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Cellular and molecular mechanism controlling collective glial cell migration in drosophila

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Ecole Doctorale
des Sciences
de la Vie
et de la Santé

STRASBOURG

Thèse de Doctorat

pour obtenir le grade de Docteur de
l'Université de Strasbourg
Discipline: Biologie Moléculaire et Cellulaire

présentée par

Arun KUMAR

Cellular and Molecular Mechanism Controlling Collective Glial Cell Migration in *Drosophila*

Soutenue le 28 juin 2013 devant le jury composé de

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Résumé

Les mécanismes cellulaires et moléculaire contrôlant la migration collective des cellules

INTRODUCTION:

Le bon fonctionnement des réseaux neuronaux dépend des interactions entre les neurones et les cellules gliales. Alors que de nombreux efforts ont été faits pour comprendre les interactions entre les neurones, moins est connu sur la nature des interactions entre les cellules gliales ; ceci est due à la complexité du système nerveux des vertébrés, qui comprend plus de cellules gliales que de neurones. Cependant, le système nerveux de la drosophile à un rapport neurones-cellules gliales faible, ce qui fait de cet animal simple un modèle idéal pour évaluer ce concept. J'ai utilisé des approches génétiques à résolution cellulaire pour disséquer les mécanismes cellulaires et moléculaires de la migration collective des cellules gliales *in vivo*. Durant ma thèse j'ai donc contribué à l'étude de deux aspects.

OBJECTIFS, RESULTATS :

1) Quel est le rôle des interactions homéostatiques sur la migration des cellules gliales ?

La migration collective est un processus directionnelle et coordonné qui probablement implique une dynamique entre les interactions cellulaires. Durant le développement, les cellules gliales de l'aile de la drosophile fournissent un outil important pour l'étude de ce processus. Durant le développement des pupes, le nerf sensoriel de l'aile (nommé L1) est enveloppé lors de la migration directionnelle d'une chaîne de cellules gliales. Il a été démontré que les cellules présentes à la pointe de la chaîne des cellules gliales contrôlent le mouvement de la chaîne se comportent comme des pionnières. Au cours des deux premières années de ma thèse, j'ai montré que les cellules gliales pionnières ne peuvent pas se déplacer individuellement, la présence des cellules suiveuses étant primordiale pour leur survie. Nous avons également constaté que les interactions cellule-cellule à la pointe de la chaîne migratoire sont nécessaires pour l'efficacité et l'intégrité de la chaîne (Figure. 1)

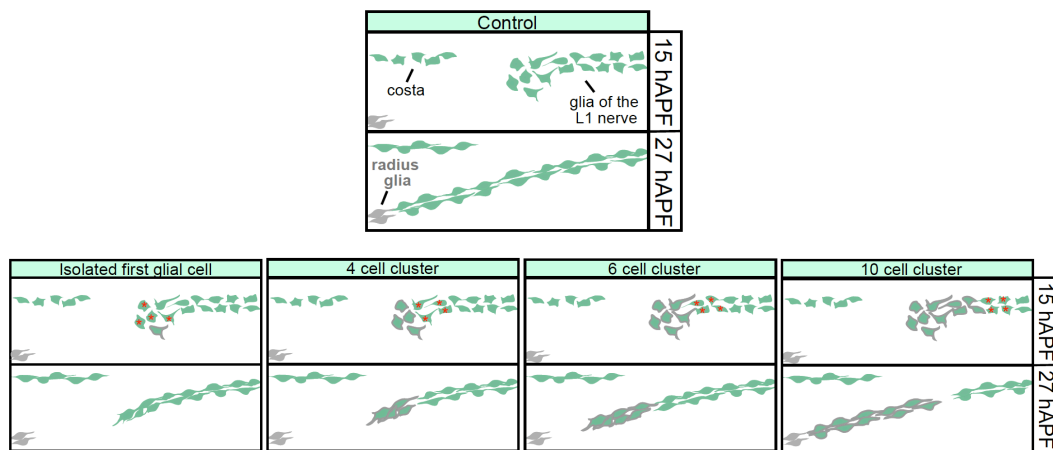


Figure 1

2) Quel est le rôle de la N-cadhérine dans le mouvement collectif ?

Les molécules d'adhésions sont des facteurs clés pour les interactions cellulaires. Il a aussi été démontré que les interactions cellulaires via les cadhérines jouent un rôle primordial durant la morphogénèse et les processus métastatiques (Berx, 2009; Stepniak, 2009). Afin de clarifier la cascade moléculaire impliquée dans la migration des cellules gliales, j'ai analysé le rôle de N-cadhérine, en utilisant des mutations spécifiques de gain et de pertes de fonctions ainsi que des time-lapse dans tout l'organisme.

J'ai démontré que la protéine N-Cadherine est exprimée dans les cellules gliales de l'aile et que le dosage de cette protéine affecte la migration des cellules gliales au niveau de cet organe. L'analyse d'ailes fixées issues de ces animaux ont permis de révéler que la

surexpression de N-Cadherine provoque un retard important dans la migration des cellules gliales par rapport aux animaux contrôle, et l'opposé se produit quand on réduit le niveau de N-Cadherine en utilisant des ARNi (**Figure 2**)

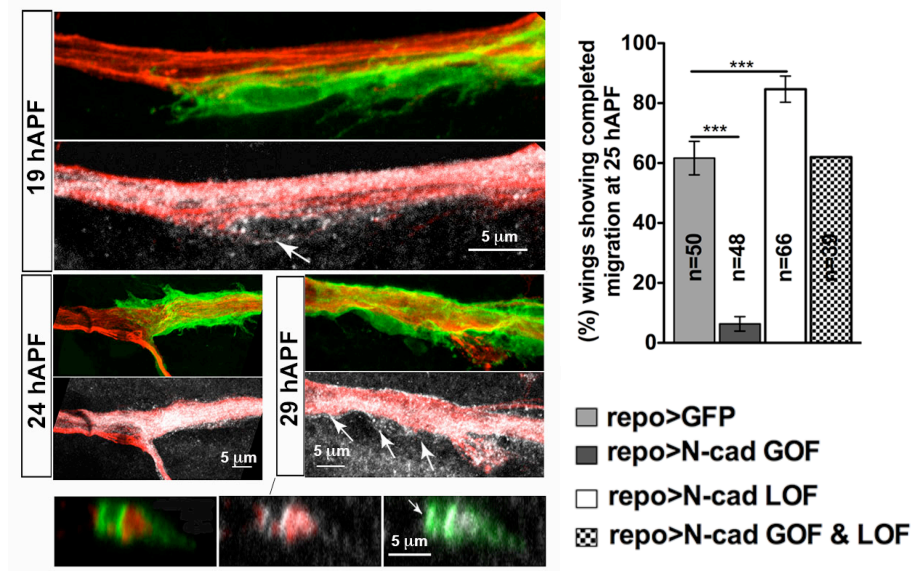


Figure 2

Ces résultats intéressants m'ont encouragé à faire des analyses poussées pour comprendre les voies moléculaires impliquées dans ce processus. Le domaine cytoplasmique des Cadherines est associé à des adaptateurs comme la b-Catenine, appelée Armadillo chez les mouches, qui à son tour se lie à une a-Catenine, une molécule qui contient un domaine de liaison à l'actine. Donc, les Cadherines constituent la plateforme moléculaire reliant l'adhésion

cellulaire à un remodelage du cytosquelette d'actine. Des données récentes suggèrent, que contrairement aux hypothèses précédentes montrant que la fixation de α -cat à un β -cat induit l'accumulation de α -cat dans les membranes, inhibant ainsi la nucléation du complexe ARP2/3 actine et la migration cellulaire et la dynamique membranaire; notre hypothèse est que le niveau de N-Cad affecte la migration en empiétant sur Arm et que l'accumulation de α -cat dans la membrane et ainsi sur le remodelage du cytosquelette (**Figure 3**).

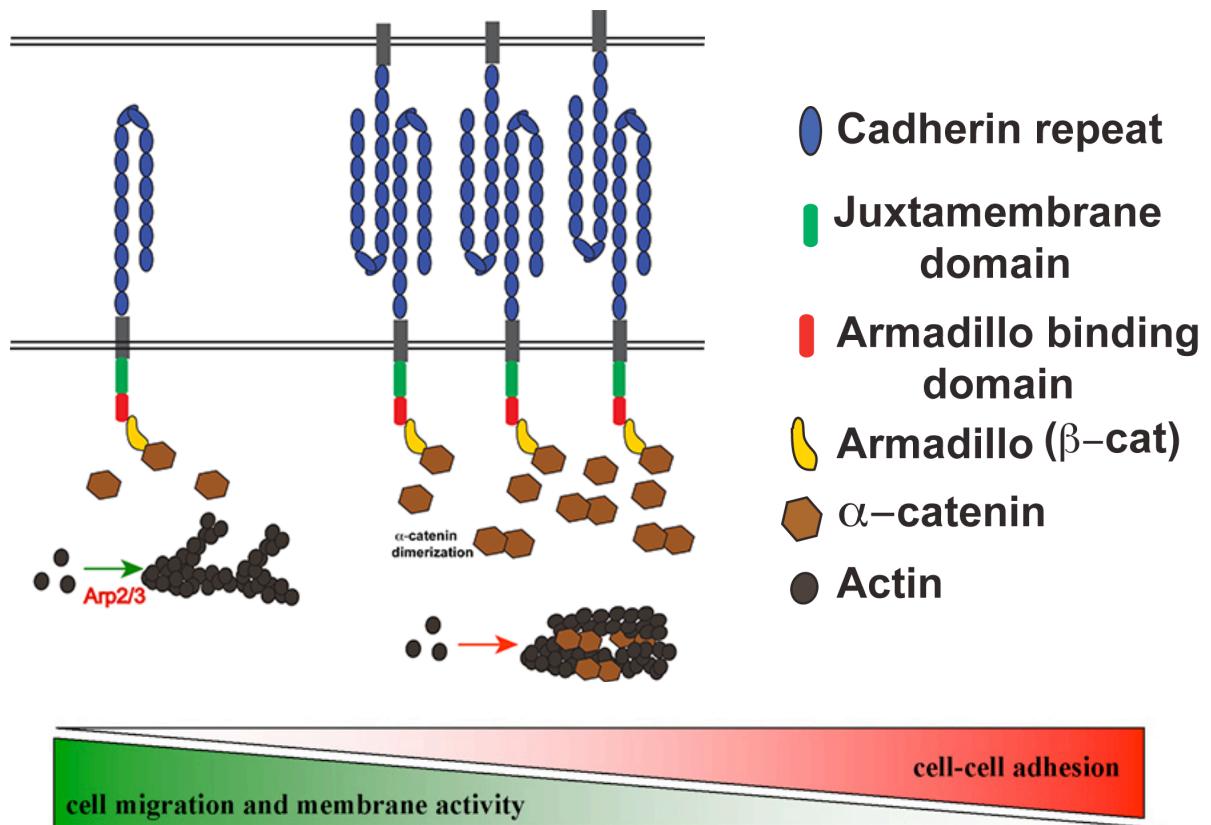


Figure 3

Pour tester cette hypothèse, j'ai altéré génétiquement le niveau de N-Cad dans les cellules gliales de l'aile et j'ai analysé la répartition différentielle des molécules adaptatrices par immunomarquage ainsi que par immunoblot. En effet, la surexpression de N-Cad déclenche une accumulation très élevée de Arm et de a-Cat. En outre, les animaux qui expriment le transgène N-Cad ARNi montrent une diminution des niveaux de ces mêmes molécules. En résumé, La N-Cad contrôle négativement la migration cellulaire et que son dosage est d'une importance capitale pour l'efficacité de ce processus (**Figure 4, 5**)

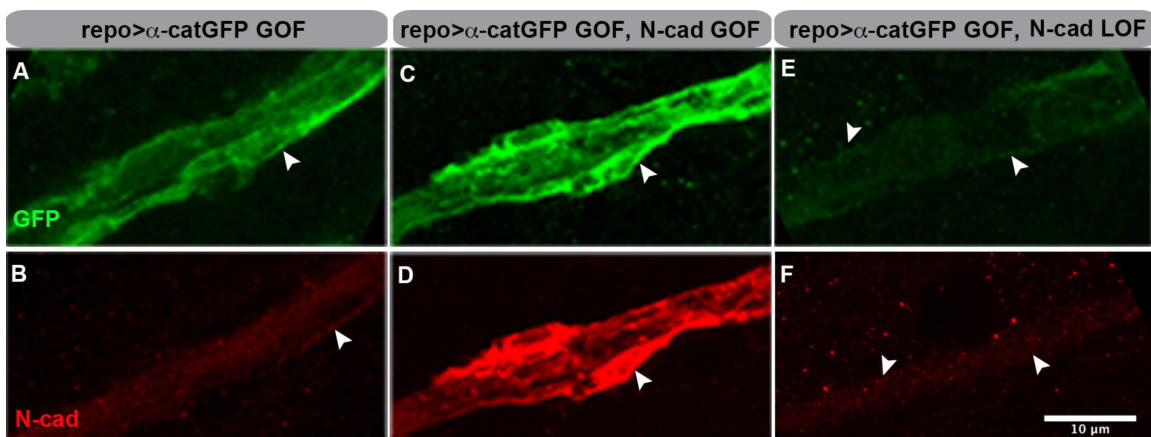


Figure 4

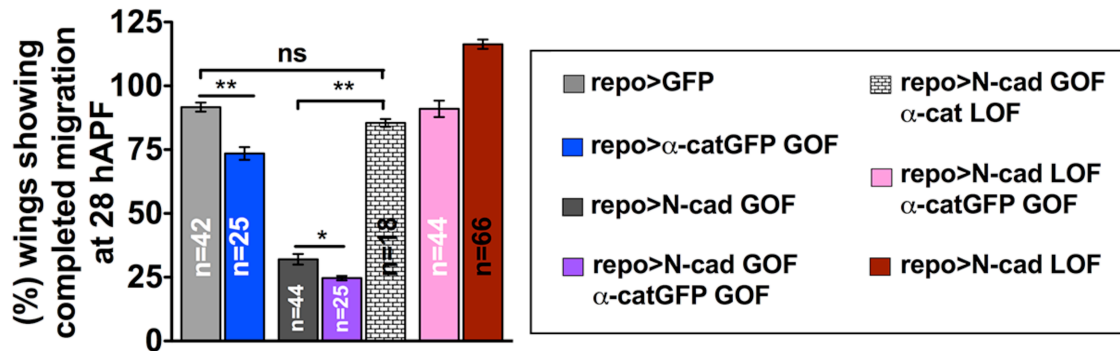


Figure 5

Pour comprendre comment N-cad module la migration cellulaire, j'ai décidé de suivre la dynamique de l'actine lors de la migration des cellules gliales, en réalisant des time-lapse à haute résolution par microscopie confocale. Des analyses quantitatives ont révélées que le nombre et la complexité des filopodes diminuent dans les animaux surexprimant N-cad par rapport aux animaux contrôles (**Figure 6**).



Figure 6

Comme N-cad contrôle négativement la migration des cellules gliales, je me suis demandé si la protéine CYFIP neutralise l'effet de N-cad. CYIFP/Sra1 est une sous-unité du complexe WAVE/Scar qui est nécessaire pour la nucléation de l'actine, Arp2/3 dépendante. On s'attendait à ce que l'augmentation du niveau de CYFIP accélère d'avantage la migration des cellules gliales. En effet, la surexpression de CYFIP dans la glie sauve les défauts de migration induits par la surexpression de N-cad, de faite que les animaux qui surexpriment les deux protéines, N-cad et CYFIP se comportent comme les animaux contrôles (**Figure 7**).

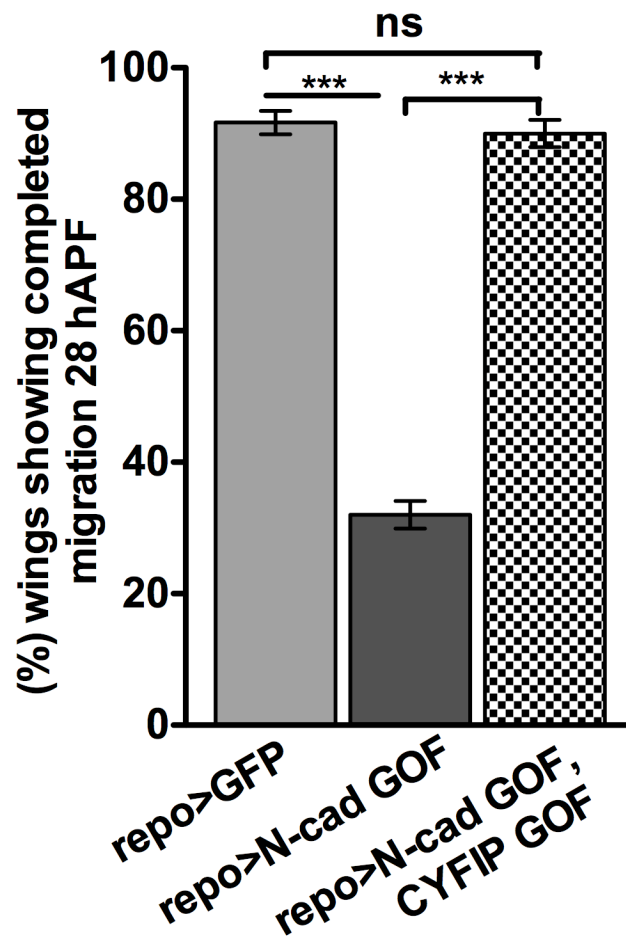


Figure. 7

En combinant les connaissances au sujet des interactions cellulaires et les voies moléculaires régulant la mobilité cellulaire, on ouvre de vastes perspectives quant à la coordination de la migration cellulaire. Par conséquent, l'investigation des événements qui se déroulent au cours de la migration cellulaire sera d'une grande importance pour la biologie du développement, ainsi que pour la compréhension de plusieurs maladies.

Conclusions:

En résumé, mes données révèlent les bases du mécanisme contrôlant la migration cellulaire collective : 1) les cellules du front de migration interagissent entre elles en amont et en aval et 2) N-cad est nécessaire pour une migration optimal de la glie.

Summary

The functionality of the complex neural network depends on the interactions between neurons and glia. While many efforts have been made to understand the neuron-neuron interactions, less is known about those amongst glial cells. Due to the complexity of the vertebrate nervous system, which comprises manifold more glia than neurons, it is hard to tackle the role of glia-glia interactions. The nervous system of *Drosophila*, however, has a lower glia-neuron ratio, which makes this simple animal an ideal model. I use genetic approaches at cellular resolution to dissect the cellular and molecular mechanisms of glial collective migration in vivo. Thus, during my thesis, I have been focusing on two aspects of glial chain movement.

Objectives

I) Cellular mechanisms controlling collective migration

What is the role of homeostatic interactions amongst migrating glia?

Collective migration is a directional and coordinated process that likely implies dynamic cell-cell interactions. Glia in the *Drosophila* developing wing provides an eminent tool to study this process (Aigouy et al., 2004) and previous work has shown that cell-cell interactions play an important role (Aigouy et al., 2008)). I have further analyzed the role of glia-glia interactions using cell ablation and confocal time-lapse approaches.

II) Molecular mechanisms controlling collective cell migration

1) What is the role of N-cadherin (N-cad) in the collective movement?

Adhesion molecules (Silies and Klambt, 2011);(Schwabe et al., 2009) (Togashi et

al., 2009) are key players in process requiring cell interactions. Cadherin-mediated cell-cell adhesion is also implicated in cell migration during both morphogenesis and cancer metastasis (Berx, 2009; Stepniak, 2009). Since cadherins are widely used during development, I have analyzed the role of N-cad in glial migration by using glial-specific gain and loss of function mutations as well as time-lapse in the whole organism.

2) What is the role of the Gcm glial determinant in collective migration?

Results

Objective I

During pupal development, the L1 sensory nerve of the *Drosophila* wing is ensheathed by a glial chain. By 17-18 hours After Pupa Formation (hAPF), glial cells start moving on the nerve towards the central nervous system and complete migration by 26-29 hAPF. Cells present at the tip of the glial collective have been shown to control the timely movement of the chain and behave as pioneers (Aigouy et al., 2008). I have investigated the importance of community effects on collective cell migration. I have shown that pioneer glia cannot move as single cells and require the presence of follower cells for survival. However, isolating clusters of glia (6-7 cells) present at the tip from the rest of the chain, are able to reconstitute a functional migrating chain without the vicinity of neighboring cells. However, a cluster of 10 cells or more fully reconstitute a migratory chain. These data illustrate that the cell-cell interactions at the chain tip control efficiency and integrity of the migratory chain.

Objective II

During the collective movement, the glia adheres to each other. This suggests that cell-cell adhesion might be at work in keeping cells together. I have investigated the role of a cell adhesion molecule, N-cad in glial collective migration. I have shown for the first time that N-cad is expressed in wing glial cells and that changing its dosage affects migration. To achieve this, I have altered the amount of N-cad by specifically overexpressing N-cad (N-cad GOF) or N-cad RNAi (N-cad LOF) UAS reporters in glial cells using the repo-Gal4 driver. The analysis of fixed wings has revealed that N-cad overexpression triggers a strong delay in completion of migration compared to control animals and that the opposite happens with the RNAi knock down line.

These encouraging results have prompted me to analyze the molecular pathway involved. The cytoplasmic domain of cadherins is associated with adaptors such as β -catenin (in flies, Armadillo or Arm), which in turn binds γ -catenin (γ -cat), a molecule that contains an actin-binding domain. Cadherins therefore represent the molecular platform linking cell adhesion to actin cytoskeleton remodeling. Recent data suggest that, binding of γ -cat to β -cat leads to the accumulation of γ -cat at the membrane, thereby inhibiting the ARP2/3 actin nucleation complex and cell migration/membrane dynamics (Benjamin and Nelson, 2008). Our hypothesis is that the levels of N-cad affect migration by impinging on Arm and γ -cat accumulation at the membrane and therefore on cytoskeleton remodeling.

To test this hypothesis, I have genetically altered the amount of N-cad in wing glial cells and analyzed the differential distribution of the adaptor molecules by immunolabeling and Western blot. My data indicate that N-cad overexpression triggers

elevated accumulation of Arm and β -cat. In addition, animals expressing the N-cad RNAi transgene show decreased levels of the same molecules. In sum, N-cad negatively controls cell migration and that its dosage is of paramount importance for the efficiency of this process.

To investigate how N-cad modulates cell migration, I have decided to follow actin dynamics in migrating glial cells using high-resolution and high resolution, high-speed confocal time lapses. Quantitative analyses have revealed that the number and the complexity of filopodia decreases in N-cad GOF compared to that observed in control animals. As N-cad negatively controls glial migration, I have then investigated whether CYFIP counteracts the effect of N-cad. CYFIP/Sra1 is a subunit of the WAVE/SCAR complex required for Arp2/3 dependent actin nucleation (Takenawa and Miki, 2001). The expectation is that increasing the levels of CYFIP may accelerate glial migration. Indeed, first data show that CYFIP overexpression in glia rescues the migratory defect induced by N-cad overexpression, as *N-cad, CYFIP CO-GOF* animals (simultaneous overexpression of *N-cad and CYFIP*) behave like control animals.

In Summary, my data show some basic mechanism controlling collective cell migration: 1) cells at the front of the collective interact with each other through anterograde and retrograde bidirectional interaction. 2) N-cad appears necessary for timely movement of glial community.

Résumé

Le bon fonctionnement des réseaux neuronaux dépend des interactions entre les neurones et les cellules gliales. Alors que de nombreux efforts ont été faits pour comprendre les interactions entre les neurones, moins est connu sur la nature des interactions entre les cellules gliales ; ceci est due à la complexité du système nerveux des vertébrés, qui comprend plus de cellules gliales que de neurones. Cependant, le système nerveux de la drosophile à un rapport neurones-cellules gliales faible, ce qui fait de cet animal simple un modèle idéal pour évaluer ce concept. J'ai utilisé des approches génétiques à résolution cellulaire pour disséquer les mécanismes cellulaires et moléculaires de la migration collective des cellules gliales *in vivo*. Durant ma thèse j'ai donc contribué à l'étude de deux aspects.

Objectifs

1) Quel est le rôle des interactions homéostatiques sur la migration des cellules gliales ?

La migration collective est un processus directionnelle et coordonné qui probablement implique une dynamique entre les interactions cellulaires. Durant le développement, les cellules gliales de l'aile de la drosophile fournissent un outil important pour l'étude de ce processus. Durant le développement des pupes, le nerf sensoriel de l'aile (nommé L1) est enveloppé lors de la migration directionnelle d'une chaîne de cellules gliales. Il a été démontré que les cellules présentes à la pointe de la chaîne des cellules gliales contrôlent le mouvement de la chaîne se comportent comme des pionnières. Au cours des deux premières années de ma thèse, j'ai montré que les cellules gliales pionnières ne peuvent pas se déplacer individuellement, la présence des cellules suiveuses étant primordiale pour leur survie. Nous avons également constaté que

les interactions cellule-cellule à la pointe de la chaîne migratoire sont nécessaires pour l'efficacité et l'intégrité de la chaîne.

2) Quel est le rôle de la N-cadhérine dans le mouvement collectif ?

Les molécules d'adhésions sont des facteurs clés pour les interactions cellulaires. Il a aussi été démontré que les interactions cellulaires via les cadhérines jouent un rôle primordial durant la morphogénèse et les processus métastatiques (Berx, 2009;Stepniak, 2009). Afin de clarifier la cascade moléculaire impliquée dans la migration des cellules gliales, j'ai analysé le rôle de N-cadhérine, en utilisant des mutations spécifiques de gain et de pertes de fonctions ainsi que des time-lapse dans tout l'organisme.

J'ai démontré que la protéine N-Cadherine est exprimée dans les cellules gliales de l'aile et que le dosage de cette protéine affecte la migration des cellules gliales au niveau de cet organe. L'analyse d'ailes fixées issues de ces animaux ont permis de révéler que la surexpression de N-Cadherine provoque un retard important dans la migration des cellules gliales par rapport aux animaux contrôle, et l'opposé se produit quand on réduit le niveau de N-Cadherine en utilisant des ARNi.

Ces résultats intéressants m'ont encouragé à faire des analyses poussées pour comprendre les voies moléculaires impliquées dans ce processus. Le domaine cytoplasmique des Cadherines est associé à des adaptateurs comme la b-Catenine, appelée Armadillo chez les mouches, qui à son tour se lie à une a-Catenine, une molécule qui contient un domaine de liaison à l'actine. Donc, les Cadherines constituent la plateforme moléculaire reliant l'adhésion cellulaire à un remodelage du cytosquelette d'actine. Des données récentes suggèrent, que contrairement aux hypothèses précédentes montrant que la fixation de a-cat à un b-cat induit l'accumulation de a-cat dans les membranes, inhibant ainsi la

nucléation du complexe ARP2/3 actine et la migration cellulaire et la dynamique membranaire; notre hypothèse est que le niveau de N-Cad affecte la migration en empiétant sur Arm et que l'accumulation de a-cat dans la membrane et ainsi sur le remodelage du cytosquelette.

Pour tester cette hypothèse, j'ai altéré génétiquement le niveau de N-Cad dans les cellules gliales de l'aile et j'ai analysé la répartition différentielle des molécules adaptatrices par immunomarquage ainsi que par immunoblot. En effet, la surexpression de N-Cad déclenche une accumulation très élevée de Arm et de a-Cat. En outre, les animaux qui expriment le transgène N-Cad ARNi montrent une diminution des niveaux de ces mêmes molécules. En résumant, La N-Cad contrôle négativement la migration cellulaire et que son dosage est d'une importance capitale pour l'efficacité de ce processus.

Pour comprendre comment N-cad module la migration cellulaire, j'ai décidé de suivre la dynamique de l'actine lors de la migration des cellules gliales, en réalisant des time-lapse à haute résolution par microscopie confocale. Des analyses quantitatives ont révélées que le nombre et la complexité des filopodes diminuent dans les animaux surexprimant N-cad par rapport aux animaux contrôles. Comme N-cad contrôle négativement la migration des cellules gliales, je me suis demandé si la protéine CYFIP neutralise l'effet de N-cad. CYIFP/Sra1 est une sous-unité du complexe WAVE/Scar qui est nécessaire pour la nucléation de l'actine, Arp2/3 dépendante. On s'attendait à ce que l'augmentation du niveau de CYFIP accélère d'avantage la migration des cellules gliales. En effet, la surexpression de CYFIP dans la glie sauve les défauts de migration induits par la surexpression de N-cad, de faite que les animaux qui surexpriment les deux protéines, N-cad et CYFIP se comportent comme les animaux contrôles.

En resume, mes donnees revelent les bases du mecanisme controlant la migration cellulaire collective : 1) les cellules du front de migration interagissent entre elles en amont et en aval et 2) N-cad est necessaire pour une migration optimal de la glie.

Abbreviations

AJ: adherence junction
Arm: Armadillo
Arp2/3: actin-related protein 2/3
BBB: blood brain barrier
BC: border cell(s)
beta-gal: beta-galactosidase
bnl: branchless (Drosophila homolog of FGF)
btl: breathless (Drosophila homolog of FGFR)
cdc42: cell division control 42
CNS: central nervous system
CXCR: chemokine CXC motif receptor
DCE: distal cellular extension
E-cad: epithelial (E-) cadherin
ECM: extracellular matrix
EGF: epidermal growth factor
EGFR: EGF receptor
elav: embryonic lethal abnormal vision
FRT: FLP recognition target
FGF: fibroblast growth factor
FGFR: FGF receptor
GBS: Gcm binding site
gcm: glial cell missing
GFP: green fluorescent protein
GOF: gain-of-function
GPCR: G-protein coupled receptor
HRP: horseradish peroxidase
L1: L1 nerve/vein in the wing
L3: L3 nerve/vein in the wing
LCE: long cellular extension
LLP: lateral line primordium
LOF: loss-of-function
MAPK: mitogen-activated protein kinase
MDCK: Madin-Darby canine kidney cells
MTOC: microtubule organizing center
NC: neural crest
N-cad: neuronal (N-) cadherin
NCAM: neuronal cell adhesion molecule

PI3K: phosphatidylinositol 3 kinase
PIP2: phosphatidylinositol-4,5-bisphosphate
PIP3: phosphatidylinositol-3,4,5-trisphosphate
PKC: protein kinase C
PNS: peripheral nervous system
PDGF: platelet-derived growth factor
PDGFR: PDGF receptor
PVF: PDGF/VEGF related factor
PVR: PVF receptor
PTEN: phosphatase and tensin homolog
repo: reversed polarity
rac1: ras-related C3 botulinum toxin substrate
rhoA: ras homolog gene family, member A
ROCK: Rho-associated, coiled-coil containing protein kinase 1
RMS: rostral migratory stream
RNAi: RNA interference
RTK: receptor tyrosine kinase
SC: Schwann-cell
SCP: Schwann-cell precursor
SDF-1: stromal-derived factor 1
SO: sensory organ
SOP: sensory organ precursor
SVZ: subventricular zone
TSM: twin sensilla of the margin **UAS:**
upstream activating sequence **VEGFR:**
vascular-endothelial growth factor **VEGFR:**
VEGF receptor
WASP: Wiskott-Aldrich syndrome protein
WAVE: WASP-verprolin homolog (SCAR)
wg: wingless (Drosophila homolog of wnt)
wnt: wg-int
PKC: atypical protein kinase C

Introduction

1. The discovery of cells and cell migration

Robert Hooke was perhaps one of the most important scientist from the 17th century, reported that life's smallest structural units were small boxes or "cells". This was one of the important discoveries in the history ever that marked the beginning of what was called the '**cell theory**' or the notion that '*all living things are composed of cells*'. The next advancement in the evolution of microscopy was brought by a Dutch scientist Anton van Leeuwenhoek. He was probably the first person to see live microorganism through the magnifying lenses that he developed. During those years he wrote a series of letters to Royal Society describing animalcules that observed through simple microscope.

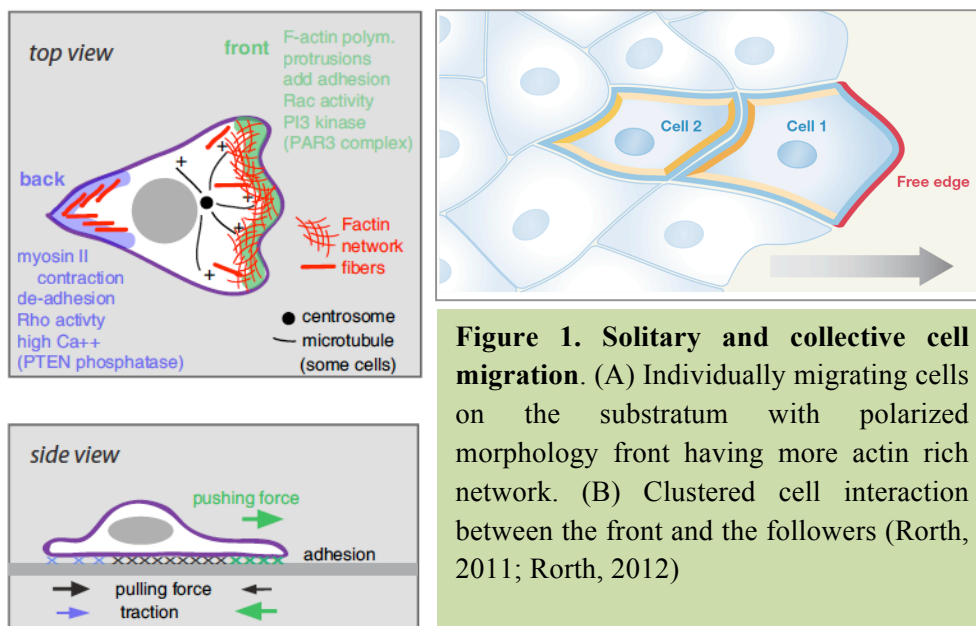
These early innovation and advances in the field of microscopy by Robert Hooke and Leeuwenhoek opened doors to look at microorganism and single celled creatures that have since been discovered. One of the most exciting discoveries made by these microscopist was unraveling animal cells that are capable of undergoing cell migration. With advances in the technologies over time have given more insight into understanding mechanistic behavior of cell movement.

