Deciphering intrinsic and extrinsic machinery underlying collective glia migration using Drosophila as a model organism

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Abbreviations

2D: Two-dimensional

3D: Three-dimensional

Ago: Archipelago

ADAM2: A disintegrin and metalloprotease 2

ALL: Anterior lateral line

BBB: Blood brain barrier

CNS: Central nervous system

DAM ID: DNA adenine methyltransferase identification

DCC: Deleted in colorectal cancer

DB: DCC binding domain

DD: Death domain

EGFR: Epidermal growth factor receptor

E-cad: Epithelial-cadherin

ECM: Extracellular matrix

FGF: Fibroblast growth factor

Fra: Frazzled

FNIII: Fibronectin type III repeats

Glide/Gcm: Glial cell deficient/Glial cell missing

GFP: Green fluorescent protein

GOF: Gain-of-function

GTP: Guanosine triphosphate

GBS: Gcm binding site

hAPF: hours after puparium formation

HRP: Horseradish Peroxidase

ISN: Intersegmental nerve

Ig: Immunoglobulin

KD: Knockdown

LOF: Loss-of-function

LG: Longitudinal glia

LGB: Longitudinal glioblast

MARCM: Mosaic Analysis with a Repressible Cell Marker

N-cad: Neuronal-cadherin

NetB: Netrin-B

NetA: Netrin-A

NC: Neural crest

OB: Olfactory bulb

PG: Peripheral glia

PNS: Peripheral nervous system

PLL: Posterior lateral line

Pnt: Pointed

PcG: Polycomb group

RNAi: RNA interference

Repo: Reversed polarity

RMS: Rostral migratory stream

SC: Schwann cell

Slimb: Supernumerary limbs

SVZ: Subventricular zone

SDF1: Stromal-derived factor 1

SN: Segmental nerve

SO: Sensory organ

SOP: Sensory organ precursor

STAT: Signal transducer and activator of transcription

TSM: Twin sensilla of the margin

TN: Transverse nerve

Ttk: Tramtrack

Ub: Ubiquitin

UAS: Upstream Activating Sequences

Unc5: Uncoordinated-5

UPD: Unpaired

WT: Wild type

Résumé

La capacité remarquable des neurones et des cellules gliales à migrer collectivement sur de longues distances assure l'architecture finale du cerveau. Ce processus est extrêmement dynamique et dépend non seulement de l'interaction entre les cellules mais aussi de la présence de facteurs de transcriptions spécifiques au sein de la cellule migrante (Friedl and Gilmour, 2009; Gupta and Giangrande, 2014). Les protéines d'adhésion comme les cadhérines et les chimioattractants/chimiorépulsifs sont connus pour réguler et guider la migration (Cai et al., 2014; Kumar et al., 2015; von Hilchen et al., 2010). Si le mode d'action de ces molécules a été extensivement étudié, les cascades de signalisation qui déclenchent le chimiotropisme sont loin d'être élucidées. Au cours de mon doctorat, j'ai analysé la régulation et le rôle d'un récepteur des chimioattractant au cours de la migration de la glie. Pour ceci j'ai utilisé le modèle du développement de la chaine gliale dans l'aile de la drosophile qui représente un outil de choix pour étudier les mécanismes moléculaires régulant la migration collective (Aigouy et al., 2008; Aigouy et al., 2004; Berzsenyi et al., 2011).

Objectifs

(I) L'une des propriétés principales des cellules pendant la migration collective est leur habilité à rester connectées fonctionnellement et à ne jamais s'isoler de la chaine (Berzsenyi et al., 2011; Gupta and Giangrande, 2014; Rorth, 2009). Ceci suggère un rôle important des molécules d'adhésions dans les processus qui nécessitent une interaction intercellulaire. Les cadhérines sont des récepteurs transmembranaires connus pour leurs rôles dans la régulation de l'adhésion intercellulaire pendant le développement (Friedl and Gilmour, 2009; Harris and Tepass, 2010). Dans un premier temps, j'ai observé le rôle de la protéine d'adhésion N-cadhérine (N-cad) dans la migration collective.

