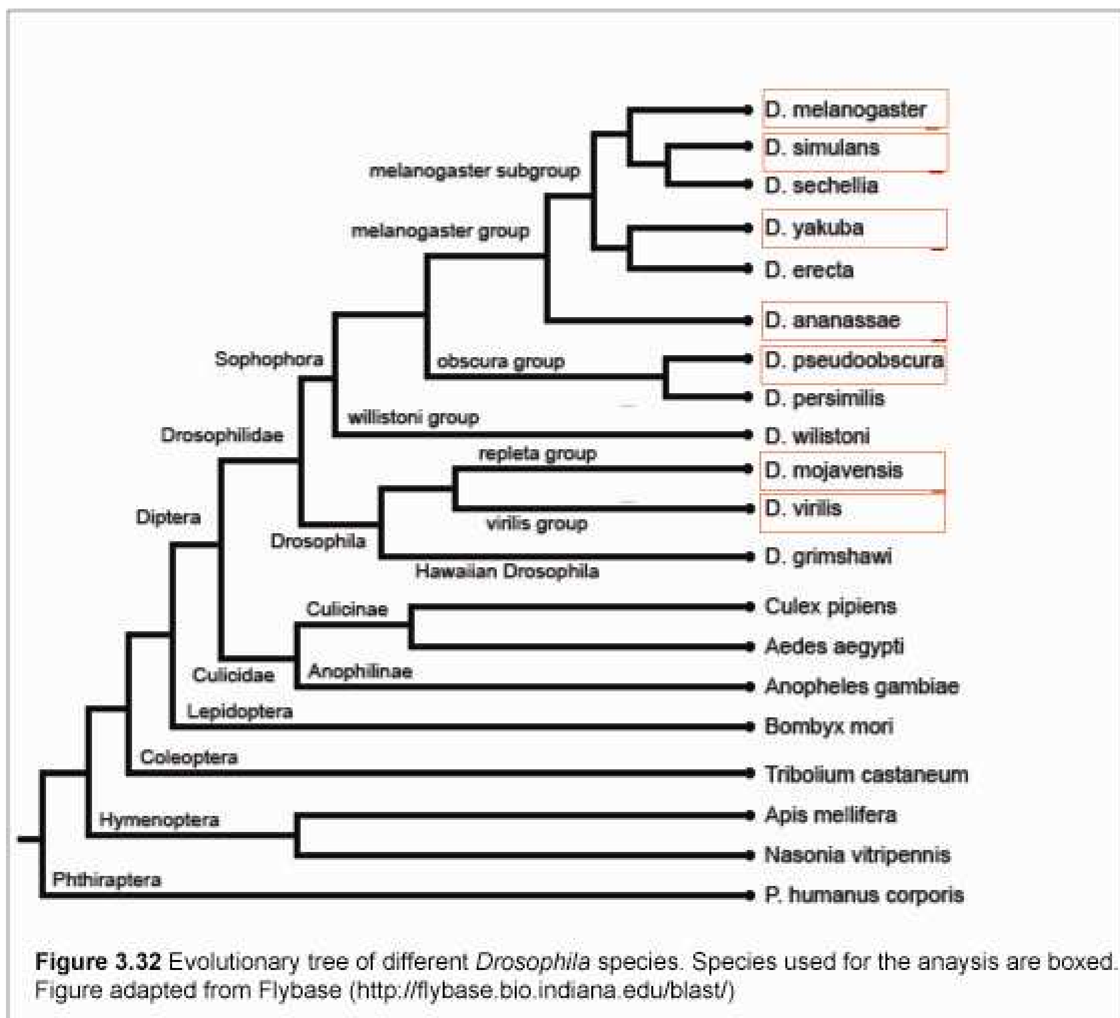


Figure 3.32 Evolutionary tree of different *Drosophila* species. Species used for the anaysis are boxed. Figure adapted from Flybase (http://flybase.bio.indiana.edu/blast/)

Laurence Ettwiller helped me to analyze the conservation of those sequences. The program that she wrote took those aligned sequences form different species and gave a conservation score to the sequence according to the number of conserved sites in different species (Figure 3.33). I selected a subgroup of genes with good conservation score as possible direct targets of Mal-D.

sequence	best conservation score	position (motif)
CG31015	6	3552 bp
		3552 bp
CG3217	5	3659 bp
		6528 bp
CG7225	4	7326 bp
CG4178	4	5973 bp
CG1921	4	764 bp
CG7504	3	3690 bp
CG16987	2	6152 bp
CG9270	2	3199 bp
CG3841	2	6383 bp
CG31762	2	6237 bp
CG17090	2	5404 bp
CG7542	1	5636 bp
CG13324	1	3031 bp
CG8579	1	7544 bp
CG10527	1	643 bp
CG31038	1	5421 bp
CG12891	1	2406 bp
CG32159	1	6510 bp
CG12057	0	8176 bp
		9488 bp
CG4807	0	1035 bp
CG31075	0	2705 bp
CG17324	0	6719 bp
		7718 bp
CG31446	0	7599 bp
CG10475	0	852 bp
		8692 bp
CG30282	0	670 bp
CG10062	0	4896 bp
CG10262	0	2985 bp
CG18030	0	7798 bp

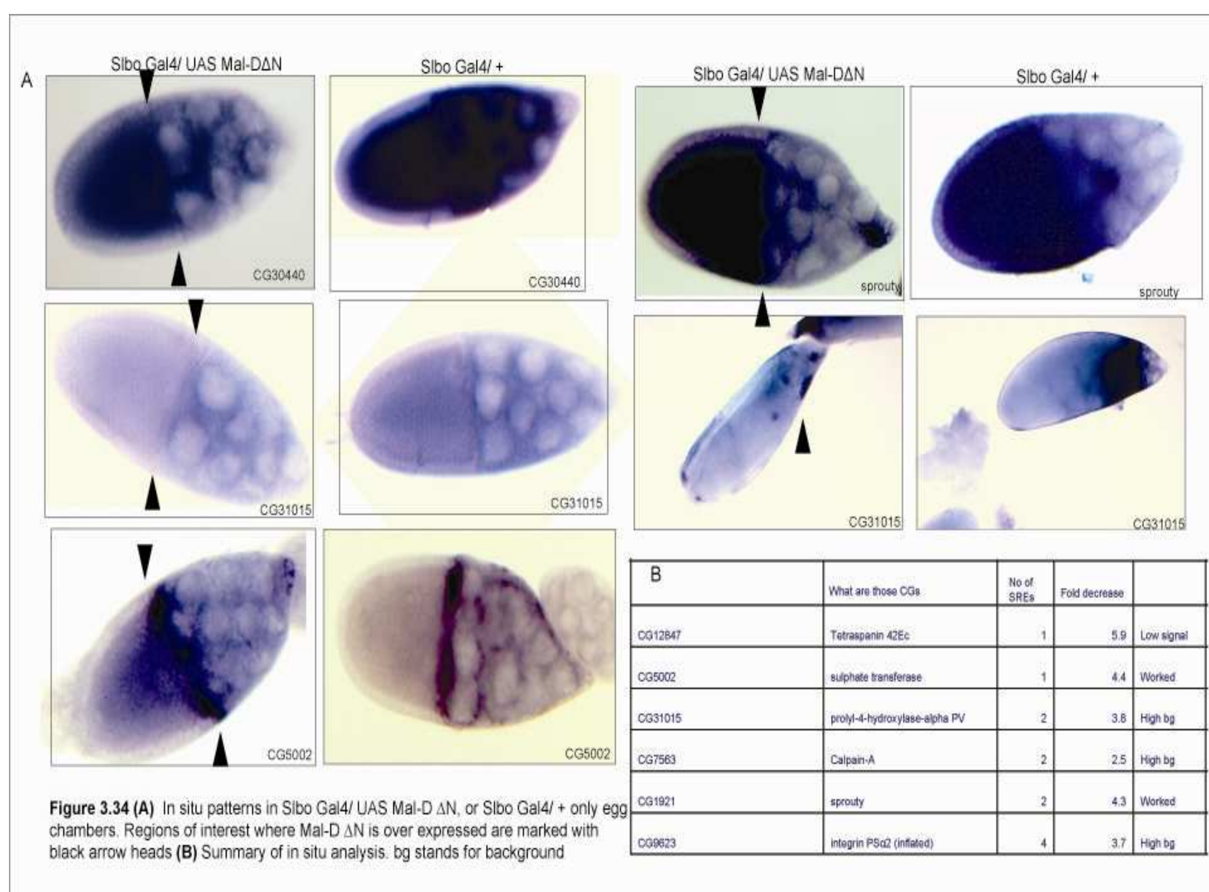
Figure 3.33 Conservation scores of the genes with SREs within the 10Kb upstream region

3.3.5.2 In situ analysis by over expressing Mal-D ΔN

I selected genes that have many SREs in their upstream region, and with high conservation score to test whether they can be directly regulated by Mal-D. In order to test whether those genes were direct targets of Mal-D I tried to ectopically over express constitutive active Mal-D by using slbo Gal4 driver in the follicular epithelium and in border cells and conduct in situ hybridization for those selected genes. The idea behind

this was that if a gene is direct target of Mal-D, it should get up regulated ectopically in response to constitutive active Mal-D. I added two additional criteria in order to filter the list of genes with SREs. The first criterion was that the gene should be strongly down regulated in mutant border cells. The second criterion was that the gene should not have maternal expression (I used BDGP in situ database to get the information about the maternal expression). A high maternal expression would create high basal signal in the germline which would make it harder to observe the signal from follicular epithelium. I selected 6 genes to conduct in situ hybridization analysis.

I have not observed an increase of signal in the follicular epithelium which was the region of ectopic expression in none of the samples but in many samples high background was problematic to give a conclusive result (Figure 3.34 A).



B	What are those CGs	No of SREs	Fold decrease	
CG12847	Tetraspanin 42Ec	1	5.9	Low signal
CG5002	sulphate transferase	1	4.4	Worked
CG31015	prolyl-4-hydroxylase-alpha PV	2	3.8	High bg
CG7563	Calpain-A	2	2.5	High bg
CG1921	sprouty	2	4.3	Worked
CG9623	integrin PSα2 (inflated)	4	3.7	High bg

Figure 3.34 (A) In situ patterns in Slbo Gal4/ UAS Mal-D ΔN, or Slbo Gal4/ + only egg chambers. Regions of interest where Mal-D ΔN is over expressed are marked with black arrow heads **(B)** Summary of in situ analysis. bg stands for background

I proceeded by doing antibody staining with 4 genes that have available antibodies: Vismay, Supercoiling Factor (SCF), Sprouty (sry) and Integrin αPS2 (inflated, if) to test whether the decrease that I see on the RNA level in mal-D $^{\Delta 7}$ border cells is cell autonomous. The way I did this was to generate border cell clusters that consist of both

wild-type and *mal-D* mutant cells and I tested whether the antibody staining goes down in the cells mutant for *mal-D* compared to wild-type cells. For *if* I could not detect the expression of the protein with the monoclonal antibodies that I had. Other proteins did not show a decrease in expression in mutant cells (Figure 3.35).

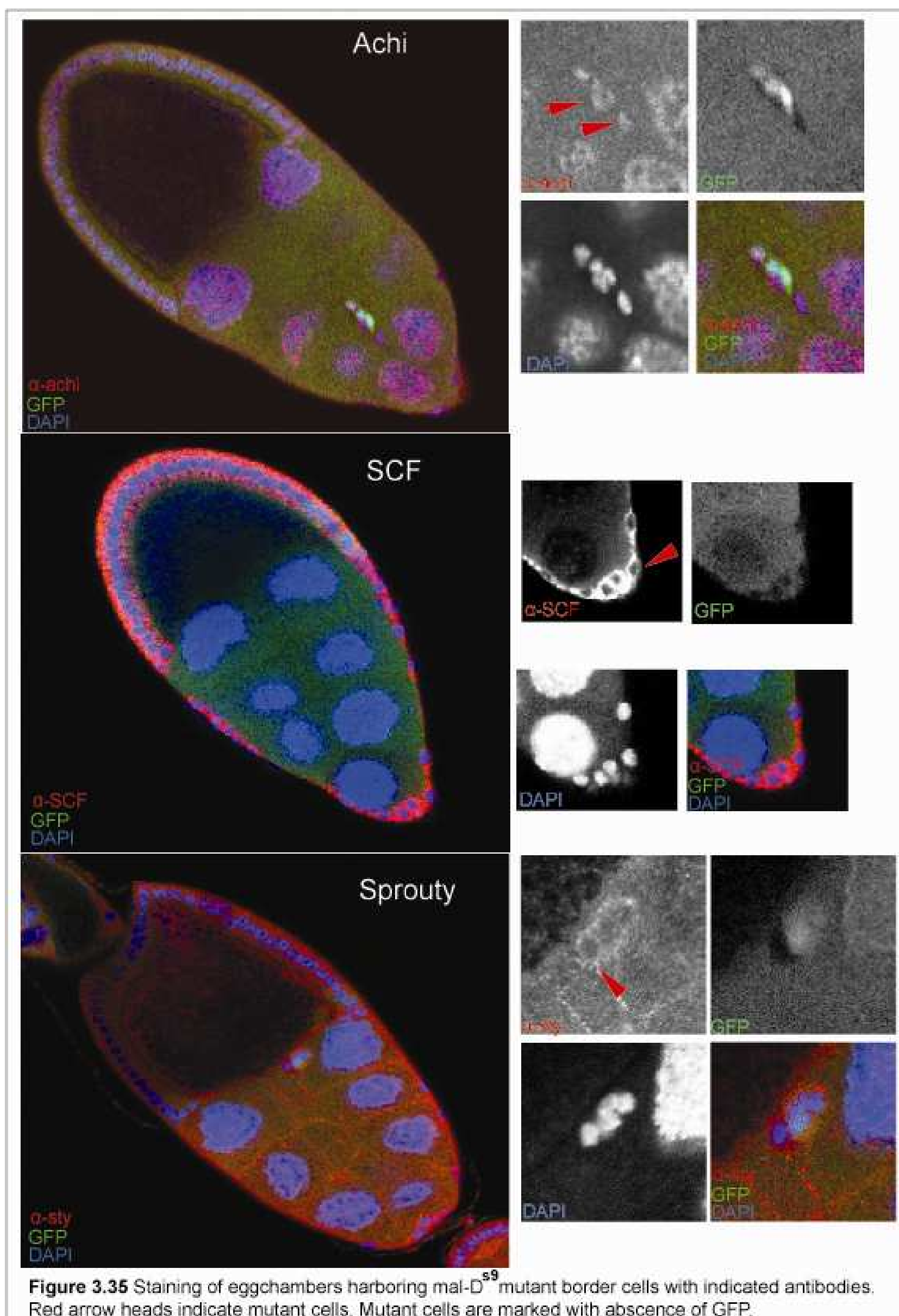


Figure 3.35 Staining of eggchambers harboring mal-D^{s9} mutant border cells with indicated antibodies. Red arrow heads indicate mutant cells. Mutant cells are marked with abscence of GFP.