2. Introduction to cell migration

Cell migration is a spectacular and widespread phenomenon observed in wide variety of biological processes and has interested biologist for long time. It is a fascinating behavior observed in most living organisms where they migrate. Regrouping individuals of the same kind is frequently an advantageous strategy to survive, forage and face predators. In a similar manner, single celled organism or metazoan cells also migrate

to reach their final destination thereby contributing to the success and survival of the community.

Cells can migrate individually or collectively. Cell motility entails dynamic events such as cytoskeletal rearrangements, chemotaxis and directional sensing and the current understanding on how single cell movement is regulated on molecular and morphological level is quite well extended. (Ridley et al., 2003); (Van Haastert and Devreotes, 2004); (Insall and Machesky, 2009); (Raftopoulou and Hall, 2004). While a migrating single-cell organism processes the input and output signals itself and translates them for its own movement (**Fig. 1A**), the members of collectively moving communities interpret the information on a single cell and transmit the signals and guidance cues to their neighbors (**Fig. 1B**). This supramolecular organization requires a dynamic exchange of information between the cells that are present at the migrating front and those that are at the trailing part. This antero- and retrograde signaling defines the directionality and contributes to the success of migration.



Since a number of biological processes such as immunity wound healing, embryo development or cancer metastasis rely highly on cells moving in concert, it is very crucial to dissect the basic process underlying collective cell migration. While guided information for the single has been studied most extensively in a simple environment, our knowledge on collective migration is far less understood despite its potential relevances to development and diseases.

2.1 Single cell migration

2.1.1 Prokaryotes directional migration

Directed cell migration is in fact one the most ancient features of life and it is widely distributed amongst prokaryotes (Adler, 1975); (Berg and Brown, 1974); (Wadhams and Armitage, 2004). Motility provides simple and excellent mechanism to adapt to the external environmental changes that are defined by external cues such as temperature, wavelength of light, pH or osmolarity. For example, in an ever-changing environment it is essential that these organisms sense and respond to appropriately to the external stimulus. The possible responses include active movement towards or away from an environment.

Chemotaxis is essential for survival; if it fails to reach their proper destination the organism dies and therefore, mechanisms processing chemotactic signals have been optimized through evolution. The bacterial chemotactic system of *E.coli* has been studied in great detail (Berg and Brown, 1974); (Rubik and Koshland, 1978); (Kollmann et al., 2005). The movement of the bacteria is carried out by the flagella rotation. When rotating in coordinated fashion, the flagella promote the forward movement of the cells while

uncoordinated triggers in tumbling on the same spot. Directed movement of the bacteria and signal transduction is determined by intracellular motor proteins that elicit the clock or counter clock rotation of the flagella at regular interval (Blair, 1995). As a result bacteria to undergo a ‘random walk’ creating a path of migration generated by alternating phase of migration and tumbling. In order to perform directional migration, bacteria recruits receptors at the tip of its cells to probe the environment for the ligands such as ions, osmolarity or wavelength of light. These receptors signal to the intracellular chemotaxis machinery to either reduce or prolong the rotational switch time interval. When they encounter an increase in chemo-attractant concentration, the receptors prolong the direction of migration towards the source (Blair, 1995).

2.1.2 Cell migration of unicellular eukaryotes: How do cell move?

The complex cell migration process involves dynamic events such as polarization, cytoplasmic protrusions, substrate adhesion and retraction. These processes need precise signal integration to relay the forward movement of the single cell to reach its destination. During the cell motility cycle, the migrating cell produces protrusions that adhere to the underlying substratum and perform contraction at its rear end to move cell forward ([Fig. 2A-D](#)).

Our present day understanding of cell migration sought for composite studies derived from different cell types and environment. In general, typical cell migration is a cyclic process (Lauffenburger and Horwitz, 1996). The initial response of a cell to a migration-promoting agent is to polarize, in a way that leading and trailing edges are defined. The triggering event leading to polarization can be either chemotactic (soluble

factors), mechanical signal from environment or adhesion receptor mediated signaling (Petrie et al., 2009).

Cell surface interaction and signaling cascade underlie the bases of cell shape change and regulate cell migration. On the molecular level, polarity is established once the external stimulus is perceived by the cell surface receptor such as G-Protein

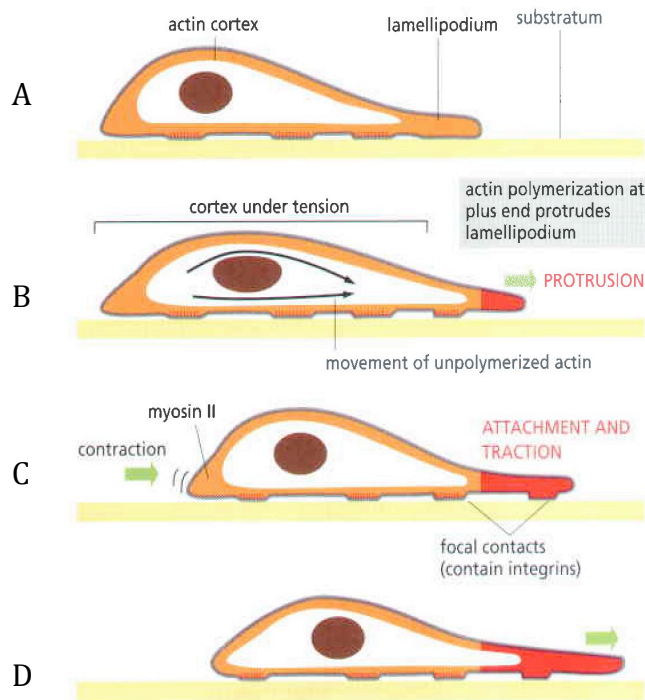


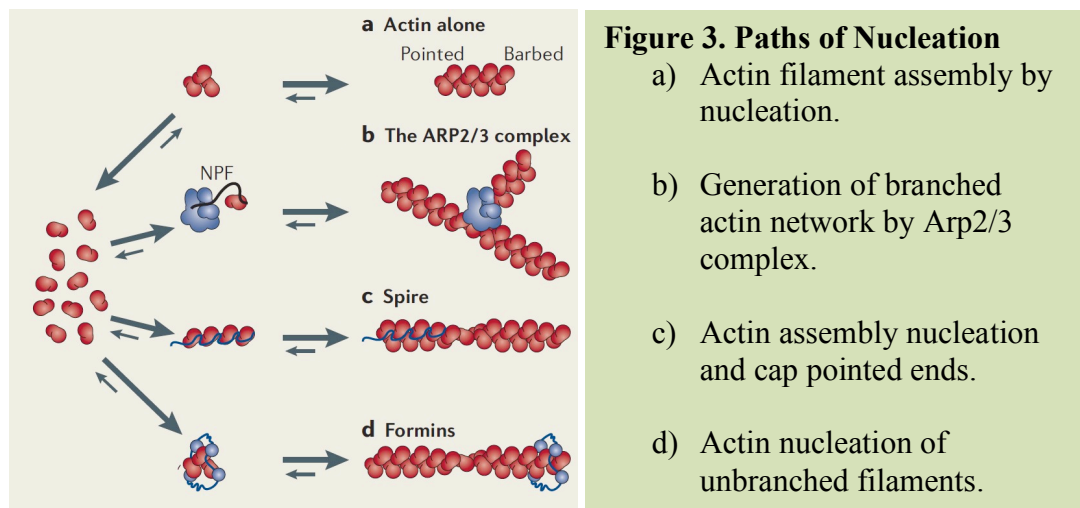
Figure 2. The scheme of cell migration. A single cell moving across a two-dimensional substrate. **A**, Cell attaches to the substratum. **B**, Polarization produces protrusion at leading edge. **C**, Strong focal contact generation at the front and rear end contraction allow cell to move forward. **D**, New forward directed protrusion is formed to start the next cycle.

Coupled receptors (GPCR) (Van Haastert and Devreotes, 2004). Heterotrimeric G proteins (G-alpha, -beta /gamma subunits) constitute one of the most important components of cell signaling cascade. GPCRs perceive many extracellular signals and

transduce them to G proteins, which further transduce these signals intracellularly to appropriate downstream effectors and thereby play an important role in various signaling pathways (Parent et al., 1998); (Servant et al., 2000).

One such molecule activated upon GPCR signaling at the membrane is Phosphatidylinositol triphosphate (PIP3), which is a result of phosphorylation of PIP2 by the phosphoinositid 3-kinase (PI3K). PIP3 is implicated at the front of the migrating cells. PIP3 phosphatase and PTEN is mainly present at the rear end to and contribute in regulating levels of PIP3. Once the polarity is achieved, reorganization of the microtubule-organizing center (MTOC), and Golgi apparatus takes place within the cells. Polarization is then maintained further by Rho GTPase family proteins (Cdc42, Rac), integrin and actin cytoskeleton complexes.

Actin assembly provides driving force for the cell motility and powers protrusions such as lamellipods and filopods by polymerizing just under the plasma



membrane. The actin nucleation promoting factors, WASP and WAVE/SCAR complex, are the key players contributing to actin polymerization (Takenawa and Miki, 2001) and

the central pillar of actin network is the seven subunit of Arp 2/3 complex. The biochemical, structure- function relations of this complex have been studied quite extensively in in vitro models (Goley and Welch, 2006). Upon activation by the nucleation promoting factors Arp2/3 nucleates actin daughter filament as a branch off of the existing mother strand thereby contributing to enhance branched and exploratory structure that are required for the sensing guidance cues form surrounding and promoting cell movement (**Fig. 3b**). Another nucleator molecule, Spire is conserved metazoan protein. Spire nucleates actin assembly by remaining associated with pointed end and thereby inhibiting depolymerization (**Fig. 3c**). The nucleator formin is responsible for the formation of unbranched actin bundles (Goley and Welch, 2006); **Fig. 3d**). The localization and the functional role of the ARP2/3 complex formation has been explored and confirmed to large extent (Nicholson-Dykstra and Higgs, 2008); (Rogers et al., 2003); (Steffen et al., 2006) and its loss has severe effects on viability in variety of organisms. Typically, genetic loss of function of ARP2/3 complex is detrimental for yeast and *Dictyostelium* and mouse knockouts produce preimplantation lethality (Yae et al., 2006); (Zaki et al., 2007).

While lamellipodia and filopodia are seen as the main protrusion players that cells produce when moving on surfaces, other structures are also important for the cell motility. For an active movement, the cell has to transmit mechanical forces to the underlying substratum or extracellular matrix (ECM) in order for success of cell progression. Adhesions between underlying substrate are mainly mediated by the integrin family (Lauffenburger and Horwitz, 1996) of the transmembrane receptors. Tractional forces are essential to move the cells forward are borned from the integrin that hook up

with the ECM (Lauffenburger and Horwitz, 1996). Integrins are heterodimeric proteins consisting of α , β chains and a large binding domain at their extracellular part. Prior to contact with ECM, integrin heterodimers are mostly inactive. Therefore, their activation is essential to initiate interaction with cytoskeleton for the efficient signal relay (Ridley et al., 2003). Like any other pathway affecting the actin cytoskeleton, integrins interact with members of the Rho family of small GTPases and Protein kinase C (PKC) to promote adhesion assembly (Kinbara et al., 2003). There is high degree of asymmetry in distribution of integrin: fast moving cells have high integrin turn over compared to slow moving cells (Ridley et al., 2003).

To migrate, cell bodies must modify their shape and stiffness to interact with the surrounding environment. The signaling cascade inside the cell is driven by the interaction of actin filament with a myosin II that generates forces to translocate. The myosin II is positively regulated and activated by Myosin Light Chain kinase (MLCK) or Rho kinase (ROCK) (Ridley et al., 2003). In order to retract the rear end, adhesion between cell and substratum has to be released and to achieve Focal adhesion kinase (FAK), ERK and Src are recruited at the site of adhesion.

The Extracellular Matrix (ECM) is a network of glycoproteins and proteoglycans that is organized into three-dimensional tissue-specific structures (Larsen et al., 2006); (Nelson and Bissell, 2006). The ECM is remodeled by a diverse array of mechanisms, including protein synthesis, proteolysis, and contraction (Larsen et al., 2006). Dynamic alterations in the structure and composition of the ECM, termed ECM remodeling, occur at different stages of development, during tissue repair and remodeling, and as a result of many pathologies and malignancies (Larsen et al., 2006); (Nelson and Bissell, 2006). The

dynamic reciprocity exists between cells and their surrounding ECM. Integrins constitute a family of transmembrane molecules that are the major receptors for ECM proteins (Hynes, 1987; Hynes, 2002). The intracellular portion of integrins associates with a plaque of structural proteins that mediate the linkage of the ECM to the cytoskeleton and signaling proteins that regulate cell function (Geiger et al., 2001). The adhesion between the motile cell and the substrate needs to be dynamic since prolonged presence of attachment cannot allow efficient cell translocation. Integrins and thus adhesions are subject to extensive assembling, disassembling and recycling processes.

So far I have presented the different modes and mechanism of cell migration. In the following section, I will describe various examples of single cell migration that are widely studied.

2.1.3 Primordial germ cell migration (PGC)

Primordial germ cells (PGC) are the cells that arise early in development and produces sperms and eggs (Kunwar et al., 2006); (Wylie, 1999). PGCs actively navigate during development to meet the somatic gonadal support cells. This observation along with the fact that germ cells are critically important for sustaining a species makes them interesting and important to study this process. In the following section, I am going to summarize the basic processes controlling cell movement.

PGC migration in *Drosophila*

In *Drosophila*, PGCs are the first cells to be formed as a tightly packed cluster located at the posterior end and are passively invaginate into midgut by the process of

gastrulation (Santos and Lehmann, 2004); (Starz-Gaiano and Lehmann, 2001); **Fig. 4a-b**). At this point, the PGC start to migrate towards the gonad by traversing the midgut epithelium (Callaini et al., 1995); (Jaglarz and Howard, 1995); **Fig. 4c**). Once PGC pass through the gut epithelium, they reorient on the surface of mesoderm (**Fig. 4d**). The directional signal for the epithelial transmigration requires the activity of two-lipid phosphate phosphatase activity called Wunen and Wunen2. In double mutant of *wunen/wunen2*, PGC exit the gut normally but migrate randomly (Starz-Gaiano and Lehmann, 2001); (Zhang et al., 1996) while overexpression of these genes in the mesoderm results in repulsion from the target site (Starz-Gaiano and Lehmann, 2001).

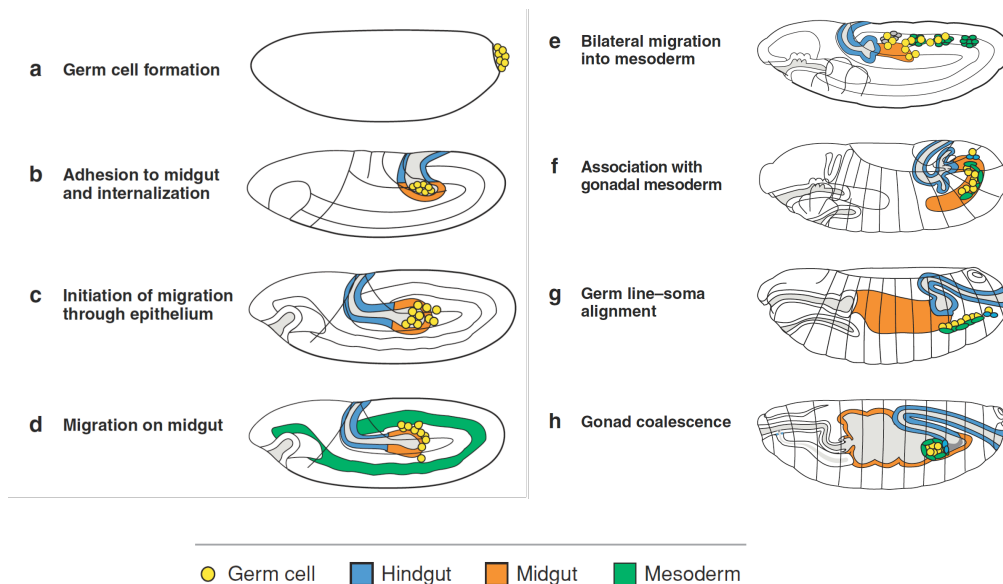


Figure 4. Stages of PGC migration in *Drosophila*. (a-b) PGC located at the posterior pole of the embryo internalized by midgut epithelium. (c) Germ cell starts to migrate out of the midgut. (d) Migration of germ cells on the surface of the midgut towards mesoderm. (e-f) germ cell migrate to reach gonad mesoderm. (g-h) germ cell associate with gonadal mesoderm to form gonads.

In supplement to the directional cues generated by two genes *Wunen/Wunen2*, it has been proposed that 3-Hydroxy-3 methyl-glutaryl-Coenzyme A reductase (HMGCoA) activity is required for the production of a germ cell attractant (Van Doren et al., 1998) **Fig. 4e-f**. Once the germ cells have arrived in the region of gonadal mesoderm, they begin to associate with somatic cells and cluster until they finally coalesce tightly into a gonad (Jaglarz and Howard, 1995); **Fig. 4g-h**).

2.1.4 Cell migration in *Drosophila* haemocytes

Drosophila haemocytes are the blood cells that constitute the cell mediated innate immune response in flies and actively participate in the removal of the apoptotic bodies or corpses during development (**Fig. 5**).

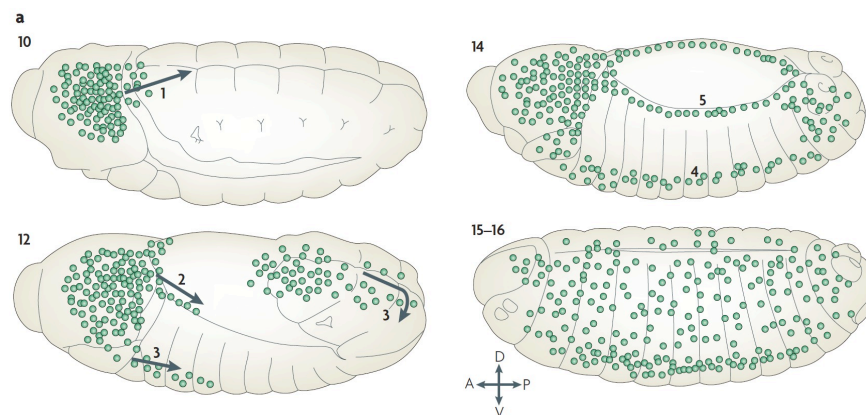


Figure 5. Migration during development. During embryogenesis, haemocytes develop from head mesoderm and populate the fly embryo. (1) Haemocytes exit the head region and infiltrate the germ band. (2) Haemocytes infiltrate more towards posterior end from the head region. (3) Migration of haemocytes towards one another from posterior and anterior end. (4) Haemocytes continue to migrate anteriorly and posteriorly until they meet. (5) Haemocytes migrate more posteriorly on dorsal side of the embryo (Wood and Jacinto, 2007; Benjamin and Nelson, 2008).

During embryogenesis, haemocytes originates as a population of cells from the procephalic (head) mesoderm and begin to disperse across the embryo. As the embryonic plasmatocytes are highly motile, they possess large, polarized, actin-rich filopodia and lamellipodia. During late stages, when there is massive movement of the cells throughout the embryo, these protrusions are highly dynamic and continually extend and retracts as cell explore their extracellular environment (Wood and Jacinto, 2007); **Fig. 5**).

Haemocytes guidance cues

The expression profiles of the PDGF/VEGF receptor (PVR) and its putative ligands PDGFs/VEGFs (PVFs) have shown that PDGF/VEGF signaling controls the migratory behaviors of the *Drosophila* haemocytes (Cho et al., 2002). Upon specification, haemocytes expresses (Cho et al., 2002); (Heino et al., 2001). PVR loss-of-function in developing blood cells results in cells that differentiates and initiate migration correctly but fail to disperse uniformly. While the mutation for one *Pvf* genes show no effect on blood cell migration, however, inactivation of all three *Pvf* genes produces similar phenotype as mutants for the receptor (Cho et al., 2002). In addition, ectopic expression of *Pvf* results in misrouting of haemocytes, showing a clear-cut involvement of the PDGF/VEGF pathway in guided migration of developing blood cells (Cho et al., 2002).

2.2 Collective cell migration: an *invivo* approach

Coordinated cell migration has many features in common with individual cell migration, but also has some unique identities. Some features might be restricted to one while others are more widespread. Cell migration is strictly regulated process and deregulation may results in severe developmental and pathological abnormalities. For

example, one of the most critical issues associated with malignant transformation is the acquisition of mobility, a situation wherein cells can detach away from the site of primary tumors and invade the neighboring tissue/organ to form secondary metastasis (Berx and van Roy, 2009); (Hazan et al., 2004).

Recent advances in high-resolution microscopy and powerful genetic tools have improved our understanding of key features of collective cell migration and revealed some possible underlying themes in this complex behavior. This has allowed us to accumulate significant amount of information about collective cell migration in several model system (Rorth, 2009); (Friedl and Gilmour, 2009; Weijer, 2009) (Friedl et al., 2012);(Ridley et al., 2003).

In the following section, I will describe some of the most widely used model systems (mammals, Zebrafish and *Drosophila*) for collective migration.

2.2.1 Rostral migratory streams (RMS)

To construct complicated neural networks, neuroblasts have to migrate towards their final destination. In the adult rat brain, new neurons are continuously generated in the neurogenic niche located near ventricle. These newly generated cells actively migrate towards their destination, i.e. the olfactory bulb (OB), via a highly specialized migratory route called the rostral migratory streams (RMS), (Murase and Horwitz, 2004; [Fig. 6](#)). Neuronal precursors in the RMS form a continuous chain by homophilic interaction where they navigate through the tunnel-like structure formed by a network of astrocytes and glial tube (Whitman et al., 2009). This process of migration is widespread in rodents, (Lois and Alvarez-Buylla, 1994) (Kornack and Rakic, 2001; Curtis et al., 2007). The migration of neuronal precursors is controlled by multiple secretory signals such as

repulsive factors in septum and chemoattractive factors in the OB (Coskun and Luskin, 2002);(Whitman et al., 2009). Furthermore, cell-cell adhesion and the ECM also significantly affect the mode, speed and direction of migration (Friedl et al., 2004).

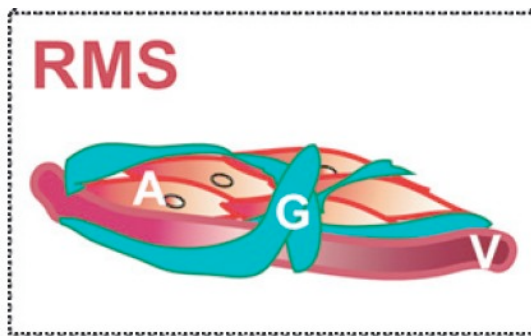


Figure 6. Rostral Migratory stream in the adult mouse brain. (A) Newly born neurons in subventricular (SVZ) zone migrate to the OB through the RMS. (G) Glial tube. (V) Blood vessels (Sun et al., 2010).

Cell-cell adhesion plays a critical role in the process of collective migration. It is known that neuroblasts in the RMS form chain-like associations with gaps or adherens junctions with neighboring cells (Miragall et al., 1997); (Marins et al., 2009) for their efficient migration. NCAM is a major cell adhesion molecule expressed in the migrating neuroblasts (Hu, 2000). NCAM loss of function in the adult mice not only exhibit reduced OB and neuroblast clustering (Hu et al., 1996) but also show severe migratory defects during embryogenesis, suggesting that NCAM-dependent chain formation is important for RMS migration. NCAM in RMS neuroblasts are highly polysialylated (Hu et al., 1996) and removal of the PSA from NCAM causes chain dispersion both in vivo and in vitro (Chazal et al., 2000); (Battista and Rutishauser, 2010). While NCAM deficient mice show severe migratory defects, removal of PSA promotes migration suggesting that Polysialylation plays a critical role in the maintenance of the neuroblast chain. Furthermore, N-cadherin, like other cell adhesion molecule plays an important

role in forming cell cluster and regulating cell differentiation. N-cad is abundantly expressed in chain migrating cells in RMS (Yagita et al., 2009).

3. Models for collective cell migration

3.1 Zebrafish as vertebrate model to study collective migration

One of the most widely used vertebrate models so far is the Zebrafish *Danio rerio* lateral line to study the process of cell migration, which is highly stereotyped. The naturally transparent embryo of the fish allow direct *in vivo* visualization of the different physiological and mutant conditions (White et al., 2008).

3.1.1 The migrating lateral line primordium (LLP)

The Zebrafish lateral line sensory system responds to variety of external stimulus including water current, prey detection and avoidance and sexual courtship. The development of the Zebrafish lateral line system offers several features, such as simple *in vivo* imaging and genetic tractability, which make it an attractive model system for studying collective cell migration behavior during organogenesis (Ghyssen and Dambly-Chaudiere, 2004). It is comprised of a series of mechanosensory hair cell organs (neuromasts) that are deposited throughout the skin by the posterior lateral line primordium (LLP), a cohesive mass of more than 100 migrating cells. The moving structure has intrinsic polarity with a front consisting of many motile cells and group of differentiating cells and ceasing the backward movement. The polarity of the structure requires a combination of FGF signaling that promote the differentiated states and counteracting Wnt front signal (Fig. 7). The two signals affect one another by secreting activators or inhibitors. This signaling appears necessary to maintain the tissue polarity during movement (Aman and Piotrowski, 2008).

The directionality or the guidance of the primordium is defined by the expression of a Zebrafish homolog of the chemokine stromal-derived factor 1 (SDF-1), which the primordium detects through the expression of its receptor CXCR4 that appears essential for directed migration (David et al., 2002) and knockdown of either the ligand or receptor results in a similar strong defect in LLP migration (David et al., 2002; Li et al., 2004). Another SDF-1 receptor CXCR7 is expressed and required in the rear cells of the primordium (Dambly-Chaudiere et al., 2007); **Fig. 7**). This confirms that SDF-1 is the major cue that determines and guides the migration of the primordium.

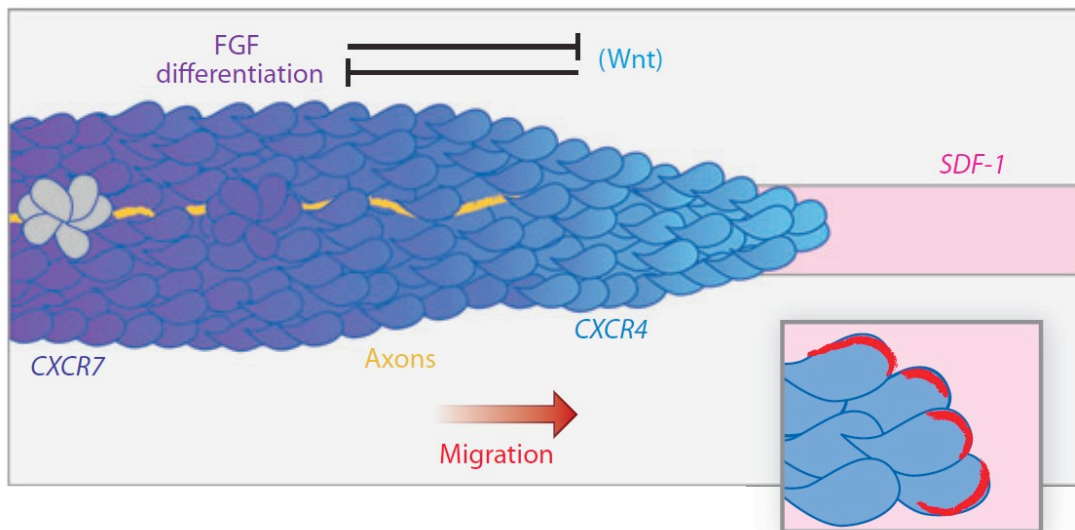


Figure 7. The migrating lateral line primordium (LLP). At the back, cells are differentiating in clusters into lateral line organs (gray) and ceasing migration in a Fibroblast Growth Factor (FGF)-dependent way; cross-regulation of FGF and Wnt may help keep the group polarized with non-differentiated cells in the front. Guided movement of the LLP requires the ligand Stromal-Derived Factor (SDF)-1 to define a path and the receptor CXCR4 in front cells. Without CXCR4, cells are motile, but the LLP does not move forward (CXCR7 may perform a similar function in rear cells). The enlarged region shows how the intrinsic polarity of the LLP makes each front cell polarized; in combination with a permissive strip of SDF-1 expression, this can give precisely directed migration (Rorth, 2009).

3.2 *Drosophila* tracheal cell migration

Tracheal development represents a very interesting case in which cell migration not only leads to the repositioning of cells within the organism but also sculpts the three-dimensional appearance of the entire organ (Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003; Ribeiro et al., 2003). Only a limited number of cells display a migratory behavior. The exceptional features of the developing tracheal system with regard to cell movement relies in the fact that cells migrating in different directions remain firmly connected with each other throughout the migratory process, ultimately giving rise to a branched tubular network. The respiratory system of *Drosophila* develops from ten clusters of ectodermal cells, the tracheal placodes that invaginate to form the tracheal pits on both sides of the embryo. Upon invagination each of these structures sprouts successively into finer branches to generate a tree like structure. Remarkably, the entire branching process occurs exclusively by cell migration and changes in cell shape without undergoing cell proliferation (Metzger and Krasnow, 1999; Affolter and Shilo, 2000).

3.2.1 Guidance and signaling pathway controlling cell migration

The molecular mechanisms controlling tracheal cell migration are now well established. It has been known for quite some time that a FGFR (Fibroblast Growth Factor Receptor) homolog and its ligand Branchless are critical determinants of the tracheal branching pattern (Sutherland et al., 1996). The secreted FGF- like ligand Branchless (Bnl) binds to the Breathless FGF receptor on nearby tracheal cells inducing collective cell migration (Glazer and Shilo, 1991); (Klambt et al., 1992). In addition, FGF signaling also stimulate the receptor tyrosine kinase (RTK) activity and downstream signal

transduction cascade involving Ras, Raf and a cytoplasmic protein encoded by *stumps* (*Dof*, downstream of FGF; (Glazer and Shilo, 1991). Expression of branchless is highly dynamic and its expression is necessary for the formation of the filopodia at the tip (Ribeiro et al., 2003). Thus, tip cells show distinct morphology and have inherent potential to sense the cues from its extracellular environment. In addition to the involvement of FGF in controlling direction sprouting of tracheal bud, the guided migration of tracheal cell is regulated by BMP (Bone Morphogenic)- like DPP (Decapentaplegic) pathway (Vincent et al., 1997; Ribeiro et al., 2003). DPP is thought to control cell rearrangements (e.g., intercalation), cell shape changes or adhesive properties of the cells specific to the dorsal branch. Misexpression of the pathway results in misguided tracheal growth from its migratory route (Ribeiro et al., 2003).

3.3 Border cell migration in *Drosophila*

Border cell migration performs well-defined, invasive and directional cell migration during *Drosophila* oogenesis (Starz-Gaiano and Montell, 2004); (Rorth, 2002). Border cells are the group of about eight cells that originate from the most anterior part of the egg chamber and which after detaching from the monolayer follicular epithelium invade the germ cell cluster. Border cell migrate as a group, on and in-between the nurse cells in the direction of oocyte. When they reach the oocytes, border cells migrate and reside over the oocyte nucleus. Border cells contribute to the formation of the micropyle, which allows the entry of sperms (Montell, 2001; Rorth, 2002).

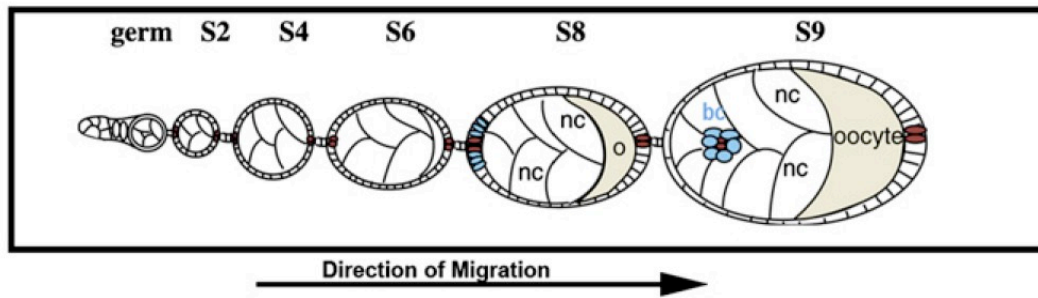


Figure 8. Schematic of oogenesis through stage 9 (Prasad and Montell, 2007).