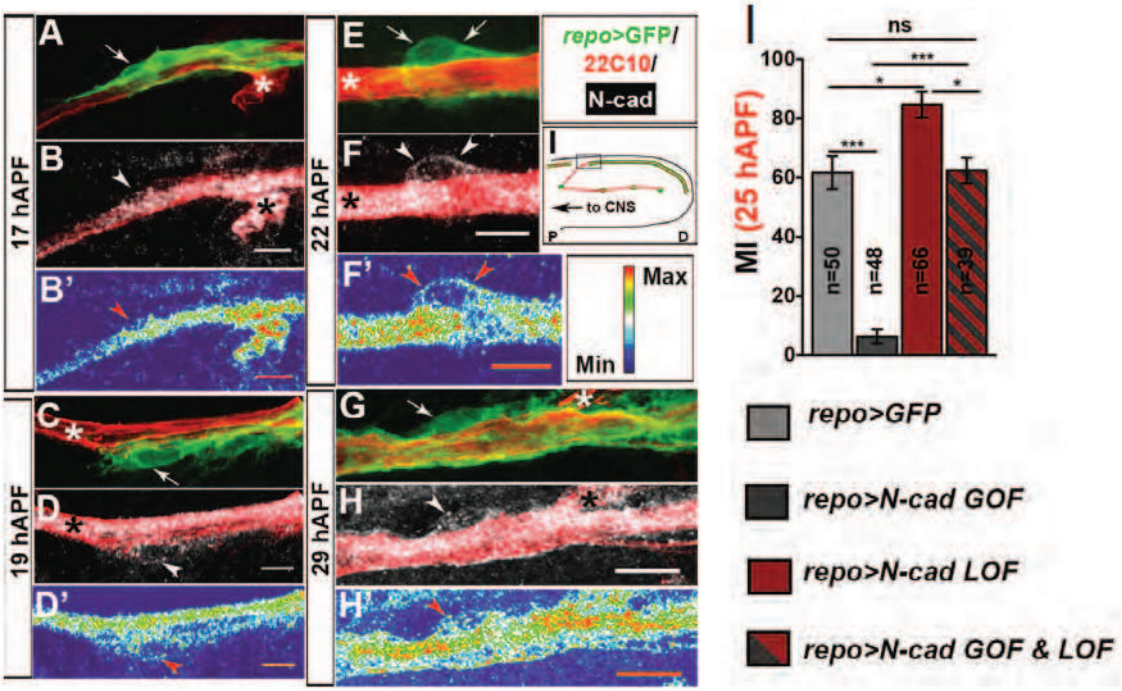
(II) Ensuite, le sujet principal de mon doctorat a été de caractériser une cascade chimiotropique au cours de la migration de la glie. Les facteurs de transcription qui régulent les récepteurs des chimioattractants ne sont pas connus . Pour cette raison j'ai analysé 1) l'impact de Frazzled (Fra), le récepteur du chimioattractant Netrine dans la migration gliale, 2) la régulation de l'expression de Fra par le facteur de transcription Glide/Gcm, qui constitue le déterminant glial.

Mes données démontrent pour la première fois le rôle direct joué par un déterminant précoce de la différentiation de la glie sur un mécanisme tardif comme la migration collective. L'intégration des voies de signalisation autonomes (Gcm) et régulatrices (Fra) assure un contrôle de la migration à l'endroit et au moment requis pour construire un système nerveux opérationnel.