This may have several reasons. First of all in the mal-D $^{\Delta 7}$ mutant flies that I used to collect the border cells for array analysis all the cells in the ovary are mutant for *mal-D*. The effect that I saw in the border cells could result from the effect of mutation in the germline or in the follicle cells, meaning the effect could be non cell-autonomous. Second possibility is that the reason that I see those genes down-regulated in mal-D $^{\Delta 7}$ border cells is that those border cells do not migrate the full migration path, and compared to the wild-type cells on average they end up in positions further from the EGF and Pvf sources, which is the oocyte. *sprouty* and *argos* are two genes regulated by EGF signaling in a negative feed back fashion (Golembo et al., 1996; Reich et al., 1999). The presence of *sprouty* and *argos* in my list of genes downregulated more then two fold in each repeat can indicate this.

Some of the genes in my list are good candidates for explaining the phenotype that I observe in Mal-D mutant border cells. I focused on those genes to test whether they may explain the phenotypes observed in Mal-D mutant border cells.

3.3.6 CG30440

3.3.6.1 CG30440 encodes for a rhoGEF

Rho family small GTPases are well known regulators of Actin cytoskeleton. They are active when they are bound to GTP and when they hydrolyze their GTP to GDP, they become inactive. GTP Exchange Factors (GEFs) catalyze the exchange of GDP to GTP, thus activate the GTPases. Thus the loss of function of a GEF for Rho family GTPases may cause decrease in F-Actin levels. Because of the F-Actin decrease phenotype of Mal-D, I focused on CG30440 which encodes a putative RhoGEF and was down-regulated about 6 fold in each repeat in mutant border cells.
There were no available mutants for this gene, so I generated an RNAi construct in flies by cloning part of this gene in inverted repeats linked by a hairpin. (Bao and Cagan, 2006).

3.3.6.2 CG30440 RNAi causes border cell migration phenotype when it is highly expressed.

In order to test whether CG30440 has a role in border cell migration, I overexpressed the RNAi construct that I generated with Actin Flipout Gal-4 system (AFG4) and analyzed border cells that are expressing RNAi, marked with the presence of GFP. (See materials and methods). When I overexpressed three copies of my UAS RNAi construct I observed a migration delay phenotype. (Figure 3.36) It is a mild phenotype that is not seen in the flies expressing UASGFP only with AFG4 system that were treated in parallel (Figure 3.36). Moreover this phenotype got more severe in sensitized flies having one copy of the endogenous gene removed by using a deficiency, Defnap8 (Figure 3.36). An important point to be made here is that this deficiency contains two of the genes from my list of interesting genes that are down-regulated in *mal-D* mutant border cells according to my array results, *CG30440* and *CG1344*, mentioned more in detail later. Deficiency alone did not cause any migration delay phenotype.

Moreover both the deficiency alone and the RNAi with the deficiency caused breakage of border cells, albeit with low frequency, which is a phonotype that is specific for *mal-D* mutant border cells (Figure 3.37). RNAi with defficiency caused cytoplasmic blobs in 3 out of 20 border cell clusters that were analyzed and defficiency alone caused cytoplasmic blobs in 5 out of 50 border cell clusters that were analyzed. Over expression of GFP, without deficiency or RNAi construct did not cause any blobbing in 50 border cell clusters analyzed. It should be indicated that since the deficiency alone did not cause border cell migration defect, the assignment of blebbing versus cytoplasmic protrusions that still were bound to cell body was more challenging. I counted cytoplasmic extensions without any discernible connection to the cell body with high magnification imaging in Deficiency alone sample as blobs.

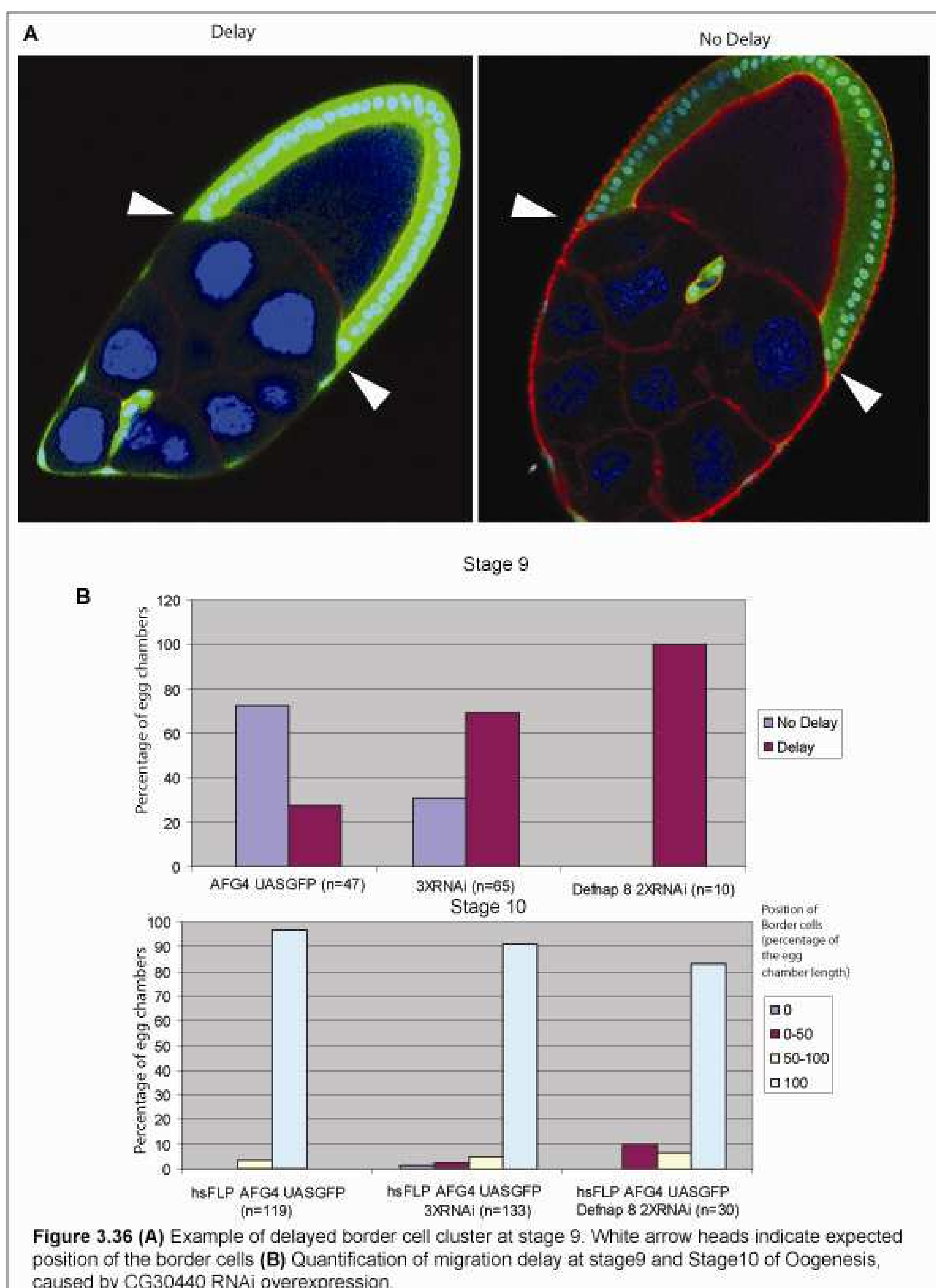


Figure 3.36 (A) Example of delayed border cell cluster at stage 9. White arrow heads indicate expected position of the border cells **(B)** Quantification of migration delay at stage9 and Stage10 of Oogenesis, caused by CG30440 RNAi overexpression.

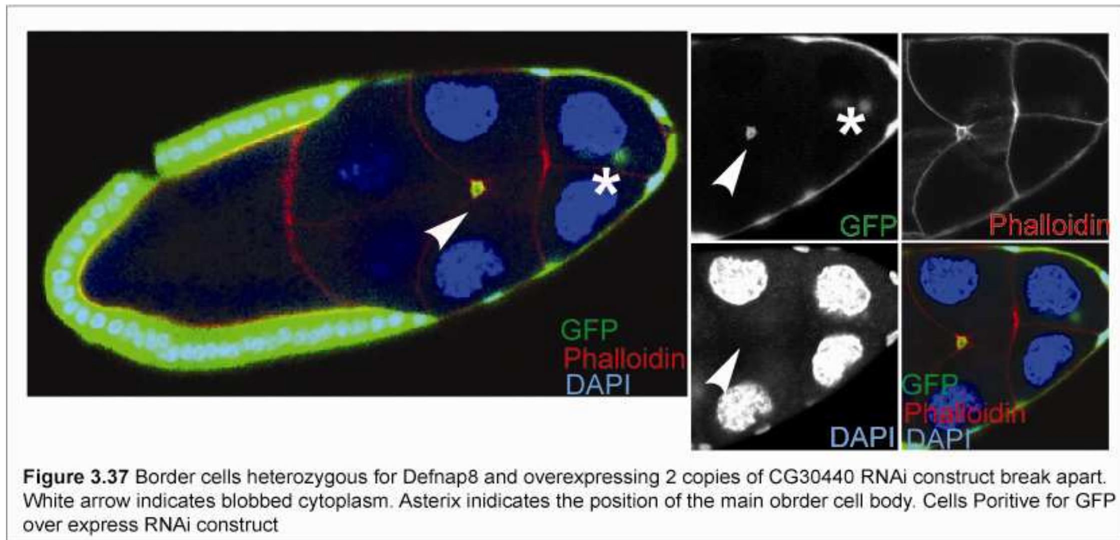


Figure 3.37 Border cells heterozygous for Defnap8 and overexpressing 2 copies of CG30440 RNAi construct break apart. White arrow indicates blobbed cytoplasm. Asterix inidicates the position of the main obrder cell body. Cells Poritive for GFP over express RNAi construct

The fact that the border cells heterozygous for deficiency have the breaking border cells phenotype but not the delay may indicate that this process is more easily perturbed than the whole migration process. Moreover the fact that RNAi alone causes the migration delay but does not cause the breakage of border cells indicate that the two processes can be caused by different genes.

In order to analyze if CG30440 RNAi caused any defects in F-Actin level I over-expressed that construct in follicle cells and analyzed apical F-Actin levels. *mal-D* mutation causes decrease in the F-Actin levels in follicle cells (Somogyi and Rorth, 2004). In follicle cells over-expression of CG30440 RNAi did not cause any F-Actin decrease. (Figure 3.38)

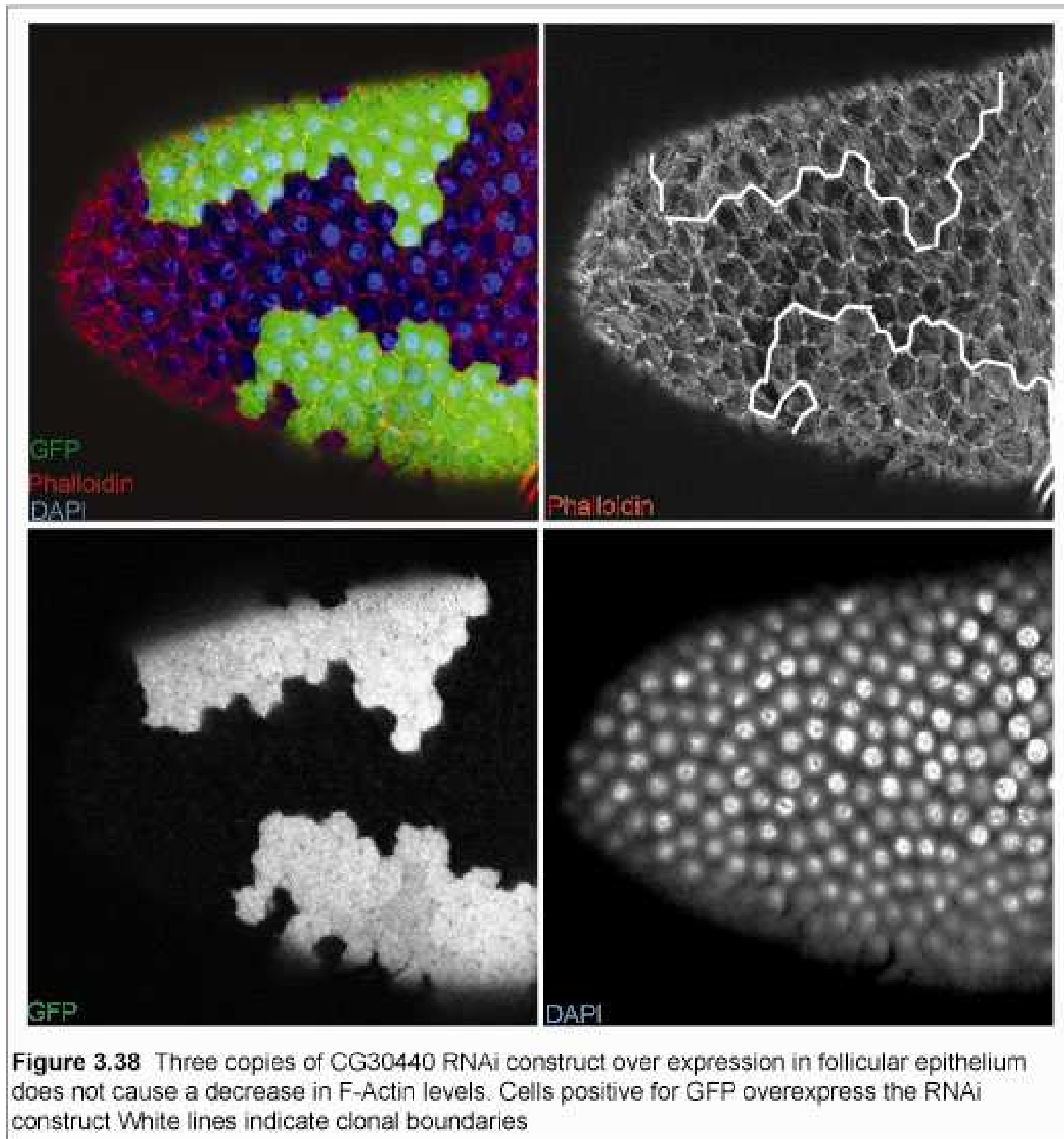


Figure 3.38 Three copies of CG30440 RNAi construct over expression in follicular epithelium does not cause a decrease in F-Actin levels. Cells positive for GFP overexpress the RNAi construct White lines indicate clonal boundaries

I currently generate an RNAi construct against CG1344 the other gene in the deficiency. This way I may test whether the knock down of this gene by itself can cause the border cells to break. CG1344 supposedly expresses a kinase. The mammalian homologue of CG1344 has been shown to interact with Ezrin protein (Sullivan et al., 2003).