3.3.1 Getting off the starting blocks (Cell motility)

For any racer, the key to a good start can be getting off the block at just right time. Similarly, the timing of migration during development can be of great importance. The timing of border-cell migration seems to be regulated by a steroid hormone named Ecdysone (Bai et al., 2000). The *Drosophila* ecdysone receptor is constituted by the heterodimeric complex of Ultraspiracle (Usp) and Ecdysone receptor (EcR). Usp and *taiman*, a steroid hormone co-activators function together with ecdysone receptor in a ligand dependent manner to regulate border cell migration cell autonomously (Bai et al., 2000). The *taiman* (*tai*) locus encodes oestrogen like receptor coactivator called AIB1 (amplifies in breast cancer 1), which is overexpressed in a significant amount in breast and ovarian cancer (Anzick et al., 1997). Mutation in *tai* locus causes severe border cell migration defects.

3.3.2 Cell motility and guidance

Once the cells are specified, they formed a functional cluster to initiate collective migration to reach their final destination i.e. the oocytes. It has been shown that two redundant pathways are involved in guiding border cells during the first phase of the

migration. Simultaneous inhibition of the *Drosophila* EGF and PDGF/VEGF receptors strongly inhibits border cell migration to the oocyte, showing that signaling through the two receptors is required for border cell migration (Duchek et al., 2001). At least one ligand for the PDGF/VEGF receptor (PVF1) and one ligand for the EGF receptor (Gurken) are produced by the oocyte during the migration and could thus in principle serve as guidance factors. Two other ligands for the EGF receptor (Spitz and Vein) are produced by follicular cells next to the oocyte and could therefore also be used as guidance factors (**Fig. 9**). Uniform overexpression of these ligands in the germline as well as constitutive activation of the receptors inhibits the migration, indicating that those signaling pathways are not only permissive for the migration, but indeed serve as guidance signals for border cells (Duchek et al., 2001). Activation of the guidance receptor PVR stimulates actin polymerization, through a signaling pathway involving the small GTPase Rac and the Rac activator Mbc (Duchek et al., 2001).

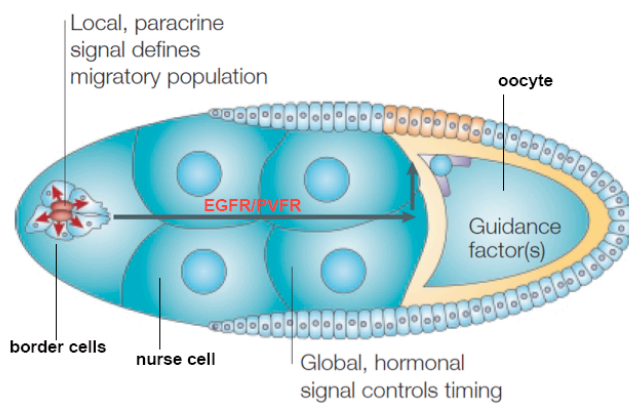


Figure 9. Signaling pathway controlling border cell migration. Presence of steroid hormone Ecdysone (blue), red indicate signaling from JAK-STAT pathway, yellow represents expression of Pvf1. Purple show Gurken expression and orange show EGFR ligand (Spitz and vein) (Montell, 2003).

In response to the guidance signal, border cells show a long cellular extension (LCE) required for initiation of migration. The LCE works in coordination with Myosin VI that seems to be required for proper border cell migration. Myosin VI promotes LCE formation and functions as “pathfinder”, helping the border cell cluster to move forward towards the oocyte (Rorth, 2002); (Montell, 2003).

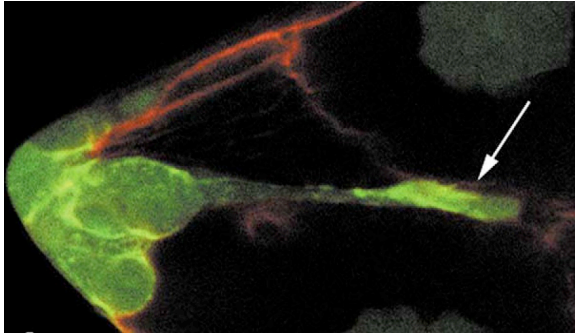


Figure 10. Egg Chamber expressing a GFP-actin fusion protein in border cell showing prominent long cytoplasmic extensions (arrow) (Ribeiro et al., 2003).

4. *Drosophila*: Swiss army knife of genetic and developmental studies

Collective migration refers to the cell migrating together in sheets, clusters, streams, and other multicellular arrangements. It is observed frequently in animals *in vivo*, both as a part of normal morphogenetic programs or in pathological situation. I would like to discuss the relevance of collective glial cell migration in flies using *in vivo* approach using developing wing. The wing is a simple tissue and so is the organization of glia. The collectively migrating behavior of the wing glia is well characterized and documented. Thanks to the improved imaging techniques (*in vivo* microscopy and use of GFP derivatives) and genetic tools that have advances the understanding of collective migration in different model systems.

4.1 The Life Cycle of *Drosophila melanogaster*

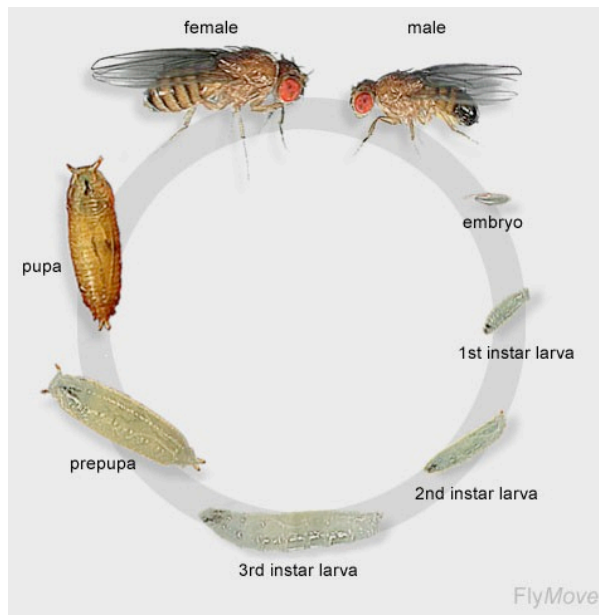


Figure 11. *Drosophila* life cycle

The life cycle of *Drosophila*, from a fertilized egg to an adult is around ten days at 25⁰C and twenty days at 18⁰C. Embryogenesis takes about one day followed by three successive larval stages. At the end of this stage, the animal is transformed into pupae where metamorphosis begins and last for around 5 days to gives rise to adult fly (Fig. 11).

4.2 *Drosophila*: a powerful genetic approach

During the past years, *Drosophila* has gained lot of popularity because of the genome sequence (Adams et al., 2000), its rapid life cycle, relative ease to handle and the genetic tool that are available for its study. The fly's genome permits the most sophisticated gene manipulation of any of the known metazoan organisms. Here, I will present some of most widely used fly specific tools:

4.3 Gal4-UAS System

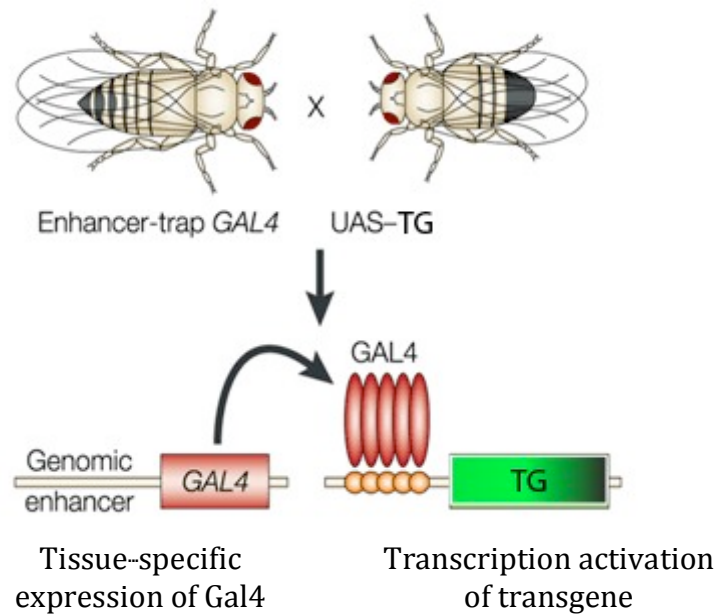


Figure 12. Directed gene Expression in *Drosophila*.

The development of the binary Gal4-UAS system by Brand and Perrimon has proven to be one of the most significant technical advances in the field of *Drosophila* genetics (Brand and Perrimon, 1993). This system allows the ectopic expression of target gene in a cell or tissue specific manner. Two transgenic fly lines are generated, in the first; a transgene placed downstream of Upstream Activating Sequences (UAS), which constitute the Gal4-binding sites. Gal4 is a yeast regulatory protein and a transcription activator (Laughon et al., 1984), the UAS transgene is specifically activated upon crossing the UAS line with the transgenic line carrying the Gal4 transgene, also known as driver (Fig. 12).

4.4 Fluorescent proteins and its implication in biology

Green fluorescent protein (GFP) from Jellyfish *Aequorea victoria* and its homolog from diverse marine animals are widely used for biological studies (Tsien, 1998; Shimomura, 2005). GFP is an unusual protein in that it is brightly fluorescent. As it is a protein, cells can be transfected with genetic markers and induced to express directly, or, to express chimeric protein comprising GFP-tagged to protein or motif of interest. Therefore, can be used to track the protein within the living cell or protein localized at the level of membrane.

Variants of GFP, with different colors have been useful for simultaneous comparison of multiple protein fates, developmental lineages and gene expression levels. These variants have emission wavelength and spectral separation that allow for multiple labeling. These include the Cyan fluorescent protein (CFP), YFP and dsRed (Heim and Tsien, 1996; Ormo et al., 1996; Matz et al., 1999) to make few examples, which emit wavelength in the region of cyan, yellow and far red respectively. The advent of fluorescent proteins has not only allowed us to label the cell but also detect protein-protein interactions.

5. Motile glial cells

Glia and neurons are the two major components of the Central or Peripheral nervous systems (CNS and PNS) respectively, in which the information is relayed in highly coordinated and well-organized manner. To construct these complicated networks, glia and neurons have to migrate either solitary or as collectives, over a long distance to reach their final destination (Valiente and Marin, 2010); (Marin et al., 2010); (Klambt,

2009). I will present the main properties of glial cells as well as their migratory behaviors, focusing PNS glia in *Drosophila*

5.1 General introduction to glial cells

Increasing complexity through evolution demands the establishment of more sophisticated nervous systems. Therefore the two neural components: glia and neurons distribution varies across evolution (Klambt, 2009) and becomes more difficult to analyze in vertebrate. Glial cells are implicated in arrays of functions. Glia are often viewed as merely 'glue' supporting neurons, but they also have guidance cues to the growing axons for proper targeting and in clearing neuronal corpses in neuronal pruning. Glia also provide neurotrophic factors to neurons; recycle neurotransmitters at the synapse and have been found to play a role in courtship behavior (Levine, 2008). In vertebrates glia provides an efficient insulation of the nervous system via the myelin sheath. In addition, astroglia in the CNS are part of the synapse and regulate efficient synaptic transmission (Pfrieger, 2009).

5.2 *Drosophila* peripheral glial cell

In *Drosophila*, both neurons and glia are derived from the neuroblasts. Differentiation of lateral vs midline glial cells in *Drosophila* relies upon the gene *Glide/Gcm* transcription factor (Hosoya et al., 1995) (Jones et al., 1995) (Vincent et al., 1996). *Gcm* acts as a binary switch between the glia and the neuronal fates: in *gcm* mutant animals almost all presumptive glia adopt the neuronal cell fate. Conversely, ectopic expression of *Gcm* induces the glial fate at the expense of neuronal fate (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1997). Several genes have been identified as downstream targets of *Gcm* and the best characterized among them is *repo* (*reverse*

polarity) and is necessary for the maintenance of the glial cells. *repo* encodes a homeobox transcription factor that remains active throughout the life of the animal and that *repo* mutations not affect glial fate determination but the glial differentiation (Campbell et al., 1994); (Halter et al., 1995); (Xiong et al., 1994).

5.2.1 PNS glial cell migration during embryogenesis

The PNS comprises sensory neurons, which project their axons into the CNS, and axons of motoneurons that navigate across the lateral body wall to innervate the target muscles.

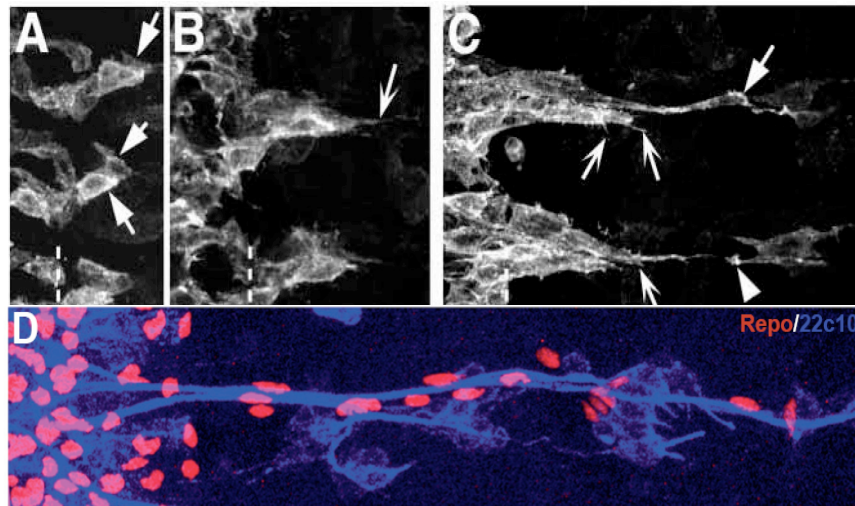


Figure 13. Immunolabelings of *Drosophila* embryonic peripheral glial cells. (A) Glial cells form cone shaped structure in each hemisegment and proliferate at the lateral edge of the CNS (arrow). (B) Glial cells migrate to periphery. The leading glia extends cytoplasmic processes (concave arrow). (C) Leading edge further moves to reach the target (arrowhead; Sepp, 2003). (D) Immunolabeling of the Peripheral Nervous System at late stage. 22c10 (blues): sensory neurons; Repo (red): glial cell nuclei (von Hilchen et al., 2008).

These axons fasciculate to form peripheral nerves that are covered by glial cells. Some glia arises from sensory organ precursors (SOP) of the periphery (Schmidt et al., 1997)

while others originate from the CNS/PNS boundary (von Hilchen et al., 2008) from where they migrate in a chain to occupy highly stereotyped positions along the nerve. Like vertebrate glia, *Drosophila* glial cells never overtake the navigating axonal growth cones (GC). The identity of individual glial cells in a well-defined genetic background (**Fig. 13D**) while the function of pioneer glia (**Fig. 13B**) have now been well established (von Hilchen et al., 2008).

5.2.2 Glia migration in the larval eye imaginal disc

The complex eye originates from a structure called the eye imaginal disc. During the larval stages, glial cells migrate collectively from the optic stalk into developing eye along the photoreceptor axons. These axons provide the navigating substrate for the glial migration (Silies et al., 2007). For the coordinated migration, the glia specific FGF8 like Pyramus ligand transduce signal through the Heartless FGF receptor (Franzdottir et al., 2009). Htl is expressed broadly in the glia and promote glial projection following the photoreceptor axons. When glia cells reach the nascent photoreceptor axon that expresses FGF8 ligand (Thisbe), glial cell start expressing sprouty (a negatively regulator of FGF) that eliminates Htl/FGFR activity. As a result migration process is halted and promotes the glial differentiation (Franzdottir et al., 2009).

5.2.3 Developing pupal wing glia migration

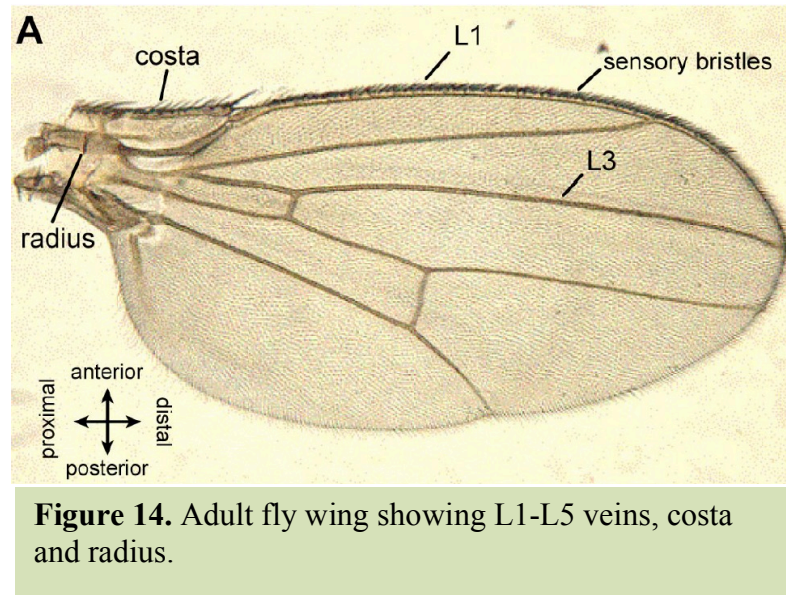
Drosophila developing wing provide excellent tool to study cell migration and has several advantages: Embryonic glia move along the motor and sensory components that are derive from CNS and PNS that move very fast. These glial need precise control over space and time to monitor the migratory process. Wing glia provide a simple tool to

study this process as its developmental process is long, wing is simple tissue and highly tractable with the advance genetic and imaging facility. Fly wing has well defined nerves that are purely sensory component and more importantly, it has no effects of maternal contribution.

5.2.4 Wing development

Animal shape and size is controlled with amazing precision during development. There are three major processes that contribute to tissue growth in a coordinated manner: cell division, cellular growth and cell survival. Each of these cellular processes is controlled by specific and highly conserved regulatory pathways that are employed in response to specific need of tissue. *Drosophila* provides a premier genetic model to study the animal growth that shed a light on how body and tissue size are regulated. The flies are holometabolous insects that undergo complete metamorphosis to produce an adult organism that is quite morphologically distinct from its larva. *Drosophila* utilizes the imaginal disc, the proliferating larval epithelial cells that form the adult appendages of the fly. Imaginal disc are the epidermal sac like structures derived from the cell cluster that were left aside during embryogenesis.

The fly wings are developed from the wing imaginal disc upon eversion. The mature wing is enclosed in a flattened pouch like structure consisting two apposed epithelia and carrying five longitudinal (L1-L5; [Fig. 15](#)) veins along antero-posterior. Amongst these five veins, only two (L1 and L3; [Fig. 14](#)) are innervated (Hartenstein and Posakony, 1989) (Huang, 2009).



5.2.5 Peripheral wing development

The peripheral nervous system composed of thousand of sensory organs that are widely distributed over wings responsible for collecting the stimulus from the environment and transmit signal to the central nervous system. There are two types of sensory organ present at wing blade: the external/ mechanosensory organ and chemosensory bristles (Hartenstein and Posakony, 1989). Along L3 vein, there are 6 companiform sensilla are present, however large number of sensory organs can be located along the wing margin. In addition, there are two also two-companiform sensilla present along L1 called TSMs (Fig. 19).

The sensory organs are developed from a complicated network of events where proneural genes are selected from proneural cluster, within which a single cell is selected to become a sensory organ precursors (SOP). These SOP are then single out from the epidermis via process called lateral inhibition, mediated by the Notch (N) signaling pathway (Hartenstein and Posakony, 1990) (Artavanis-Tsakonas et al., 1999). Upon

specification of SOP, it undergoes series of asymmetric cell division to give rise to different cells that constitute a sensory organ. These SOPs then produces glia and neurons (Giangrande, 1995) (Van De Bor et al., 2000) **Fig. 15**).

The sensory axons present at the along the wing margin are clustered together in nerve called L1 nerve whereas the neurons along L3 form L3 nerve. The L1 and L3 axons navigate towards the proximal part of the wing to reach the CNS where the L1 joins the radius (Murray et al., 1984). At the margin of the wing around 12 hAPF, the L1 neurons extend its projection towards CNS that forming a continuous sheath by 16-17 hAPF.

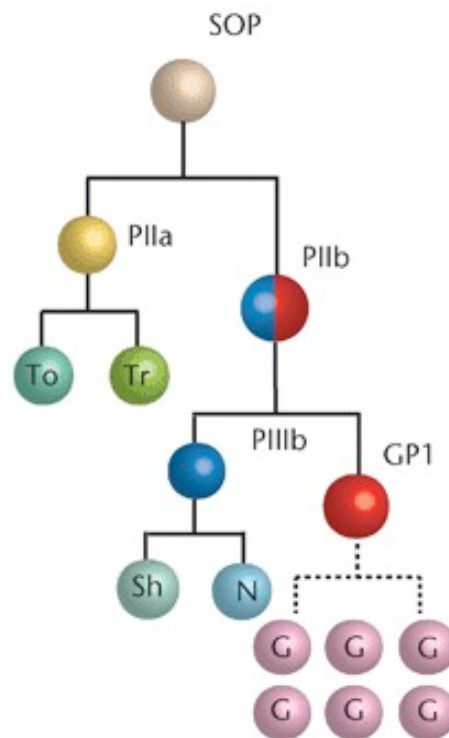


Figure 15. Wing gliogenic Sensory organ lineages. Sensory organ precursors undergo division to produce second order precursor PIIa and PIIb. PIIa divides to produce Tormogen cells (To) and Trichogen (Tr). PIIb gives rise to PIIIb (Sh: shaft and N: neuron) and a glial precursor (GP1).

These neurons can be marked with specific markers, for example, nuclear labeling by Elav protein (Embryonic lethal abnormal vision; (Murray et al., 1984), membrane specific protein HRP (Horseradish Peroxidase; (Jan and Jan, 1982) and microtubule associated protein 22c10/Fustch (Hummel et al., 2000). During development, glial cells arises from the sensory organ precursors and glial specific protein Gcm can be detected as early as 9 hAPF followed by then repo positive glial cells. The glial present along the margin of the wing undergo differentiation and proliferation in a manner where the distal cells appear earlier than the proximal ones and gliogenesis in the wing persist until 15-17 hAPF.

5.2.6 Glial cell migration

Glia present on the anterior margin are known to move extensively from the place of origin to reach their final destination, often as migrating group of cells. During development, neurons send their axons towards proximally located CNS then glia migrates on axonal bundle and cover it. This process of glial collective migration is well characterized the lab (Aigouy et al., 2004) (Aigouy et al., 2008). Laser assisted confocal video microscopy and cell selective destruction (UV Laser) provides direct evidences to show cell migration and proliferation. The glial cell present along L1 and L3 undergo proliferation very occasionally. This proliferation process is more evident along L3 nerve because of the limited number of glia where as along L1, high density of glia makes it difficult to follow individual behaviors of migrating cells (Aigouy et al., 2004); (Van De Bor et al., 2000). Proliferation process however is not regulated by the glia-glia interaction but migration process is indeed influenced by the glial contacts. When some of progenitor specific cell present along L3 are removed, the rest of the glia undergo

proliferation and occupy the position. The axon provides the navigating substrate for the migrating cells. When axons are misrouted, the glial cell followed the misguided neuronal path. In addition, glial cell migration can be followed in the absence of neurons. When Notch mutant (N^{ts1}) receptor is absent throughout the wing sensory organ development, all lineages are transformed into glia and move in a continuous chain as compared to control wings (Aigouy et al., 2004). These, clearly showing that glia are endowed with intrinsic migratory potential. Altogether, these observation indicate that neuron-glia interaction affect the direction of migration but not the cell motility.

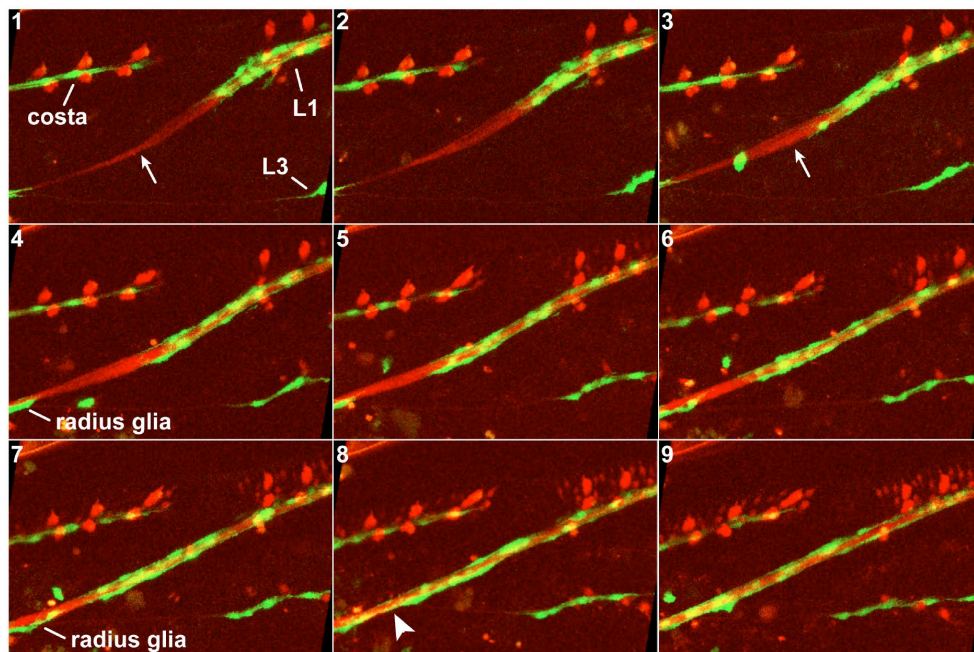


Figure 16. Glial cell migration along axons. Snapshots show the projection from time-lapses on UASGFP/+; repoGal4/elav-dsRed wing in which neurons are labeled in red and glial cells in green. The movie begins at 19 hAPF and glia starts moving progressively and completes migration upon joining the proximally located radius glia (arrowhead; (Aigouy et al., 2004).

One of the most peculiar features of glial cell is that they tend to form a chain of cells. This suggests that glial cells display affinity for axons as well as other glial cells. Most of

the above mention results were obtained by analyzing the behaviors of glial cells present along L3 nerve because it contains few neurons and glia making it easier to follow the dynamic process. While on the other hand glia cells present along L1 are more densely populated and unsheath axon by the time. The glial cells initiate/start to as chain along L1 nerve at around 18 hAPF and completes migration upon joining radius glia by 26-29 hAPF (Aigouy et al., 2004); [Fig. 16, 17](#)).

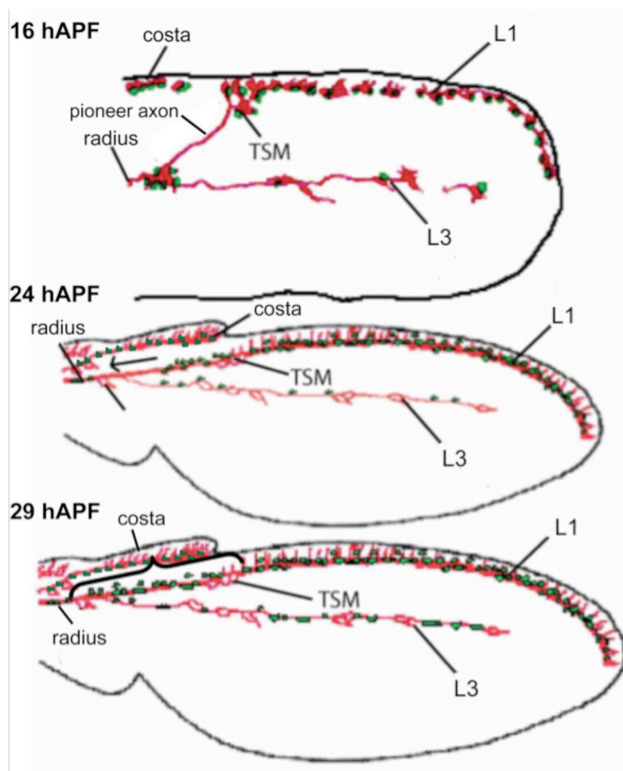


Figure 17. Schematic representation of *Drosophila* wing migration. Glial cells are shown in green, neurons in red and twin sensilla of the margin. At 19 hAPF neurons forms a continuous structure along L1 and L3 nerve. By the 24 hAPF glia has moved and by 29 hAPF completes migration by joining at radius.

Glial cells present at the anterior margin of the wing contain morphologically distinct population of cells within the chain: a proximal front (tip cells) region and the distal tip region present at the distal end of the anterior wing margin.

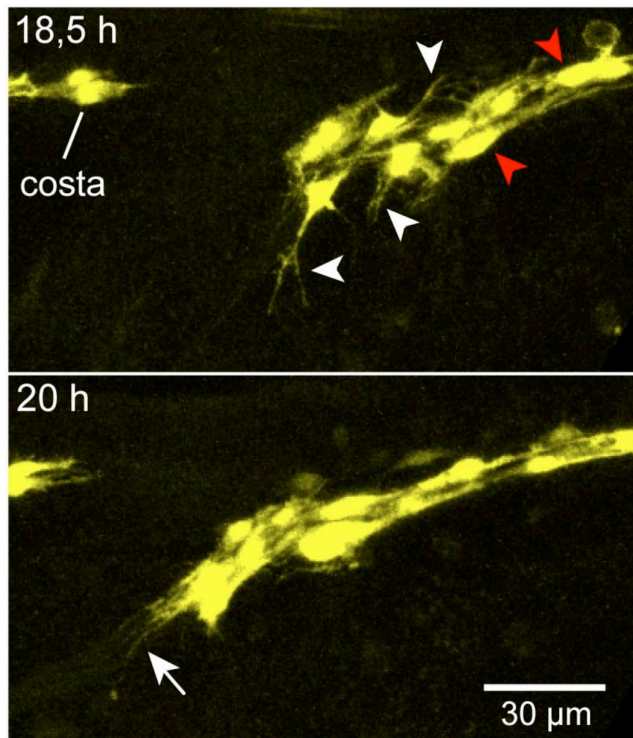


Figure 18. Glial chain tip at the initiation of migration (18 and 20 hAPF). Glia is marked by GFP (yellow). Tip cells display elaborate cytoplasmic process in all direction (white arrowheads) while cell behind do not (red arrow head). Upon initiation, cells at front project filopodia in the direction of migration.

At the time of initiation of migration, the proximal most glia of the L1 nerve display long exploratory filopodia, actin based extensions that grow and retract in all directions (white arrowheads, [Fig. 18](#)) where as other cells distal to the tip region do not display dynamic morphology and are aligned along the axon (red arrowheads, [Fig. 18](#)). Collective glial migration is characterized by the coordinated behaviors and highly motile cells present at the migratory front. The cells that are present at the tip explore the environment and provide the directional cues for the rest of the cells. The presence of the pioneer population likely provides the forces that allow the efficient movement of large group of cells stretching over long distance. Interestingly, it has been shown that ablating the first four cells (pioneers) at the tip of the chain severely delays migration compared to those

that were not ablated (control) (Aigouy et al., 2008). Therefore, presence of pioneers at tip chain is critical for coordinated glial cell migration. In addition to the leader role, MARCM clones have revealed a key feature of pioneers that interact with the followers by sending long and stable process at the rear end called distal cytoplasmic extension (DCE). The DCE has several implications: the antero- and- retrograde interaction promotes soma translocation and ensures the chain integrity for efficient cell migration (Aigouy et al., 2008; [Fig.19](#)).

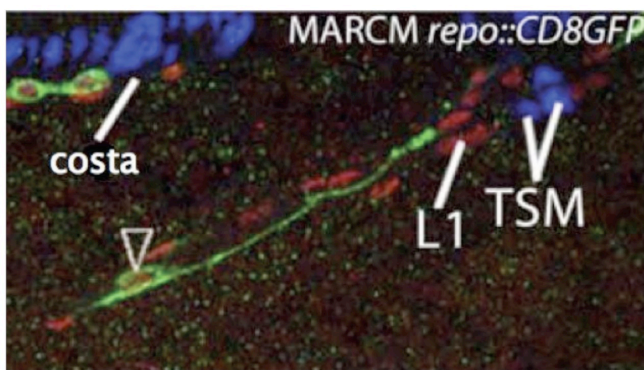


Figure 19. Pioneer cells extending DCE (Distal cellular extension) towards follower glia. Neurons are stained with anti-elav (blue), glia nuclei (red) and GFP marks the clone by MARCM method (Aigouy et al., 2008).

6. Cadherin mediated cell-cell adhesion

Normal tissue development, remodeling, and homeostasis require tight control over cell adhesion to the extracellular matrix (ECM) and to neighboring cells (Gumbiner, 1996). Cadherins are transmembrane receptors that mediate cell-cell adhesion (Yap et al., 1997a). The adhesive role of cadherins underlies many morphogenetic processes and requires their interaction with the actin cytoskeleton (Delon and Brown, 2007; Gumbiner, 2005). Cellular responses to cell adhesion are regulated by the underlying protein complexes that link adhesion receptors to the cytoskeleton of the cell (Geiger et al., 2001; Yap et al., 1997a). ECM proteins have been shown to affect the expression, localization, and composition of cell-cell adherens junctions (Chen and Gumbiner, 2006; Liu and

Senger, 2004; Sakai et al., 2003; Wang et al., 2006). The interactions between the cell-ECM and cell-cell adhesion systems that occur during tissue morphogenesis are not completely understood.