Résultats (I)

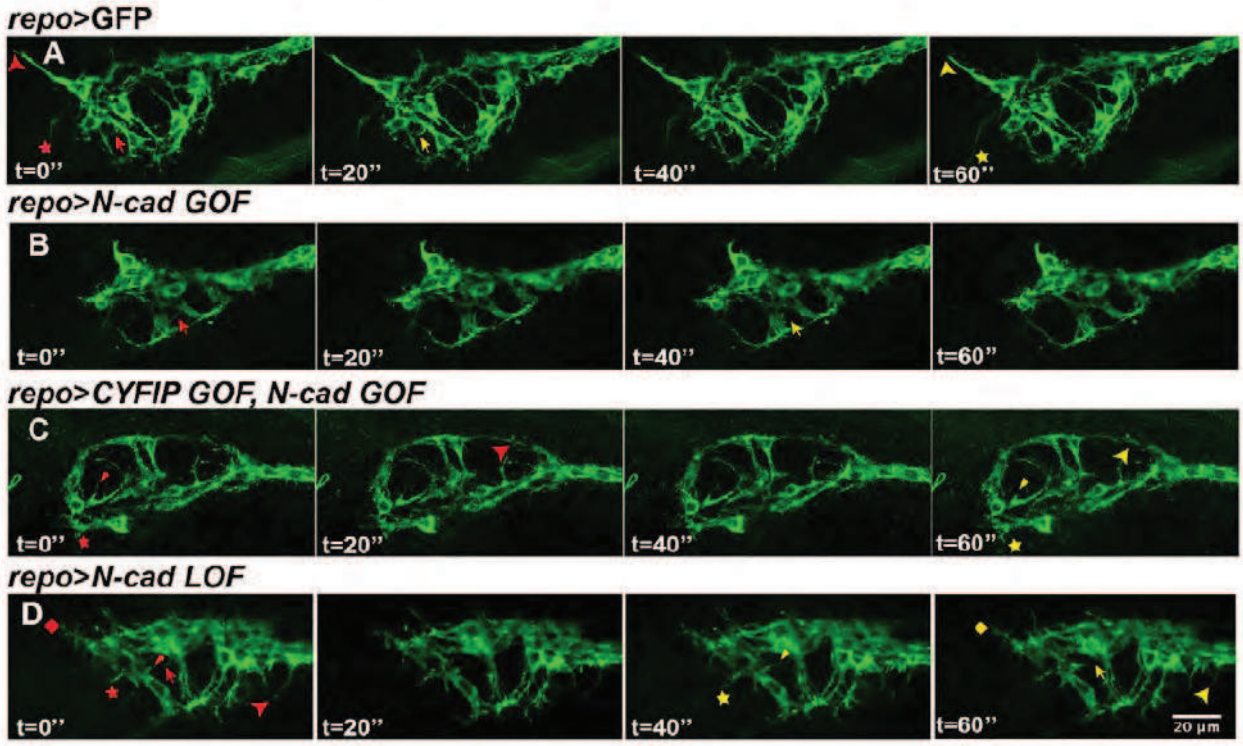
Une des caractéristiques principales des cellules gliales pendant la migration collective est l'adhésion. En effet, les cellules gliales sont liées les unes aux autres quand elles migrent. Sur cette base, nous avons observé le rôle de la molécule d'adhésion cellulaire N-cad dans la migration collective de la glie. Nous avons montré que N-cad est exprimée dans la glie de l'aile et que la quantité de N-cad a un impact direct sur l'efficacité de la migration (Figure 1).