3.3.7 Integrin PS2α (inflated) is not required for border cell migration

One of the genes that was seen to be regulated by Mal-D according to my transcriptional profiling was Integrin PS2α. Integrins are well known adhesion molecules that are important for cell- extracellular matrix adhesion. They are known to play role in different migration systems in Drosophila and in mammalian models. Inflated is a gene that was identified because of the mutation effect that causes separation of dorsal and ventral surfaces of the wing, thus causing blisters in the wing surface, reminiscent of DSRF mutant phenotype.

I decided to test whether *if* was important for border cell migration and if the loss of function of *if* can mimic Mal-D mutant phenotypes. I selected if ^{b4} allele for generating loss of function situation. if ^{B4} is a null allele of inflated which results from a deletion of the coding region of the gene. if ^{B4} is a homozygous lethal mutation. I recombined if^{B4} allele with FRT19A chromosome which would give me the chance of generating homozygous mutant cells in an otherwise heterozygous animal. I generated mutant clones with Mosaic analysis with a repressible cell marker (MARCM) technique. With this method, I mark the mutant cells by the over expression of GFP. This would give me the chance of looking both for the integrity and for migration phenotype of the border cells. When I generated mutant clones of if ^{B4} I did not observe any delay either in stage 9 or in stage 10 of oogenesis. Moreover there was no blobbing of border cells, meaning that the integrity of the clusters was not perturbed.

4. Discussion

4.1 Different means of Mal-D regulation

Border cells undergo a developmentally regulated invasive migration during the oogenesis of *Drosophila melanogaster*. During their migration process they accumulate Mal-D in the nucleus (Somogyi and Rorth, 2004). The accumulation of nuclear Mal-D has been previously shown to be regulated by a migration related signal, since border cells mutant for *slbo* do not accumulate nuclear Mal-D on their own, but if they are pulled in a migrating cluster by wildtype cells they then can accumulate nuclear Mal-D (Somogyi and Rorth, 2004). In this study I analyzed the requirements for the migration related signal and nuclear accumulation of Mal-D by using an antibody against HA tag that is added at the C-terminus of endogenous protein. This approach made it possible to have a lower detection limit compared to our old antibody results and unraveled the presence of nuclear Mal-D accumulation in follicle cells and stretched cells, albeit in lower levels than migration related signal induced nuclear accumulation of Mal-D in the nuclei of border cells.

4.1.1 Profilin effect

In our system *chic* mutation causes an interesting phenotype in Mal-D accumulation. *chic* mutant border cells not only have decreased nuclear Mal-D levels but also half of the cases have increased cytoplasmic signal. In wild-type cells most of the border cells show either low staining all over the cell body of the cell, mildly increased in the nucleus or strong nuclear staining with low staining in the nucleus. The accumulation in the cytoplasm is not observed in wild-type border cells. This brings further questions such as whether the shifting of subcellular localization of Mal-D in our system is the critical step of the regulation, or the regulation by enlarge goes through the levels of Mal-D, in other words goes through the stabilization of Mal-D protein in migrating cells. It is noteworthy that in wild-type border cells in half of the cases one observe strong nuclear accumulation of Mal-D (NMI 2 and 3). Myocardin is regulated by its tissue specific expression and is

nuclear in the cells where it is expressed (Wang et al., 2001). Interestingly Mal-D behaves like Myocardin in the muscle context where it is observed nuclear in differentiated muscle. Mal-D is the only MRTF family orthologue in *Drosophila*. One possibility is that either by alternative splicing (although there is no annotated alternative splicing events) or through its association with different factors Mal-D behaves like MAL or Myocardin in different cells of *Drosophila*.

chic causes a decrease in nuclear Mal-D levels in follicle cells and stretched cells too. Thus Profilin does not seem to have a direct role in migration-related signal processing. Presence of Profilin is a general requirement for the cell to have nuclear Mal-D. The effect of Profilin on SRF has been shown in mammalian cell culture system (Sotiropoulos et al., 1999). There, it has been suggested that Profilin sequesters most of the G-Actin in the cell. When there is no Profilin, G-Actin level that can bind to Mal-D and keep it cytoplasmic increases. Alternatively Mal-D nuclear export rate increases when G-Actin level increases. There is another actin sequestering protein in the cells called β thymosin 4 (or ciboulot in Drosophila). It was shown to act together with Chickadee in the *Drosophila* brain morphogenesis (Boquet et al., 2000). It would be interesting to test double mutants of *ciboulot* and *chic* to test whether exclusion from the nucleus phenotype can get more dramatic.

Although the accumulation of over expressed Mal-D in the cytoplasm suggests that in overexpression scenarios nuclear transport can be the limiting factor, there is no evidence that shows that in endogenous levels nuclear translocation of Mal-D is limiting. Alternatively there may be two different pathways that act on Mal-D concomitantly. One effect stabilizes the protein therefore increasing its concentration and the other one increasing the nuclear accumulation of the protein. The fact that we do not see only cytoplasmic accumulation in the wild-type situation suggests that those two pathways should be tightly coupled.

4.1.2 Rho effect

Unlike the need for Rho GTPase activity in tissue culture cells, border cells were not found to require Rho for accumulating nuclear Mal-D (Miralles et al., 2003). Rho is not

required for follicle cells to accumulate nuclear Mal-D either. On the other hand stretched cells fail to accumulate nuclear Mal-D when they are mutant for *rho*. This can be caused by different reasons. One probable reason is the presence of a different GTPase that acts redundantly to Rho in the border cells in terms of Mal-D nuclear localization, and the absence of this factor in stretched cells. In fact it is known that Rac GTPase is important for actin polymerization downstream of activated RTKs in border cells (Duchek et al., 2001). Over expression of constitutive active PVR causes an over accumulation of F-Actin and this effect can be reversed if Myoblast city (mbc) which is a Rac GEF is mutated at the same time (Duchek et al., 2001). Interestingly Rac has been suggested that to regulate Mal-D activity in the tracheal terminal cell outbranching event (Han et al. 2004). Looking at Mal-D localization in *mbc* mutant border cells may be informative.

Although over-expression of constitutive active Diaphanous can drive Mal-D to the nucleus in border cells and follicle cells it looks like the endogenous protein is not essential for the nuclear accumulation of Mal-D in those cells. Again the possibility that there is redundancy in terms of Mal-D regulation remains. Moreover although stretched cells require Rho for the nuclear accumulation of Mal-D they do not require Diaphanous which suggests that the signaling pathway may go thorough the activity of ROCK. Indeed in fibroblasts, application of force can drive MAL to the nucleus and induce the expression of smooth muscle specific genes in MAL dependent way, and this is disrupted by usage of Rock inhibitor drugs (Zhao et al., 2007). Rock has been shown to be an important regulator of myosin II in many contexts. It would be interesting to see whether myosin II signals to Mal-D in stretched cell in the stage where Mal-D goes to the nucleus. It would be interesting to analyze stretched cell mutants where stretched cell fate specification occurs normally but stretching or adhesion remodeling in response to stretching is defective such as in *fringe* mutants (Grammont, 2007).

The stage specificity of stretched cell nuclear Mal-D is interesting. Stage 9 of oogenesis is the stage where the main body follicle cells move towards oocyte and change their morphology from cuboid to columnar epithelial morphology and cause stretching of the stretched cells. An interesting speculation is that this change of morphology and pulling force coming from the migration of main body follicle cells cause the nuclear

accumulation of Mal-D. Further analysis for determining whether Rho-ROCK-Myosin II pathway has a role in regulating Mal-D in stretched cells would give interesting results on the regulation means of Mal-D.

4.1.3 *shg* and *slbo*

Border cell clusters that are formed by wild-type and *slbo* mutant cells do not accumulate high nuclear Mal-D levels with the same frequency as wild-type cells. This may mean that some of the genes activated by Slbo may in part be important for the processing of migration-related signal. In fact Slbo activates the transcription of multiple genes important for Actin remodeling, cell adhesion, and cell signaling. (Borghese et al., 2006). Cadherin mutant clones which only lack the adhesion on the substrate can accumulate high levels of nuclear Mal-D in frequencies comparable to wild-type, showing the importance of migration-related signal.

What is sensed by the migrating border cells to increase nuclear Mal-D levels is still not clear. One possibility is the increase of cytoskeletal tension due to pulling from other cells of the cluster. In fact this kind of mechanism has been suggested to change behavior of cells in different contexts. Mechanical tension can be sensed by the cells by the changes in focal adhesions and focal complexes (reviewed in (Bershadsky et al., 2003), by stretching in adherens juctions, or by specialized mechanosensor ion channels (reviewed in (Gillespie and Walker, 2001). Tension on the cells can drastically influence the cell signaling. Experiments with human mesenchymal stem cells showed for instance that plating them on micro patterned substrates, to force them to stretch or get round can influence their differentiation in osteoblast or adipocyte fate. Moreover this effect goes through regulation of RhoGTPase. (McBeath et al., 2004) Differing matrix elasticity on which hMSCs are plated can be inductive in the differentiation of those cells as well. In a series of experiments plating hMSCs on soft matrices caused them to differentiate in neurons, on stiffer matrices caused them to form muscles and most rigid matrices caused them to become bone tissue (Engler et al., 2006). Inhibiting non muscle myosin II is enough to block the instructive role of the matrix on the cell fate specification, meaning that the signaling goes though the activity of myosin (Engler et al., 2006). A recent paper showed that applying force on fibroblast through elastic beads coated with integrin

substrate can activate Rho and cause Rho-Rock-LIMK cofilin pathway which in turn sends MAL to the nucleus (Zhao et al., 2007).

4.1.4 DSRF effect

bs mutant border cells, but not other cell types, show increased nuclear Mal-D. There may be several reasons for this. Many transcription factors are regulated by ubiquitination of active transcription factor and subsequent degradation executed by Proteosome. In fact myocardin has been shown to be sumo modified and this sumoylation has been shown to be important for its regulation (Wang et al., 2007). Moreover MAL is sumoylated in three sites and it causes it to be less stable and slightly less nuclear (Nakagawa and Kuzumaki, 2005). On the other hand the effect of *bs* mutation on nuclear localization of Mal-D is seen in border cells specifically. We know that the protein is active in the follicle cells, since there is a decrease of F-Actin levels in the absence of it in this tissue (Somogyi and Rorth, 2004). A mutation that uncouples possible activation induced degradation should in principle increase Mal-D levels in follicle cells as well. Another finding that is contradictory to the idea of lack of degradation of Mal- D in *bs* mutants comes from the fact that the levels of the protein in *bs* mutant cells is not over accumulated and in the same cluster sometimes a border cell that is wild-type can have the same amount of nuclear Mal-D as *bs* mutant border cell. In other words in *bs* mutant border cells Mal-D seems to be stuck in the high nuclear state.

An attractive idea for explaining the border cell specificity is the presence of a feedback loop. In this scenario what may happen is that the border cells start their migration and get the migration-related signal. They send Mal-D to the nucleus in order to counteract the effect of migration by increasing the F-Actin levels. Then Mal-D and DSRF activate some key factors that increase F-Actin levels and quench the signal. In *bs* mutant Mal-D goes to the nucleus because of the migration-related signal but as there is no DSRF it cannot transcribe those key factors, and the signal is not quenched. For the moment I do not know whether the increase in the frequency of border cells with high nuclear Mal-D is dependent on the presence of the migration-related signal. Another alternative scenario that can explain why the increase of the frequency in nuclear Mal-D accumulation in *bs* is border cell specific is that border cells are different from follicle cells. Maybe there are some transcription factors, or Mal-D interactors present in border cells but absent in

follicle cells. One key experiment to test whether border cell specific increase in frequency of higher nuclear Mal-D comes from the border cell fate or from the presence of the migration-related signal is to make border cells where all the cells lack both DSRF and DE-cadherin. In this situation border cells are fully differentiated as border cells, but they do not receive the migration-related signal. Thus if they still have more frequent nuclear Mal-D accumulation it means that the effect we see is border cell fate specific. If on the other hand we do not see accumulation of Mal-D in the nucleus, this would mean the effect is migration-related signal dependent and there is a feedback loop.

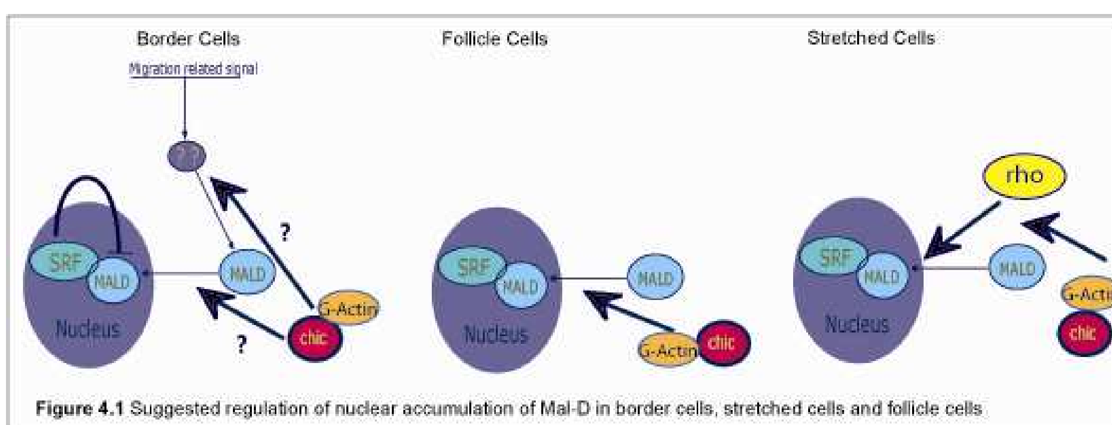


Figure 4.1 Suggested regulation of nuclear accumulation of Mal-D in border cells, stretched cells and follicle cells

My experiments showed that Mal-D can be recruited to the nuclei of different cell types in the developing *Drosophila* egg chamber. Border cells accumulate the highest amount of nuclear Mal-D while they are migrating, where as stretched cells accumulate nuclear Mal-D transiently while the egg chamber is growing, and follicle cells accumulate nuclear Mal-D in all stages of oogenesis. The results of different mutant clone experiments pointed out that the nuclear accumulation of Mal-D in those different cell types can be genetically dissected (Figure 4.1).

4.2 Mal-D function

My results showed that Mal-D/ DSRF can form a transcriptional couple in *Drosophila* both in S2 cells and in vivo over a reporter gene. Moreover in follicle cells Mal-D ΔN activity on F-Actin levels goes through DSRF. Judging from both loss of function and gain of function experiments Mal-D and DSRF seem to express together some actin regulating proteins (Somogyi and Rorth, 2004). In fact in other organisms MRTFs and SRF were shown to collaborate to induce actin regulating factors suggesting that the ancestral role of MAL and SRF was related to actin regulation (Morita et al., 2007; Sun et al., 2006a).

There are other tissues where I can see the nuclear accumulation of Mal-D. In muscle cells the protein is always strongly nuclear. This is reminiscent of the myocardin protein. On the other hand Mal-D maternal and zygotic mutant embryos do not show any obvious muscle morphology defects, but the hatched larvae are sluggish which can be caused by a functional defect in body wall muscles (Kalman Somogyi personal communication). I observed nuclear Mal-D staining in the germ line as well. As germline mutant eggchambers develop normally without problems (Kalman Somogyi personal communication) the function of nuclear accumulation of Mal-D in germline cells is unclear.