Migrating cells are in constant contact with their substrate extracellular matrix (ECM) (Yap et al., 1997a), which can either be stably localized, or move with the migrating cells. Therefore, unraveling the basic mechanisms of cell-cell or cell-ECM interactions is relevant to understand coordinated cell migration.

6.1 Adherens Junctions

The type I “classical cadherins” (Munro and Blaschuk, 1996) mediate one of the most integral and ubiquitous types of cell-cell adhesion. Adherens junctions are found in most kinds of tissue throughout the body and can be formed by one or more cadherin subtypes in any given cell type (Takeichi, 1988). It is well established that the adhesive function of adherens junctions relies on their interaction with actin microfilament network of the cell (Gumbiner, 2005; Yap et al., 1997b), although the details of this connection are still a subject of intense debate and research (Gates and Peifer, 2005). Adhesive forces are critically important and prevent animal tissues from dissociating into their component cells. These forces must be dynamic and strength is needed to maintain stable cell association under external stress, for example: tissue morphogenesis, body movement or cell movement. Therefore, dynamic changes in cell adhesion are required to establish new cell contacts during cell movement, tissue renewal and wound healing. In order to form a stable connection or to be able to communicate, the cells need to establish a link at the membrane. Adherens junction (AJ) is one such component that fulfills this requirement where it links membrane and cytoplasm at discrete contact regions.

Adherens junctions consist of two basic adhesive units: a) the cadherin/catenin and b) nectin/afadin complexes. Broadly speaking, these adhesive complexes link a homophilic recognition event with the underlying actin cytoskeleton and distinct contributions of each complex to AJ structure/function have emerged.

6.1.1 Cadherin/catenin clustering at AJs

The first identified members of the cadherin superfamily, or classical cadherins, have been characterized by their ability to mediate calcium dependent adhesion between cells (Hyafil et al., 1980; Hyafil et al., 1981), hence their name. In vertebrates, there are 20 members that have been identified as classical cadherin while the *Drosophila* genome encodes only 3 classic cadherins (epithelial-, Neuronal- and Neuronal 2-cadherin) (Hill et al., 2001). Adhesion is mediated by the cadherin ectodomain that engages an identical molecule on the surface of adjacent cells (homophilic, trans-interaction). Cadherin clustering, however, also form cis- dimers within the same transmembrane. They undergo lateral clustering forming a stable adhesive interface (Trojanovsky, 2005; Kovacs and Yap, 2008).

The cytoplasmic domain of classic cadherins is highly conserved in length, sequence and interacting partners across species (Takeichi, 2007). Catenins provide a key link from cadherin to actin cytoskeleton. Here, I will discuss the main activity of cadherins in AJ and cytoskeleton formation. The cytoplasmic domain of cadherins can be divided into a juxtamembrane domain and a C-terminal domain. The C-terminal domain binds to β -cat (Ozawa et al., 1989; Ozawa et al., 1990). This binding is required to link cadherins to the actin filaments (Ozawa et al., 1990). The juxtamembrane contains

binding sites for members of the p120-catenin family, which is involved in lateral clustering of cadherins (Yap et al., 1997a); **Fig. 20**).

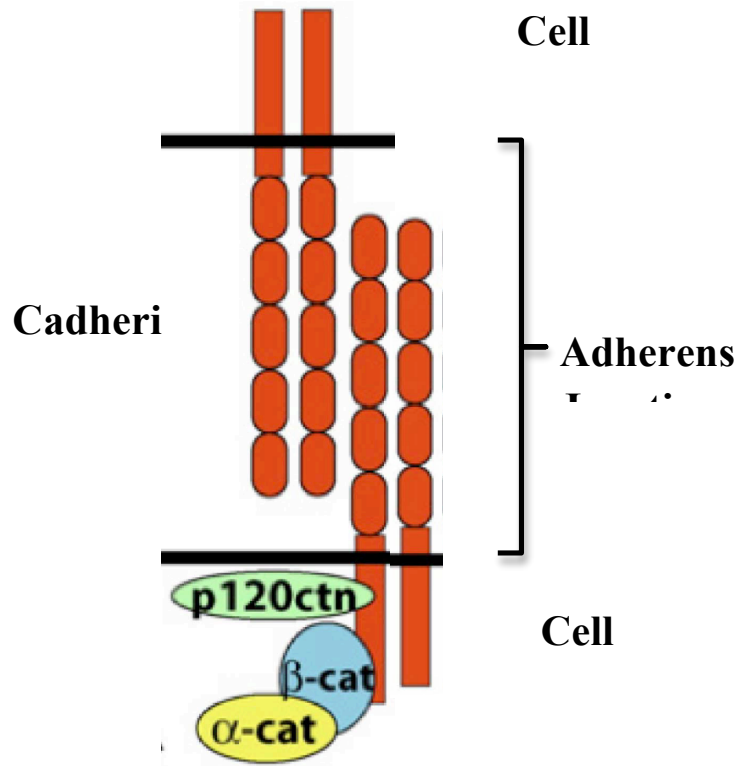


Figure 20. Cadherin/catenin complex in cell adhesion (Niessen and Gottardi, 2008).

6.1.2 β -catenin (Arm) and γ -catenin- linking cadherin to actin

The catenins constitute the major group of proteins that associate intracellularly with the cadherin cytoplasmic tail (Ozawa et al., 1989; Aberle et al., 1994; Huber et al., 2001) that begins in the endoplasmic reticulum and is required for the effective surface transport of cadherins through the biosynthetic pathway (Chen et al., 1999; Lock et al., 2005). Once at the plasma membrane, the cadherin- β -cat complex rapidly recruits γ -cat that is linked to actin filaments (Ozawa et al., 1990; Bajpai et al., 2008). γ -cat binds to

actin directly or through interaction with other actin binding protein called \langle -actinin or vinculin (Knudsen et al., 1995; Rimm et al., 1995; Weiss et al., 1998). \textcircled{R} -cat is constituted of 12 Armadillo repeats that bind to the C-terminal region of cadherin (Hulsken et al., 1994; Pai et al., 1996), while the N-terminal domain interacts with \langle -cat (Pai et al., 1996). \langle -cat cannot bind simultaneously to \textcircled{R} -cat and \langle -cat homodimers have higher affinity for actin while monomers readily bind to \textcircled{R} -cat (Koslov et al., 1997; [Fig. 21](#)).

\langle -cat is essential for AJ formation and function and it operates at the interface between the cadherin- \textcircled{R} -cat complex and the actin cytoskeleton (Huber et al., 2001; Benjamin and Nelson, 2008). In addition, the cadherin-catenin complex contains binding information for \langle -cat required for the formation of a ternary complex. \langle -cat binds to actin filament *in vitro* (Rimm et al., 1995) and is critical for the actin polymerization process *in vivo* (Vasioukhin et al., 2001). More importantly, it was assumed that \langle -cat is the direct linker between cadherin- \textcircled{R} -cat to the actin cytoskeleton forming a quaternary complex. (Yap et al., 1997a). However, recent studies have shown showed that \langle -cat binding to cadherin- \textcircled{R} -cat and actin cytoskeleton is mutually exclusive. This is due to an allosteric switch in the conformation of \langle -cat, in which \langle -cat monomers preferentially bind the cadherin- \textcircled{R} -cat complex while homodimers binds actin filaments. According to this view, clustered cadherin- \textcircled{R} -cat complexes at the membrane result in high local \langle -cat concentration, which eventually impinges onto the actin cytoskeleton changes (Drees et al., 2005; Yamada et al., 2005; [Fig. 21](#)). Cadherin also associates to other cytosolic proteins that in general act as positive regulator of cadherin function. One such example is p120-cat (p120^{ctn}) (Yap et al., 1998; Niessen and Gottardi, 2008). The cadherin-p120

interaction counteracts cadherin endocytosis and degradation and thus promotes cell surface abundance of cadherin (Ireton et al., 2002; Davis et al., 2003).

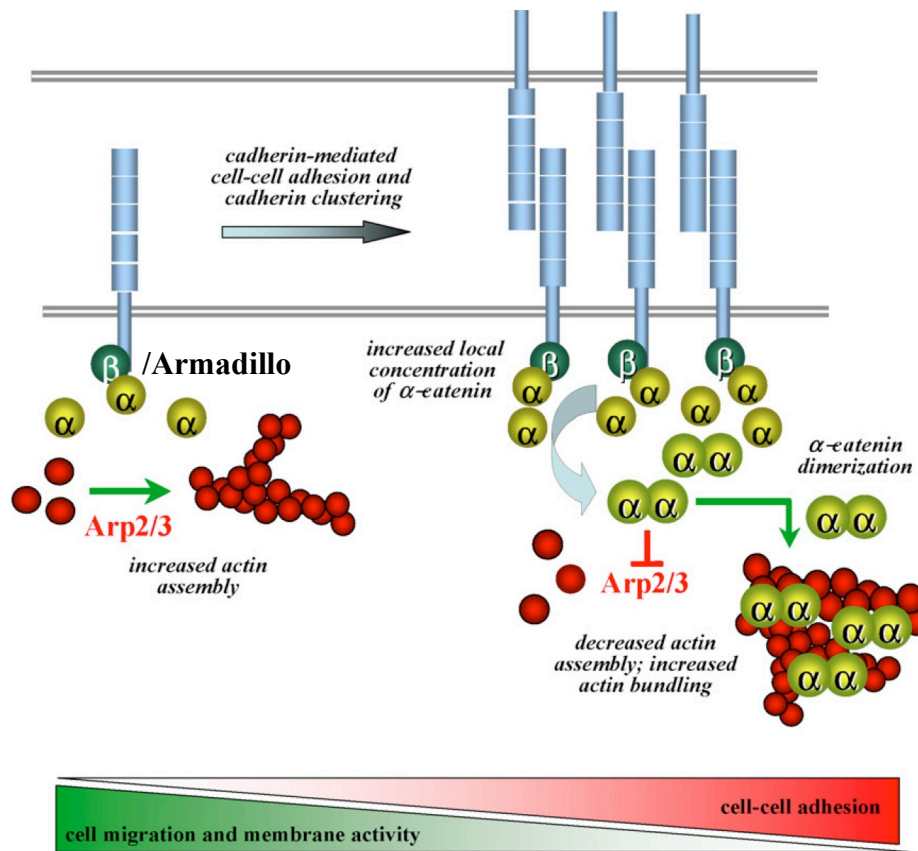


Figure 21. Schematic explaining the cadherin- β -cat mediated cell adhesion. β -cat do not establish a direct contact with cadherin and actin. Instead, increase in the concentration of β -cat by cadherin clustering may lead to the dissociation of β -cat forming homodimers. These homodimers inhibit the activity of the Arp2/3 nucleation complex, which is known to promote actin branching a process required for cell motility (Hosoya et al., 1995; Niessen and Gottardi, 2008).

In

sum, the cadherin- β -cat “core adhesive” complexes constitute components that mediate homophilic interaction at the membrane and regulate actin dynamics via β -cat in the cytoplasm.

6.1.3 The nectin-afadin adhesion complex

Nectin is a member of the IgG superfamily of calcium-dependent adhesion molecules. Like cadherins, members of nectins family also form lateral homodimers that engage in homophilic and heterophilic manner. The cytoplasmic domain of nectin interacts with an actin binding protein known as AF6/afadin (Takahashi et al., 1999; **Fig. 22**), thereby providing an alternative way to couple AJs to actin. Afadin is known to interact with Ras/Rap-family GTPases as well as other actin binding protein and ζ -cat (Yokoyama et al., 2001). Although, in mammalian system, Nectin /Afadin seems to play a crucial role in promoting assembly of cadherin based AJs (Sawyer et al., 2009). However, in *Drosophila* this function of Nectin/Afadin has not been identified (Sawyer et al., 2009).

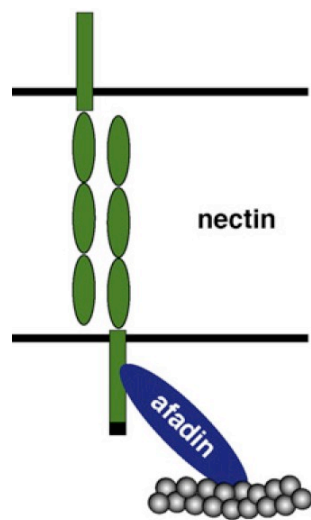


Figure 22. Core structural components for adhesion and junction formation. Nectin homophilic adhesion receptors either directly or indirectly associate with actin filament via afadin (Niessen and Gottardi, 2008).

Similar to ζ -cat, afadin knockout reveals an essential role in epithelial organization (Ikeda et al., 1999; Yokoyama et al., 2001). Thus, like the cadherin- ζ -cat adhesive unit, the nectin-afadin complex mediates intercellular adhesion and actin association.

6.2 From the AJs to the Cytoplasm

6.2.1 β -catenin/Armadillo signaling

The elephant in the room is β -cat. In vertebrates, β -cat was identified as a binding partner for classical cadherin via the cadherin cytoplasmic tail (Takeichi, 1988; Ozawa et al., 1989). In contrast, the *Drosophila* homolog of β -cat, *armadillo*, was identified initially as a component of the Wg/Wnt signaling pathway both in vertebrate and invertebrate (Peifer and Wieschaus, 1990; Peifer et al., 1991). β -cat participates in both adhesion and signaling functions in a mutually exclusive way: linking to cadherin at the plasma membrane or localized in the cytosol (Daugherty and Gottardi, 2007). In absence of Wg/Wnt, multiple phosphorylation in the protein results proteasome-mediated degradation. The phosphorylation depends upon additive action of Adenomatous Polypous Coli (APC), Axin protein and kinases: Glycogen synthase Kinase 3beta and it Casein Kinase I (CK I). In presence of Wg/Wnt, GSK3beta is inhibited as a consequence β -cat accumulates and promotes transcription of Wg/Wnt target genes by binding to TCF (T-cell Factor) in the nucleus. (Fig. 23). Mammalian homolog of *Drosophila* Legless (Lgs) also acts as the switch between cell adhesion and Wg/Wnt signaling.

In Wg/Wnt signaling, β -cat acts independently of cadherin (Daugherty and Gottardi, 2007). β -cat can function as a transcriptional co-regulator, cooperating with transcription factors of the TCF (T-cell factor) family to induce gene expression (Dierick and Bejsovec, 1999). Key to the role of β -cat in Wnt signaling is the regulation of the cytosolic pool of β -cat that is available to enter the nucleus and thereby modulate transcription.

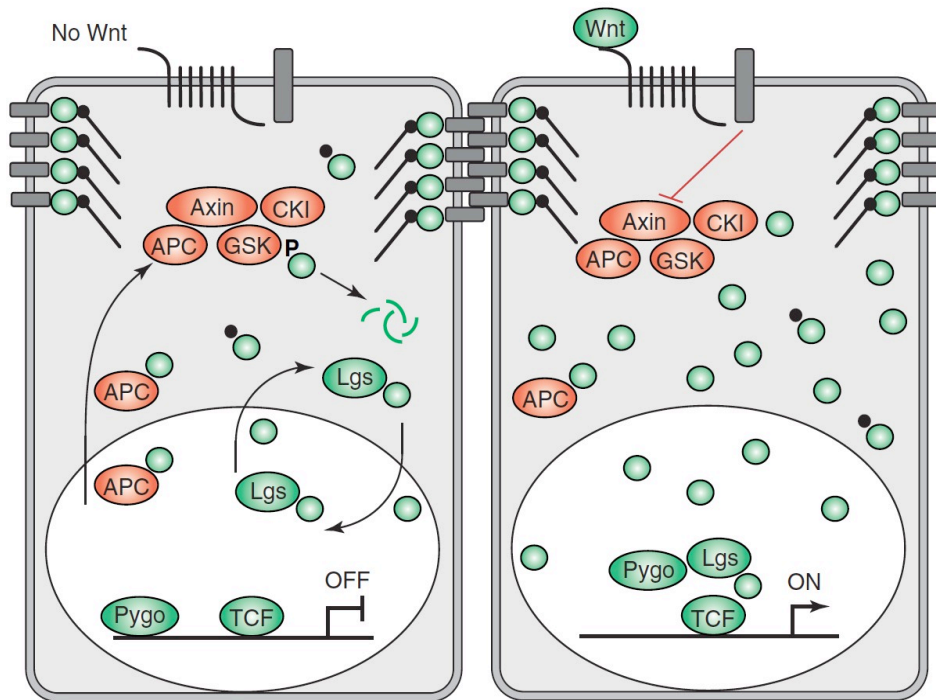


Figure 23. The Wg/Wnt signaling cascade. Bars, E-cadherin; green dots, β -cat/Armadillo; black dots, α -cat; thin black lines, actin. In the absence of Wg/Wnt signaling (left), the Axin complex is active and phosphorylates β -cat/Armadillo, triggering degradation; β cat/Armadillo is inactive and TCF transcription is off. Wg/Wnt stimulation activates the transcription of Wnt target genes in the nucleus (right).

6.2.2 Adherens Junction and associated signaling pathway

Beside cadherin- α -cat complexes, there are other factors involved in the adhesion complex that contribute to the regulation of developmental cellular signaling pathways. One mode of signaling from the AJs involves a direct activation of RTK leading to specific tyrosine phosphorylation. The interaction of RTKs with the cadherin can be direct where N-cad activation promotes motility by FGF-dependent activation of FGFR, suggesting that N-cad interact with FGFR and stabilizes it on the membrane (Suyama et al., 2002). Echinoid/Nectin can also interact with EGFR signaling pathway during

Drosophila eye development (Bai et al., 2001).

Taken together, these studies have provided significant amount of information showing that the cadherin- β -cat complexes are not only involved in the formation of adherens junctions but also provide a link to the developmental signaling pathways.

6.3 Roles of classic cadherin during development and diseases

The cadherin superfamily consists of the transmembrane protein containing conserved extracellular repeats. The superfamily members show high degree of variability in the structure of the extracellular and cytoplasmic domains as well in the

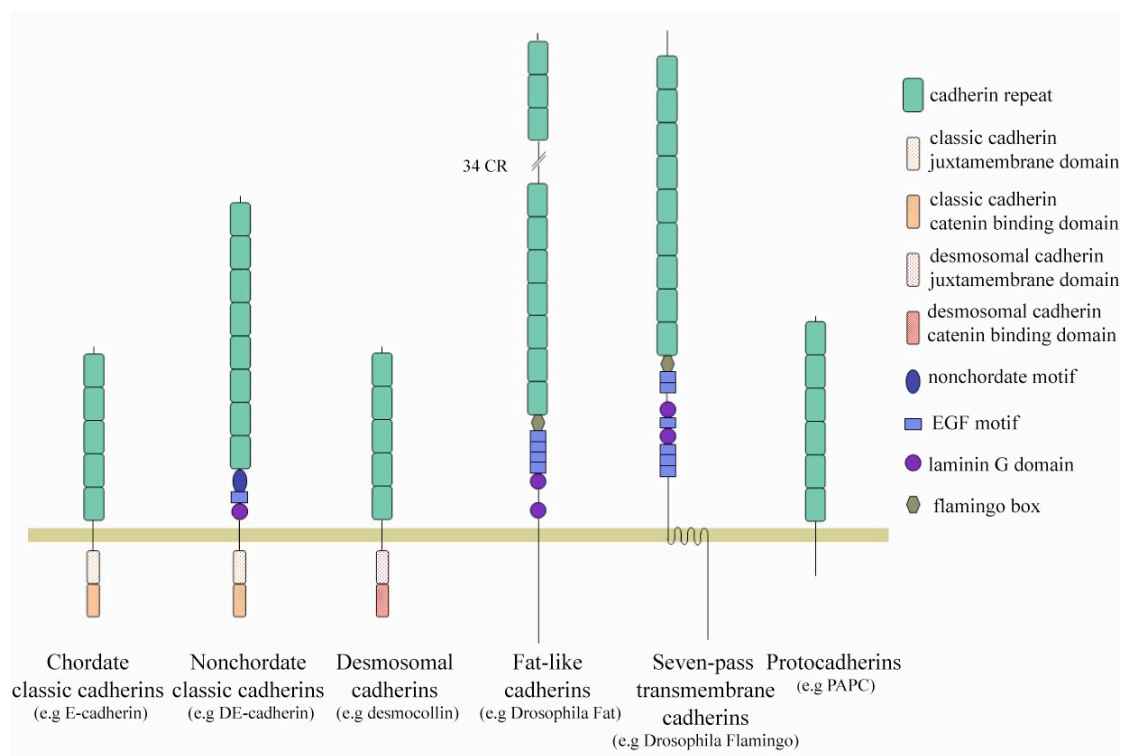


Figure 24. The cadherin superfamily. Five cadherin subfamilies showing their composition. Classical cadherin, Fat-like cadherin and seven pass transmembrane cadherins are found in vertebrates and invertebrates. Desmosomal cadherin and proto cadherins are found only in chordates.

number of transmembrane domains (Harris and Tepass, 2010). The superfamily also contains other well known classic cadherin namely: desmosomal cadherins which are linked to intermediate filaments via proteins of the plakin family; protocadherins, which constitute the largest among the cadherin subfamily and found only in chordates; Fat-like cadherins and seven transmembrane family domain proteins such as *Drosophila* Flamingo (Adler et al., 1998). Most of the cadherin super family show high degree of variability in terms of their structures of the extracellular and cytoplasmic domains as well as in the number of transmembrane domain that it contain (Fig. 24). The *Drosophila* genome contains 3 main classical cadherins that are involved in various developmental and morphogenetic processes. E cadherin was first identified as an adhesive molecule required for compaction during early embryonic development (Hyafil et al., 1980). In *Drosophila*, DE-cadherin null mutant show severe defects in epithelial cells such as ventral epidermis that undergo important cell rearrangement during embryogenesis (Tepass et al., 1996). In the E-cadherin knockout, mice embryos die before implantation due to “decompaction” that corresponds to loss of adhesion (Larue et al., 1994; Riethmacher et al., 1995). Therefore, E-cadherin is required both for establishing and maintaining proper adhesion. Finally, cadherin regulation is not only essential during development but also in disease conditions. Loss of E-cadherin is observed during most epithelial cancers and significantly contribute to proliferation at early stage of tumorigenesis as wells as during epithelial to mesenchymal transition (Gottardi et al., 2001).

6.4 Tissue integrity and nervous system development

Cadherins have been implicated in a wide variety of roles rather than just simply maintaining epithelial integrity. The epithelial cells of multicellular organisms possess a well-defined architecture, referred to as basoapical polarity that coordinates the regulation of essential cell features. Cadherin loss of function are the cause of most carcinoma and contributes to the proliferation at the early stages of tumorigenesis as well as transition from benign to malignant tumors. Invasive behavior has been thought to be due to the decreased cell-cell adhesion. However, in some tumor lines, both proliferation and invasiveness can be suppressed by E-cadherin in an adhesion-independent manner (Gottardi et al., 2001).

In addition to their role in apicobasal polarity, cadherin members have been implicated in another forms of cell polarity known as planar cell polarity (PCP). PCP describes the orientation of a cell within the plane of epithelial cell layer. PCP has been extensively studied in variety of epithelia; *Drosophila* wing has remained a primary experimental system to study it. There are several genes including *frizzled (fz)* that are required for normal wing hair polarity (Gubb and Garcia-Bellido, 1982). Other studies revealed that the protocadherins (*fat*) and Dachshous (*Ds*) are the component of second signaling pathway that is implicated in normal wing PCP (Eaton, 2003).

Cadherin have been shown to play a diverse role than simply maintaining epithelial integrity. In particular, E-cadherin is sufficient to establish polarity in cultured cells (McNeill et al., 1990). Similarly, DE-cadherin has been implicated in migrating cells onto a cellular substratum. In many cases, both in mammals and in *Drosophila* cadherins have been shown to mediate adhesion between somatic and germline cells

(Godt and Tepass, 1998; Jenkins et al., 2003). DE-cadherin is required for adhesion of migrating border cells along the surrounding nurse cells (Niewiadomska et al., 1999). Loss of DE-cadherin function results in slow border cell migration. During *Drosophila* oogenesis, DE-cadherin is required in oocytes and in the follicular cell for positioning of the oocyte into the egg chamber (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998). One possible explanation for this is that BCs are in constant contact with their surrounding (nurse cells). In absence of E-cadherin border cells probably donot generate enough traction force to push them forward through nurse cells. While in the wings axons provide the well-defined navigating substrate upon which glia migrate.

Finally, cadherin and their regulation are not only essential during development but also contribute to proliferation at early stages of tumorigenesis. Invasive behavior is thought to be due to decreased cell-cell adhesion (Gottardi et al., 2001). In conclusion, cadherin form a “core cohesive” complex with catenins that mediate hemophilic interaction at the membrane. The cytoplasmic part of the cadherins appears necessary for mediating the interaction between adherens junction and actin cytoskeleton.

*Materials &
Methods*

List of *Drosophila* strains used

Genotype	Abbreviated names
gcmrA87/CyOtwi-GFP	gcm>GFP
gcmGal4,UASGFP/CyO	
gcmGal4,tubgal80ts/ CyO; UASGFP/UASGFP	gcm,tub80ts
gcmN7-4/CyO-GFP	N7-4
UAS-F18A (III)	
gcmGal4,UASGFP,repoGal80/CyO; UASGFP/UASGFP	gcm,repogal80
UAS ricin/CyO-GFP; tubgal80ts/tubgal80ts	UAS ricin
UAS N-cadherin (X)	N-cad GOF
UAS N-cadherin RANi (II)	N-cad LOF
UAS actin42GFP/Tb	
UAS α -cat GFP (III)	
UAS actin42GFP/sb	
UAS CYFIP (III)	CYFIP GOF
UAS CYFIP RNAi (III)	CYFIP LOF

Immunolabeling

The anterior and posterior ends of staged pupae were cut off in 4% PFA PBS (paraformaldehyde in phosphate buffer saline) and pupae were fixed at 4°C for 1 hour. The pupae were pulled out from the puparium case, the cuticle over the wing was removed and the wings were pinched off the body in PBT (PBSTriton-X100 0,3%). After quick washes in PBT, the wings were incubated in PBT-NGS (5%normal goat serum in PBT) for 20 minutes at room temperature on shaker. Then, incubated in primary antibodies (diluted in PBT-NGS) overnight. After three washes in PBT, the wings were in secondary antibodies for two hours diluted in PBT-NGS at room temperature. Following a final wash in PBT the wings were mounted on slides in Aqua-Poly/Mount medium (Polysciences Inc.).

List of Antibodies used

Primary Antibody: mouse- α -Repo (1:800), mouse- α -22c10/Futsch (1:1000), rat- α -Elav (1:800), rabbit- α -HRP (1:600), chicken- α -GFP (1:1000), mouse- α -Armadillo (1:50), rat- α -alpha-cat (1:50), rat- α -N-cad (1:50), rabbit- α -beta-gal (1:500)

Secondary Antibody: Secondary antibodies (raised against mouse, rat, rabbit or chicken, and coupled to Cy3, Cy5 or FITC fluorescent dyes) were purchased from Jackson ImmunoResearch Laboratories and used in 1:500 dilutions in PBT-NGS.

Laser Scanning Microscopy

Images of immunolabeled samples were taken with SP2 confocal microscopes. GFP/FITC excitation: 488 nm, emission: 498-551 nm; Cy3 excitation: 568 nm and emission: 648-701 nm; and Cy5 excitation: 633 and emission: 729-800 nm. z-series of

images were collected at step-size between 0.2 and 1.5 μ m. z-stack projections of confocal images, optical z-section representation, image rotations and color-coding were obtained using ImageJ Software (NIH). Figures were prepared in Adobe Illustrator/Photoshop.

Fast Confocal Microscopy

For time-lapse movies, pupae were staged and dissected according to (Aigouy et al., 2004; Classen et al., 2008). The pupae were observed with Leica SP5 upright confocal microscope equipped with a heating stage ($25\pm 2^\circ\text{C}$) and GFP was excited at 488 nm with emission at 498-551 nm. Time-lapse movies on the control and experimental animals were taken at an interval of 20 sec and processed by Adobe Illustrator, Photoshop and Image J software.

Chapter 1

Cellular mechanisms controlling glia migration.

Collective cell migration is a widespread developmental process where cells move extensively to ensure proper tissue and organ formation. The members of these migrating communities move in the same direction and stay in more or less close proximity to each other (Rorth 2007, 2009, 2012; Friedl, 2009). Some of them behave as leaders and others as followers (Aigouy, 2004). A lot of recent studies have focused on how these populations are specified or what are the external cues that drive the movement of collective, however, much less is known about the homeostatic interactions amongst these cells. Glia in the *Drosophila* developing wing provide an excellent tool to study this process (Aigouy, 2004) and previous studies have shown that cell-cell interactions play an important role (Aigouy, 2008, Berzsenyi, 2011). However, these investigations left many interesting questions unanswered.

When we address the issue of collective cell migration, we refer to it as the movement of several cells in close proximity. In different model systems, moving groups display different sizes: the *Drosophila* border cell cluster comprises 6-10 cells while the Zebrafish lateral line primordium is constituted by several hundreds of cells. Can the cells sense whether they are part of a big or small community? Does this have any impact on migration if a cell is in contact with several or just a few ones?

In this section we have addressed these questions by studying the homeostatic interactions amongst the glial cells at the front. I will present the results that were obtained by analyzing migration upon UV-ablation of cells at the tip of the chain. We show here that pioneers cannot move in isolation. Furthermore, upon ablation at different positions in the tip region, we have separated cell cluster of different sizes (1, 4 and 6-10 cells). These *de novo* formed communities display migratory behaviors that depend on cell number. This work has been carried out in collaboration with a

senior PhD student and has led to a publication in an international, peer reviewed journal.

Homeostatic Interactions at the Front of Migration Control the Integrity and the Efficiency of a Migratory Glial Chain

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In metazoans, cell migration often occurs in a collective manner: the cells move while physically and functionally connected to their neighbors. The coordinated and timely movement of the cells eventually ensures the proper organization of tissues, and deregulation in such a process contributes to the development of severe diseases. Thus, understanding the cellular mechanisms underlying coordinated cell movement is of great interest in basic and medical science.

The developing *Drosophila* wing provides an excellent model to follow the chain migration of glial cells *in vivo*. Cells at the tip of the glial collective have been shown to control the timely movement of the chain. In the present study, we show that while pioneers trigger chain migration, they cannot move as single cells. We also show that isolating cell clusters at the chain tip restores the formation of smaller migratory communities. Interestingly, the migratory efficiency of these *de novo* formed communities depends on the number of cells and progressively improves as the size of the cluster increases. Thus, homeostatic events at the migratory front control community integrity, efficiency, and coordination, emphasizing the importance of interactions and cell counting in fine-tuning collective processes.

Introduction

The correct pattern of complex tissues like the vertebrate nervous system arises from the coordinated migration of cells that are connected to each other (Marín et al., 2010). Defects in this collective process lead to neural diseases, and disruption of tissue cohesiveness may result in the mobilization of tumor cells (Friedl et al., 2004; Valiente and Marín, 2010). During the last decade, we have gained important insights on the molecular specification of leader versus follower cells at the tip of a migrating cohort (Ghabrial and Krasnow, 2006; Hellström et al., 2007). “Supracellular” molecular structures have also been recently shown to control migratory community integrity and coordination (Friedl and Gilmour, 2009). The cellular bases and the role of the homeostatic interactions occurring at the migratory front remain, however, poorly understood, due to the dynamic and complex nature of such interactions.

In the present study, we address this issue *in vivo*, in a system that allows tracing of collectively migrating cells. Using targeted ablation, we previously showed that four cells at the tip of a glial chain in the *Drosophila* wing act as pioneers (Aigouy et al., 2008).

We here show that pioneers cannot move in isolation. Furthermore, upon ablation at different positions in the tip region, we have separated cell clusters of different sizes. These *de novo* formed communities display migratory behaviors that depend on their cell number. A four-cell cluster migrates less efficiently than a six-cell cluster, which migrates less efficiently than a ten-cell cluster, a migratory community that recapitulates the features of an intact chain. Moreover, the four-cell cluster tends to reestablish contact with the neighboring chain more efficiently than the six-cell cluster, whereas the ten-cell cluster moves as an independent collective.

Thus, bidirectional interactions and cell-counting mechanisms at the chain tip control the efficiency of migration and the integrity of the community, two main aspects of collective migration.

Materials and Methods

Dissection, time-lapse and immunolabeling were as described previously (Aigouy et al., 2004, 2008; Soustelle et al., 2008). Animals of either sex were analyzed (total $n = 79$ time-lapses: 18 controls; 17 early ablations, 8 late ablations; 9 single-cell, 10 four-cell, 11 six-cell, and 6 ten-cell clusters). To keep a predetermined number of cells isolated, we sometimes performed a second round of ablations because new glia emerged nearby the cluster.