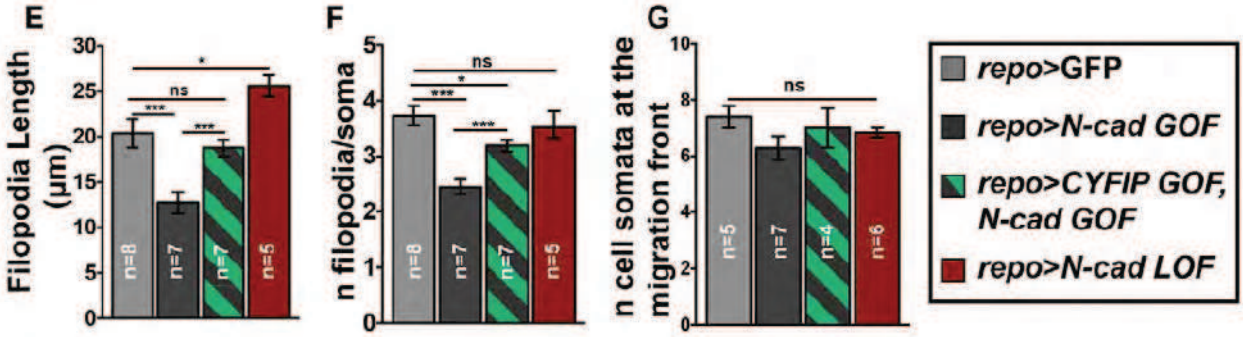
Nous avons utilisé la surexpression de N-cad (gain of function, GOF) et la répression de N-cad par RNAi (Knock Down, KD) pour montrer que N-cad inhibe la migration : la migration est fortement ralentie dans les animaux N-cad GOF et accélérée dans les animaux N-cad KD (Figure 1I). Ceci suggère que N-cad régule et assure l'efficacité de la migration de la chaîne gliale au moment requis. Nous avons aussi montré que dans les GOF de N-cad, la glie exprime Armadillo (la β-catenine) et l'alpha-catenine, des molécules qui contrôlent la dynamique de l'actine et la formation du cytosquelette. Ceci a été confirmé par une analyse qui a montré la présence de filaments d'actines plus courts et moins nombreux dans les cellules gliales en migration lorsque la N-cad est surexprimée (Figure 2). De plus, nous avons trouvé que l'effet du GOF de N-cad sur la migration de la glie est inhibé par CYFIP, un membre du complexe de nucléation de l'actine WAVE/SCAR



<u>Figure 1: Expression et role de N-cad.</u> (A-H') Ailes de Drosophila à different stades marquees avec anti-22c10 pour marquer les neurones (rouge), anti-N-cad (blanc) et anti-GFP (cellule gliale, vert) dans la lignée transgénique *repoGal4,UAS PHGFP* (repo>GFP). Les flèches indiquent les cellules gliales, les têtes de flèches blanches et rouges indiquent le marquage N-cad dans les cellules gliales. Les astérisques blanches indiquent les neurones et les asteriques noires le signal N-cad dans ces cellules. **(I)** Phénotype de migration des cellules gliales en condition de sur-expression de N-cad (GOF) et de répression (LOF) dans la glie en utilisant le transgène repo>GFP.

Cette étude a été publié en 2015 (Kumar et al., 2015). Ma contribution a été de caractériser le phénotype des mutants de N-cad et d'autres molécules impliquées dans la migration en utilisant des clones MARCM et d'autres outils génétiques.





H

Filopodia Length (µm)			
repo>GFP	*repo>N-cad GOF*	*repo>CYFIP GOF N-cad GOF*	*repo>N-cad LOF*
20.35	12.74	18.75	25.47

I

Number of filopodia/soma			
repo>GFP	*repo>N-cad GOF*	*repo>CYFIP GOF N-cad GOF*	*repo>N-cad LOF*
3.74	2.47	3.10	3.2

Figure 2: Impact de la N-cad sur la dynamique du cytosquelette d'actine. **(A-D)** Images confocales d'aile de Drosophile prise à 20 s d'intervalle montrant l'organisation du cytoplasme de la glie sur le front de migration des cellules gliales dans des animaux de génotype suivant : repo>GFP (control); repo>N-cad GOF; repo>CYFIP GOF N-cad GOF et repo>N-cad LOF. Les flèches rouges, les triangles, les carrés et les étoiles indiquent la

forme initial et la position du filopodium, les symboles en jaune montrent le même filopodium après réorganisation. **(E)** Quantification de la longueur des filopodia (en mm) lors de l'initiation de la migration dans tout les génotypes. **(F)** Nombre de filopodia present par cellule pour les génotypes indiqués. **(G)** Nombre de cellules present sur le front de migration. **(H, I)** Résumé des données montrées dans les panneaux E et F. A noté : la lignée UAS Actin42A GFP a été utilisée dans cette figure pour suivre la dynamique de l'actine.