The reason for Mal-D mutant phenotype is not very clear. Among the targets that I got from profiling analysis there were no genes that could directly explain the phenotype. In a previous genome wide expression profiling approach, comparing border cell transcriptome to follicle cells, Lodovica Borghese found that there was a group of muscle related genes that were up regulated specifically in border cells compared to follicle cells (Borghese et al., 2006). Due to the role of MRTF Family of transcription factors in muscle development in the mammals one possibility was that those genes could be targets of Mal-D. In my expression profiling experiment I could not find this group of

genes down regulated in Mal-D mutant egg chambers. Probably the effect that Lodovica observed goes through a different border cell specific transcription factor such as Six4.

The upstream regions of the genes that were down-regulated by Mal-D in my expression profiling experiments did not show an enrichment of SREs. This may indicate that many of the genes that I found are due to secondary effects. This is to be expected. The transcriptional changes that are resulting from the lack of a single transcription factor is masked by the fact that the border cells are mutant and cannot migrate to their final destination in Mal-D mutant. In fact the expression profiling comparing wild-type and slbo mutant border cells was not enriched for direct targets of slbo. (Borghese et al., 2006) Moreover there is a technical problem about the expression profiling experiments, reflected by the fact that even in the wild-type border cell collection, different repeats do not show strong correlation. This means that there may be false positive genes in my list of potential Mal-D, DSRF targets. This technical problem was not seen in prior expression profiling experiments done with border cells in our laboratory. The differences between the two experiments were the usage of a different FACS machine (although the parameters are kept the same), the usage of a different kit for doing two step amplification, and the usage of Affymetrix Drosophila Version 2 arrays intead of version 1. Maybe those changes increased the background signal of different genes and caused the observed problems.

The fact that decreasing the level of some of my putative targets gives rise to border cell migration delays and more specifically to border cell blebbing is encouraging and may indicate that my list has really direct targets of Mal-D. The lack of antibodies against those proteins makes it difficult to address whether those are direct targets of Mal-D.

4.3 Conclusions and Future Perspectives

Mal-D/DSRF cooperation in border cell migration is important for the cells to keep their cellular integrity. Their joint activity is regulated by a migration related signal in border cells. My results indicated that *bs* mutant border cells keep Mal-D nuclear longer and have higher nuclear Mal-D accumulation frequency. This may indicate a feed back loop. A detailed analysis of Mal-D expression profiling results in order to determine a factor

that can regulate Mal-D in a feed back loop would be important. Profilin on the other hand is important for nuclear Mal-D localization in all cells. It suggests that actin is a permissive factor for nuclear accumulation of Mal-D and when it is free of Profilin it can block Mal-D entry to the nucleus. Identification of more genes that have roles in this regulation would be the key in order to understand clearly the mechanism of Mal-D regulation.

The presence of a good tag in the endogenous levels of the protein is a very powerful tool. The presence of a perfect control which is the same starting sample from flies without the tag makes Mal-D 9HA ideal to do biochemistry experiments with. Mal-D 9HA can be used both for understanding the regulation of Mal-D and for determining its targets. Using HA antibody in combination with Mal-D 9HA in co-immunoprecipitation experiments one can identify the kinds of posttranslational modifications on Mal-D and binding partners of Mal-D. One particular cell type where it can be particularly interesting is the muscle where Mal-D is seems to be constitutively nuclear. One can isolate Mal-D 9HA from muscle cells in order to identify how Mal-D is regulated to be always nuclear in those cells. For determining direct targets of Mal-D, Mal-D 9HA can be used for chromatin immunoprecipitation experiments.

Mal-D regulation in border cell migration is a dynamic process. It would be exciting to analyze this process live. With the recent advancement in real time imaging of border cell migration, it is now possible to visualize border cell migration. Generating GFP knock-in in the Mal-D locus may provide means to observe Mal-D dynamics in the migrating cells. Particular questions are how fast the accumulation of Mal-D is and how many times a given cell gets increased Mal-D during the migration.

Live imaging of *mal-D* mutant border cells can be rewarding too. Observation of the kinetics of blob formation in *mal-D* mutant would be key to answer questions about the factors that are missing. After determining the factors regulated by Mal-D, in order to prevent lost of integrity of border cells, one can visualize the localization and kinetics of that factor in order to understand better the nature of strengthening of the protrusion.

5. Materials and Methods

5.1 Cloning

5.1.1 Primers and oligos

Primer name	Sequence (5' to 3')	Length	TM
CG30440BHI-for	ATT AGG ATC CAG CAA CTA CAT TGC GTC	27mer	63.4 ˚C
CG30440RI_rev	GAT TGA ATT CCG CCA GCC GCA G	22mer	64.0 ˚C
CG30440RNAi_for	ATT ATC TAG AAT GTC TGC TCC CAA GAT GC	29mer	63.9 ˚C
CG30440RNAi_rev	TAA TTC TAG AGC TCG CGA TTG AAT TCC G	28mer	63.7 ˚C
IfHomshXba_for	ATT ATC TAG ACG GTG CAG CTG AAG GAG	27mer	65.0 ˚C
IfHomshXba_rev	TAA TGA ATT CGG ATC CGT AGG CTT AGC TGG AC	32mer	68.2 ˚C
MalCtermSalTerm_for	TTG CAA TGG ATC CAT TGA ATC CTC G	25mer	61.3 ˚C
MalCtermKpnTerm_rev	ATT AGG TAC CGA CTG TAA AAT CTC CCG	27mer	63.4 ˚C
XhoHAAmp_for	ATT ACT CGA GAT GGA TCT CCA CCG CG	26mer	66.4 ˚C
SalHAAmp_rev	ATT AGT CGA CTC CGC CAT GAG C	22mer	62.1 ˚C
KpnMultHAs_for	ATT AGG TAC CGG AGG TAG CTT ATC GAT AC	29mer	65.3 ˚C
KpnNotMultHAstp_rev	ATT AGG TAC CGC GGC CGC CTA CCC CTC GAG GTC GA	35mer	>75 ˚C
AscMal3UTR_for	TTT GGC GCG CCT AGG CGG TTT TAT GTA TTC ATA TGG	36mer	70.6 ˚C
AscMal3UTR_rev	AAT TAG GCG CGC CAC ACC AAA GCC AGA TGG	30mer	70.9 ˚C
MalCtermseq	GAG GAG GAA TGG GCG TGG ACA A	22mer	64.0 ˚C
MALDKI_UTR	CGC GAG TGC CAT TGT TTG GCT TGT TTT CG	29mer	68.1 ˚C
MALDKI_EX	CAG CGA TCT GCT GAA GGC	18mer	58.2 ˚C
pW25_F_wto3'flank	GCA AAC ACA ATC ACA CAA ATG TGC	24mer	68.4 ˚C
pW25_R_wto5'flank	AGT GAG AGA GCA ATA GTA CAG AGA GG	26mer	62.5 ˚C
SREamp_Not	ATT ATG CGG CCG CTA GTG GAT CAG ATG TCC	30mer	69.5 ˚C
SREamp_Pst	ATT ACT GCA GCT AGT GGA TCA GAT GTC C	28mer	65.1 ˚C
SREamp_Spe	TTA TTA CTA GTC CGG GGG ATC GGA TG	26mer	64.8 ˚C
SREamp_Sal	TTA TTG TCG ACC CGG GGG ATC GGA TG	26merr	68.0 ˚C
GbeSense	AAT TAT TGG AAC CGG TTA TGC GAG GAA TTC ATT A	34mer	64.7 ˚C
GbeAsense	AGC TTA ATG AAT TCC TCG CAT AAC CGG TTC CAA T	34mer	67.1 ˚C
SreNotchSpsense	CTA GTA TTG TCC ATA TTA GGA CTT ACT TTC AGC TCG GCC ATA TTA GGG CCA CAT TGT CCA TAT TAG GGC CAG TCT AGA TTA CAT A	85mer	> 75 ˚C

SreNotchSpAsense	GAT CTA TGT AAT CTA GAC TGG CCC TAA TAT GGA CAA TGT GGC CCT AAT ATG GCC GAG CTG AAA GTA AGT CCT AAT ATG GAC AAT A	85mer	> 75 ˚C

5.1.2 Cloning Mal-D 3XGFP

pRm-Mal-D-GFP (Pernille Rørth) vector was cut with Not I enzyme and relegated with a self ligating short oligo that destroys NotI site and generates a KpnI site. (GGCCGGTACC) Oligo was heated to 95 ˚C for 5 minutes and let cool in the room temperature. 1:10 diluted oligo was added to the ligation of NotI digested pRm-mal-D-GFP plasmid. Insertion of the oligo was tested by cutting with Asp718I. Modified pRm-Mal-D-GFP plasmid was cut with Asp718I and XbaI along with pEGFP-NI 3XGFP (kind gift from Natalie Daigle, Ellenberg Laboratory). 3XGFP cassette was ligated to cut pRm-Mal-D-GFP plasmid. The whole construct was subcloned with EcoRI and NotI sites to pUAST, pCasper4 Tub, and pCasper4 Arm vectors.

5.1.3 Cloning Mal-D 9HA

3 Hemagglutinin tag (HA tag) was PCR amplified from pHW vector and cloned in SalI, XhoI sites of the pBsIISK vector by using XhoHAAmp_for, and SalHAAmp_rev. SalI and XhoI sites are compatible to ligate to one another, for this reason cut vector was dephosphorylated for 10 minutes after restriction enzyme digestion with Alkaline Phosphatase. Resulting vector was ran on gel for isolation. pBsIISK vector was used as an intermediate vector as it is an easy vector to manipulate. I generated this way pBsIISK-3HA vector. I cloned PCRed 3HA, cut with XhoI and SalI to pBsIISK-3HA vector cut with SalI and Alkaline phosphatase treated. This way when SalI SalI ligation occured it created a new SalI site and when SalI XhoI ligation occured it killed the SalI site there, making it possible to use the resulting vector for another round of 3HA addition. Directionality of the construct was tested with restriction enzyme digestion. I repeated this step in order to get pBsIISK-9HA and pBsIISK-12HA. I continued with 9HA plasmid. 9HA cassette was PCR amplified with primers that added a stop codon at 3' end of the construct, KpnNotMultHAstp_rev and KpnMultHAs_for. This amplified cassette was cloned into a new pBsIISK plasmid that contained the last 100 base pairs of Mal-D cDNA (before stop codon). This region was PCR amplified and cloned into SalI

KpnI sites of pBsIISK with primers MalCtermKpnTerm_rev and MalCtermSalTerm_for. The rest of the 5' homology arm was prepared in another pBsIISK vector. BglII/ SalI region of pBs-Mal-D cDNA (From Pernille Rørth) was subcloned to pBsIISK vector BamHI/SalI sites. cTerminal 100 bps fused to 9HAs with a stop codon was subcloned to the remainder of the homology arm with SalI/KpnI digestion and the whole cassette wais cubcloned in NotI site in pW25 vector. 3' Homology arm was directly PCRed from genomic DNA with AscMal3UTR_rev and AscMal3UTR_for primers and subcloned in AscI site of pW25 vector.

5.1.4 Cloning of SRE repoters

Annealing oligos containing 3 serum response factor binding sites were used. Oligos were: Sense :
GATCGGATGTCCATATTAGGACATCTGGATGTCCATATTAGGACATCTGGATGTCCATATTAGGACATCT
Antisense:
GATGAGATGTCCTAATATGGGACATCCAGATGTCCTAATATGGACATCCAGATGTCCTAATATGGACATCC
Oligos were annealed by mixing equal amount of oligos (2 milimoles), heating them to 95 ˚C and letting them slowly cool. This ligated oligo was cloned into the BamHI site of pBsSKII plasmid. Blue white selection was done on Amp^+ plates coated with 40 μl 2% X-Gal. As the number of nucleotides in the oligo that I used (71) was not divisible by 3 this caused a frame shift in the β Gallactosidase gene of pBsIISK vector. β Gallactosidase gene creates a blue non soluble product by using X-Gal. Whereas bacteria transformed with plasmid that self ligated had a functional β Gallactosidase gene, thus created blue colonies on X-Gal coated plates, bacteria transformed with plasmid with insert did not have a functional β Gallactosidase gene, and created white colonies on X Gal coated plates. I selected three white colonies for Miniprep. 3SREs were subcloned in pCasperAUGβGal plasmid as NotI/XhoI fragment from pBsIISK-3SRE.

New Notch reporter with notch reporter spacing sites was cloned with annealing the oligos SreNotchSpAsense and SreNotchSpsense the same way. This oligo was cloned in the BamHI XbaI sites of pBsSKII. This approach killed BamHI site in the binding region.

I continued to clone new trimers of the SREs with XbaI digestion and cloning in the XbaI cut alkaline phosphorylated pBsIISK with multiple SREs in it. This way I generated multimers of SREs in pBsSKII vector. Gbe region was annealed the same manner from the oligos GbeAsense and GbeSense, Gbe was cloned in pBsIISK vector in EcoRI/ HindIII region. This step was repeated to get 2 repeats of Gbe. Different numbers of SREs were cloned in NotI/PstI of the resulting pBsIISK Gbe (1 or 2) plasmids. Resulting fragments were subcloned in NotI/XhoI sites of pCasperAUGβGal vector.

if enhancer reporter was cloned by PCR with IfHomshXba_rev and IfHomshXba_for into EcoRI/XbaI site of pBsIISK. It is subcloned into pCasperAUGβGal with SpeI and BamHI.

5.1.5 Clonining of CG30440 RNAi

cDNA for *CG30440* was obtained from Drosophila Genomic Research Consortium (DGRC) cDNA library from Genecore facility. The clone number is LD43457. For cloning RNAi construct I used Cagan Lab protocol (Bao and Cagan, 2006). I used PCR to amplify the first 500 nucleotides of the cDNA with primers that introduce XbaI sites, CG30440RNAi_for and CG30440RNAi_rev. Then I used TOPO cloning to have this fragment into pCRII TOPO vector. This fragment was cut with XbaI to clone in pGEM-WIZ vector cut with AvrII and alkaline phosphatase treated for 10 minutes, generating pGEM-WIZ-1X30440repeat. pGEM-WIZ vector contains bipartite multiple cloning sites that are separated with *white* gene intron. Expression of inverted repeats with this intron causes splicing of the intron this forming a double stranded RNA which then generates RNAi effect. Second copy of 500bps was ligated in NheI cut and alkaline phosphatese treated pGEM-WIZ-1X30440repeat plasmid, generating pGEM-WIZ-2X30440repeat. The directionality of repeats was confirmed with restriction enzyme digestion with EcoRI. Head to head oriented inverted repeats were chosen for subcloning into pUAST vector in XbaI/XhoI sites.