The *UAS-Apoliner* strain was from J. P. Vincent (MRC, London, UK). Antibodies were m-anti-22c10 (Developmental Studies Hybridoma Bank, 1:1000) and Cy3-anti-mouse (Jackson Laboratory, 1:500). Images and movies were processed using ImageJ (NIH). Statistical analysis was done by Student's *t* test; bars indicate SEM. For semiquantitative analyses, distances were calculated manually and then transformed into micrometers upon considering the used magnification.

Results

Dynamics of glial chain migration

The L1 sensory nerve of the developing *Drosophila* wing is ensheathed upon directional migration of a glial chain (Gian-

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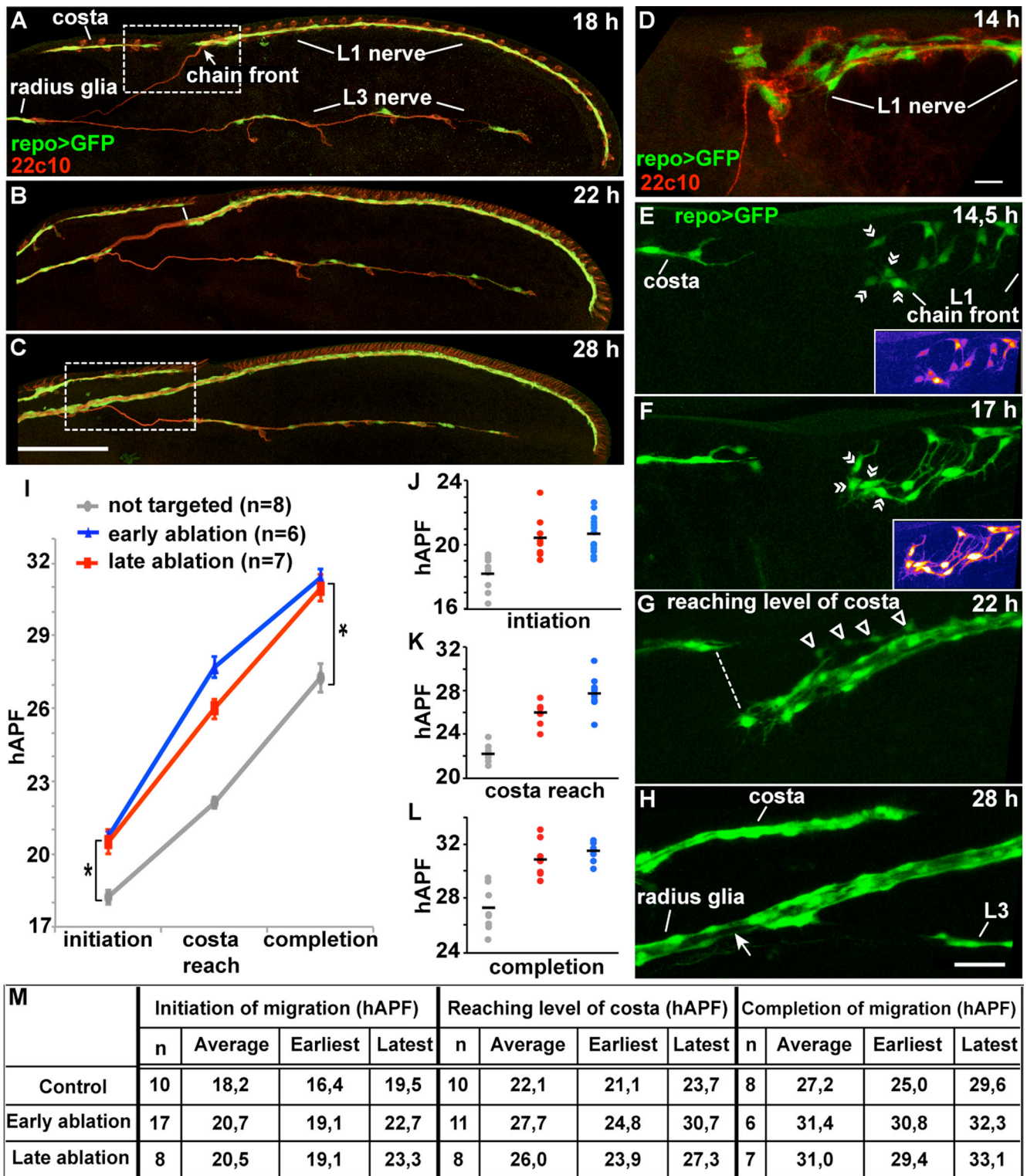


Figure 1. Glial chain migration in a control wing and upon early/late ablation. **A–D**, Immunolabeling showing wing glia (green) and neurons (anti-22c10, red) at different stages: onset of migration (**A**), reaching level of the costa (**B**), completion of migration (**C**), 14 hAPF (**D**). In all panels, proximal is to the left, anterior to the top. **E–H**, In this and in following figures, snapshots show projections from time-lapses on *repo>GFP* wings. **E–H** correspond to the regions outlined in **A** and **C**, respectively. **E**, Before migration, glia show simple morphology and low GFP level (color-coded inset). **F**, By 17 hAPF, GFP intensity is higher (inset) and cells extend filopodia. The four double arrowheads (**E**, **F**) indicate the position of the ablated cells. **G**, By 22 hAPF, the migration front reaches the level of the costa. Open arrowheads show new glia appearing on the margin. **H**, The glial chain completes migration (arrow), joining radius glia. **I**, Graph representing migration efficiency in control wings and in wings in which the first four cells were ablated early or late (**p* < 0,05, *n* = wing number). **J–L** show the raw data: **J** refers to migration initiation, **K** to reaching level of costa, and **L** to migration completion. The x-axis shows three experimental conditions: controls (gray circles), late-ablated wings (red circles), and early-ablated wings (blue circles). The y-axis indicates stages as hAPF. Each circle represents one sample; horizontal bars, average values. **M**, Ablation data summary. The first column indicates the experimental conditions, the second, the third and the fourth columns indicate the stage of achievement (hAPF) of migration initiation, reaching the level of costa and completion. For each column, from left to right: wing number, average, earliest and latest stages. Bars: **A–C**, 100 μ m; **D**, 10 μ m; **E–H**, 30 μ m.

grande, 1994; Aigouy et al., 2004, 2008). During this period, the two wing epithelial blades are widely separated at the position of the L1 vein, creating a large cavity in which the nerve and the glia move (Fristrom et al., 1993). First the neurons send their axons proximally toward the CNS, then glia migrate along the axonal bundle and cover it (Fig. 1*A–C,E–H*). By 17–18 h after pupa formation (hAPF), glial cells start moving proximally along the nerve as shown by their soma translocation (Fig. 1*A,F*), and by 22 hAPF the chain front reaches the level of the nerve on the costal vein (Fig. 1*B,G*). The glial collective completes migration by 26–29 hAPF, upon joining the glia on the proximally located radial nerve (Fig. 1*C,H*).

Cells at the migration front display a network of exploratory filopodia and are indispensable for the proper timing of chain migration, since elimination of the first four cells at migration onset (17–18 hAPF) induces severe delay in glial coverage of the axonal bundle (Aigouy et al., 2008). Despite these dynamic features, pioneer cells are not observed in isolation; that is, they do not migrate independently from the collective. To address the issue of collective integrity, we decided to separate a tip cell from the chain and asked whether it has the potential to move in isolation. Unfortunately, at migration onset, the chain tip displays a complex organization (Aigouy et al., 2004, 2008), making it difficult to isolate cells. We therefore asked whether ablations could be performed at early stages, when tip cells may have a simpler organization. We could visualize glia as early as 14.5 hAPF, using *UAS-GFP; repogal4* (*repo > GFP*) transgenic flies that express nuclear and cytoplasmic GFP in all wing glia. At this stage, tip glia are located close to the neuronal somata and show simple morphology (few filopodia, low GFP intensity), and individual cells can be identified since they are arranged in a looser manner than at later stages (Fig. 1*D*). Between 14.5 and 17 hAPF, glia develop filopodia and accumulate GFP; limited, passive migration may be observed (Fig. 1*E,F*), mostly due to morphogenetic changes (wing extension and growth) occurring in this period. Because this initial modest shift is mostly passive, we focused on the active glial migration occurring after 17 hAPF.

Upon eliminating the first four cells at 14.5 hAPF, we found that early ablations affect chain migration like late ablations (18 hAPF) do. To define the overall migration efficiency, we performed very long confocal time-lapses and calculated the percentage of wings showing glia at a given position. We identified three steps: initiation (somata leaving the initial position), maintenance (reaching the level of costa), and completion of migration (reaching glia on the radius) (Fig. 1*A–C,I–M*). The delay in migration was similar between early and late ablations. In both cases, wings showed an initial delay of 2 h in average and chains kept migrating at a slower pace compared with control chains (average 4 h delay by migration completion) (Fig. 1*E–M*). The relatively small variability among the different samples shows the robustness of the migratory behavior at each step.

In sum, the pioneer activity is already detected by 14.5 hAPF, making it possible to analyze tip cell behavior upon early ablations.

Migration of individual pioneer cells requires contact to the collective

To determine the nature and role of the homeostatic interactions at the migratory front, we separated the first cell from the rest of the chain. We produced a gap upon targeted ablation and followed the migratory phenotype of the isolated cell as above. We used the *repo > GFP* ($n = 4$) or the *UAS-Apoliner; repogal4* (*repo > Apo-GFP*) transgenic line ($n = 5$), to score for apoptotic pathway activation. In *repo > Apo-GFP* flies, the glial membrane-tethered GFP localizes to the nucleus upon caspase activation (Bardet et al., 2008). Upon ablation of neighboring cells, the isolated cell did not emit the filopodia network typical of a pioneer and was eventually reached by the chain (Fig. 2*A–E*). In three cases, the isolated cell assumed rounded morphology and eventually underwent blebbing several hours after targeting (Fig. 2*A–E*). This suggests that the cell isolated from the chain entered the apoptotic pathway and hence could not migrate. In six cases, however, the isolated cell did not die and yet failed to migrate, subsequently getting incorporated into the slowly moving chain (Fig. 2*F–J*). The defective migration of apoptotic and non-apoptotic isolated cells suggests that connectivity controls both survival and cell movement. An experimental asset in which we were able to not completely separate the first cell confirmed this: the cell still in contact with neighbors distal to the gap was rapidly joined by the chain and recovered the migratory phenotype ($n = 2$) (Fig. 2*K–N*). The chain then completed migration with a delay comparable to the first four-cell ablated glial chain (Figs. 1*I–M*, 3*P*).

Thus, pioneer cells are controlled by the migratory community.

The size of the cluster determines its migratory efficiency

As tip cells cannot move in isolation, we asked whether separating a cluster of tip cells from the chain had a distinct effect and ablated cells behind the first four pioneer glia (Figs. 3*A–E*, 4*A–F*).

The isolated four-cell cluster did not initiate migration on time (Fig. 3*A–E,P–S*). Just after ablation, it exhibited a rather

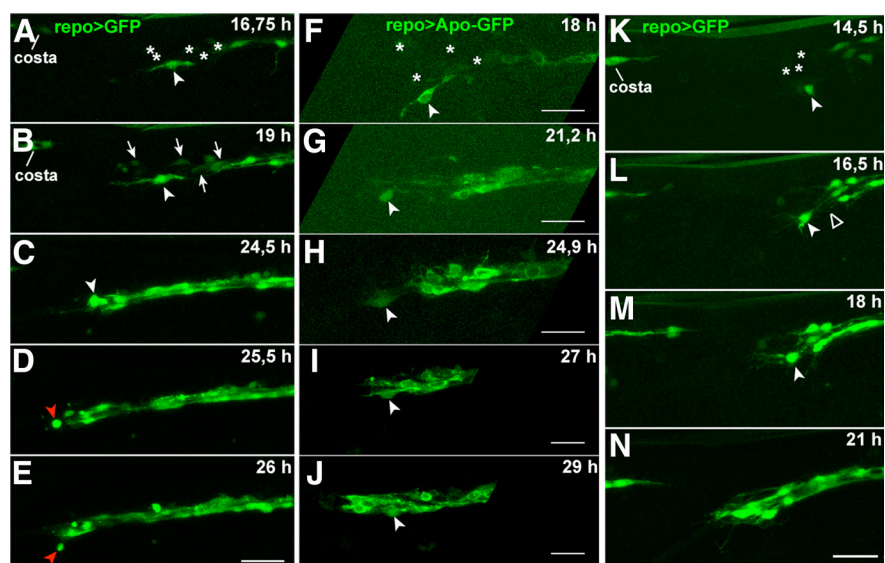


Figure 2. Single cell behavior upon isolation. The first cell is isolated by ablating cells behind it (asterisks in *A, F, K*); genotypes are indicated. *B*, Arrows indicate the UV-targeted, dying, cells. Several hours after isolation, the left-alone cell (white arrowhead) shows rounded morphology (*C*), then undergoes blebbing (red arrowheads in *D, E*). In *F–N*, the isolated cell eventually reintegrates the chain, despite initial caspase activation (nuclear GFP) (*G–I*). *L*, Empty arrowhead shows that the gap between the isolated cell and the chain is rapidly filled in this case and the joined cell resumes migrating. Bars: *A–E, K–N*, 30 μm ; *F–J*, 20 μm .

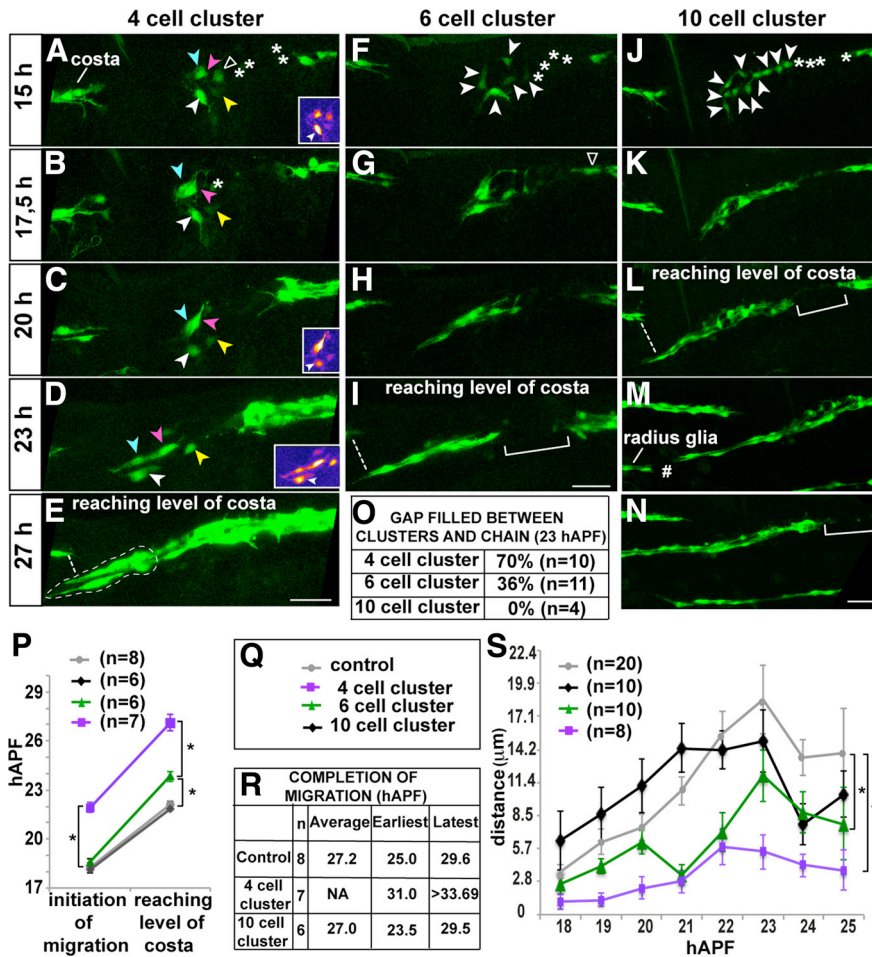


Figure 3. Migration efficiency upon isolating cell clusters of different sizes. Cell clusters are isolated upon UV-targeting (asterisks) **A–E**, Four pioneer isolation (colored arrowheads). The cyan⁺/magenta⁺ cells (arrowheads) were initially identified as one cell; however, as it became obvious that they were two, the cell indicated by the open arrowhead was eliminated (asterisk in **B**). The remaining four cells start migrating at ~22 hAPF and reach the costa by 27 hAPF (**E**) (see dashed line). GFP intensity initially decreases in one cell (white arrowhead, color-coded insets). GFP intensity and migration efficiency progressively recover (**D**, **E**). **F–I**, Six-cell cluster (**F**, arrowheads). To keep the cluster separated from the rest of the chain, additional ablation was performed (open arrowhead in **G**). The gap between the separated group and the chain persists until 23 hAPF (bracket), when the cluster has reached the costa (**J–N**). The ten-cell cluster (arrowheads) starts migrating and reaches the costa by 20 hAPF, as control chains. Note in **K** that a targeted cell still bridges the gap before disappearing at a later stage (**L**, **M**). The cluster has almost completed migration by 23 hAPF (#) and by 27 hAPF is still separated from the distal chain (bracket in **N**). **O**, Percentage of wings in which the isolated clusters and the distal chains have reestablished contact by 23 hAPF. **P**, **Q**, Graph representing migration efficiency in control chains and in isolated clusters. Symbols as in Figure 1*I*. **R**, Ablation data summary. For the four-cell clusters, migration was not always complete by the end of the time-lapse; therefore, the average time was not assessed (NA). **Q**, **S**, Graph showing the distance (μm, *y*-axis) covered each hour (hAPF, *x*-axis) by the soma of the proximal-most cell in control chains and in isolated clusters. Each point represents the average value, bars represent SEM. Bars, 30 μm.

simple morphology and the cell somata were not aligned along the axons. In some cases, the GFP intensity decreased in cells of the cluster (Fig. 3*A–C*, white arrowhead), suggesting that their overall health was affected; however, they subsequently resumed projecting cellular processes, aligning along the nerve, and recovered GFP expression. The cluster slowly resumed migration and was joined by the rest of the chain (Figs. 3*A–E*, *O*, 4*C*, *D*). The reconstituted chain completed migration with a delay as above (Figs. 1*I–M*, 3*P–S*). Thus, the four-cell cluster can migrate, albeit inefficiently.

The different behavior of the isolated first cell versus the four-cell cluster prompted us to ask whether the size of the cellular unit influences its functionality. We increased the number of cells in the isolated cluster and separated six to seven cells from the rest of the chain (Figs. 3*F–I*, 4*G–K*). Strikingly, the glia of the isolated

group started to extend filopodia at the proper time before migration and accumulated the GFP as in control wings. The six-cell cluster started migrating on time compared with control chains and kept moving proximally while disconnected from the chain (Fig. 3*F–I*, *P–S*). Despite the obvious gap between the group of cells and the followers, the six-cell clusters formed a new chain and moved proximally (Fig. 3*H*, *I*), reaching the level of the costa later than control chains but earlier than the four-cell clusters (Fig. 3*I*, *P*, *S*). Eventually joined by the cells distal to the gap, the reconstituted glial chain reached the radius glia, albeit delayed compared with control chains (data not shown). In one-third of the cases, the six-cell clusters rapidly reestablished contact with the chain (vs two-thirds for the four-cell clusters) (Figs. 3*O*, 4*I*). Interestingly, disconnected and reconnected six-cell clusters reached the level of the costa with a similar delay (compare Figs. 3*I*, 4*J*) and both seemed more prone to migrate as a new chain than four-cell clusters (compare Figs. 3*I*, 4*K*; 3*D*, 4*D*).

Finally, isolated ten-cell clusters at the chain front moved as intact, control chains: they started migrating, reached the level of the costa, and finished migration as not-targeted glial collectives (or even before), even though they stayed disconnected (Figs. 3*J–S*, 4*L–Q*).

To evaluate migration efficiency in a semi-quantitative manner, we tried photoactivatable-GFP transgenic lines (Murray and Saint, 2007) to follow the dynamics of individual cells over several hours. While this technique allowed us to trace small, epithelial cells, the signal/background ratio is too low for large and dynamic cells such as glia (S. Berzsenyi, unpublished observation). We therefore used conventional GFP lines and determined the average distance covered each hour by the soma of the proximal-most cell. This parameter

provides the net cellular movement and is not affected by the fast changes and the variability of cytoplasmic protrusions. In control animals, cells initially migrated slowly, at the time of highly exploratory behavior. The speed of migration progressively and significantly increased until 23 hAPF and then slightly slowed down (Fig. 3*S*; data not shown). A similar profile was observed in the ten-cell clusters. Interestingly, the migration of the four-cell clusters was significantly slower at each time point and that of the six-cell clusters, initiated as the controls, slowed down precociously and subsequently recovered normal speed (Fig. 3*S*).

Thus, the ten-cell cluster shows migratory features that recapitulate those of a control chain; the four-cell clusters perform poorly in migration and tend to stay with the rest of the chain; the six-cell clusters behave in an intermediate manner.

Migratory behaviors around the gap reveal homeostatic interactions at the chain tip

The ablations behind the clusters allowed us to analyze the behavior of cells proximal and distal to the gap, shedding new light onto the collective migratory process.

One explanation for chain directionality is that contact inhibition enforces the proximal migration of tip cells. We have indeed shown that ablation in the middle of a migratory community releases contact inhibition, leading cells proximal to the gap to move backwards (Aigouy et al., 2004). If the same were true at the chain tip as well, inhibition would be lost when groups are isolated from the rest of the chain, accounting for the poor performance of small clusters in proximal migration. Regardless of the size of the restored community, however, the cells of the clusters do not migrate toward the gap (Figs. 3, 4). It is unlikely that the presence of cell debris and/or hemocytes prevents backward migration because we did see distal cells subsequently filling the gap (Figs. 3A–E, 4B–F). These data strongly suggest that cells at the chain tip actively move in a directional manner.

Interestingly, the cells distal to the gap did not become pioneers: they did not show the highly dynamic filopodia network of such cells and did not migrate as the tip cells of control chains or ten-cell clusters (Fig. 4A,G,L). Indeed, semi-quantitative analyses showed that their migratory speed resembles that of the four-cell clusters (Fig. 4A). After the first hours, the distal chain migrated slightly more efficiently upon a four-cell than upon a six- or ten-cell cluster isolation (white arrows in Fig. 4B–D,H–K,M–P). This is likely explained by the fact that cells at each side of the gap slowly tried and reestablished physical contact, a process that was more successful when the cluster was small and not too motile (in some ten-cell cluster samples, the gap persisted until completion of migration; Figs. 3N,O, 4P).

Together, these data suggest that the isolated clusters do not move in response to contact inhibition and that the homeostatic interactions that finely tune the efficiency/integrity of the collective depend on the size of the cluster.

Discussion

A pioneer cell cannot move in isolation

Collective migration is a complex phenomenon in which cells constantly and actively interact with each other. In the present study, we have explored the role of specific cell interactions in the glial chain of the developing *Drosophila* wing. We show that the pioneer cells belong to a community and that they are not able to switch from collective to individual migratory behavior. Interest-

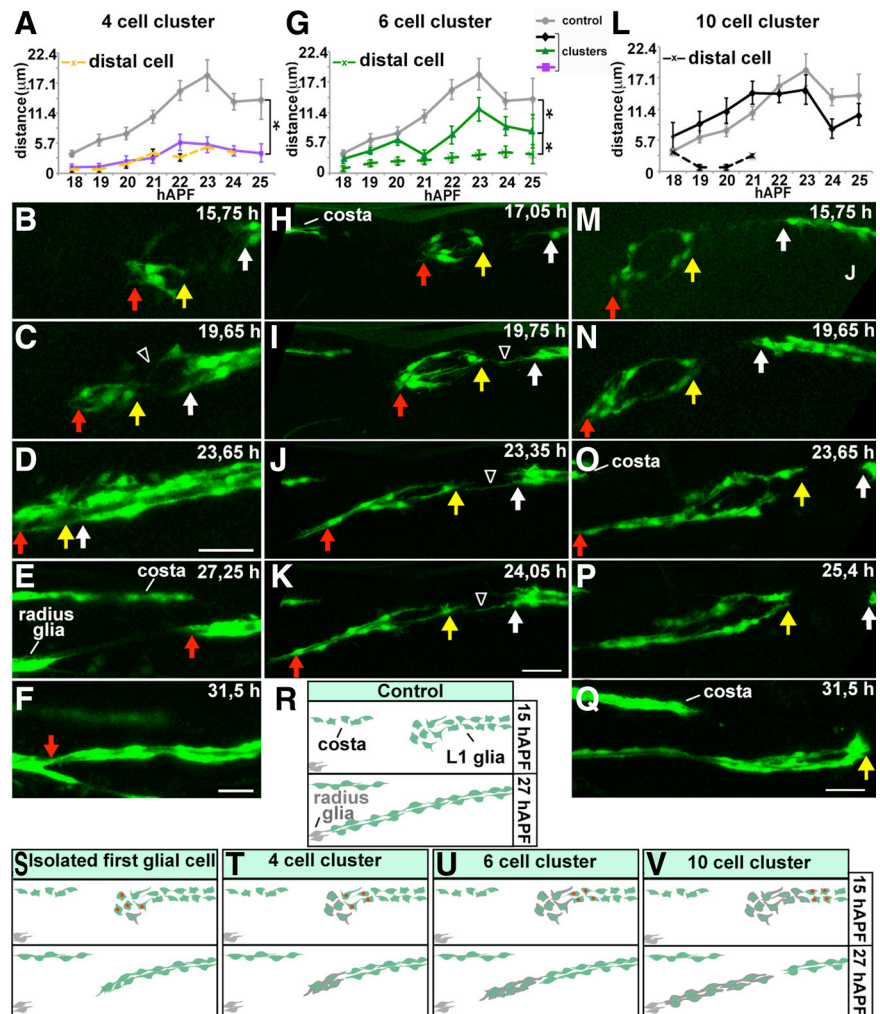


Figure 4. Migration dynamics in the gap region upon isolating clusters of different sizes. **A, G, L**, Semi-quantitative analysis as above showing the migratory behavior of clusters and cells distal to the gap. Distal cells in **L** could only be followed for a few hours, after which the stage was shifted to trace the rapidly moving cluster. **B–F, H–K, M–Q**, Red and yellow arrows indicate proximal and distal cluster edges, respectively, and the white arrow, the proximal edge of the chain. Empty arrowheads indicate filopodia reestablishing connection. The relative positions of the arrows reveal the cell behavior in the gap region in four (**A–F**), six (**G–K**), and ten (**L–Q**) cell clusters: in the first two cases, the gap is filled by 23 hAPF, whereas in the ten-cell cluster it persists until late (yellow arrow in **M–Q**). This cluster moves more efficiently than the four- and the six-cell clusters, the XY microscope stage was therefore moved twice and once, respectively (check the costa reference point). Chain migration is indicated by white arrows in **B–D, H–K, and M–P**. **H–K** show an extreme example of contact reestablishment: the proximal-most cells of the cluster send extensions backwards, seeking for contact with the chain. Similarly, a cell in the chain sends a long extension proximally. Strikingly, a cell in the cluster lags behind the others and bridges the gap between the cluster and the chain. **R–V**, Schematic summary. Gray cells are radius glia, red asterisks indicate the UV-targeted cells, and green cells outlined in gray show the isolated one (**S**), four (**T**), six (**U**), and ten (**V**) cell(s). Scale bars, 30 μm .

ingly, this kind of transition has been observed in pathological conditions such as with primary melanoma explants *in vitro* (Hegerfeldt et al., 2002) and breast cancer cells *in vivo* (Giampieri et al., 2009). Also, a recent study has revealed the behavior of single mesendoderm cells of a zebrafish embryo in which a transplanted cell is able to migrate directionally isolated from its collective (Arboleda-Estudillo et al., 2010). Our data strongly suggest that collective migration adopts different strategies depending on the context: tumor cell plasticity is likely due to the metastatic nature (Friedl et al., 2004) and mesendodermal cells may constitute a quite homogeneous population, whereas glia at the tip are clearly morphologically and functionally distinct from those within the chain. This shows the importance of analyzing different models *in vivo*.

Homeostatic interactions at the tip of the migrating chain: sensing number?

Our data show that a cluster of tip cells is able to move directionally and that its migratory efficiency improves with the size of the cluster (Fig. 4*R–V*). The poor migratory efficiency of the small clusters might be an indirect consequence of pioneer fate changes, due to lack of contact with follower cells. If that were the case, however, the behavior of the four- and six-cell clusters should be similar. Indeed, we previously showed that the pioneer potential fades as the distance from the chain tip increases, the fifth and the sixth cells still contributing to it in a mild manner (Aigouy et al., 2008). The progressive improvement of the migratory features according to the cluster size rather leads us to propose that quantitative mechanisms are at work at the chain tip; that is, signaling pathways are reinforced/amplified by the increasing number of cells. We propose that cell-counting mechanisms provide robustness in complex behaviors, like the homeostatic interactions sensing cell density that allow microorganisms to make collective decisions (Boyen et al., 2009).

Several molecular mechanisms may control the observed behaviors. For their known role in migration or axonal guidance, potential pathways are those of RTK receptors and ligands, netrins and molecules controlling the extracellular matrix. Indeed, tip cells may not leave the chain because they must be provided enough trophic factors for their functioning. Also, small, isolated clusters may be unable to interpret shallow gradients of guidance cues that require integration across a tissue's length. Furthermore, the size of the cluster may be important to ensure directionality in dense environments, such as when cells migrate through the extracellular matrix. The identification of a collective mechanism at the chain tip sets the basis for studying the molecular nature of the “community effect,” ensuring that cells belonging to one collective stay together and migrate efficiently.

It is now accepted that collective migration at suboptimal conditions may lead to diseases as severe as mental retardation and epilepsy (Valiente and Marín, 2010). Collective behaviors require the integration of complex pathways, and eliminating one of them does not necessarily produce clear-cut phenotypes, often hampering the use of genetic analyses. Our work shows that eliminating specific cells allow the analysis of subtle but scorable phenotypes. A current challenge is to provide computational models for collective migration (Vargas and Zaman, 2011); however, the value and significance of such models heavily relies on our knowledge of this complex and dynamic process. In the future, it will be of great interest to incorporate parameters that take into account the homeostatic cell interactions of the kind we have described, to faithfully simulate collective migration.

Notes

Supplemental material for this article is available at [ftp://ftp-igbmc.u-strasbg.fr/pub/angela/SARAMOVIES_JNS.zip](http://ftp-igbmc.u-strasbg.fr/pub/angela/SARAMOVIES_JNS.zip). Time-lapses related to data shown in this article. This material has not been peer reviewed.

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Discussion

Collective migration is a complex phenomenon in which cells constantly and actively interact with each other. In the present study, we have explored the role of specific cell interactions in the glial chain of the developing *Drosophila* wing. We show that the pioneer cells belong to a community and that they are not able to switch from collective to individual migratory behavior. Interestingly, this kind of transition has been observed in pathological conditions such as with primary melanoma explants *in vitro* (Hegerfeldt et al., 2002) and breast cancer cells *in vivo* (Giampieri et al., 2009). Also, a recent study has revealed the behavior of single mesendoderm cells of a zebrafish embryo in which a transplanted cell is able to migrate directionally isolated from its collective (Arboleda-Estudillo et al., 2010). Our data strongly suggest that collective migration adopts different strategies depending on the context: tumor cell is likely due to metastatic nature (Friedl, 2004) where as glia at the tip are clearly morphologically and functionally distinct from those within the chain. This shows the importance of analyzing different models and to do that *in vivo*, in the whole animals, whenever possible.

Our data show that the cell cluster present at the tip of the migratory chain is able to move directionally and that its migration efficiency increases upon increasing cell number in the isolated cluster. When pioneers present at the tip of the chain are isolated, cells show poor migration efficiency. This might be an indirect consequence of the pioneer fate change due to the lack of antero- and retrograde signaling from the follower cells. Indeed, when we increase the size of the migratory cluster by increasing the number of cells isolated from the rest of the chain, migration efficiency increases. The progressive increase in the migration efficiency according to cluster size leads us to propose that quantitative mechanisms are at the work at the chain tip.

We propose that cell-counting mechanisms provide robustness in complex behavior like homeostatic interactions sensing cell density that allow microorganism to make collective decisions (Boyen, 2009).

In sum, we show that, although pioneers trigger chain migration they cannot migrate as an individual cells.

Chapter 2

Manuscript in preparation

Title

N-cadherin mediated interaction controls glial cell migration in
Drosophila

Keywords

Collective migration, glial cells, N-cadherin

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SUMMARY

Cell migration is essential and highly regulated process. During development, glia and neurons have to migrate, in most cases as collectives, over long distances to reach their final destination. Defective migration leads to severe human diseases including mental retardation. Collective migration is highly stereotyped and efficient, however the complexity of cell interactions and their dynamic aspect make the *in vivo* analysis of this process a major challenge. We investigate the impact of the levels of N-cadherin (N-cad) expression on glial cells migration in developing wing. We show that N-cad is dynamically expressed in glial cells. When N-cad is overexpressed (gain-of-function), migration occurs slowly as compared to what observed in control animals. Conversely, N-cad depletion (loss-of-function) triggers acceleration in glial chain migration. We show that the level of N-cad affects migration by impinging on actin cytoskeleton remodeling.

INTRODUCTION

The nervous system represents the most complex tissue in the body and is composed of glia and neurons that migrate over long distances to reach their final destination. In some cases cells migrate in isolation, in others they migrate in clusters, as chains, streams and sheets (Berzsenyi and Giangrande, 2010; Gilmour et al., 2002; Klambt, 2009; Lemke, 2001; Marin et al., 2010; Rorth, 2003; Valiente and Marin, 2010). The second mode of migration, also called collective migration, indicates that the final architecture of the nervous system depends on complex and dynamic cell interactions. For the simplicity of its genome and nervous system *Drosophila* represents an ideal model to dissect the molecular bases of these interactions.

Adhesion molecules play an important role in collective events (Schwabe et al., 2009; Silies and Klambt, 2010; Togashi et al., 2009) and adhesion complexes mediate links between individual cells that eventually keep tissues and organs in a well-defined shape.

Major player in cell adhesion is provided by the Ca^{++} dependent family of cadherins, primarily involved in homophilic interactions between opposing cells (Arikkath and Reichardt, 2008; Giagtzoglou et al., 2009; Kiryushko et al., 2004). The physical connection between cells via cadherins contributes to morphogenetic movements and cell migration (Harris and Tepass, 2010). Increase in the cadherin function in the glioma cell lines leads to significant decreased in their invasive behavior (Asano et al., 2004), while decrease of cadherin function induces an increase in cell migration (Rappl et al., 2008). Here we address the cell-specific role of the Cadherin pathway (Benjamin and Nelson, 2008) using a system that allows tracing of collectively migrating cells by time-lapse in the whole animal. Glia in the developing *Drosophila* wing migrates over axons in a chain like manner and sends exploratory filopodia (Aigouy et al., 2008; Aigouy et al., 2004). Each member of the community is closely attached to the neighboring cells and glia-glia interactions tightly control migration extent, efficiency and coordination, highlighting the importance of interactions amongst the members of the migratory cohorts. In the vertebrate and in the *Drosophila* nervous systems, the most abundant classic cadherin is the Neural (N)-cadherin (N-cad) (Fung et al., 2008; Stepniak et al., 2009). During *Drosophila* embryogenesis, N-cad starts to be expressed in the mesoderm and in its derivatives, the myoblasts and myotubes (Iwai et al., 1997). Then, it appears in the developing

neural cells and accumulates in the axons of the central nervous system (CNS) (Iwai et al., 1997). In the larva, N-cad is expressed in the neuropile of the central brain ((Fung et al., 2008), as well as in the visual system (Iwai et al., 1997). In the latter study, the authors argue that N-cad is not expressed in glial cells.

Here we show that N-cad is dynamically expressed in glial cells of the developing wing and is necessary for their timely migration along the axons. When N-cad is overexpressed (gain-of-function), migration occurs more slowly than in control animals. Conversely, N-cad depletion (loss-of-function) triggers the acceleration of glial chain migration. Cell migration depends on actin cytoskeleton dynamics that depends on the WAVE/SCAR nucleation complex (Patel et al., 2008). We here show that the levels of N-cad affect actin dynamics and that overexpressing *CYFIP*, a member of the WAVE/SCAR complex, counteracts the effects of N-cad overexpression. This *in vivo* study in the whole animal clarifies the role of N cad onto collective migration and helps understanding the impact of this cell adhesion pathway in morphogenesis and cancer metastasis (Berx and van Roy, 2009; Stepniak et al., 2009).

MATERIALS AND METHODS

Fly stocks and genetics

Flies were raised at 25 °C. *repoGal4* (V. Auld) was used to drive the expression of *UAS ncGFP* (nc: nuclear and cytoplasmic) (C. Desplan), *UAS PHGFP* (fusion protein of pleckstrin homology domain of PLC-TM and the GFP) (A. Zelhof); *UAS N-cad* (T. Uemura); *UAS N-cad RNAi* (VDRC stock center); *UAS Arm* (Bloomington stock center); *UAS N-cad* ⊗ *Arm* (S. Yonekura); *UAS <-catGFP* (M. Affolter); *UAS Actin42AGFP* (J. Casanova); *UAS CYFIP* (N. Harden) in glia.

***in vivo* Imaging**

Dissection, time-lapse and immunolabeling were as described previously (Aigouy et al., 2008; Aigouy et al., 2004; Soustelle et al., 2008). Fast imaging of glial cells was performed on SP5 Leica confocal microscopes equipped with hybrid detectors. The GFP labeled region in the wing was selected and scanned in the z-axis using the 488 nm laser at a time interval of 20 sec. z-stack projections for time-lapse movies were obtained using Image J software. Images were annotated using Adobe Photoshop and

Illustrator; movies were converted to QuickTime format using Image J. Statistical analysis was done by Student's *t* test; bars indicate s.e.m. For semiquantitative analyses, distances were calculated manually and then transformed into micrometers upon considering the used magnification.

Electron microscopy

Sample preparation

High pressure freezing: Wings were dissected in cold PBS and transferred to 200 μ m deep flat carriers (Leica) filled with a 1:1 mixture of 20% bovine serum albumin (Sigma). Cryo-immobilization was performed in Leica EMPACT-2 high pressure freezing apparatus. Freeze substitution was processed in Leica AFS for 60 hours at -90°C in 1% osmium tetroxyde, 0.5% uranyl acetate, 0.5% glutaraldehyde and 2% water in pure acetone. Temperature was then slowly raised to -30°C at a 3°C/h rate. After 6 hours at -30°C, samples were extensively rinsed with pure acetone and infiltrated in graded concentration of Epon 812. When the concentration of the resin reached 70%, the temperature was gradually raised to 20°C. The infiltration was then finished by three incubations in pure Epon (2 hours each). Blocs were left for 48 hours at 60°C for polymerization. Ultrathin sections (50 to 70nm) were collected on carbon/formvar coated copper slot grids, contrasted with uranyl acetate and lead citrate. Images were acquired with an Orius1000 CCD camera (Gatan) mounted on a Philips CM12 transmission electron microscope operated at 80kV.

Chemical fixation: The wings were dissected in PBS buffer and immediately immersed in the fixative consisting of 2,5% glutaraldehyde and 4% formaldehyde in 0.1M pH 7.4 phosphate buffer (PB). After a minimum fixation time of 2 hours, the samples were rinsed with PB and postfixed for 1 hour in 1% osmium tetroxyde at 4°C. After several rinses in distilled water, the samples were dehydrated in graded series of acetone (50, 75, 90, 95, 100%) and infiltrated with epoxy in pure acetone (25, 50, 75 and 100%). The wings were flat embedded as described in (Kolotuev et al., 2010), between two sheets of aclar (EMS) and left to polymerize for 48 hours at 60°C. Targeted ultramicrotomy was used to systematically section the wings in the same region. Ultrathin sections (60nm) were collected on electron microscopic grids.

Immunogold labeling

Resin infiltration that was performed with graded concentration of Lowicryl HM20 monostep (EMS). The resin was polymerized under UV light at -50°C for 48 hours and at room temperature for 48 hours. Ultra-thin sections were collected on carbon/formvar coated nickel slot grids and processed for immunogold labeling on the Leica EM-IGL automate. The immunogold experiment was performed in PHEM buffer (60mM PIPES, 25mM HEPES, 20mM EGTA, 2mM MgCl₂, pH 6.8) as follows: 3 rinses in PHEM; blocking in 0.1% bovine serum albumin (Sigma), 0.1% Fish Skin Gelatin (FSG, Aurion) in PHEM for 30 minutes; 1 hour incubation in primary antibody (rat- α -N-Cad) diluted 1/2 in 0.1% FSG; 6 rinses in PHEM; 1 hour incubation in 6nm gold-coupled goat- α -rat IgG (Jackson ImmunoResearch); 6 rinses in PHEM; post-fixation in 1% glutaraldehyde and extensive rinsing in distilled water. Grids were slightly contrasted with uranyl acetate before observation.

Western blot assay

Protein expression was detected from 30-40 μ g of whole wing protein lysate (extracted by freezing-thawing cell pellet in 400mM KCl, 25mM Tris HCl, pH 7.9, 10% glycerol) by using the following primary antibodies: chicken- α -GFP (ab13970, Abcam, 1:5000 working dilution), rat- α -N-cad (DN-Ex#8, DSHB, 1:20), mouse- α -Arm (N27A1, DSHB, 1:200), rabbit- α -Actin (A2066 Sigma Aldrich, 1:5000). Rabbit- α -chicken HRP, donkey- α -rat HRP, goat- α -rabbit HRP and donkey- α -mouse HRP (Jackson ImmunoResearch) were used as secondary antibodies (1:10000). 40 μ g of the wing extract (produced from the control and from the following crosses: 1) *repo>GFP X UAS N-cad/+*; 2) *repoGal4> α -catGFP X UAS N-cad/+* and 3) *repoGal4> α -catGFP/UAS N-cad RNAi* were fractionated on gradient acrylamide gels (Invitrogen), transferred onto nitrocellulose membrane and probed with respective primary antibodies. Signal was detected with Pierce ECL western blotting substrate (Thermo Fisher Scientific, Waltham, MA) using appropriate HRP-conjugated secondary antibodies (1:10000, Jackson).

Reverse Transcription and qRT-PCR

Total RNA was purified from 1) *repoGal4> α -catGFP*; 2) *repoGal4> α -catGFP/UAS N-cad* 3) *repoGal4> α -catGFP/UAS N-cad RNAi* wings by TriReagent (MRC),

reverse transcribed by SuperScriptII reverse transcriptase (Invitrogen) using a mix of random hexamers (6 μ M) and oligodT primers (5 μ M), and analyzed by quantitative PCR (qPCR) machine Roche LightCycler480 with Syber Green (Roche) Master mix. For each gene, expression levels were automatically calculated (LightCycler480 Software, release 1.5.0) by calibration to gene-specific standard curves generated on input cDNAs. Collected values, normalized to Actin5C amount, derive from three amplification reactions, each performed on three independent experiments.

Immunolabeling and antibodies

The staged pupae were fixed in 4% PFA PBS (paraformaldehyde in phosphate buffer saline) at 4°C for 2 hours. The pupae were dissected in PBT (PBS Triton-X100, 0.3%). After quick washes in PBT, the wings were incubated in PBT-NGS (5% normal goat serum in PBT) for 20 minutes at room temperature on planar shaker. Then, the samples were incubated in primary antibodies (diluted in PBT-NGS): mouse- α -Repo, mouse- α -22c10, rat- α -Elav, rabbit- α -HRP, chicken- α -GFP, rabbit- α - β -gal, rat- α -N-cad, rat- α -alpha-cat and mouse- α -Arm overnight. After three washes in PBT, the wings were incubated in secondary antibodies: mouse, rat, rabbit or chicken coupled to Cy3, Cy5 or FITC fluorescent dyes for 2 hours diluted in PBT-NGS at room temperature. Following a final wash in PBT the wings were mounted on slides in Aqua- Poly/Mount medium (Polysciences Inc.).

RESULTS

N-cad is expressed in the *Drosophila* peripheral glial cells

At the anterior margin of the developing *Drosophila* wing, glial cells form a migratory chain along the so-called L1 sensory nerve and move towards the CNS (**Fig. 1**; Aigouy et al., 2004). The cells at the chain front play a crucial role in leading and promoting the movement of the glia as a collective (Aigouy et al., 2008) and homeostatic glia-glia interactions ensure that the cells at the front do not leave the chain, even though endowed with migratory potential (Berzsenyi et al., 2011). Cell ablation followed by time-lapse analyses also indicate that glia-glia interactions control the coordination and the efficiency of migration at the front and within the chain (Aigouy et al., 2008; Aigouy et al., 2004).

In the present study we sought to characterize the molecular bases controlling coordinated glial chain migration. We identified N-cad as a molecule expressed by most or all wing glia (**Fig. 1**). N-cad starts being expressed at about 19 hAPF (hours After Puparium Formation), at the time glia start moving, and its expression increases until the end of the migration period (i.e. 29-30 hAPF) (**Fig. 1A',C',D'**). N-cad also accumulates in the axons at seemingly similar amount over time (**Fig. 1A',D'**, from 19 to 29 hAPF).

To verify whether N-cad is expressed in other peripheral glia (PG), we performed immunolabeling on *Drosophila* embryos and confirmed that peripheral (arrowhead, **Supp. Fig. 1A,B**) but not central glia express N-cad (**Supp. Fig. 1C**).

Thus, wing glia expresses the N-cad adhesion molecule in a timely manner over the migratory period.

The levels of N-cad affect the efficiency of glial chain migration

The presence of N-cad prompted us to investigate its possible role in the migratory process. Because the expression of N-cad is not restricted to glia, we specifically modified its levels in glia by using the *repoGal4* driver and established a protocol in which we could compare glial migration efficiency in different genetic backgrounds. To follow the position of the glial cells we crossed the glial driver with the *UAS GFP* transgenic line (*repo>GFP*) and each genotype, we fixed and dissected wings ($n \geq 40$) at 29 hAPF. By this stage all wings of control animals (*repo>GFP*) show completed migration, i.e. the glial chain has reached the proximally located radius glia (**Fig. 1L**; Berzsenyi et al., 2011). We then assessed and compared the percentage of wings displaying completed glial migration in the different backgrounds.

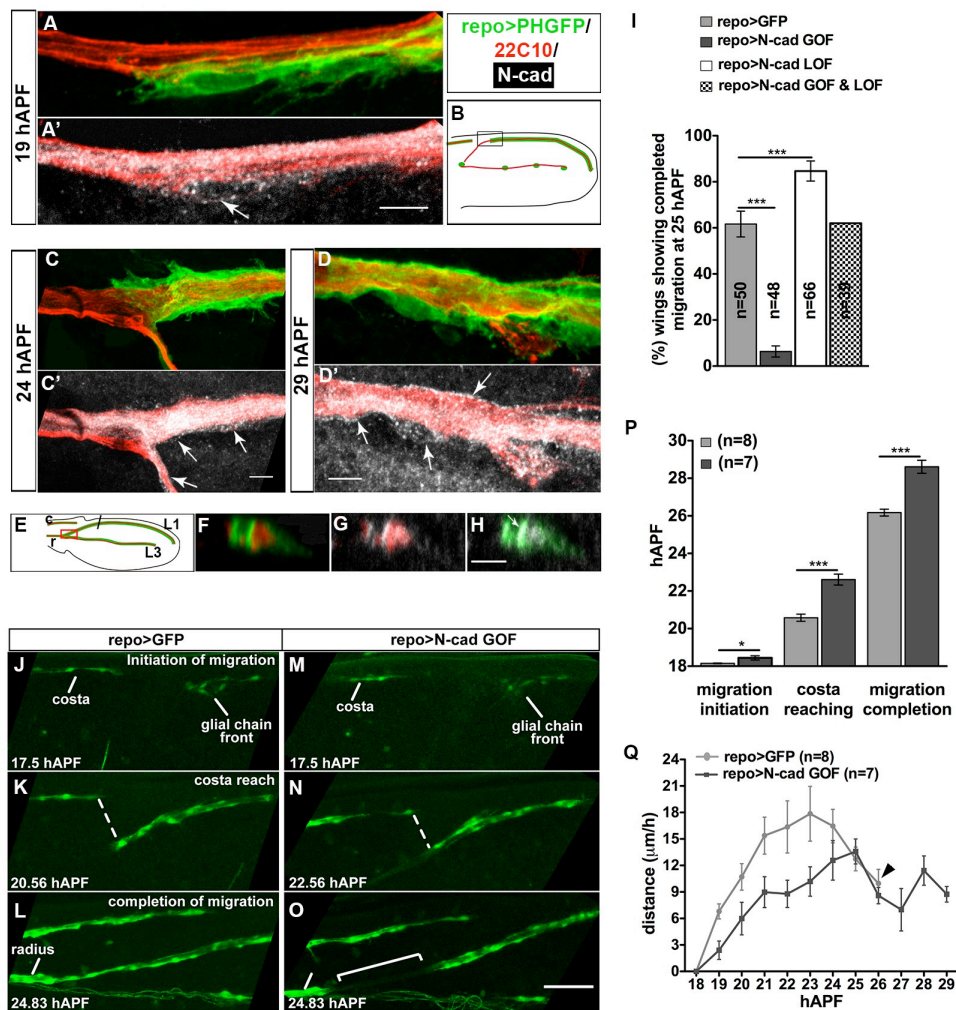
First, we overexpressed N-cad (gain-of-function or GOF: *repo>N-cad GOF*), upon crossing *UAS N-cad* flies with the *repo>GFP* line and verified that this results in a strong increase in N-cad levels in glia (compare **Supp. Fig. 2A,B**). Interestingly, N-cad overexpression significantly decreases glial migration efficiency compared to what observed in control wings (*repo>GFP*) (**Fig. 1I, J-O**).

When we knocked down N-cad using a *N-cad RNAi* transgene (loss-of-function, LOF: *repo>N-cad LOF*), the N-cad signal was lost on the glial cell membrane but not on that of neurons (**Supp. Fig. 2C**). By counting the number of

repo>N-cad LOF wings with completed glial migration, we observed a significant increase in migration efficiency compared to what observed in control wings (**Fig. 1I**). Importantly, when we co-expressed the *UAS N-cad* and the *UAS N-cad RNAi* transgenes (*repo>N-cad GOF* and *LOF*), the migration efficiency of the glial chain was restored to control levels, confirming that N-cad overexpression plays a regulatory role and that the RNAi effects are specific (**Fig. 1I**).

Glial cells proliferate as they move, therefore the number of cells increases over the migration period and cell death occurs only occasionally (Aigouy et al., 2004). To assess whether N-cad overexpression affects glial proliferation/death, we used the panglial nuclear marker Repo (Bernardoni et al., 1998; Hosoya et al., 1995; Lee and Jones, 2005; Vincent et al., 1996) to count the number of glia along the L1 nerve of control and *repo>N-cad GOF* wings at 29 hAPF. N-cad overexpression does not alter the number of glial cells (control: 90 ± 6 cells; *repo>N-cad GOF*: 85 ± 10 cells, $n=5$, $p=0.42$), indicating that the migratory phenotype observed in *N-cad GOF* wings is not due to changes in the size of the chain. To further investigate whether N-cad acts cell-autonomously in glial migration, we analyzed the morphology of the axons in *repo>N-cad GOF* and *repo>N-cad LOF* wings. Since the axons grow in close proximity to the glial cells, the excess or reduction of N-cad in glia may influence the navigation of the neuronal processes, which may trigger indirect glial migration defects. We compared the advancement of axon growth in GOF, LOF and control wings and observed no detectable difference in axon/bundle morphology and organization (**Supp. Fig. 3**).

These observations indicate that the glial levels of N-cad affect chain migration.



N-cad slows down the migration of the glial chain

To gain insights into the kinetics of glial migration in control and N-cad overexpressing wings, we followed the glial chain by time-lapse confocal microscopy. The migratory process was subdivided in three phases (Berzsenyi et al., 2011): the earliest one describes migration initiation; the intermediate one identifies the time at which the glial chain reaches the level of the costal nerve; the latest phase refers to migration completion, upon connection of the chain with the proximal glia located on the radius nerve (**Fig. 1J-L**). We found that, in average, a glial chain overexpressing N-cad reaches the level of the costa and completes migration 1.3 hours later than the control chain (**Fig. 1M-O**). To evaluate the migration efficiency in a semi-quantitative manner, we determined the average distance covered each hour by the first cell soma present at the front of the chain. The analysis of wild type animals revealed that the speed of migration progressively and significantly increases until 23 hAPF, and then it slows down until migration completion (**Fig. 1Q**). This

late, slow phase, suggests that the chain front may be able to sense some kind of STOP signal(s). It is possible that radial glia, send such inhibitory signal(s) since L1 glia move towards the more proximal located radial glia (Aigouy et al., 2008).

When compared to control wings, glial cells overexpressing N-cad start moving at the same time, but at a lower speed. Interestingly, the slow phase is delayed (25 hAPF, **arrowhead, Fig. 1Q**), further confirming that the chain front may sense the proximity of target glial cells.

In sum, these data suggest that glial cell speed is influenced by cell interaction and does not only rely on an internal clock. They also suggest that appropriate levels of N-cad in the glia control their timely movement in the wing.

Ultrastructure of the wing nerve and glial cells

Since N-cad is expressed in the neurons and in the glia of the pupal wing, it may be a key component of axon-axon, glia-glia and/or axon-glia adhesion. We therefore analyzed control and N-cad misexpressing animals at the ultrastructural level. The fly wing is composed of two juxtaposed epithelia that are separated at the position of the veins and constitute five veins (**Fig. 2A'**; Murray et al., 1984). The L1 nerve bundle is composed of several hundred axons and is wrapped by the glial cytoplasmic processes (**Fig. 2A**). First, we wanted to visualize the N-cad in control animals, however, the available antibody did not allow us efficient immunogold labeling in *repo>GFP* control wings. Therefore, we labeled a *repo>N-cad GOF* nerve. In this case, we were able to detect the N-cad molecules at the interface of two glial membranes (pink arrowhead, **Fig. 2B**). Interestingly, we found multivesicular bodies (MVB, membranous sacs containing numerous small endocytic vesicles) in the glial cells is labeled with several \langle -N-cad gold particles (white arrow; **B'**). N-cad is also localized between two axons (blue arrow; **B''**) and axon and glia (orange arrow, **B''**). This suggests that N-cad in the glial cells undergoes turnover through the endocytic pathway.

Second, we analyzed whether N-cad overexpression produces morphological alterations at ultrastructural level. Cadherins constitute structural components of the adherens junctions (AJs) (Niessen and Gottardi, 2008), thus, it is possible that the number of the AJs is affected upon N-cad overexpression or downregulation in the glial cells, resulting in altered adhesive properties. This change in the strength of glial

adhesion to the neighboring glia and/or to the axons may account for the migratory phenotype we observed in *repo>N-cad GOF* and *repo>N-cad LOF* wings. Thus, we decided to compare the number of AJs present in control, *repo>N-cad GOF* and *repo>N-cad LOF* nerves (**Fig. 2C-F**). Since the other abundant AJ-forming cadherin, E-cadherin, is not expressed by wing glia or neurons (**Supp Fig. 4**), N-cad likely constitutes the major cadherin of the AJs present in those cells.

We analyzed 8 control and N-cad overexpressing wings, seven sections from each sample, and seven control and N-cad downregulating wings, four sections from

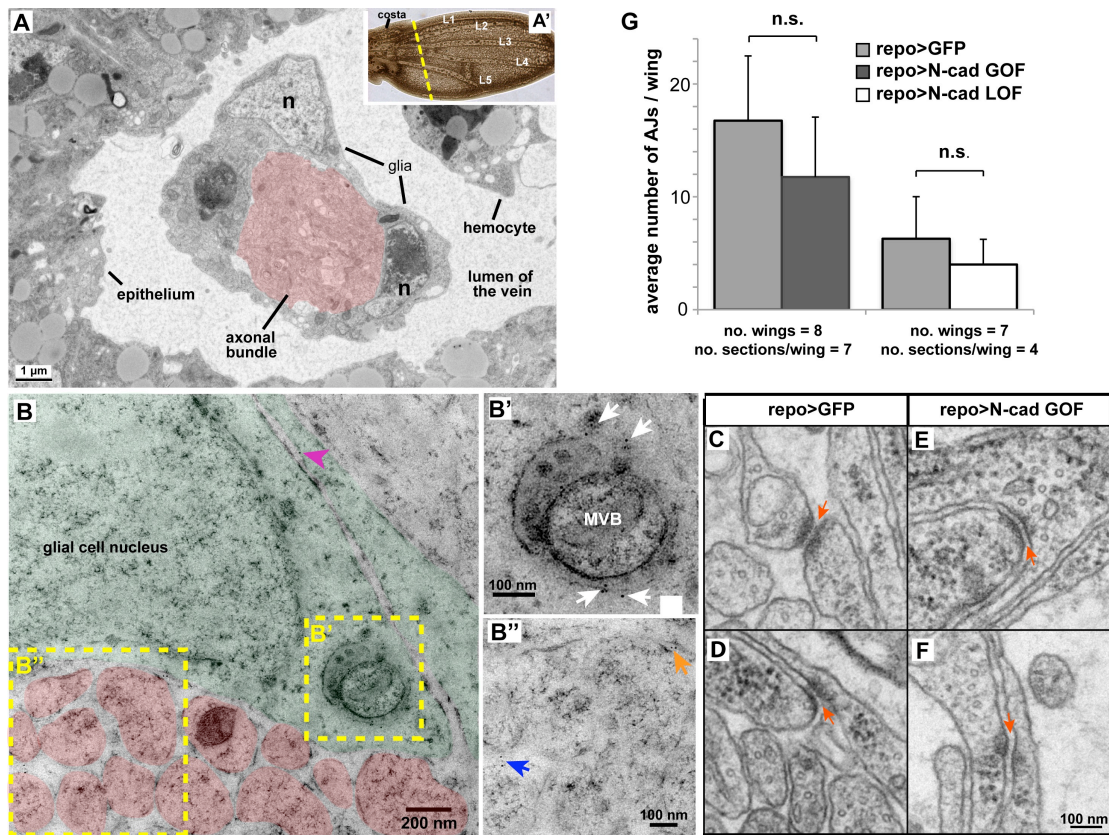


Figure 2: Ultrastructure of the L1 wing nerve and glial cells in *Drosophila*
(A) Ultrathin cross section of the L1 nerve at 29 hAPF at the level of the yellow dashed line indicated in the inset wing. **(A')** The axonal bundle (red shading) is surrounded by glial cells. “n” indicates the nucleus of the glial cell. **(B)** N-cad immunogold-labeling on ultrathin section of a *repo>N-cad GOF* nerve. Glial cell processes are shaded in light green, and axons are highlighted in red. Pink arrowhead points to a gold particle located between two glial membranes. Yellow rectangles designated as **(B)** corresponds to the magnified images in panel **B'** and **B''**. **(B')** Multivesicular body (MVB) in the glial cell is labeled with several anti-N-cad gold particles (white arrows). **(B'')** N-cad is localized between two axons (blue arrow) and between axon and glial cell (orange arrow). **(C-F)** Ultrastructural organization of control and *repo>N-cad GOF* nerves, the red arrows point to adherens junctions (AJs). **(G)** The graph shows the quantification of the number of AJs in control *repo>GFP*, *repo>N-cad GOF* and *repo>N-cad LOF* nerves.

each sample. We calculated the average number of AJs per wing found between glial cells and observed no significant difference between control and N-cadherin misexpressing wings (**Fig. 2G**).

These data suggest that the altered levels of N-cad do not significantly affect the AJ abundance between glial cells.

Organization and function of cadherin/catenin complexes

One of the key factors associated to classic cadherins is β -catenin (β -cat) or Armadillo (Arm) in *Drosophila* (Yap et al., 1997). Arm binding to cadherins appears to protect the cadherin cytoplasmic domain from proteolysis *in vitro* (Huber et al., 2001) and to enhance the efficiency of endoplasmic reticulum to cell surface transport (Chen et al., 1999). We therefore analyzed the effects of *N cad GOF* on Arm in the glial cells. First, we immunolabeled *repo>GFP* control animals with an α -Arm antibody. As expected, Arm is widely expressed in the wing epithelium at cell-cell contacts (**Fig. 3F**). Glial Arm labeling is relatively weak and could be best visualized at the glial membrane that faces the vein lumen (arrowhead, **Fig. 3B**). Upon comparing the expression profile in control and *repo>N-cad GOF* glial cells (**Fig. 3J-O**) we found that Arm accumulates at high levels at the cell membrane in N-cad overexpressing glial cells, (arrowhead, **Fig. 3N**). This is in line with previous data showing that different levels of cadherin at the membrane can regulate the accumulation of β -cat/Arm in the cell (Goichberg et al., 2001; Orsulic et al., 1999). Interestingly, the levels of Arm depend on the N-cad doses, which we could verify by using a weaker *repoGal4* driver. In N-cad overexpressing glial cells, Arm accumulates at high levels, as confirmed by Western Blot analyses (**Fig. 3P**). These data strongly suggest that the N-cad mediated phenotypes involve Arm.

Arm is a dual function protein as it acts in adhesion as well as in Wnt/Wg signaling and indeed can be detected at the cell membrane, in the cytoplasm or the nucleus (functioning as a transcription cofactor in the Wg/Wnt pathway). Its actual localization and concentration within the cell eventually defines which function of β -cat/Arm (adhesion vs. signaling) dominates. The fact that, neither in neurons nor in glial cells could we detect nuclear localization of Arm, suggest that Wg signaling is not very prominent (**Fig. 3A-I**). Nevertheless, to elucidate the mode of action of Arm in collective migration, we overexpressed it in the glial cells and observed strong

accumulation in the nucleus (compare **Fig. 3N, R**). In these conditions, however, glial migration efficiency is similar to that of control wings. Since it is known that in the activated Wnt/Wg pathway Arm acts in the nucleus, these data further suggest that the Wg pathway does not influence the migration progression of glial cells.

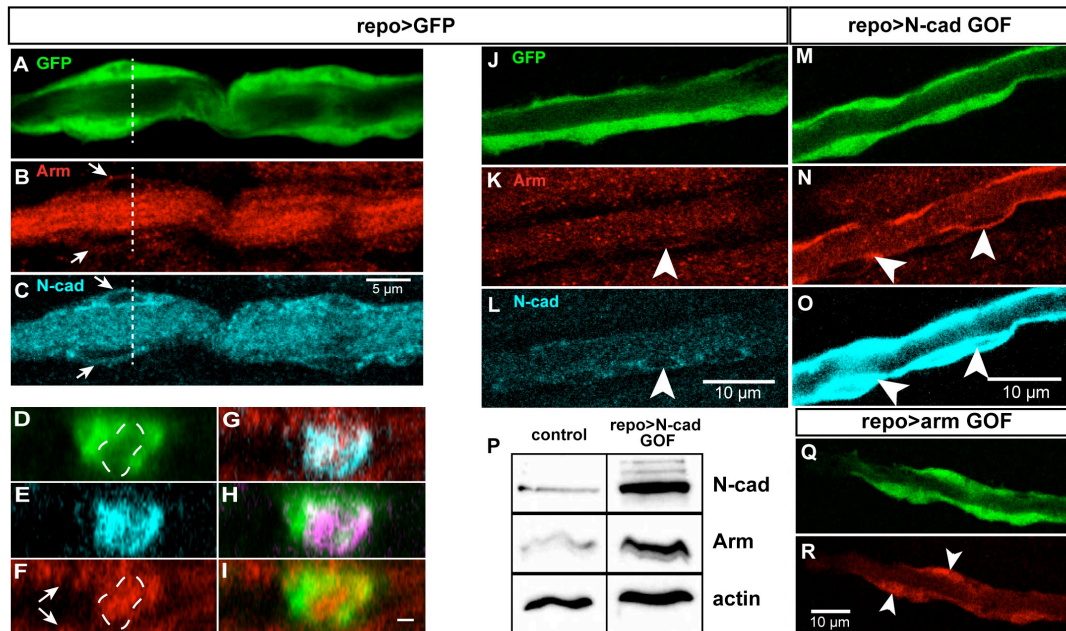


Figure 3: Armadillo (Arm) expression upon N-cad overexpression

(A-C) Maximum projections of *repo>GFP* animals labeled for GFP (green) stained for Arm (red) and N-cad in (cyan). Arrow shows the membrane localization of Arm (B) and N-cad (C). (D-I) represents the z-cross-section of the nerve. Arm is also localized in the wing epithelial cells (F; arrows) surrounding the glial cells (I). (J-O) maximum projection of confocal stacks showing the intensity of Armadillo labeling in control and *N-cad GOF* glial cells. In (M-O), the Arm in the *N-cad GOF* shows high accumulation at the membrane (arrowhead, N) compared to the control (arrowhead, K). Note that (P) immunoblot showing the increased N-cad and Arm protein level upon overexpression of N-cad compared to control animals. In (R), Arm GOF animals showing nuclear localization of Arm (arrowhead).

N-cad affects migration via the actin cytoskeleton-remodeling pathway

Drosophila N-cad is a multi domain transmembrane protein in which the extracellular region contains 16 cadherin repeats and the intracellular part consists of a juxtamembrane domain and an Arm-binding domain. To clarify the mode of action of N-cad, we overexpressed a construct in which the Arm binding domain is deleted (*N-cad ΔArm*) (Fig. 4A) under the control of the *repoGal4* driver. Immunolabeling analyses revealed that, while overexpression of the full length N-cad transgene

triggers high levels of N-cad and Arm (**arrowhead, Fig. 4I-J**), overexpression of the *N-cad ΔArm* transgene results in high levels of N-cad but normal levels of Arm (**compare Fig. 4B-D, E-G**). Moreover, overexpressing *N-cad ΔArm* in the glia does not affect migration efficiency (**Fig. 4K**). We conclude that the cytoplasmic domain of N-cad participates in regulating glial cell migration.