5.2 Drosophila Genetics

5.2.1 Fly Husbandry

Flies were grown on standard corn meal molasso agar prepared in the Fly Kitchen facility in EMBL (12g agar, 18 g dry yeast, 10 g soy flour, 22g tunip syrup, 80 g malt extract, 80 g corn powder, 6.25 ml propionic acid, 2.4 g methyl 4-hydroxybenzoate (Nipagin) per liter) All crosses were set in 25 degrees unless indicated otherwise, in vials containing a few grains of dry yeast in order increase the fertility. Approximately 18 hours prior to dissection, female flies were put in vial containing wet yeast along with a few male flies in order to boost the oogenesis.

For larval heat shock experiments the vials were submerged in water bath set to 37 ˚C (1hr for mitotic recombination clones, 30 minutes to induce flip out clones). For larval heat shocks to induce mitotic recombination larvae were heat shocked on days 3, 4 and 5 after egg laying, once per day. For adult heat shocks flies were put in vials with wet yeast over night prior to the day of adult heat shock. Flies were transferred into empty vials and heat shocked in those empty vials in order to boost heat transfer. Adult heat shocks were done by submerging flies in empty vials in 37˚C water bath. Flies were transferred in vials containing wet yeast afterwards in order to optimize oogenesis. 2,5 days after the adult heat shock flies were dissected.

5.2.2 List of Fly strains

The list of used fly strains can be found below. Most of the fly strains that were used were inherited from Kalman Somogyi. Some of the used stocks were present in our laboratories stock collection and were neither requested nor recombined to FRT by me.

mal-D$^{\Delta 7}$, *c522 Gal4* and mal-D$^{\Delta 7}$, *UAS-Actin-egfp* stocks that were used for generating mutant border cells for transcriptional profiling analysis were recombined by Kalman Somogyi.

Fly stock	Description	Source
mal-D ^{S2}	Amino acid replacement: Q675@	Pernille Rorth (Somogyi and Rorth, 2004)
mal-D ^{S9}	Amino acid replacement: L659@.	Pernille Rorth

		(Somogyi and Rorth, 2004)
mal-D ^{S5}	Amino acid replacement: Q736@.	Pernille Rorth (Somogyi and Rorth, 2004)
mal-D ^{F2}	Frameshift mutation at position A1364	Pernille Rorth (Somogyi and Rorth, 2004)
mal-D $^{\Delta 7}$	Imprecise excision of the P element deleting sequences from -257 to +1066 relative to the transcription start site.	Pernille Rorth (Somogyi and Rorth, 2004)
UAS mal-D-ΔN	UAS drive expression of a 5' truncated Mrtf protein starting at a.a. 171 immediately downstream of the three RPEL motifs.	Pernille Rorth (Somogyi and Rorth, 2004)
Tub mal-D	Tubulin promoter driving expression of Mal-D cDNA	Pernille Rorth (Somogyi and Rorth, 2004)
UAS-Actin-egfp	UAS drive expression of Actin-EGFP	Pernille Rorth (Fulga and Rorth, 2002)
slbo gal4	Gal4 driver downstream of *slbo* enhancer	Pernille Rorth (Rorth et al., 1998)
slbo ^{8ex2}	Deletion, null mutant of allele of *slbo*	Pernille Rorth (Rorth, 1994)
shg ^{R69}	Deletion, null mutant of allele of *shg*	Ulrich Tepass (Godt and Tepass, 1998)
chic ^{221}	Deletion, null mutant of *chic*	Bloomington
bs ^{14}	Amino acid replacement: Q102@. Mutation lies before MADS domain.	Markus Affolter (Fristrom et al., 1994)
UAS dia CA	UAS driving the expression of dia with the C-terminal a.a.1029-1091 (the predicted autoinhibitory domain) have been removed.	Simone Becarri (Beccari, 2003)
GawCc522 Gal4	Gal4 Driver Specific for border cells	Bloomington
hsFLP hsISCE-I	Stock required for excision of pW25 and creating double stranded breaks	Bloomington
dia ^{5}	Loss of function allele of *dia*	Bloomington
rho ^{72O}	Deletion removing translation start site	Bloomington
Defnap 8	Defficiency that removes region 41D2-42A7	Bloomington
if ^{b4}	Deletion that removes part of Integrin PSα2 resuting a suggested frameshift	Nick Brown (Brown, 1994)

5.2.2 GAL4/UAS system

Adapting yeast Gal4 transcriptional activator into fly made it possible to over-express any transgene that is cloned downstream of Upstream Activator Sequence (UAS) (Brand and Perrimon, 1993). Expression of Gal4 gene in a given enhancer makes it possible to have spatial control on gene expression (Figure 5.1).

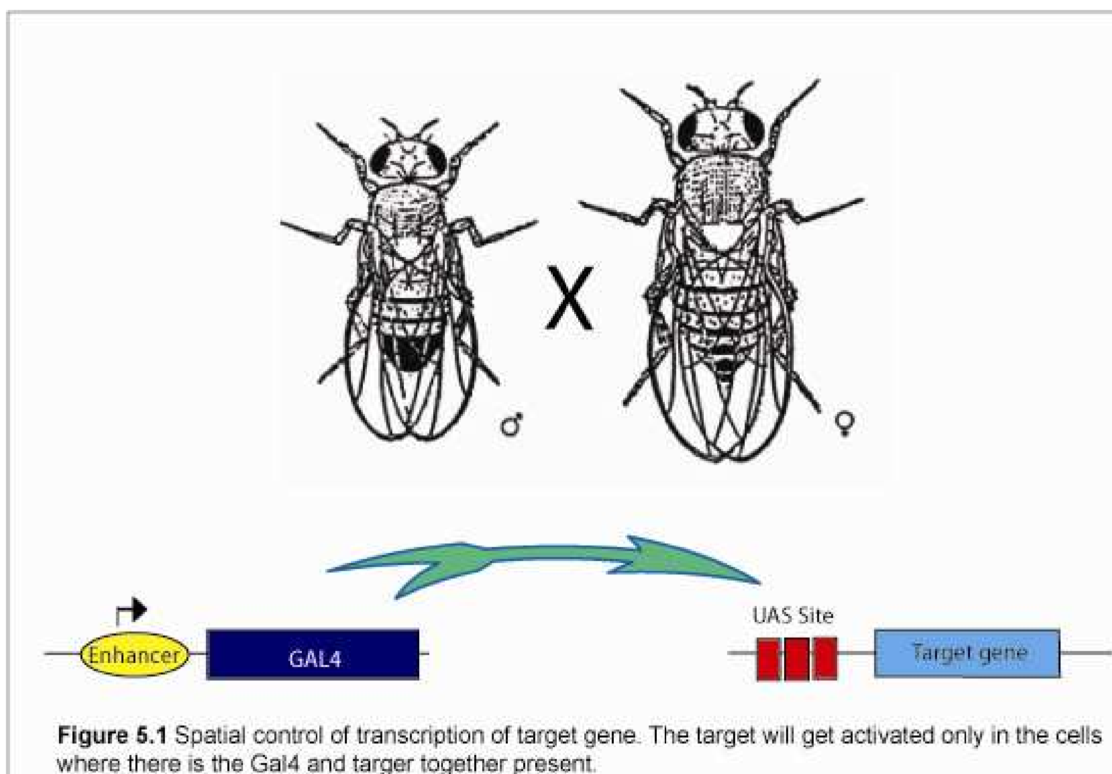


Figure 5.1 Spatial control of transcription of target gene. The target will get activated only in the cells where there is the Gal4 and targer together present.

Actin flipout Gal4 System is a modification of UAS Gal4 system that provides temporal specificity to the Gal4 UAS induction. In this system Actin promoter drives a cassette containing stop codon and that is flanked by FRT sites, followed by GAL4 gene. Expression of FLIP gene under heat shock promoter, by switching the flies to 37˚C for thirty minutes induces the flipping of the cassette containing the stop codon, thus permitting the expression of GAL4 gene. The flies that start the expression of the Gal4 gene are marked by the expression of GFP with UASGFP construct. This system provides temporally specific high over expression.

5.2.3 Generation of mosaic clones

In flies it is possible to induce cells homozygous for a mutation in an otherwise heterozygous animal FLP/FRT system was used (Golic, 1991). The mutant was

recombined onto a suitable FRT chromosome according to the genomic location of the mutation (Xu and Rubin, 1993). This generated FRTxx m chromosome (xx=19 for genes on 1st chromosome, 40 and 42 for genes in left and right arm of 2nd chromosome respectively and 80 and 82 for genes in left and right arm of 3rd chromosome respectively). Flies harboring FRTxx m could then be crossed to flies with hsFLP; FRTxx UbiGFP. UbiGFP encodes GFP ubiquitously in flies. The progeny of this cross has hsFLP; FRTxx UbiGFP/ FRTxx m genotype. If the progeny is subjected to heat shock in either larval or adult stages (see 4.2.1) it causes the induction of FLP gene which recognizes FRT sites and induces recombination between them. If this happens during mitosis some of the events result in the formation of a homozygous mutant daughter cell and a homozygous wild-type daughter cell, which can be discerned by GFP expression. Wild-type cell will have 2 copies of GFP, and thus have high GFP expression whereas mutant cell will have no GFP and will look devoid of GFP. (Figure 5.2)
Alternatively this system was used by losing not the GFP, but Gal80 which binds and neutralizes the action of Gal4, in a technique called Mosaic Analysis with A Repressible Marker (MARCM) (Lee and Luo, 1999). Using Tub Gal4, UAS GFP in this background gave possibility of marking the mutant cells with the presence of GFP.

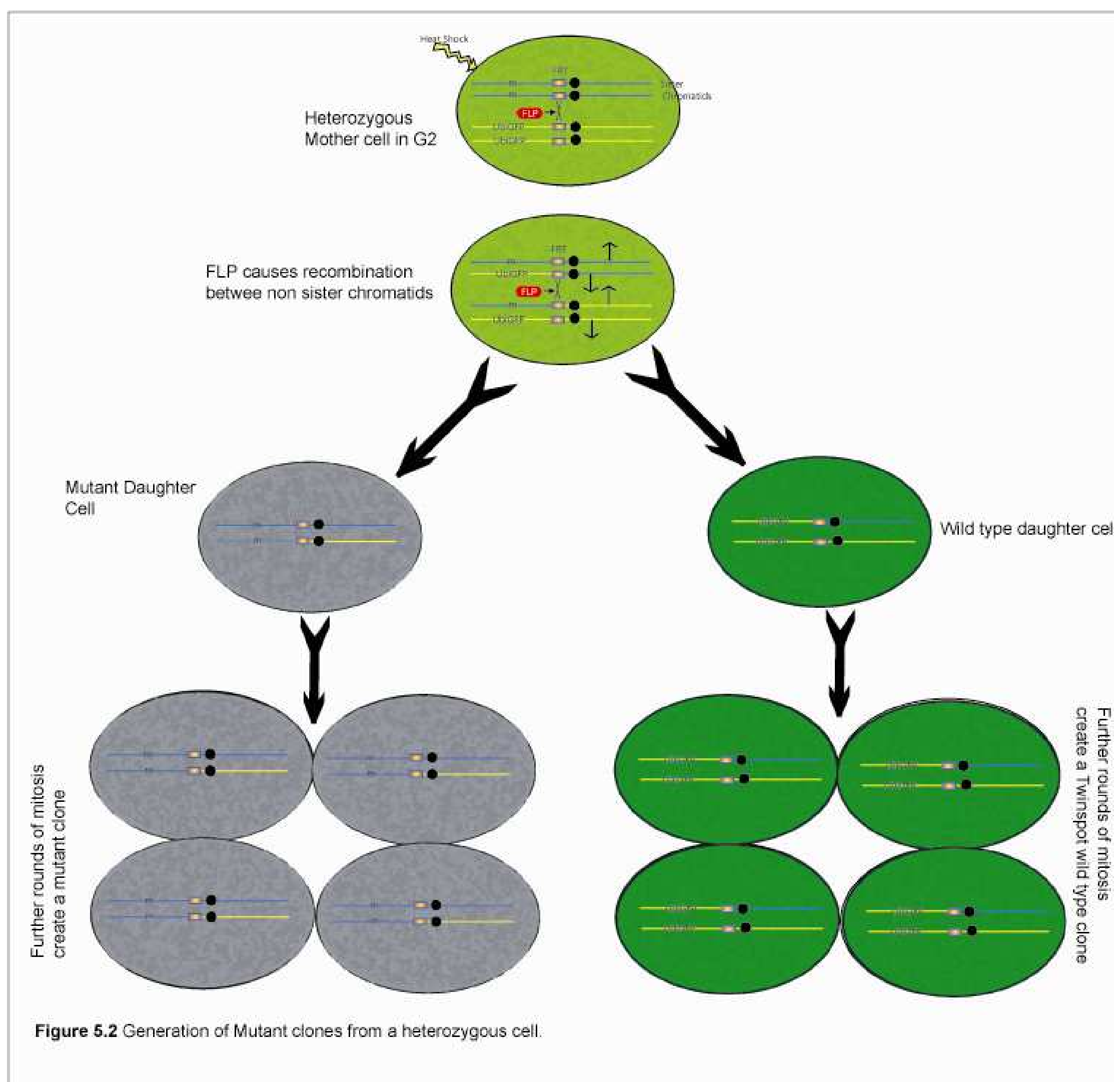


Figure 5.2 Generation of Mutant clones from a heterozygous cell.

5.2.4 Generation of Mal-D 9HA with homologous recombination

The homologous recombination technique in *Drosophila* aims to generate linear homology regions that flank White selection marker in order to generate homologous recombination and simultaneously mark the event by expression of White (Gong and Golic, 2003). Linear fragments are generated by the activity of FLP and I-SCEI enzymes. pW25 vector that was used for the homologous recombination, has two distal FRT sites that are positioned so that the induction of recombination between those sites causes excision of the construct from its genomic insertion site and generate a round plasmid. I-SCEI is a sequence specific endonuclease that cuts the plasmid resulting from the action of FLP, and generate double stranded breaks that in low frequency can attract the DNA

repair machinery and drive the formation of homogous recombination. Cloning of the pW25 vector was explained in section 4.1.2. Transgenic flies were generated with this vector. One transgenic line in first chromosome was obtained. This line is crossed to hsFLP,hsISCE-I, e/TM2 flies and the progeny was larval heat shocked. Virgins with mosaic eye color, because of the activity of hsFLP, from this cross that hatched form this cross that were heat shocked were collected and crossed with TM2/TM6 males, in about 150 single fly crosses. (Figure 5.3) This generated 9 independent events. Males resulting from those vials were crossed to TM2/TM6. 2 of those events mapped to 2nd chromosome that indicates non homologous jumps, since Mal-D is located in 3rd chromosome. 7 independent alleles were generated. I focused on one allele that was verified by PCR from the *white* gene embedded in the knock-in construct to the region flanking the homology arms with primer pairs MALDKI_UTR, pW25_F_to3'flank and MALDKI_EX, pW25_R_wto5'flank . (Figure 3.3)

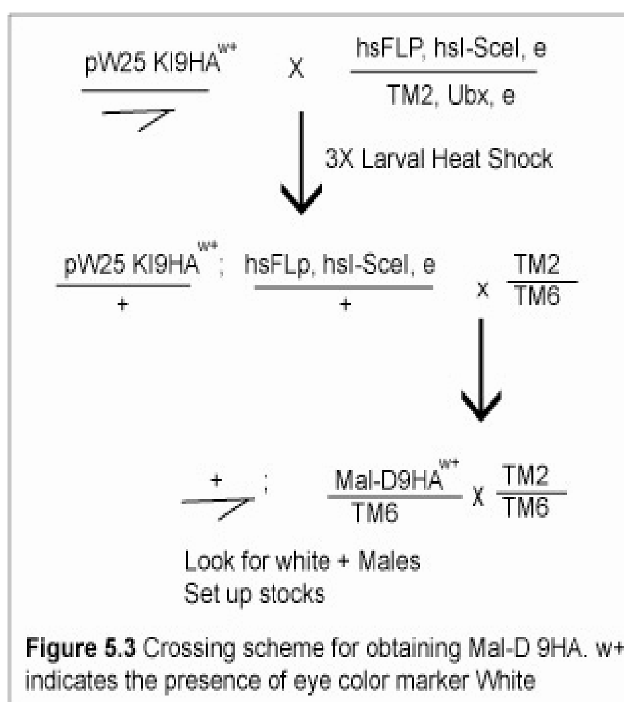


Figure 5.3 Crossing scheme for obtaining Mal-D 9HA. w+ indicates the presence of eye color marker White

Moreover the *white* cassette was excised from this line by using hs-Cre transgenic flies generated by Lodovica Borghese. *White* cassette in pW25 vector is flanked by LoxP sites which are targets of Cre site specific recombinase. Induction of Cre in the fly results in the excision of the *White* cassette. The region where *White* cassette was removed was PCRed and sequenced. Sequencing did not show any mutations in the coding region. It showed that removal of white cassette did not cause any mitations. It left one copy of the loxP site which was to be expected.

5.3 Staining protocols

5.3.1 X Gal staining

Ovaries were dissected in ice cold PBS and fixed for 10 minutes in 0.5% Glutaraldehyde. After 3 brief washes with PBS + 0.1 Triton X100 (PT) and a further wash of 30 minutes in PT, ovaries were incubated in staining buffer containing 0.4% X-Gal. Incubation was done at 37˚C in dark until the signal was apparent which was usually overnight for endogenous reporters and about 2-4 hours for reporters in Mal-D ΔN over expression conditions. Ovaries were then washed once with PBS and then mounted in 50% glycerol in PBS. Images were then taken with a digital camera attached to a Zeiss Axiopod microscope.

5.3.2 Phalloidin DAPI staining

Ovaries were dissected in ice cold PBS and fixed with 4% Paraformaldehyde (PFA) for 15 minutes on a rotator in room temperature. Ovaries were then briefly rinsed three times with PT and washed for an additional 30 minutes in PT. Then they were pipetted several times first with blue pipette tip and then yellow pipette tip in order to separate the egg chambers and remove them from muscle sheet. Ovaries were then incubated in PT with Rhodamine conjugated Phalloidin (1:500 from Molecular Probes), and DAPI (1μg/ml) for 1 hour in room temperature on a rotator in dark. After the incubation samples were washed twice with PT and twice with PBS. The samples were then mounted in 80% Glycerol in PBS containing 0.4% n-propyl gallate (NPG). All images were scanned with confocal microscopy.

5.3.3 Antibody staining

For detecting Mal-D9HA I used either mouse monoclonal Antibody (HA.11) or rat monoclonal antibody (3F10). Both of them gave similar results and staining patterns. For mouse anti HA the primary and secondary antibodies are preadsorbed by dissecting about

50-100 flies without the epitope, fixing and processing them as I would do for the Mal-D9HA samples (explained below) until the addition of antibody. Antibody was applied 1:25 in 500 µl overnight in blocking buffer at 4˚C on a rotator. Next day the preadsorbed antibody is recovered by centrifugation at 14000 Rpm of the readsorbing sample for 30 minutes at 4˚C and isolating the supernatant. Preadsorbed antibody would be used for 1:1000 final dilution (1:40 further dilution) on the samples to be stained. For stainings with either of the antibodies, secondary antibody (Cy5 conjugated anti mouse or anti rat antibodies from Jackson Scientific) was preadsorbed the same way as well. The final dilution for secondary antibody on the sample is 1:300 (1:12 further dilution).

For staining of Mal-D9HA samples I used a staining method optimized by Katrien Janssens. Samples were dissected in Grace's cold medium (Invitrogen) with 4% PFA in it for 10 minutes. Then ovaries were punctured with forceps in order to let the fixative enter the sample for 5 minutes and samples were incubated on a rotator at room temperature for 15 minutes. After fixation samples were rinced briefly three times with washing buffer (WB) (50mM Tris-HCl pH 7.4 and 150 mM NaCl (TBS), 0.1% NP-40 (Igepal), 1mg/ml Bovine Serum Albumin (99% purity, Sigma Aldrich)) The washing buffer was prepared fresh each day for dissections. After three rinses samples were washed 30 minutes in WB. Then samples were pipetted up and down several times in order to dissociate them. Then they were blocked on blocking buffer (BB)(same as WB except for 5mg/ml BSA rather than 1mg/ml) for 30 minutes. Primary antibody was added overnight at 4˚C on a rotator. The next day samples were washed 4 times 30 minutes in WB. Then blocked again 15 minutes in BB. Secondary antibody was then applied along with Rhodamine-Phalloidin (1:500) and DAPI (1µg/ml) for 2 hours in dark at room temperature on a rotator. At the end of incubation with secondary antibody sample was washed 4 times 10 minutes in WB and rinsed twice with WB and twice with TBS and mounted in 80% Glycerol in PBS containing 0.4% n-propyl gallate (NPG).
Guinea pig α-Achi antibody was used 1:1000 (Gift from Richard Mann), Rabit α-Sty antibody was used Rabit 1:1500 (Gift from Mark Krasnow), α βGal antibody (Cappel) was used 1:1000, Rabit α SCF (Gift from Susumu Hirose) antibody was used 1:300. 2˚ antibodies were Cy5 conjugated antibodies against the species IG chains (Jackson Scientific)

5.3.4 In situ Hybridization

In situ hybridization method was optimized by Georgina Fletcher and Juliette Mathieu (Borghese et al., 2006). DGRC Genomic clones for CG30440 (LD43457), CG10966 (rdgA, GH23785), CG9623(if, GM12416), CG1921(sty, RH67029), CG3217 (LD32354) and CG31015 (prolyl-hydroxylase 4, RE70601) were obtained from Genomics Core Facility. 6µg of plasmids were digested with 3µl of SalI and BglII in the case of CG30440, MunI and BglII in the case of rgd A, BglII in the case of if, XhoI and NotI in the case of sty, HindIII and BglII in the case of CG3217, HindIII and NotI in the case of Prolyl-hydroxylase4 in order to generate linearized constructs to use in probe synthesis. Linearized DNA was isolated using phenol chloroform extraction and precipitation.
1 µg of linearized DNA was mixed with 2.5 µl 10X DIG (Roche), 2.5 µl 10X buffer (Roche, preheated to 37 ˚C) 1µl Rnase block and 1.5 µl polymeraze (Sp6 for CG30440, rgd A, if and CG3217 and T3 (Roche) for sty and Prolyl-hydroxylase) and filled up to 25 µl with DEPC H_2O. This mixture was incubated at 37 ˚C for 3 hours. At the end of 3 hours 1µl of RNAse free DNAse (Quiagen) was added on the reactions to stop them and they were incubated for another 45 minutes in 37 ˚C. RNA probe was precipitated by using LiCl (Ambion) precipitation method. RNA was resuspended in 20 µl 2X SSC 50% formamide. One day prior to hybridization day preHybridization buffer was prepared. 10% Boehringer Block (BB) was thawed along with torula RNA in a separate tube. 5ml of 10% Boehringer Block, 25 ml Formamid, 12.5 ml 20XSSC (3 M NaCl; 0.3 M Na-citrate), 1ml DEPC H_2O, 5 ml Torula RNA (10mg/ml), 100 µl Heparin (50 mg/ml), 250 µl 20% Tween, 500µl 10% CHAPS, 500µl 0.5 M pH8 EDTA were mixed to generate preHybridization buffer.

Flies were dissected in DEPC PBS on ice, and fixed 20 minutes with DEPC PBS+ 4%PFA + 0.1% Tween. Samples were 4 times rinsed with DEPC PBS+ 0.1% Tween. Samples were incubated with 100% MeOH at -20 ˚C for 1.5 hours. Then ovaries were rehydrated by sequential addition of buffers with decreasing MeOH concentrations (75%, 50%, 25% and last 2 washes with PBS Tween) 5 minutes for each wash. After washes 10 µg/ml Protease K in PBS was applied 8 minutes in room temperature. Samples were washed twice with PBS + 0.1% Tween and refixed for 20 minutes with PBS + 0.1%

Tween. Samples were washed 5 times with PBS + 0.1% Tween. Meanwhile preHybridisation buffer was placed in 65 ˚C water bath. 300 µl of preHybridization buffer was added on samples and they were incubated 1.5 hours in 65 ˚C water bath. 1.5 µl probe per sample was added at the end of incubation. Samples were incubated overnight in 65 ˚C water bath. On second day samples were washed at 65 ˚C 2 times 30 minutes with 50% Formamide (FA), 5XSSC, 0.1% CHAPS, once 15 minutes with 2XSSC, 01.% CHAPS, 2 times 30 minutes 0.2 X SSC, 0.1% CHAPS. Then samples were passed to room temperature and washed 3 times 5 minutes with MAB + 0.1%Tween (1 mM maleic acid, 1.5 mM NaCl pH 7.5). Samples were blocked with 5% BB in MAB + 0.1% Tween for 1 hour. α-Dig antibody was applied in MAB + 0.1% Tween in 1:4000 dilution for 2 hours. Samples were washed 6 times 20 minutes with MAB+ 0.1% Tween. Meanwhile fresh AP buffer was prepared (100mM Tris-HCl, 100mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, pH 9.5). Samples were washed 3 times with AP buffer and stained in BM Purple 1:1 in AP buffer. Samples were incubated until signal develops.

5.4 Microarray experiments

5.4.1 Isolation of mutant larvae

Heterozygous mutant mal-D $^{\Delta 7}$ (recombined to UAS-*Actin*-GFP or c522-Gal4) virgins and males were crossed in large cages. In the progeny only homozygous mutant larvae would have both the driver and UAS construct and have green signal in the salivary glands due to the leakiness of Gal4 driver (Figure 5.4). Plates were collected overnight and kept one day at 18˚C to let the embryos develop to 1st instar stage. Then embryos were collected by using meshes and were put in 2% Tween 20 in PBS. GFP positive population was sorted by using Union Biometrica COPAS embryo sorter 500 events/ fly bottle (Figure 5.5). Daily extinction, time of flight and parameters were set each day and tested by sorting 20 larvae on a slide that was observed under microscope for expected GFP expression.

This method gave good purity and yield of mutant flies to dissect 10 days later when the pupae hatch.

I collected the flies that are 3 days old and dissected them. All the flies resulting from mutant collection had kinked bristles, serving as an internal control, showing the flies were homozygous mal-D$^{\Delta 7}$.