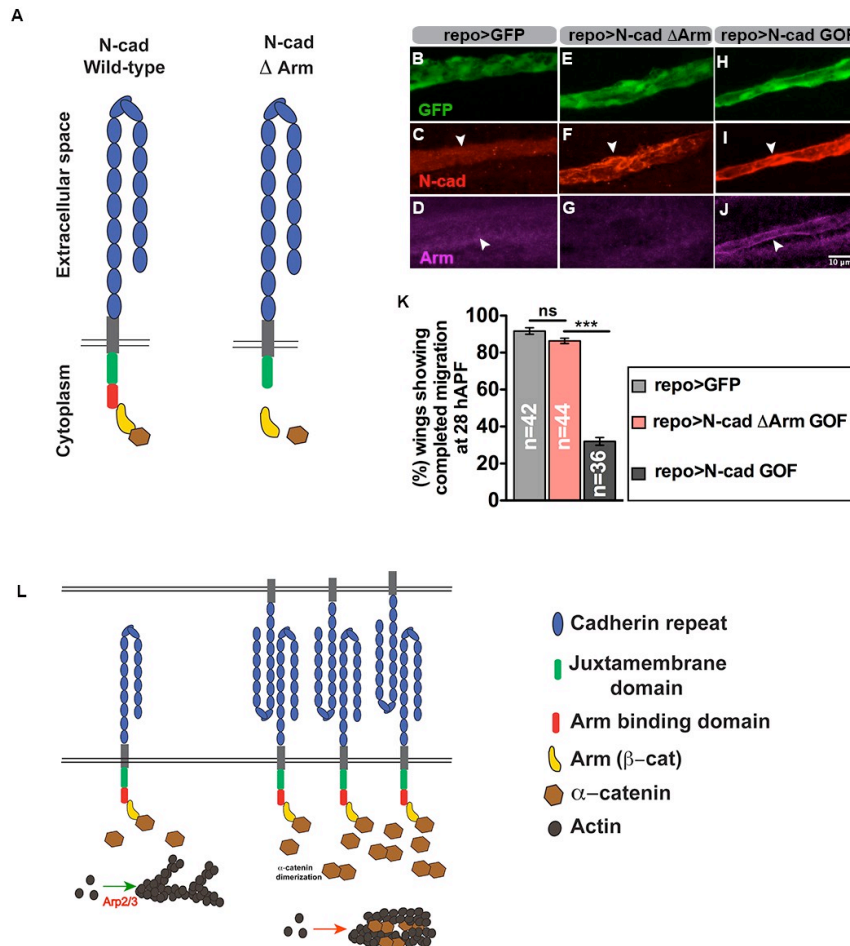


Figure. 4: Cell migration dynamics upon eliminating the cytoplasmic part (A) Schematic drawings illustrating the wild type N-cad (left) and the *N-cad ΔArm* construct in which the Arm binding domain is deleted. (B-J) Immunolabeling for GFP (green), N-cad (red) and Arm (magenta) in glia overexpressing the N-cad transgenes (*N-cad* full length and *N-cad ΔArm*) under the *repo>Gal4* driver. Maximum projection of the confocal stacks shows elevated N-cad and Arm levels (arrowhead in I, J) in *N-cad GOF*, while in *N-cad ΔArm GOF* glia no Arm can be detected (G). (K) Migration efficiency of the glial cells in the indicated genotypes. (L) Schematic drawing showing cadherin clustering upon N-cad overexpression. Bar: B-J, 10 μ m.

To understand the molecular bases of the migration defects induced by high levels of N-cad, we further analyzed the actin cytoskeleton-remodeling pathway. The

main mediator between cadherins and the cytoskeleton is provided by ζ -cat, which is able to bind both β -cat and actin molecules, thereby providing a bridge between the cell membrane and the cytoskeleton (Benjamin and Nelson, 2008). We found that overexpressing α -cat in glia (*UAS α -catGFP* transgene; **Fig. 5C**) also delays migration (*α -cat GOF*, **Fig. 5H, blue column**). In addition, overexpressing that transgene in a *UAS N-cad* background (*N-cad GOF*, ζ -cat *GOF*, **Fig. C,D,H, purple column**) aggravates the N-cad overexpression phenotype (*N-cad GOF*, **Fig. 5H, dark grey column**). Furthermore, decreasing the levels of ζ -cat in a *UAS N-cad* background rescues the migratory delay induced by N-cad overexpression (*N-cad GOF*, ζ -cat *LOF*, **Fig. 5H, patterned grey column**). Finally, overexpressing ζ -cat in a *UAS N-cad RNAi* background (*N-cad LOF*, ζ -cat *GOF*, **Fig. 5E,F,H, pink column**) suppresses the fast migration induced by loss of N-cad (*N-cad LOF*, **Fig. 5H, red column**). Such genetic interactions confirm that ζ -cat and N-cad work in the same pathway and act in the same direction, to slow glial chain migration.

Finally, and in agreement with the migratory phenotypes, the levels of ζ -cat are affected by N-cad, as shown by immunolabeling and Western blot analyses. GFP was used to monitor ζ -cat expression in flies carrying the gene fusion *α -catGFP*. The levels of the GFP increase in animals also overexpressing N-cad (*α -catGFP GOF*, *N-cad GOF*) and decrease in animals expressing low levels of N-cad (*N-cad LOF*) (**arrowheads, Fig. 5A-F and Fig. 5I**). Interestingly, we could not observe a significant difference in ζ -cat mRNA levels in *repo*> ζ -cat*GFP GOF*, *N-cad GOF* or *LOF* backgrounds compared to those observed in the control *repo*> ζ -cat*GFP GOF* animals (**Fig. 5G**). Thus, the levels of α -cat change according to Arm in response to different N-cad levels through a post-transcriptional process.

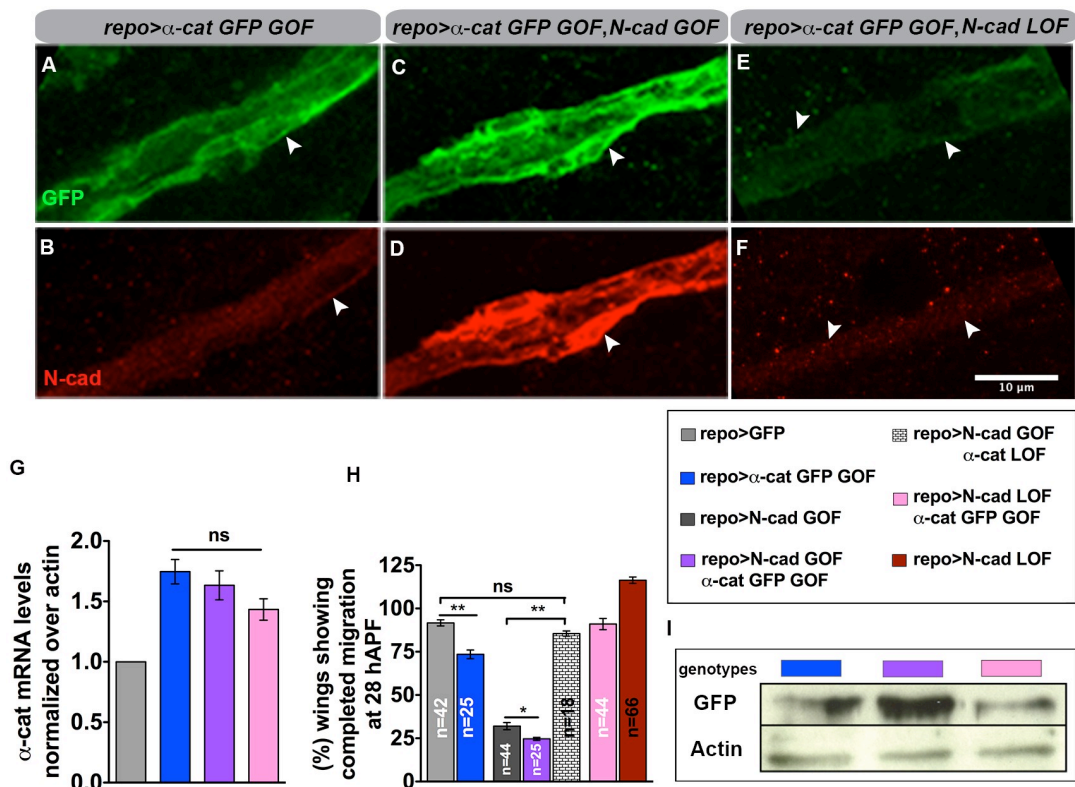


Figure 5: Distribution and function of α -cat upon gain and loss of N-cad function

(A-F) Maximum projections of wing nerves showing α -cat (green) and N-cad (red) expression upon *N-cad GOF* and *N-cad LOF* using the *repo> α -cat GFP* transgene. Note the elevated levels of GFP in *repo> α -cat GFP; UAS N-cad/+* (C, D) and the reduced amount of GFP in *repo> α -cat GFP; UAS N-cad RNAi/+* (E, F) glial cells compared to *repo> α -cat GFP/+* control wing (A, B). (G) qPCR analysis of the relative expression of α -cat mRNA normalized to *actin* mRNA upon *N-cad GOF* and *LOF* in *repo> α -cat GFP* background and in *repo>GFP* animals. (I), Immunoblot of *repo> α -cat GFP* (control) and *repo> α -cat GFP, N-cad GOF* and *N-cad LOF* for GFP expression. Actin was used as loading control. (H), The graphs represent the comparison of glial cell migration efficiency upon gain or loss of function of the indicated genes. Bar: (A-F), 10 μ m.

Rescuing actin cytoskeleton remodeling restores glial migration

It has been proposed that, at high levels of cad, when cell-cell adhesion is strong, α -cat accumulates at the membrane and the pool of α -cat molecules also increases (Benjamin and Nelson, 2008). As a consequence, α -cat homodimers form and bind actin filaments thereby preventing the activity of the ARP2/3 complex. The later is known to nucleate the branched actin filaments at the leading edge of the cells to promote migration (Benjamin and Nelson, 2008). Thus, the migratory phenotype

induced by altered N-cad levels may be due to defects in the actin nucleation pathway.

The ARP2/3 complex is activated by nucleator complexes as WAVE/SCAR, of which the CYFIP/Sra1 (*CYFIP*) adaptor is an integral member (Blagg and Insall, 2004; Stradal et al., 2004). To further validate the hypothesis that N-cad levels control cell migration by preventing appropriate actin cytoskeleton dynamics, we asked whether over activation of the WAVE/SCAR complex rescues the defects induced by high levels of N-cad. We overexpressed *CYFIP* in a *repo>N-cad GOF* background and counted the number of wings displaying completed migration. Indeed, overexpression of *CYFIP* in glia rescues the migration phenotype induced by N-cad overexpression (**Fig. 6A, green column**). In addition, we also down regulated *CYFIP* in *repo>N-cad GOF* animals and found that glia migrate even less efficiently than in wings expressing N-cad alone (**Fig. 6A, yellow column**). Interestingly, overexpression of *CYFIP* in an otherwise wild type background does not have any effect on glial cell migration, suggesting that *CYFIP* is not present in limiting amounts (**Fig. 6A, hatched column**).

In sum, we show that promoting actin nucleation is able to counteract the effects of high N-cad levels during collective migration.

Actin cytoskeleton and membrane dynamics

The above data highlight the role of N-cad in signaling to β -cat and indicate that the observed migratory phenotypes depend on actin cytoskeleton defects. To investigate these phenotypes *in vivo*, we used high-resolution time-lapse microscopy at the stage when cells start to migrate (**arrowhead, Fig. 6A**). To follow actin dynamics of the migrating glial cells we *UAS actin42AGFP* transgene under the control of the *repoGal4* driver (**Fig. 6C-F**). We focused on the cells at the front of the chain and analyzed filopodia behavior in control *repo>actin42AGFP* wings as well as in (*repo>actin42AGFP; N-cad GOF*), in (*repo>N-cad GOF; CYFIP GOF*) and in (*repo>actin42AGFP; N cad LOF*) animals. Again assessment for actin dynamics we used TWO parameters the number of filopodia per cell soma as well as their length. We then compared the behavior of these parameters in the four genetic backgrounds. To achieve this, we have taken the isolated cell somata in each of the genotypes at the migration front. From each soma we counted the number of fibers that it contained

and measured the lengths of each fiber from the cell somata. Fiber length was calculated by multiplying the pixel length by the total length of fiber measured using Image J software.

The number of filopodia significantly decreased in *N-cad GOF* compared to control glial cells and the cytoplasmic processes are also considerably shorter in *N-cad GOF* glia (**Fig. 6C,D,G and H**). Conversely, downregulation of N-cad in glia results in increased filopodia length and extensive branching of cytoplasmic processes (**Fig. 6F,G, H**). This difference is not due to the number of glia at the front of chain, which does not change between control and *repo>N-cad GOF, actin42AGFP* wings (**Fig. 6I**).

As a measurements of actin dynamics we used fast confocal microscopy at a interval of 20 sec and analyzed the dynamic behavior of the glial processes in control *repo>actin42AGFP* and in *repo>actin42AGFP, N cad GOF* animals and found that the filopodia of the N-cad overexpressing glial cells show reduced motility.

We then investigated how *CYFIP* modulates cell migration upon N-cad overexpression *in vivo* by the same high-resolution and fast speed confocal time lapses. Quantitative analyses revealed that the number and the complexity of filopodia were comparable to those observed in control animals (**Fig. 6E,G, H**).

Taken together, these results show that the levels of N-cad tightly regulate cytoskeletal dynamics and filopodial motility in the migrating glial cells.

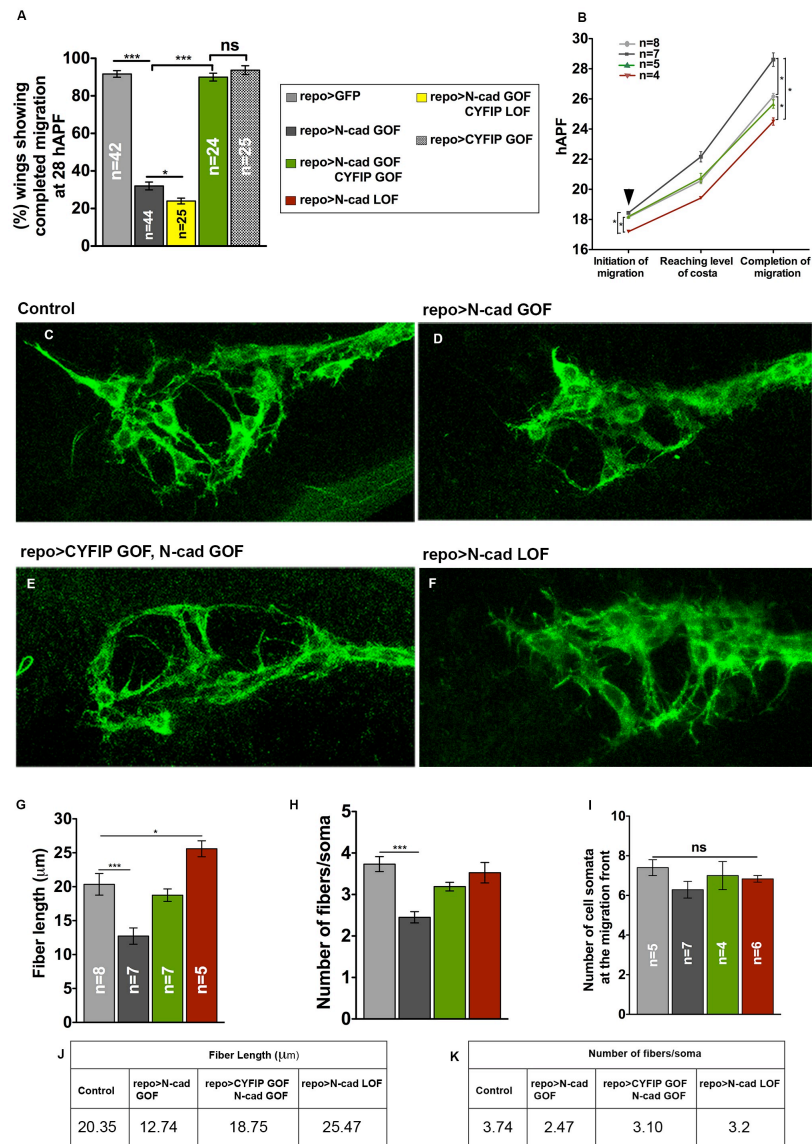


Figure. 6: Actin cytoskeleton and membrane dynamics

(A) The graphs represent the comparison of glial cell migration efficiency of the indicated genotypes. (B) Graph showing the initiation of migration, reaching level of costa and completion of migration in the indicated genotype. (C-F) Snapshots from confocal time lapse imaging show the organization of cellular protrusions of migrating glial cells at the front of the glial chain in *repo>GFP* (control), *repo>N-cad GOF*, *repo>CYFIP GOF*; *N-cad GOF* and *repo>N-cad LOF* wings. (G) The graph represents the quantification of fiber length (μm) measured from the soma in all genotypes at the time just before initiation of migration. (H) Shows the number of fibers present in each soma of the indicated genotype. (I) Graph indicates the number of cell somata present at migration front. (J, K) Quantification summary. The first table indicates the fiber length and the second fiber/soma. Bar: (C-F), 20 μm .

Discussion

Cell adhesion plays a very important role not only in providing the mechanical basis for static tissue organization (e.g. defined cell arrangement in polarized epithelium) but also in shaping the tissue by enabling plastic connections between cells. In collectively migrating cells, lower levels of cell adhesion molecule cadherin regulate speed and invasive behavior of the cells (Arboleda-Estudillo et al., 2010; Camand et al., 2012; Derycke and Bracke, 2004).

In the neural tissue, glial cells represent a very motile cell type. They migrate not only during development (i.e. from the place of birth to their final position), but, at least in the case of specific glial cell types, also in the adult. Astrocytes in the mammalian brain, for example, are able to migrate to lesions caused by injury or neurodegeneration, a process commonly known as reactive astrogliosis (Sofroniew and Vinters, 2010). In addition, tumor glial cells move through the nerve tissue, leading to the formation of glioma, the most aggressive form of tumor in the nervous system (Cayre et al., 2009). However, how the cells migrate through the dense network of neurons and glia in the CNS, is mostly still unknown. Additionally, N-cad is implicated in glioma at the early stages tumor development. N-cad overexpression in the glioma results decrease in cell adhesion that impinges on the dramatic decrease in invasive behavior (Asano et al., 2004).

Glia in the developing *Drosophila* wing provide an excellent tool to dissect the molecular and cellular processes underlying collective glial movement. Cells in the migrating collective are physically and functionally connected, therefore the adhesion between cells has to be very dynamic. In the present study we show that, in contrast to previous reports (Fung et al., 2008; Iwai et al., 1997), the cell adhesion molecule N-cad is undoubtedly expressed by the peripheral glia but not the central nervous system glia. N-cad expression in the glia increases as the cells start migrating along the underlying nerve and reaches its maximum levels by the completion of migration. This suggests a regulatory role of N-cad in wing glia migration and indeed altering N-cad levels does alter glial migration efficiency. Excess of N-cad decreases, while N-cad depletion increases migration efficiency. Moreover, we could conclude that it is the first step (i.e. initiation) of migration that is delayed upon N-cad overexpression suggesting that N-cad might put a hold on migration, whereas its depletion releases the “brake”. We believe that N-cad plays a permissive role in the glial chain

movement since we never observed a blockage in migration upon altering N-cad levels in the glia. Similarly, we never saw cells moving in isolation in wings blocking N-cad. To conclude, controlled levels of N-cad in glia are important for the efficiency of their migration.

Cadherins and Adherens junctions

Wings display AJs between glial cells, and between glia and neuronal processes, however, we did not observe significant difference in the number of AJs formed between the glial processes of control, N-cad overexpressing or N-cad downregulating glial cells. Fluorescent immunolabeling with N-cad antibody did not show punctate appearance of the N-cad signal even at very high magnification. This suggests that, while it may accumulate at slightly higher levels at AJ, N-cad is uniformly present along the cell membrane. Indeed, although cadherins are classically regarded as components of the AJs, cadherin/catenin complexes can be observed all along the cell membrane in MDCK epithelial cells (Nathke et al., 1994). *in vitro* adhesion assays with CHO cells or fibroblastic L-cells have suggested that cadherins can mediate cell-cell adhesion (Briehner et al., 1996; Nose et al., 1988). This selective adhesion of cadherin observed is due to preferential interaction between the identical cadherin molecules. The distribution of cadherin/catenin complexes might be important for the formation of contacts between migrating cells. It has been demonstrated in the case of border cells in the fly egg (Niewiadomska et al., 1999) that cadherin are strongly accumulate at the contact site between border cells and are required for migration (Niewiadomska et al., 1999). The preferential assembly of cadherins at AJ might provide anchor sites for cell translocation.

Cadherin and intercellular motility

Cell-cell adhesion together with extracellular matrix play a crucial role in maintaining cell architecture in developing and mature tissues. N-cad protein expression is shown to either downregulated (Asano et al., 2004) or upregulated (Utsuki et al., 2002) and its precise role in glial cell migration remained unanswered. Our observation made in the glia of developing wing suggests that N-cad expression is dynamic and is necessary for the timely movement of glia along axons. Using GOF and LOF approaches as well as rescue experiments, we show that N-cad is involved in

glial cell migration. Overexpression of N-cad in glia indeed leads to significant decrease in cell migration (Asano et al., 2004), whereas decrease in N-cad induces increase in cell migration (Rappl et al., 2008).

Cadherin-catenin interaction in the control of glial cell migration

A key organizer of the actin assembly at the cell membrane is γ -cat (Kobielak and Fuchs, 2004; Oda et al., 1993), which has been shown to bind either β -cat as a monomer or the actin filaments as homodimer (Drees et al., 2005; Yamada et al., 2005). Dissociation of γ -cat from the cadherin/catenin complex leads to actin bundling and suppression of Arp2/3 mediated actin assembly. As consequence, membrane dynamic, a process that is essential for cell migration is reduced (Benjamin, 2008).

The hypothesis that cadherin mediate intercellular motility, where cells move on the surface of neighboring cells, was supported by two observations (Gumbiner, 1992, 1996). First, the actin cytoskeleton is the main force generating system that promotes cell migration. Second, cadherin based cellular junction are major anchor point for actin filament at cell-cell contact site. Our investigation of the function of N-cad in glial collective migration strongly supports the hypothesis that classical cadherins can directly participate in the intercellular motility *in vivo*. *Drosophila* wing glia is the excellent system for the further analysis of cadherin based intercellular motility, as N-cad appears to be the adhesion receptor that controls glia migration.

In sum, we provide *in vivo* evidence that increased level of cadherin/catenin complex in the cell membrane causes decreased membrane motility, while low cadherin/catenin levels increase it by controlling actin cytoskeleton dynamics. (Benjamin and Nelson, 2008). In our model of moving glia in the developing *Drosophila* wing, N-cad might regulate the timing of the migration process: in the first phase of migration, low levels of N-cad allows the initiation of cell movements, while at the end of the migration, N-cad accumulates in the cell membrane and slows down cell movements.

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SUPPLEMENTARY FIGURES

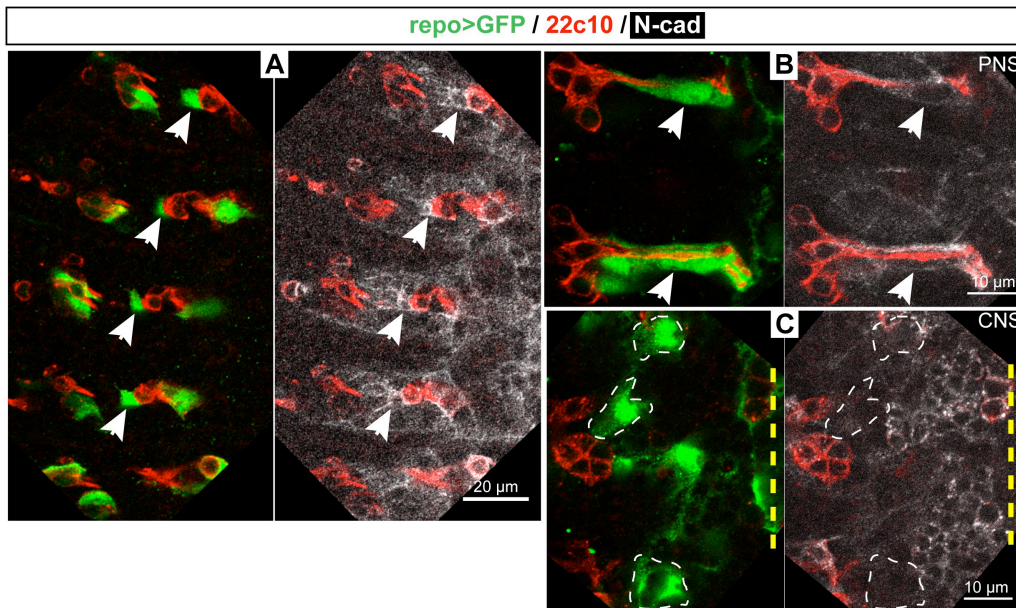


Figure. S1: N-cad is expressed in the peripheral nervous system

Confocal images of *repo>GFP* embryo immunolabeled neurons (22c10, red) and N-cad (grey) and GFP in green. (A) Lower magnification of the peripheral nerves. (B, C) Higher magnification of the glia and neurons in the PNS (B) and CNS; C, Note the expression of N-cad in the glia in the periphery (arrowheads) and the absence of N-cad in the CNS (dashed circle). Dashed line: midline. Bars: (A) 20 μm , (B) and (C) 10 μm .

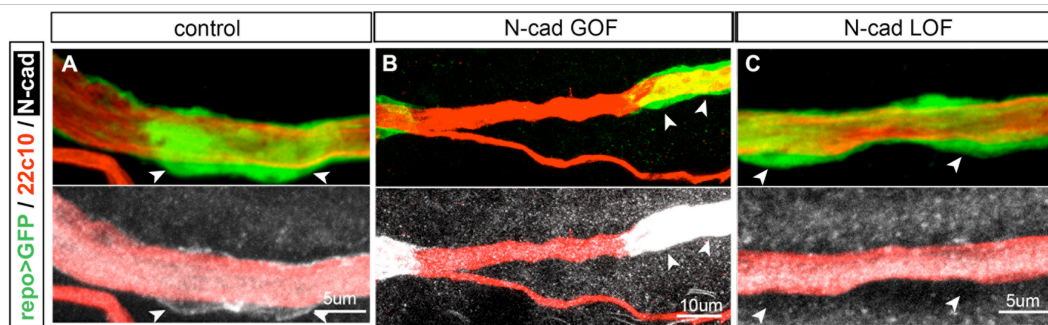


Figure. S2: Expression of N-cad upon N-cad gain and loss of function

22c10 (red) and N-cad (grey, arrowheads) immunolabeling of *repo>GFP* control, *repo>N-cad GOF*, *repo>N-cad LOF* and *repo>N-cad GOF& LOF* nerves. Bars, A and C, 5 μm ; B and D, 10 μm .

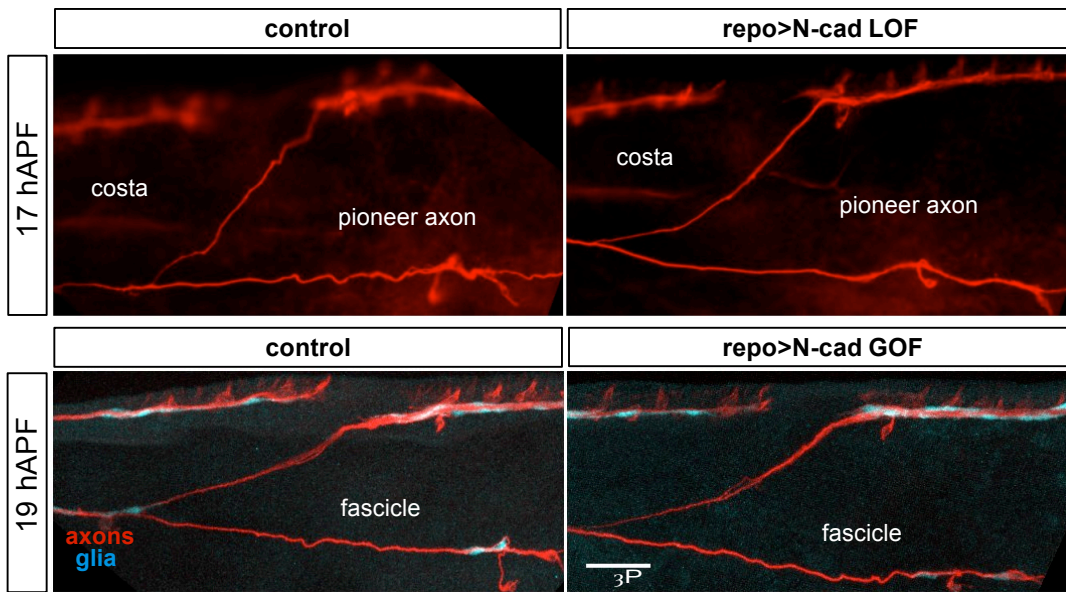


Figure. S3. Axon growth morphology in N-cad gain and loss of function
 22c10 (red) immunolabelling of *repo>GFP* control, *repo>N-cad GOF* and *repo>N-cad LOF* nerves. Bar, 30 μm.

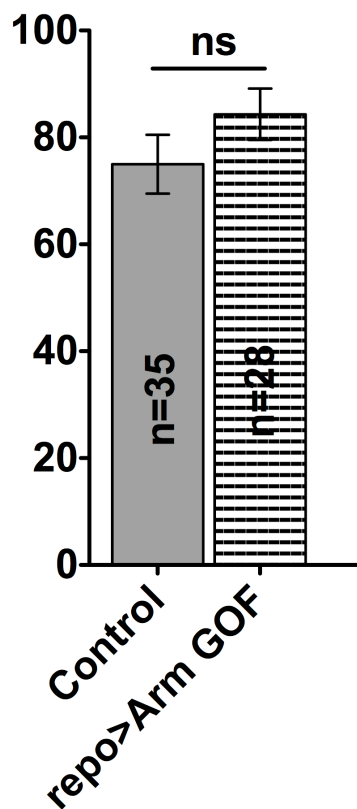


Figure. S4. Migration efficiency of the glial chain
 The graph represents the migration efficiency of glial cells upon Arm overexpression in the glial cells compared to the control *repo>GFP* cells.

Chapter 3

Role of Gcm in glial cell migration

glide/gcm encodes a protein expressed transiently at early stages of glial development. Its mutation causes presumptive glial cells to differentiate into neurons, whereas its ectopic expression forces virtually all CNS cells to become glia (Jones, 1995; Hosoya, 1995; Vincent, 1996; Van De Bor, 2000; Van De Bor and Giangrande, 2002; **Fig. 1**). Previous studies suggested that critical levels of Gcm may be essential for glial migration in embryogenesis (Ho et al., 2009), however *gcm* null mutants transform glia into neurons and are lethal, they are thus difficult to analyze for the migratory phenotype. The wing is a simple tissue so is the organization of peripheral glia and the collective migratory behavior of wing glia is well characterized (Benoit, 2004). For these reasons, I made use of the developing *Drosophila* wing to study the role of the Gcm transcription factor in collective behavior. Using conditional and weak *gcm* mutants I have found that *gcm* directly affects migration in a dosage dependent manner. When *gcm* is downregulated (loss-of-function) in glia, migration occurs slowly, while *gcm* overexpression triggers faster migration. In addition, time-lapse analyses in the mutant *gcm* background have allowed me to define its role *in vivo*.

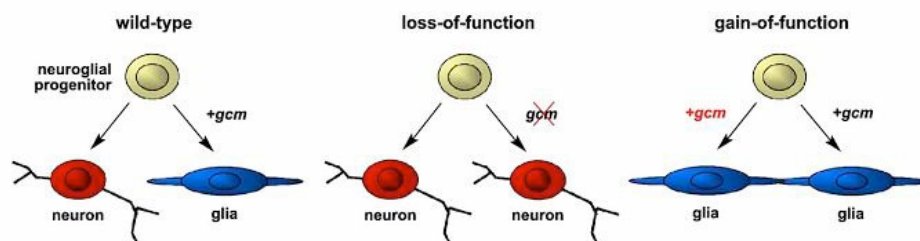


Figure 1. *gcm*: Binary switch between glia and neurons. Glia and neurons are derived from neuroblasts. *glide/gcm* loss-of-function mutation causes glial cell differentiation into neurons. Ectopic *gcm* expression results in the differentiation of glia at the expense of neurons (Jones, 1995; Hosoya, 1995; Vincent, 1996; Van De Bor, 2000; Van De Bor and Giangrande, 2002).

RESULTS:

1. Low amounts of Gcm slow down collective cell migration

To assess the role of *gcm*, I genetically altered the amounts of the Gcm protein and analyzed the migratory phenotype. Since the *gcm* null mutation is embryonic lethal and doesn't allow the analysis of wing glia, I used different hypomorphic combinations that are not lethal. *gcm-Gal4* is a weak hypomorphic allele due to the insertion of a Gal4 containing transposon into the *gcm* promoter. The *gcm-Gal4, UAS GFP* (*gcm>GFP*) stock is semiviable in homozygous condition (Kumar, in Prep). *rA87* is an enhancer trap line carrying a LacZ transposon into the *gcm* promoter and constitutes an even weaker allele than *gcm-Gal4*; *rA87* is viable in homozygous condition. Finally, *N7-4* carries a point mutation that abolishes the DNA binding activity and represents a null allele (Miller et al., 1998, Vincent, 1996). No *N7-4* homozygous flies eclose. Because *gcm-Gal4* homozygous flies are poorly viable and because *rA87* is very weak, I decided to use transheterozygous combinations and produced an allelic series: (*gcm>GFP/rA87*; *gcm>GFP/gcm>GFP*; *rA87/N7-4*): *gcm>GFP/rA87* is the weakest combination, *rA87/N7-4* is the strongest while *gcm>GFP* homozygous animals represent an intermediate condition. Other mutant combinations with *gcm>GFP* using *gcm34* (hypomorph), *gcm26* (null allele) and *N7-4* are lethal and I therefore could not use them to follow the migration process.

To clarify whether mutants have an effect on the migration of the glial cells, I compared them (LOF animals) to control animals. To assess migration efficiency, I collected the animals at the white pupae stage (0 hour After Puparium Formation, 0 hAPF) and put them at 25°C. Depending on the stage I wanted to analyze, I fixed and dissected the wings out from the cuticle. Then I performed immunolabeling on

dissected wings and visualized them under the fluorescent microscope. Then, I counted the number of wings showing completed migration (upon joining the radius glia) in control and LOF animals and represented them on graphs. Clearly, flies expressing low Gcm levels show less efficient glial migration compared to control animals. (**Fig. 2, compare red, pink and cyan with grey column**). In sum, the strong allelic combination showed severe migration defects compared to what seen in control animals.

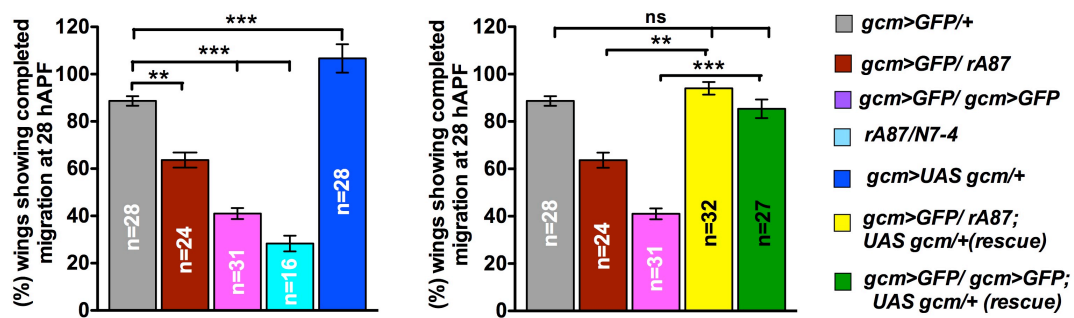


Figure 2. The graph shows the glial cell migration efficiency in the indicated genotypes. (n= number of wings; Bars=indicated genotypes; stars= SEM).

2. Gcm levels control the efficiency of glial migration

In order to further validate the hypothesis that Gcm levels control glial migration, I asked whether the overexpression of Gcm rescues the migratory defect. As Gcm overexpression is embryonic lethal (Soustelle et al., 2007; Vincent et al., 1996), I used the Gal4/UAS system combined with an approach that allows for temporal control. In the conventional Gal4/UAS system, Gal4 drives the expression in a tissue specific manner. The Gal4 protein binds to its target UAS (Upstream Activating Sequences) and activates the transcription of the fused downstream

transgene. In the TARGET (Temporal And Regional Gene Expression Targeting) system, a temperature sensitive GAL80 protein (GAL80^{ts}) represses the transcriptional activity of Gal4 at 19°C and thus prevents the expression of reporter genes fused to UAS (Fig. 3A). However, when shifted to the restrictive temperature (30°C), GAL80 repression is released, allowing Gal4/UAS to drive transgene expression (McGuire, 2003; Fig. 3B). I have analyzed the rescue in two *gcm*-LOF backgrounds (*gcm>GFP/rA87* and *gcm>GFP/gcm>GFP*) and found that reexpressing Gcm under the control of its own promoter does rescue the migratory defect found in the mutants (Fig. 2, yellow and green column). These observations indicate that the delayed migration observed in loss-of-function mutations is specific to glia. Finally, and to complement to the LOF data, flies expressing elevated amounts of Gcm (gain-of-function) in glial cells display faster migration as compared to what observed in the control animals (Fig. 2; compare grey with blue column). While in the later 92% wings show completed migration by 28 hAPF a higher percentage (106%) show completed migration upon Gcm overexpression.

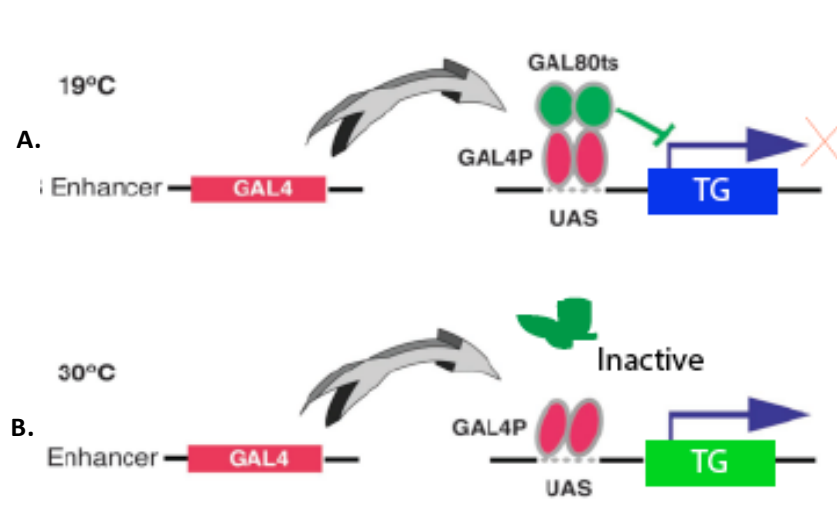


Figure 3: TARGET System: The target system allows controlled expression of a transgene in both time and space. Temporal control is achieved by GAL80^{ts} temperature sensitive transgene expressed ubiquitously via the tubulin promoter. At 19 °C, the GAL80 protein is active thereby inhibiting the activity of Gal4 to drive transgene (TG) expression (**Fig. 3A**). Upon shifting at permissive temperature (30 °C), GAL80 is inactivated, induce the transcription of the UAS Transgene (**Fig. 3B**; McGuire, 2003).

The tight balance between glia and neurons is maintained by the amounts of Gcm and it is known that loss-of-function *gcm* mutations result in the conversion of glia into neurons. One possible explanation for the

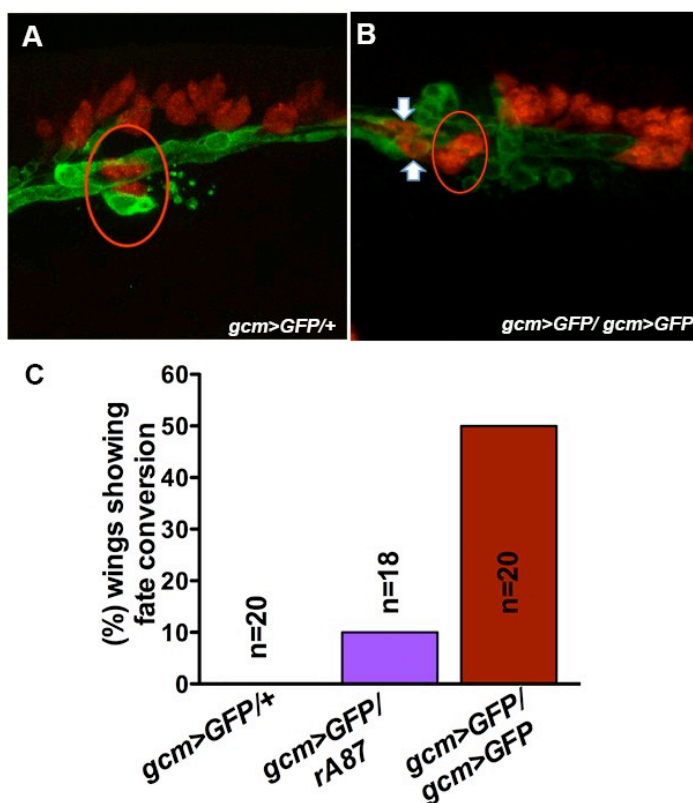


Figure 4. (A, B) Immunolabeling and confocal stacks of wing glia marked in green and neurons in red. At the margin of the wing there are two sensory neurons positioned along the margin called Twin Sensillum on the Margin or TSM neurons (red circle). Glia conversion into neurons is shown at the migration front (white arrow; **B**). The graph shows the percentage of glia to neuron conversion of the first 10 cells at the front of the chain in the indicated genotypes (**C**).

migratory phenotype in the loss-of-function background is that glia to neuron conversion affects the migration indirectly. Therefore, I inspected the glia to neuron

conversion phenotype in the different transheteroallelic combinations. Interestingly, the weak hypomorph combination (*gcm>GFP/rA87*; **Fig. 4C**) shows very mild (10%) conversion, whereas *gcm>GFP/ gcm>GFP* animals show a more consistent fate conversion rate (50%) (**Fig. 4B,C**). Nevertheless, even in a background that shows very low conversion frequency, migration is affected. Thus, fate conversion does not seem the cause of the observed migratory phenotype.

Conclusion: *gcm* affects migration in a dosage dependent manner.

3. Gcm effects on migration do not depend on glial cell number

Gcm induces the expression of genes that maintain the glial identity and allow for glial terminal differentiation. A second explanation for the observed phenotype is that the levels of Gcm control the number of glial cells and hence migration is only affected indirectly. To discriminate between direct versus indirect effects of the *gcm* LOF and GOF mutations, I made several controls. First, I counted the number of Repo⁺ cells in the LOF background and found that mutant chains contain fewer glial cells than wild type chain (**Fig. 5; compare red, pink and cyan with grey column**).

Second, I made the same analysis in a GOF background and found that the chains contain more glia (**Fig. 5, blue column**). These data suggest that number of glia correlates with migration efficiency, however this is not a completely quantitative phenotype. For example, *gcm>GFP* homozygous and *rA87/N7-4* wings display the same number of glia but different migratory phenotypes (**Fig. 5, pink and cyan column**).

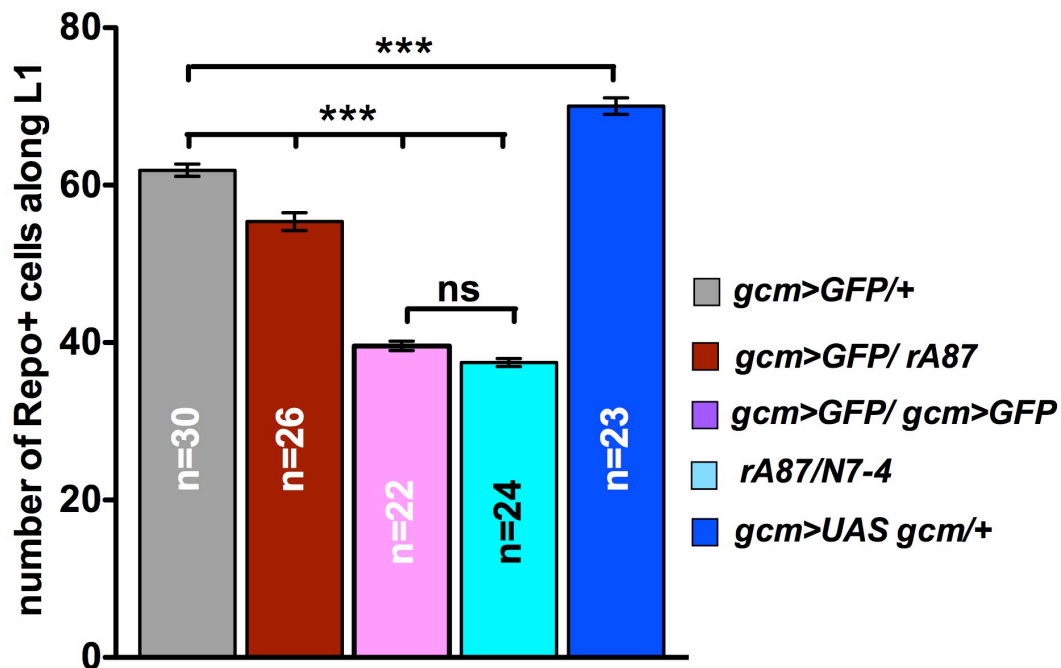


Figure 5. The graph shows the quantification of Repo+ cells in the indicated genotypes.

Third, and complementary to the above-mentioned analysis, I used a Gcm-independent approach to clarify the effect of the number of glial cells on migratory efficiency. Cell cycle regulation is a key process that affects cell number. I analyzed the migration of glia in genetic backgrounds that overexpress a protein that promotes or represses cell division, String or Dacapo, respectively (String/ *cdc25*, a gene that encodes a phosphatase that triggers the mitosis by activating the *cdc2* kinase that enables cell proliferation; Dacapo inhibit the cdk-cyclin complex thereby inhibiting the cell proliferation) (O'Farrell et al., 1989; Lehner et al., 2001). Because we know that glia finish migration in most control wings and in all Gcm GOF by 29h APF, we analyzed earlier stages to characterize the gain-of-function phenotypes. Interestingly, Gcm GOF affects migration much more drastically than the String GOF condition, even though the increase of number of glia is similar in the two backgrounds (**Fig.**

6A; grey and blue column). Clearly, increasing or decreasing the number of glia doesn't produce the same effects as Gcm overexpression or down-regulation. The cell counting assay agrees with the previous data indicating that, in addition to affecting cell proliferation (Ho, 2009), Gcm has a direct effect on glial chain migration. Similarly, Dacapo GOF induces a considerable loss of glia, a much stronger phenotype than that triggered by *gcm*-LOF (**Fig. 6B, green column**), and yet migration is only affected in the latter genotype (**Fig 6 A, green column and hatched column**).

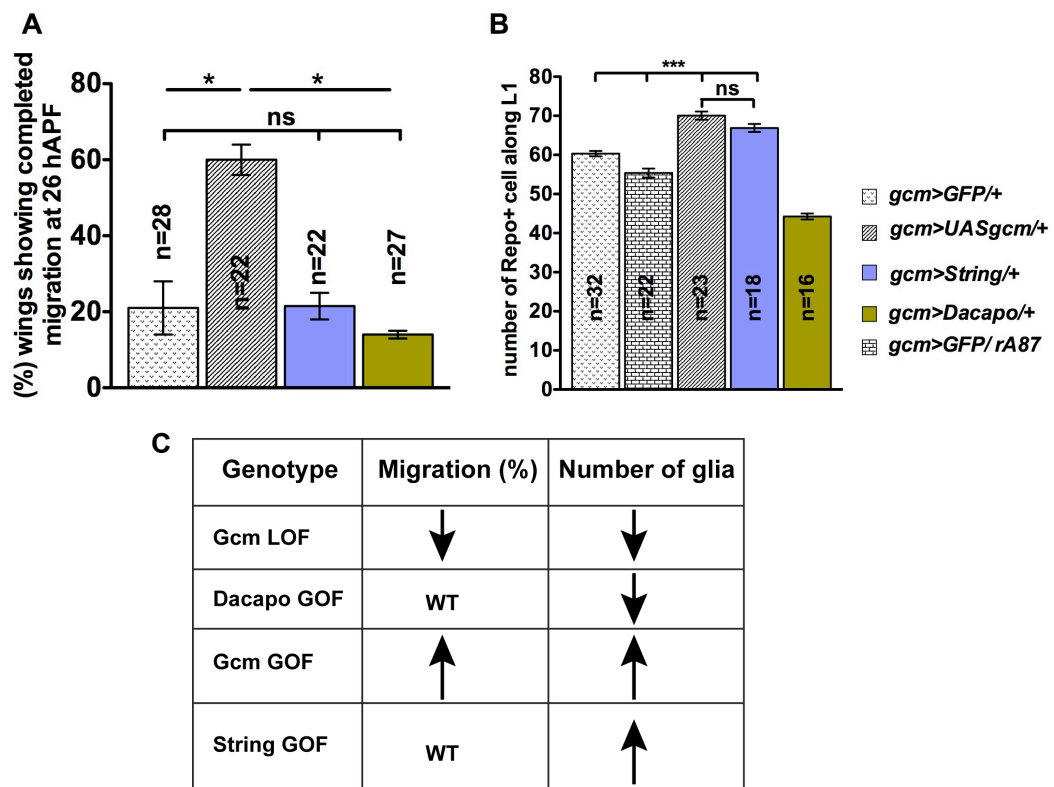


Figure 6. Correlation between migratory defects and glial cell number: (A) indicates the % of wings in which migration is finished as a readout for migration efficiency. (B) Indicates the number of glial cells in the indicated genotypes. *gcm>GFP/rA87* indicates mutations carrying low levels of Gcm (hypomorphs). GOF indicate animals in which proteins (Gcm, String or Dacapo) are specifically overexpressed in glial cells using TARGET system (as in Fig. 3). (C) Summary of migration and number of glia in the indicated genotypes.

4. Dynamics of glial migration in a *gcm* mutant background

The phenotype induced by loss of Gcm may be due to delayed initiation of migration, to slower migration throughout the process, to early arrest of migration or to a combination of these situations. To clarify the nature of the migratory defects, it is important to analyze the dynamics of glial migration by confocal time-lapse microscopy. I therefore created a hypomorphic background in which glia can be followed by time lapse. To gain insight into the kinetics of glial migration, I subdivided the glial migratory process into three phases: initiation of migration, reaching the level of costa and completion of migration (Berzsenyi, 2011, see chapter 1) and I found that in an average, *gcm*-LOF (one hour), wings show delayed migration from the beginning. (Fig. 7).

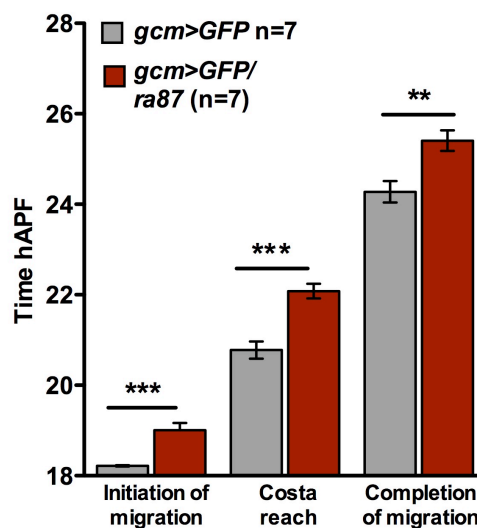


Figure 7. Migration efficiency in *gcm* mutant background

Conclusions and perspectives

Indeed, when compared to control wings, glial cells in *gcm*-LOF wings start moving later and the delay stays constant. This suggests that Gcm may a) negatively control the expression of a gene(s) that works as a brake or a “stop” signal, b) Gcm positively controls the expression of a motility factor, c) a combination of the two.

The transactivation potential of the Gcm transcription factor suggests that Gcm directly controls the expression of genes involved in migration. Previous microarray data have identified several genes that are involved in cell migration (Altenhein, 2006). These include NetrinA (NetA) and Unc5 (Altenhein, 2006, Freeman, 2003). NetA is expressed during embryogenesis by the cells of the ventral midline and serves as a guidance cue for navigating axons (von Hilchen, 2010), while Unc5 is a repulsive Netrin receptor involved in glial migration (Freeman, 2003). Because microarray may identify indirect target genes, to clarify the role of the Glide/Gcm glial determinant in collective migration, we performed a genome-wide screen searching for the direct Gcm targets. The DamID approach is based on the poor methylation of the fly genome and the use of a transgenic line expressing the DAM methylases (Southall, 2007). We fused the bacterial DAM methylase to Gcm and expressed this construct in transgenic flies. In this way, DNA methylation was induced in the vicinity of Gcm binding to its target motif (Popkova, in prep). Methylated DNA was marked with fluorochromes and hybridized to a microarray containing all *Drosophila* genome (Nimblegen). The intensity of fluorescence permits to identify the methylated enriched sequences that will likely identify Gcm direct targets. One such example is *Frazzled (Fra)*, which is a netrin receptor known to respond to the attractive signals in neurons (von Hilchen, 2010; Kolodziej et al., 1996; Hiramoto et al., 2000). To characterize the Gcm pathway that controls migration and determine how different

levels of Gcm affect this process, we will analyze the role of its target genes and their dependence on Gcm. We have also identified several other potential targets including EGFR, Innexin (Inx 2; Bauer, 2004) and (Inx7; Phelan, 2005) that are downstream to Gcm as found in DAM ID screen. This part of the project is now dealt with by a PhD student in the laboratory, Tripti Gupta.

Finally, I had previously standardized a protocol to visualize fast cell dynamics using high-resolution and fast speed confocal time-lapse microscopy (see chapter 2). Fast imaging on *gcm*-LOF animals will shed light on filopodia organization, and we will be able to ask whether they extend or retract rapidly, upon directional cues from extracellular environment as in wild type animals. In sum, *gcm* affects migration in dosage dependent manner.

The *Drosophila* glial-specific transcription factor Gcm has been considered as a typical binary switch in glial cell induction. Here I describe the involvement of Gcm in collective cell migration. Genetic analyses (Ho, 2008) have shown that Gcm amount are critical for glial cell migration. Persistence Gcm expression in the glial progenitor promotes further proliferation and cell migration while downregulation triggers delayed migratory phenotype. Expression of Gcm by the *gcm-Gal4* promotes the cell progression, in particular Cyclin transcript. During *Drosophila* retina development, these cyclin undergo proliferation. One explanation is that glial progenitor in *gcm>gcm* stay longer in the progenitor state causing delay in differentiation.

Discussion

Discussion

Glial cells represent the most motile cell types in the neural tissue. They migrate not only during development (i.e. from their place of birth to their final destination) but also during diseased condition. Cell migration process is highly stereotyped and efficient, however the complexity of cell interaction and their dynamic behavior make this process a major challenge. Here we address this important issue, using *Drosophila* developing wing as model system together with cell selective ablation and demonstrated that a minimal number of cells in group is essential to reconstitute a moving chain. By analyzing carefully we report that cluster at the tip of the chain is able to move directionally and that its migratory efficiency improves with size of the cluster. Our data also showed how timely movement of glia depends on the expression of cell-adhesion molecule N-cadherin. Defects in the functionality of these cell adhesion molecules in the glia could induce migratory defects.

Homeostatic interaction at the glial front controls cell migration

One of the key features of collectively migrating cell is that they establish a community in which cell-cell interaction eventually define a functional migratory unit. The cell-cell interaction may provide the basis for collective behavior, however, how the molecular or cellular mechanism controlling migration remains unknown.

Collective behaviors however may be explained by the exploratory structures that are present at the leading edge called the DCE or distal cytoplasmic extension (Aigouy, 2008). Interestingly, pioneers cells display a dynamic network of filopodia at the front of their soma (Aigouy, 2004) as well as the long and stable process at their rear, the DCE, in direct contact with many follower cells. These DCE may provide a physical substrate or send a directional cues to the follower cells and their retrograde interaction may insure chain integrity. Interestingly, when cells are

separated from the rest of the chain upon target ablation (cluster of 4-6 cells), they never show DCE projections towards the rear end. This may be probably due to the fact that separated cluster do not migrate as efficiently as intact chain and thus DCE don't produce long extension.

At the advent of the new and more powerful technique, morphology and behavior of single cells can be followed. One such example is the Cre/lox mediated recombination in mice that expresses several fluorescent proteins, giving rise to cells differentially labeled with multicolor (Livet, 2007). In complementary to this approach in mice, this technique is available in *Drosophila* as well called Flybow. This technique will facilitate the tracing the morphology and behavior of single cells that differentially labeled with multicolors. In addition, this will allows us to identify and follow individual cells along glial chain and gain more insight into cell-cell interaction and single cell behavior in migrating cells.

The *Drosophila* tracheal system, one-two motile tip cells trigger the outgrowth of tracheal branches (Affloter, 2008). Branched outgrowths are originally arranged in a head to tail manner. When growing branches are disconnected from the stack cells, the groups of separated cell keep moving efficiently (Caussinus, 2008). Thus, it could valuable to test how tracheal cells isolated in in different cluster behave.

Cell-adhesion between migrating cells

During development, cell move from the place of origin to their target site. While moving cells are physically and functionally connected to each other. The adhesion between the cells needed to be dynamic since cells are constantly moving and changing their position relative to each other while in epithelial cells cell adhesion are localized a specific region in the cells.

In migrating glial cells of developing wing, cell adhesion molecule N-cad expression starts at time of migration initiation and show accumulation over time in glia and neurons. The overexpression and downregulation of the N-cad function in the glia alter the migration efficiency of the glial chain. Over-expression of N-cad results in excessive accumulation of the Arm (β -cat) on the membrane that might control the collective cell migration in Wg/wnt pathway independent manner. This data show that N-cad may influence the cell migration upon interacting with other molecules and regulate the glial collective movement in the wing.

N-cad expression in PNS glia

Here I have shown that N-cad is dynamically expressed in the glial cells of the *Drosophila* peripheral nervous system in embryo and pupal wing. Immunolabeling revealed that upon N-cad overexpression, glia showed increase in the intensity while introduction of N-cad RNAi (downregulation) into glial cells clearly show dramatic decrease in the intensity of N-cad labeling. N-cad so far have been shown to be expressed in the neurons (Fung, 2008; Iwai, 1997), our data illustrate that N-cad is expressed in the PNS glia. However, E-cadherin, on the other hand, is neither expressed in the neuron nor in the glial cells of wing. During course of development, glial cells have to migrate long distance along the axonal substrate during embryonic and pupal stages (Aigouy, 2004; von Hilchen, 2008). Cell assumes different morphology while migrating i.e. cell at front of the chain show elaborate morphology while cells behind are followers. N-cad may play a role in controlling cell migration upon impinging on adhesion between migrating glia and axons. In the case of PNS glia in the wing migrate in a chain like manner over large distance, while glial cells in the *Drosophila* CNS migrate singly over very short distance (Klamt, 2009) therefore, N-cad may not required for the movement.

Like glia in the wing, Astrocytes in the mammalian brain expresses N-cad mediated adherens junction (Perego, 2002) but do not express E-cadherin. The cadherin mediated cell adhesion has been widely studied in the pathological condition such as cancer glial cell invasion while little is known about N-cad involvement under physiological conditions (Shinoura, 1995). It has also been shown that N-cad is highly expressed in the embryonic rat nerve front (Wanner, 2006) where axons interact with environment to receive directional cues. The rodent nerve is also associated with glial cells that expresses adhesion molecule N-cad at the nerve that actively navigate to their target location. N-cad show higher expression in Schwann cells precursor than the mature ones. The N-cad downregulation in glia as a precursor generate Schwann cells. Wanner et.al, illustrate that N-cad appears necessary for the glia and neuron during the embryonic nerve formation. Higher amount of N-cad show axon navigation towards the Schwann cell precursor than along Schwann cell with lower N-cad amount. However, glial cell in *in vivo* migrate on the axonal substrate (Aigouy, 2004). Thus N-cad might modulate the vertebrate glial movement along axon.

Cadherin and adherens junction

N-cad expression in the glial cells dynamically changes during development i.e. after the start of migration until completion. N-cad might play a role in the collective movement of glia. In order to test this hypothesis, I have altered the amount of N-cad by specifically overexpressing or down regulating N-cad in the glial cells. Excessive N-cad expression in the membrane cause strong delay in migration compared to control animals while opposite happens with known down line. Glial chain in both GOF and LOF eventually complete migration and forms a continuous sheath around axons. However, I don't observe a complete blockage of cell migration upon altering N-cad function in glial cells. Time- lapse microscopy revealed that N-

cad overexpressing animals show delay from the time when migration is initiated. Therefore, the data suggests that N-cad play a role in controlling glial chain movement.

Invasive behavior of some the tumors cells can be observed as a result from the downregulation of cell adhesion molecules that are essential for cell contacts. Cadherins have been implicated in many of invasive behavior of the cancerous cells (Berx, 2009). Recent studies have shown implication of N-cad in the cancer cell migration. In breast cancer, N-cad positively regulates the migration (Hazan, 2000).

In border cell cluster (BC cluster) of *Drosophila* egg chamber, E-cadherin controls migration of these cells (Niewiadomska, 1999). The border cells cluster migrate between the nurse cells and therefore expression of E-cad is necessary for the BCs and neighboring nurse cells. In the case of the BCs, lower levels of E-cadherin slow down migration process, whereas in our studies, N-cad loss-of-function (N-cad RNAi) in wing show enhance migratory efficiency. One possible explanation is that BCs are in constant contact with the nurse cells that provide the substrate during intercellular motility. Thus, in the absence of E-cadherin function, the border cells probably cannot exert enough traction to move further between nurse cells (Niewiadomska, 1999). However, in the case of the wing, the migration path is constituted by the navigating axons that provide well-defined substrate upon which glial cells slide to reach their final destination.

Interestingly, cells present at the front of both border cells and glial chain show long cytoplasmic projection in space (Aigouy, 2008; Fulga, 2002). In the case of BCs the long cytoplasmic projection/extensions (LCE) are produced by one of the eight cells that are present in the migrating cluster between the nurse cells. The distal cytoplasmic extensions are produced by the pioneer cells, project backward. Recent

studies demonstrate that either border cells or nurse cells mutant for E-cadherin, LCE is not formed by the BCs (Fulga, 2002). However, it is not clear whether absence of LCE in absence of E-cadherin is cause or its migration halt as such is the cause is yet to be understood.

Cadherin and its associated molecules

Cadherin are the key players in transducing signals at the membrane. At the cytoplasmic domain cadherin is associated with several adaptor molecules that eventually links adhesion molecule to actin cytoskeleton and regulate the membrane dynamics (Nelson, 2008). One of the key organizers of the actin assembly is the γ -cat (Oda, 1999; Kobiolk, 2004). γ -cat can bind either to β -cat as a monomer or actin filament as a homodimer (Drees, 2005) and competes with activity of Arp2/3 as potent actin nucleation molecules required for pseudopod formation. When N-cad is overexpressed in the glia γ -cat is recruited at the membrane leading to lower migration efficiency and opposite happen when N-cad is depleted from the glia.

Immunolabeling show that Arm is expressed in both glia and neuron in the wing. Arm provides a link between the cadherin and γ -cat (Oda, 1993). When N-cad is overexpressed, Arm is localized at the membrane. Arm performs dual functions. a) localizes to the adhesion complex and b) is associated with degradation complex involving Wg/Wnt pathway (Bienz, 2005). Upon Arm overexpression in the glia, Arm was enriched in the nucleus and yet has no affect on migration suggesting that Wg/ Wnt pathway may not be involved in the glial migration.

***glide/gcm* pathway in cell migration**

Gcm needs to be expressed at threshold levels and transiently to insure appropriate glial differentiation: i) Gcm positive autoregulation is necessary for gliogenesis; ii) Gcm degradation ensures the differentiation of proper glial number; iii) ectopic Gcm threshold levels induce gliogenesis at the expense of neuronal fates; iv) hypomorphic and hypermorphic Gcm backgrounds affect wing glia migration.

I have found that mutants expressing low Gcm levels show less efficient glial migration compared to the wild type, whereas animals expressing elevated levels display the converse phenotype, that is, more efficient migration. These defects cannot be simply attributed to changes in glial number, as glial chains containing fewer cells than wild type animals (due to ablation or other mutant backgrounds) reach their final destination in the due time (Berzseny et al., 2011). Recent studies show that embryos mutant for the F-box proteins Slimb and Ago show glial migratory defects and affect Gcm degradation (Ho et al., 2009). Absence of Slimb and Ago in the double mutant also caused defect in glial migration along peripheral nerves (Ho et al., 2009). One possible explanation for the delay in migration in wing is that when Gcm is not timely degraded, extra glial proliferation was found that might account for triggering acceleration in migration and imbalance between glial and neuronal fates in the nervous system. Low levels of Gcm triggers delay in migration of glial cells. This could be due to the fact that reducing Gcm in glial cells may hamper glial cell motor machinery and signaling pathway that are essential for efficient migration. Cell cycle is a key process that affects the cell number (O'Farrell et al., 1989; Lehner et al., 2001). Increasing or decreasing cell proliferation does not produce the same phenotype as Gcm hypomorph or hypermorphs indicating the fact that Gcm amount are critical for cell migration process.

Conclusion and Perspectives

My studies presented here further the understanding of community characteristic of coordinated cell migration. This data suggest that cell counting at the front of the migration is indeed a key-determining factor in collective cell movement. Isolated cell cannot migrate efficiently and dies while 4-6 cells survive but show poor migratory efficiency. Therefore, cell present in the community senses some the guidance cues that seems to be the fundamental for the formation of migratory community. However, signals that regulate the community effect are still unknown and identification and characterization will advance the knowledge in understanding collective process.

Although, migratory community display a clear difference between the cell that are present at the front and those that are present behind. One approach to understand pioneer and followers on their molecular levels will to compare gene expression profile. This approach will give detail insight to understand the molecules that are expressed by the pioneer and followers at the start of migration. What are the key molecule expressed at the time of initiation of migration process and how is it different in followers? What are the genes that are expressed when glial cells reaches it target site?

Glial cell are in constant contact while they are moving. The adhesion between migrating cell need to be dynamic since the constantly change their position. The key discovery of this work is that N-cadherin coupling is important for the timely movement of PNS glia in the developing wing. In future combining the knowledge of cell-cell interaction and homeostasis with molecular cascade controlling cell motility will provide enough insight to understand the collective process in detail and opens broader perspective to investigate nervous system disorders.

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