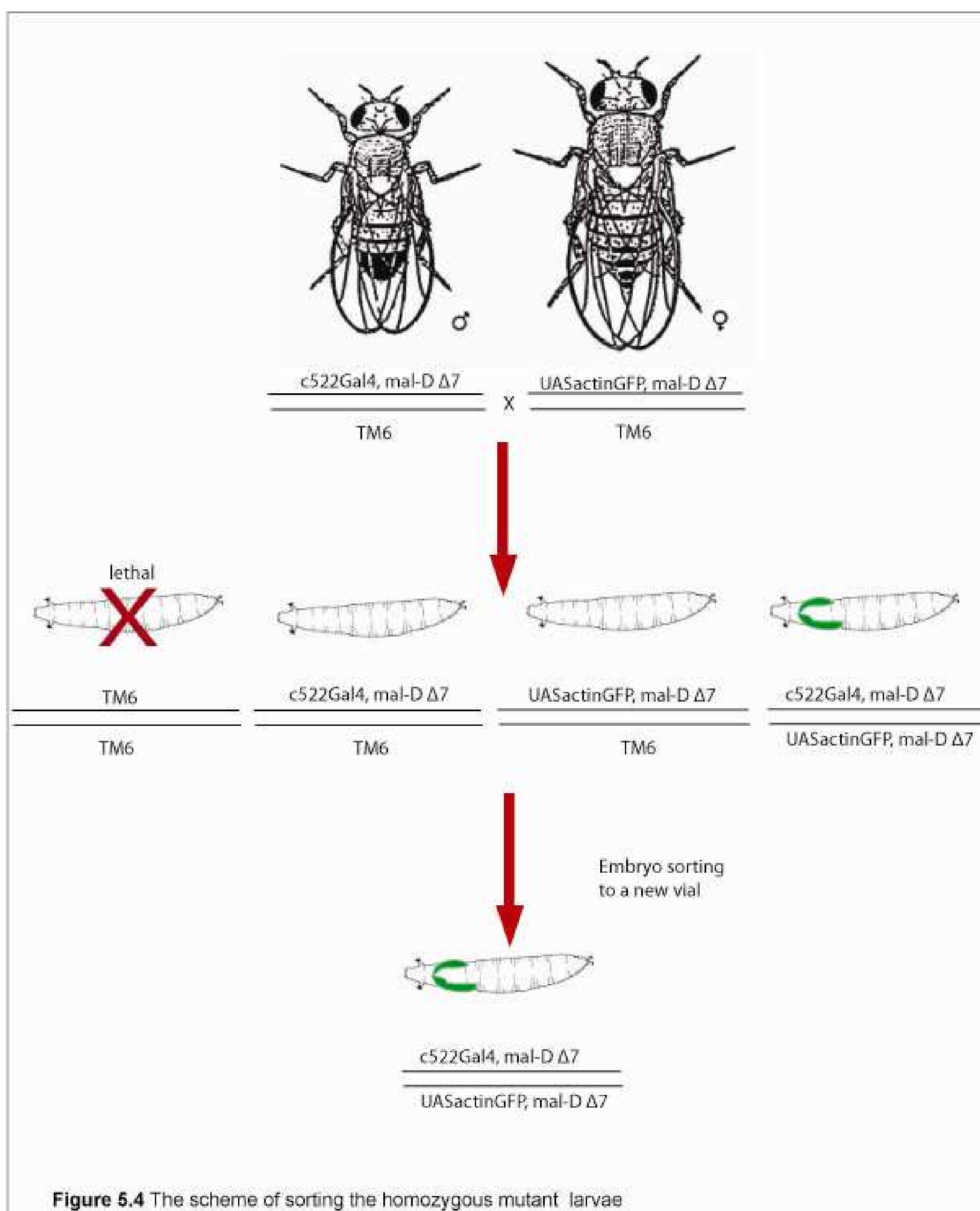


Figure 5.4 The scheme of sorting the homozygous mutant larvae

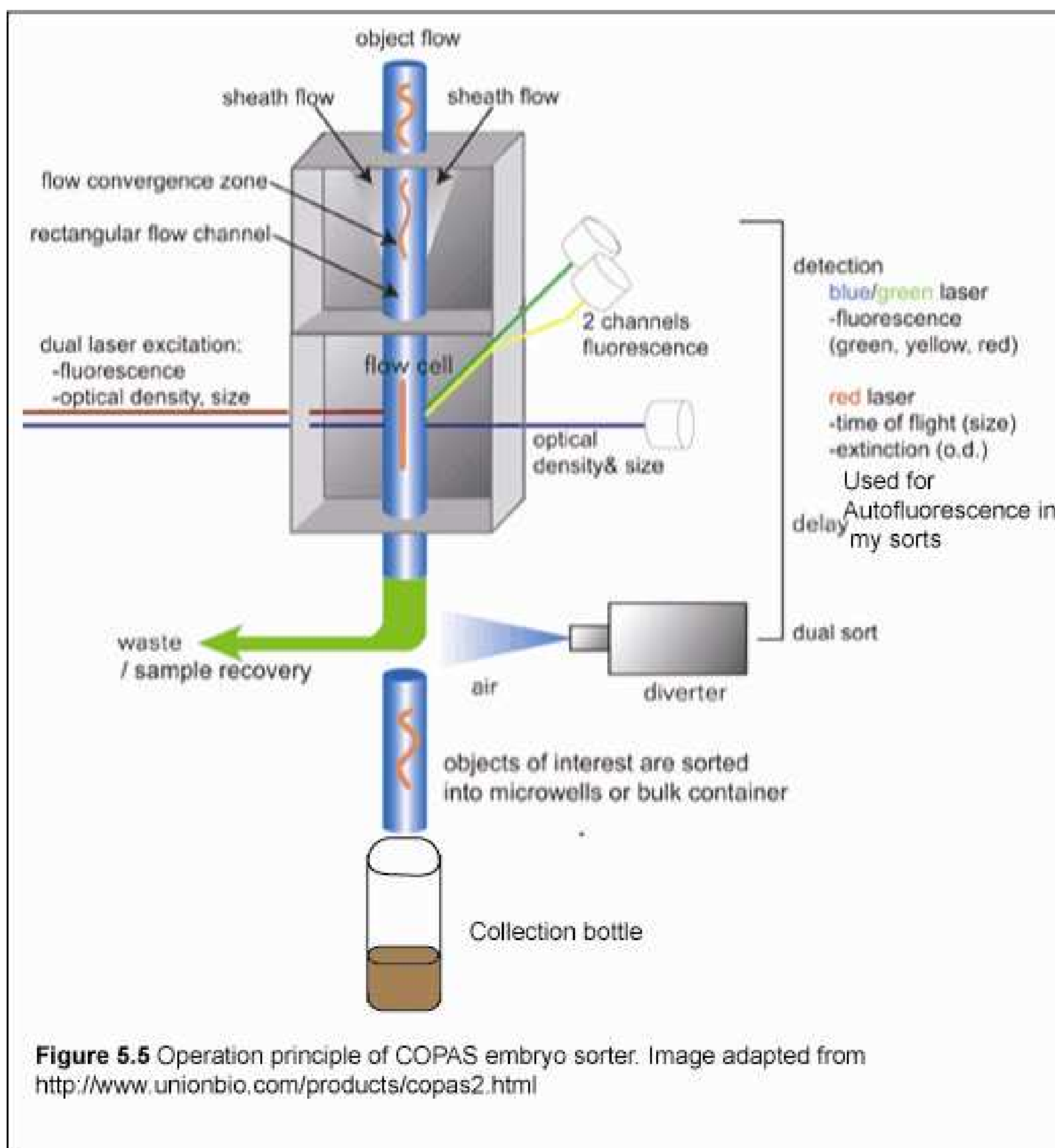


Figure 5.5 Operation principle of COPAS embryo sorter. Image adapted from http://www.unionbio.com/products/copas2.html

5.4.2 Dissection

Ovaries from 3-4 day old females incubated for 18 hours at 25˚C were dissected on ice, in cold Serum Free Medium (SFM) (GIBCO-Invitrogen). 200 females were dissected in one hour. Ovaries were then dissociated by 30 minutes of incubation in 0.9 ml of Trypsin/ EDTA (0.5%) (Sigma) + 0.1 ml of a collagenase solution in PBS (67 mg/ml). Sample was shaken every 2 minutes in order to increase dissociation efficacy. Supernatant was filtered through a nylon mesh with 62 µm grid size (Small Parts Incorporated) into tubes containing ice cold SFM + 10% FCS to a final volume of 1 ml. Cells were pelleted by centrifugation at 1300 RPM for 7 minutes and a 7 second short

spin. Pellet is resuspended in SFM + 10% FCS and kept on ice until start of sorting protocol.

5.4.3 Fluorescently Activated Cell Sorting (FACS)

The protocol to use FACS for obtaining border cells was optimized by Lodovica Borghese. The same settings were applied by Andrew Riddell in the Flow Cytometry Core Facility. Maximum of 4 sorts per day were performed.

The GFP Border cells from clusters that resolve as brightly fluorescing population in the flow Cytometer. The overall pressure was kept low with the assumption that it will maintain the cells as clusters during the sorting procedure.

The Assay was performed on a DAKO MoFlo Flow Cytometer (Dako GmbH, Hamburger Str. 181,22083, Hamburg) with Enrich 1.0 sort mode.
The primary laser was a Coherent Innova 90-6 argon ion laser (Coherent Inc., 5100 Patrick Henry Drive, Santa Clara, CA 95054 USA) tuned to 488nm. The aperture was set at 5. The beam quality was checked visually by expanding the beam with a 10X microscope objective and projecting the beam onto a wall. TEM_{00} mode was observed. The beam was carefully aligned, using an in-house alignment tool, to the MoFlo's primary optical path. The stream was carefully aligned to be perpendicular to the beam at the laser intercept point. A medium width obscuration bar was used in wide-angle light capture. The optical path was then optimised using FLOW-CHECK beads (Beckman Coulter Inc Fullerton, CA 92835 Cat No.6605359).
Low background noise from sheath is important. Becton Dickinson FACS Flow sheath was used (Becton Dickinson GmbH, Tullastrasse 8-12, 69126, Heidelberg, Germany, – Cat No 322003-). It was filtered in-line through a PALL Fluorodyne II filter O.2µm (Part No. MCY4463DFLPH4).

The sample rate was approximately 100-1000 events/sec^{-1}. The differential pressure was low to confine the border cells in the centre of the co-axial flow.

The data was analysed using DOKO Summit software.

5.4.4 Total RNA extraction from sorted border cell collections

Total RNA was isolated by using PicoPure RNA isolation kit (Arcturus, Mountain View, CA) according to manufacturers protocols. Briefly the border cells were directly sorted in an eppendorf tube containing the lysis buffer of the kit at a 1:4 volume of buffer. After the sort the sorted volume was estimated and if needed was readjusted to have 1:4 ratio. The collected samples in lysis buffere were heated at 42 ˚C for 30 minutes. The final extract was kept frozen at -80˚C until the time of pooling of different collections to obtain enough material. Pooling was done with approximately 10000 border cell cluster events (580 flies dissected) for wild-type and 12500 border cell cluster events for Δ7 (700 flies dissected) for the first array, 13400 border cell cluster events (560 flies dissected) for wild-type, 17500 events (719 flies dissected) for Δ7 for second array, and 30000 for Δ7 (1043 flies dissected), 24000 events (1100 flies dissected) for wild-type for the last array. Events correspond to single border cells or clusters of cells. Dissociation rate may change from day to day which gives difference in the number of flies dissected to number of events collected. RNA purification was performed according to the protocol of the kit. The optional DNAse treatment was performed. Total RNA preps were eluted with 11 µl of Elution Buffer and kept at -80˚C. 0.7 µl of the sample was always used to measure quantity of RNA and 0.3 µl was used to assess the quality of the RNA with bioanalyzer tool. Remaining 10 µl of the sample was used for the hybridizations on Drosophila Genome 2.0 Arrays.

5.4.5 Assessing the quality and quantity of the RNA

RNA quantity was measured by using Quant-it reagent kit (Molecular Probes) according to manufacturers instructions with a Tecan Fluorometer (Tecan Group, Switzerland) in 96 well plates (Thermo Labsystems, Finland). Briefly a standart curve was constructed by using the RNA that is provided in the kit and the quantity of the RNA was determined by comparing the signal of the sample to the corresponding point in the linear standard curve.

The quality of RNA was measured by using Bioanalyzer according to manufacturers instructions. This step is performed by Tomi Ivacevic in Genomics Core Facility. Bioanalyzer (Agilent Technologies, Palo Alto CA) is a very sensitive apparatus that can test the integrity of RNA in as low amounts as picograms.

5.4.6 Linear RNA amplification and labeling with Biotin

Two cycle linear RNA amplification and labeling was conducted in Genomics Core Facility by Tomi Ivacevic by following the manufacturers protocols for GeneChip Expression Arrays (Affymetrix, Santa Clara, CA). Genechip 2 cycle cDNA synthsis kit claims to amplify RNA in linear way starting from total RNA amounts between 10-100 ng. Starting total RNA amounts were 43.2 ng wild-type, 39.6 ng Δ7 for the first array, 45 ng wild-type, 37 ng for Δ7 for second biological repeat and 50 ng for both samples for third biological repeat. The steps that are carried out by using the kit are summarized in figure 5.6.

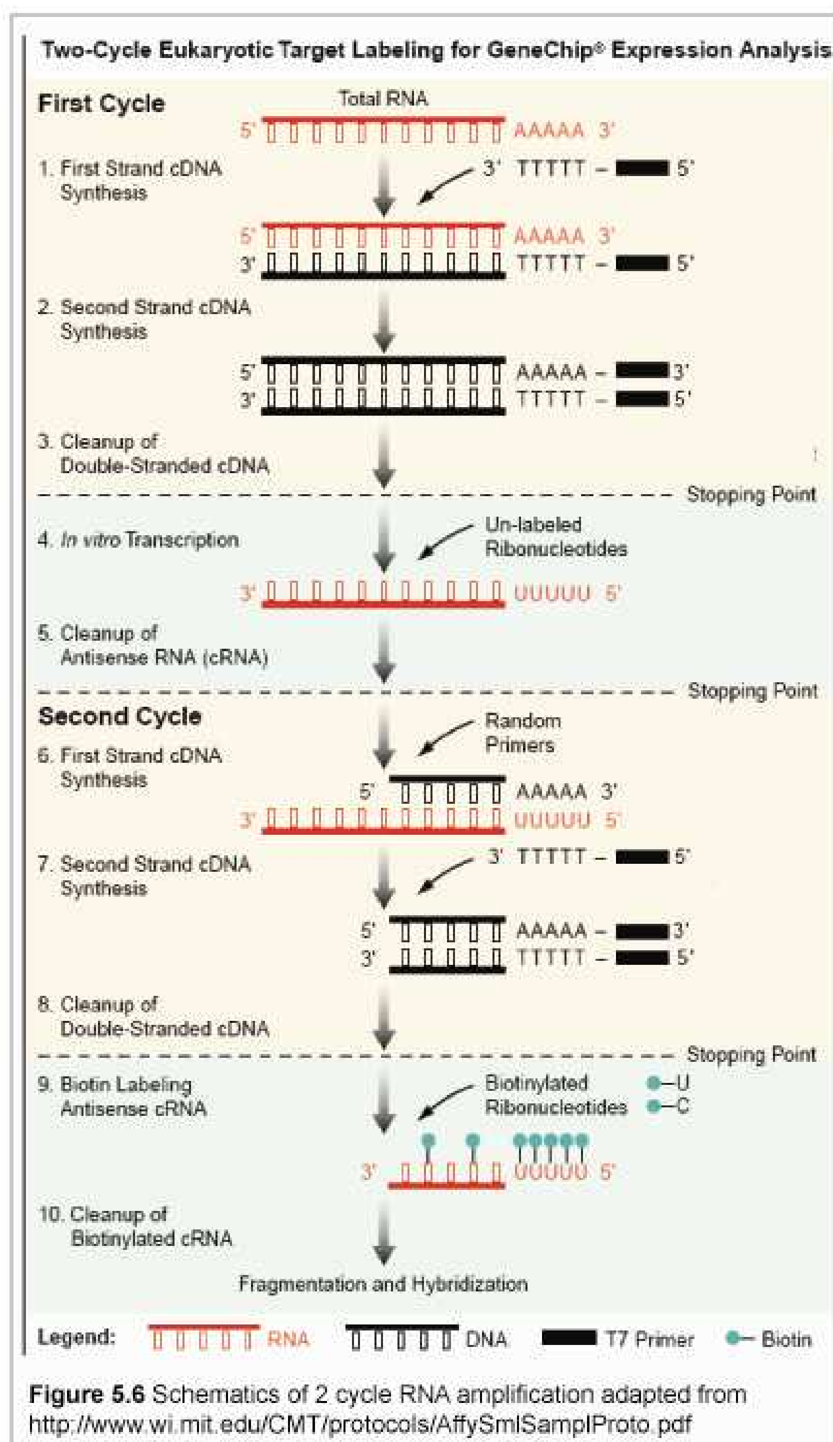


Figure 5.6 Schematics of 2 cycle RNA amplification adapted from http://www.wi.mit.edu/CMT/protocols/AffySmlSamplProto.pdf

The quantity and quality of Biotinylated RNA was assessed by using UV-Spectrometer and Bioanalyzer respectively by Tomi Ivacevic. 10 µl of fragmented, labeled RNA was placed in a hybridization cocktail and hybridized to Affymetrix Drosophila Genome 2.0 Array. Those arrays contain 18880 probe sets, measuring expression of about 18500.

Each probe set contains 14 Perfect Match, Mismatch probe pairs that are used to detect the level of abundance of a given RNA.

Images of each scanned chip were processed with the default settings of GeneChip Operating Software 1.4 (GCOS). Raw data was normalized over all the probe sets and converted to numerical data sets that were used to assign a Present, Absent or Marginal flags on each transcript. Genespring GX Software (Agilent) was used in order to analyze the data set and detect samples that were differentially regulated.

5.5 Tissue Culture

5.5.1 General Maintenance

S2 Schneider cells, which are hemocyte derived cells, were kept in flasks in SFM supplemented with 100 U/ml Penicilin-Streptomycin (Gibco) 2 mM L-Glutamine (Gibco) Cells were splitted in every 10 days by diluting 1 to 1 with fresh medium. Cells were incubated in 25 ˚C cell incubator.

5.5.2 Transfection

Transient transfections were conducted by using Lipofectin reagent (Gibco) following the supplied protocol. Briefly confluent cells from a flask were mixed 1:1 with fresh medium and plated on 6 well plates 4 ml per well. Cells are let to adhere for 1 hour. Meanwhile Plasmids were prepared by mixing them with 60 µl of SFM per sample. In a separate tube 60 µl SFM/ Sample was mixed with 1/10 of Lipofectin. The content of second tube waas added 60 µl/ sample to the content of the other tube and DNA was incubated for 30 minutes to form complexes with Lipofectin. After the incubation medium was washed away and cells were washed once with medium without antibiotics. 480µl SFM without antibiotics was added on the DNA Lipofectin mixture to make the total volume 600µl per sample. This mixture was applied on the cells. Cells were incubated 6 to 8 hours in this mixture and then medium with antibiotics was added on cells. Cells were left overnight for recovery. The following day induction was done with 700 µM final concentration $CuSO_4$ for 12 hours.

5.5.3 β Gal Activity read out

After 12 hours of induction of the constructs cells were scraped and pelleted by centrifugation at room temperature at 5000 RPM for 1 minute. Pellet was resuspended in cold 300 μl / sample Grinding Buffer (0.1 M Tris pH 7.8, 0.1 mg/ml BSA, 1 mM DTT, 0.03% sodium deoxycholate (Sigma)) Samples were vortexed in order to resuspend them and left on ice for 10 minutes. 50 μl of this mixture was taken for Western Blot analysis. The rest was spinned 5 minutes at 14000 RPM at cold room. 50 μl of supernatant was mixed with 750 μl PM2/ONPG(39 mM NaH_2PO_4, 60 mM Na_2HPO_4, 3mM $MgSO_4$, 2mM EDTA, 0.2 mM $MgCl_2$, 2mg/ml ONPG (Sigma), 100 mM β mercaptoethanol) for the detection of β Gal activity. The reaction was stopped with the addition of 250 μl 1M Na_2CO_3.

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Appendix

List of Genes that were more than 2 Fold Down regulated in mal-D $^{\Delta 7}$ border cells in all repeats

Gene Name	Common	Transcript ID_Affymetrix	WT Averge	D7 Average	Average Change
1627869_at	Ser4	CG8867-RA	3471.3	578.9	6.0
1638872_at	Hsp68	CG5436-RA	3065.8	1039.2	3.0
1635125_a_at	CG6206	CG6206-RA	2873.2	871.4	3.3
1639196_at	CG8869	CG8869-RA	2246.0	490.0	4.6
1636040_at	CG10527	CG10527-RA	2076.2	200.7	10.3
1623364_at	CG4250	CG4250-RA	1956.6	461.1	4.2
1635819_at	sty	CG1921-RC	1861.2	683.8	2.7
1628840_at	ken	CG5575-RA	1802.2	597.1	3.0
1634186_a_at	CG17119	CG17119-RA	1600.8	372.3	4.3
1629476_at	CG7542	CG7542-RA	1570.8	214.5	7.3
1630258_at	GstD2	CG4181-RA	1554.5	414.6	3.7
1639036_at	CG8857	CG8857-RC	1457.3	518.6	2.8
1623027_s_at	CG6277	CG6277-RA	1384.0	111.7	12.4
1634240_at	CG5107	CG5107-RA	1313.5	324.0	4.1
1624506_at	CG8871	CG8871-RA	1228.0	124.4	9.9
1624067_at	CG6704	CG6704-RA	1153.5	488.1	2.4
1630291_at	MtnB	CG4312-RA	1146.6	281.0	4.1
1627088_at	fit	CG17820-RA	1105.5	148.1	7.5
1636762_a_at	scf	CG9148-RA	1072.5	292.6	3.7
1640170_at	CG10311	CG10311-RA	1053.0	342.9	3.1
1630842_s_at	CG32641	CG32641-RA	1007.5	285.1	3.5
1632974_s_at	CG30015	CG30015-RA	967.3	357.3	2.7
1629843_s_at		CG2257-RA	946.5	406.3	2.3
1629313_at	CG8661	CG8661-RA	939.6	113.2	8.3
1639256_at	CG5676	CG5676-RA	881.2	303.4	2.9
1636800_at	CG13610	CG13610-RA	852.5	313.9	2.7
1629844_s_at	rap	CG3000-RA	792.4	295.5	2.7
1638929_at	CG8920	CG8920-RA	710.2	197.2	3.6
1635677_a_at	sty	CG1921-RB	707.1	162.6	4.3
1636174_at	GstD9	CG10091-RA	688.5	245.6	2.8
1630051_at	Tsp42Ec	CG12847-RA	661.0	111.8	5.9
1640329_s_at	CG6391	CG6391-RA	651.5	248.1	2.6
1641326_at	CG30118	CG30118-RA	647.9	206.5	3.1
1626011_at	CG17834	CG17834-RB	620.7	273.2	2.3
1632530_s_at	CG8776	CG8776-RD	618.9	121.5	5.1
1636240_at	CG7678	CG7678-RA	571.4	90.1	6.3
1633763_at		CG2855-RA	533.9	169.2	3.2
1639262_at	CG11669	CG11669-RA	525.4	59.1	8.9
1634549_at	CG11750	CG11750-RA	509.8	190.2	2.7
1633532_at	CG5767	CG5767-RA	508.2	127.4	4.0
1625985_at	CG31446	CG31446-RA	474.8	68.7	6.9
1641634_at	Lsp2	CG6806-RA	449.6	66.3	6.8

1626147_s_at	CPTI	CG12891-RA	427.1	107.1	4.0
1638377_x_at	CG30025	CG30025-RA	423.1	109.4	3.9
1633946_at	CG31955	CG31955-RA	403.3	47.8	8.4
1625332_at	CG14764	CG14764-RA	395.3	120.3	3.3
1634012_at	CG5002	CG5002-RA	392.0	90.1	4.4
1630187_a_at	CG6299	CG6299-RB	391.8	151.1	2.6
1625249_at	ld14	CG12664-RB	391.7	142.1	2.8
1635343_a_at		CG3217-RA	379.5	82.5	4.6
1628446_at	MtnC	CG5097-RA	373.6	80.4	4.6
1639306_s_at	CG17090	CG17090-RB	368.8	49.5	7.5
1630411_at	CG9945	CG9945-RB	367.2	129.6	2.8
1636764_at	CG31075	CG31075-RA	363.6	87.8	4.1
1629842_at	Gap1	CG6721-RB	362.9	141.1	2.6
1631555_at	CG10062	CG10062-RA	360.3	36.8	9.8
1633849_at	CG31559	CG31559-RA	358.1	140.7	2.5
1624793_at	GstD7	CG4371-RA	356.8	144.3	2.5
1635007_at	Sulf1	CG6725-RA	351.9	153.1	2.3
1627040_at	CG1344	CG1344-RA	349.1	95.1	3.7
1629625_at	CG13211	CG13211-RA	348.4	136.5	2.6
1623425_at	Buffy	CG8238-RA	340.0	107.6	3.2
1633294_at	CG2812	CG2812-RA	338.4	78.0	4.3
1636976_at	CG5322	CG5322-RA	323.3	41.3	7.8
1640386_at	wbl	CG7225-RA	321.6	96.8	3.3
1641554_at	CG16728	CG16728-RA	319.1	128.7	2.5
1634125_at	CG30440	CG30440-RA	317.5	44.5	7.1
1632461_at	CG31233	CG31233-RA	317.0	43.4	7.3
1627284_at	PH4&agr;PV	CG31015-RA	315.9	82.4	3.8
1628628_at	CalpA	CG7563-RB	311.3	122.1	2.5
1631962_at	MtnD	CG33192-RA	296.8	67.1	4.4
1639597_at	Obp44a	CG2297-RA	276.1	105.0	2.6
1635518_at	nonA	CG4211-RA	269.8	100.6	2.7
1640002_at	CG4586	CG4586-RA	239.1	83.2	2.9
1627405_at	CG14966	CG14966-RA	236.4	93.9	2.5
1640896_at	CG4462	CG4462-RA	230.1	51.0	4.5
1639469_a_at	Pu	CG9441-RB	226.2	83.0	2.7
1640217_at	CG30154	CG30154-RA	223.7	82.6	2.7
1641578_at	argos	CG4531-RA	223.3	53.9	4.1
1628655_at	CG4476	CG4476-RB	216.6	54.9	3.9
1631834_at	CG31098	CG31098-RA	216.5	81.6	2.7
1640236_at	CG4325	CG4325-RA	216.1	71.2	3.0
1626253_at	GstD4	CG11512-RA	212.8	78.0	2.7
1630218_at	CG13227	CG13227-RA	206.1	60.9	3.4
1624914_at	CG8690	CG8690-RA	203.9	55.4	3.7
1632372_at	CG31076	CG31076-RA	199.2	65.5	3.0
1625017_at	CG3732	CG3732-RA	198.9	57.0	3.5
1636287_at	CG10592	CG10592-RA	196.7	35.5	5.5
1638051_at	CG17323	CG17323-RA	189.0	69.7	2.7
1637420_a_at		CG6706-RA	182.7	52.4	3.5
1641232_s_at	CG6999	CG6999-RA	163.4	49.3	3.3
1630725_at	CG14572	CG14572-RA	162.5	24.3	6.7
1634213_at	CG13139	CG13139-RA	161.8	57.9	2.8
1636826_at	CG14072	CG14072-RA	155.9	5.4	28.9

1639454_at	CG10912	CG10912-RA	153.5	54.3	2.8
1629694_at	Pabp2	CG2163-RB	149.9	56.1	2.7
1625275_at	CG32037	CG32037-RA	146.4	45.6	3.2
1641327_at	CG9416	CG9416-RA	146.0	25.4	5.7
1638730_at	Hsp67Ba	CG4167-RA	145.3	34.6	4.2
1637534_at		CG11430-RC	143.8	37.0	3.9
1641646_at	CG5677	CG5677-RA	138.1	43.5	3.2
1634076_at	CG12057	CG12057-RA	137.4	17.6	7.8
1625366_at	rst	CG4125-RA	135.6	35.0	3.9
1637644_at	CG13813	CG13813-RA	134.7	41.2	3.3
1634139_at	Cyp301a1	CG8587-RA	133.1	11.7	11.4
1637660_at	CG5150	CG5150-RA	127.2	29.9	4.3
1628699_at	Lsp1&bgr;	CG4178-RA	126.8	27.6	4.6
1627834_a_at	aret	CG31762-RD	126.2	27.3	4.6
1635446_at	CG15043	CG15043-RA	121.5	36.0	3.4
1632744_a_at	if	CG9623-RB	120.5	33.0	3.7
1624448_at	CG6356	CG6356-RA	112.0	20.8	5.4
1640279_at	CG31869	CG31869-RA	110.7	36.7	3.0
1639110_at	CG4484	CG4484-RA	107.6	19.3	5.6
1632070_at	Ugt58Fa	CG4414-RA	105.3	31.7	3.3
1636664_at	CG1077	CG1077-RA	104.8	15.5	6.8
1638811_at	Sug	CG7334-RA	103.5	33.4	3.1
1635769_at	CG8773	CG8773-RA	101.1	16.2	6.2
1636906_s_at	CG13320	CG13320-RA	100.5	24.0	4.2
1630441_at	CG12716	CG12716-RA	99.9	9.7	10.3
1638592_at	CG6560	CG6560-RA	91.5	37.5	2.4
1628393_at	CG10039	CG10039-RA	87.9	26.5	3.3
1627946_at	CG12068	CG12068-RA	76.4	24.6	3.1
1629566_at	CG8834	CG8834-RA	75.7	9.7	7.8
1641671_at	CG2183	CG2183-RA	75.6	20.3	3.7
1625325_s_at	CG32067	CG32067-RB	75.1	7.4	10.2
1639903_at	Ser6	CG2071-RA	74.4	15.3	4.9
1636970_at	CG9394	CG9394-RA	70.7	14.2	5.0
1630124_at	CG5514	CG5514-RA	67.1	24.0	2.8
1629264_at	CG13025	CG13025-RA	64.2	24.1	2.7
1627020_at	CG11110	CG11110-RA	61.2	18.6	3.3
1625493_at	HLHm7	CG8361-RA	61.2	15.0	4.1
1639098_s_at	CG2837	CG2837-RB	61.2	10.0	6.1
1635674_at	CG6901	CG6901-RA	59.2	10.7	5.5
1634239_at	CG14205	CG14205-RA	59.2	8.2	7.2
1641039_at	CG1397	CG1397-RA	58.4	8.6	6.8
1641349_at	rdgA	CG10966-RA	55.6	18.4	3.0
1636460_at	CG10475	CG10475-RA	55.2	1.9	29.6
1629362_at	mthl8	CG32475-RA	54.1	3.7	14.5
1632582_at	CG9270	CG9270-RA	53.9	7.5	7.2
1633047_at	CG1809	CG1809-RA	51.6	13.5	3.8
1624704_at	CG4741	CG4741-RA	51.0	18.9	2.7
1632360_s_at	CG31038	CG31038-RB	49.7	11.1	4.5
1637462_at	CG13833	CG13833-RA	49.5	9.1	5.4
1631016_at	CG32822	CG32822-RA	46.2	10.7	4.3
1628503_at	CG30424	CG30424-RA	45.1	17.2	2.6

1636865_at		CG3250-RA	43.2	15.8	2.7
1632400_at	CG3841	CG3841-RA	39.3	8.4	4.7
1638211_at	CG10933	CG10933-RA	37.5	7.8	4.8
1629201_at	CG5550	CG5550-RA	35.3	6.9	5.1
1630768_s_at	vis	CG8821-RA	34.5	13.3	2.6
1630333_at	CG13338	CG13338-RA	28.6	2.2	12.8
1628656_at	CG12654	CG12654-RA	28.6	9.0	3.2
1631165_at	CG4688	CG4688-RA	26.4	1.8	14.4
1632531_at	CG5770	CG5770-RA	26.2	5.8	4.5
1636393_at	CG15005	CG15005-RA	23.9	1.8	13.0
1626922_at	CG31876	CG31876-RA	22.5	7.6	2.9
1634510_at	CG17324	CG17324-RA	22.3	4.5	4.9
1639859_at	CG15753	CG15753-RA	21.5	5.8	3.7
1632447_at	B52	CG10851-RD	20.7	5.0	4.1
1633089_a_at	lola	CG12052-RJ	19.0	5.9	3.2
1636780_at	meso18E	CG14233-RA	17.7	4.9	3.6
1626627_at	CG11718	CG11718-RA	17.6	6.5	2.7
1641674_at		S.C3L000093	31.3	10.9	2.9
1634534_at		CG30132-RA	29.5	6.9	4.3
1626887_at		Stencil:X:13639537:13636195:GENSCAN	35.1	13.9	2.5
1634247_at		HDC08957	51.1	12.1	4.2
1633386_s_at		CT37020	67.6	5.4	12.4
1628444_at		HDC18647	125.3	45.5	2.8
1638469_s_at		AY180918	224.1	71.5	3.1
1629160_s_at		GM02923	443.8	155.7	2.9