Résultats (II)

La chaîne gliale L1 commence à migrer durant la métamorphose, vers 17h après la formation de la pupe (AFP), et complète la migration à 28h AFP. J'ai utilisé les souches exprimant le rapporteur *UAS mCD8 GFP* et des vecteurs d'expressions Gal4 sous le contrôle des promoteurs *repo* (*repo>GFP*) et *gcm* (*gcm>GFP*). Ces souches m'ont permis d'observer les profils d'expressions et de manipuler l'expression de gènes précisément dans ces cellules gliales. Ainsi, j'ai découvert que Fra s'accumule dans la glie et dans les neurones adjacents à 15h APF, plus précocement que le stade auquel la migration débute (Figure 3A). Ensuite, j'ai estimé le ratio d'ailes présentant une migration complète à 28h APF. Cette analyse a montré que la migration de la glie est affectée quand l'expression de *fra* est diminuée (LOF) (Figure 3). Nous avons également démontré ceci en utilisant un RNAi ciblant *fra* (*UAS-fra-RNAi*) (Figure 3B et 3C).

En accord avec ces données, la surexpression de *fra* (*UAS fra*, GOF) augmente l'efficacité de la migration, avec de la glie qui commence à migrer plus tôt par rapport à ce qui se passe chez les animaux contrôle (Figure 3D). Il est important de noter que la co-

expression de *UAS fra* et de *UAS fra RNAi* restore l'efficacité de migration à son niveau normal. Ceci confirme que *fra* joue un rôle de régulateur de la migration et que l'effet du RNAi est spécifique à *fra*.

Pour conclure, le récepteur Fra est exprimé dans la glie et est nécessaire à la migration.

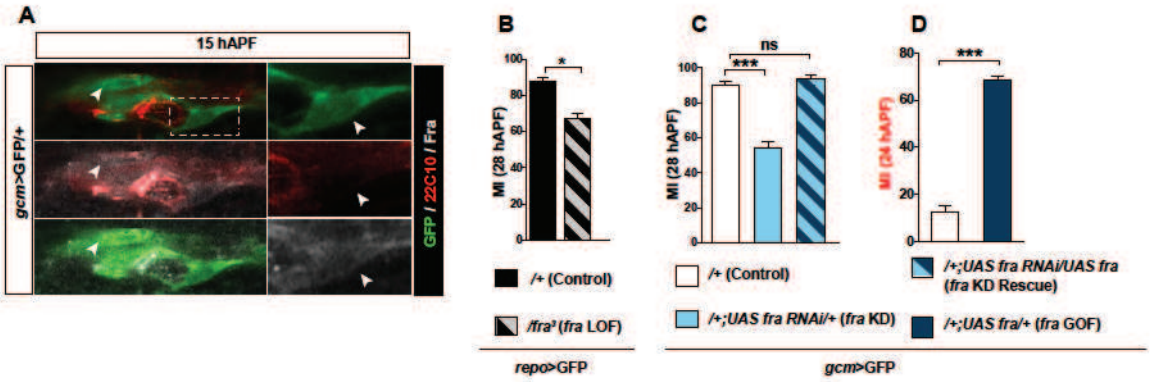


Figure 3:Expression et rôle de Fra. **(A)** Profile d'expression de Fra dans des animaux sauvages (control) et *gcm>fra* gain de fonction à 15 h après formation de la pupe (hAPF). **(B)** Graphique représentant l'indice migratoire (MI) des génotypes indiqués. La GFP cytoplasmique a été utilisée pour calculer le MI. **(C-D)** Graphiques représentant le MI dans des ailes d'animaux dans lesquels Fra a été inhibé (fra KD) ou surexprimé (fra GOF) en utilisant la lignée *gcm>GFP/+*.

L'étude des ailes des mutants de *fra* ont révélé que le phénotype est plus fort dans des animaux qui sont également hypomorphiques pour *gcm* (*gcmGal4*). Afin de comprendre ce phénotype, j'ai examiné les interactions génétiques et moléculaires plus en détail.

En effet, une étude récente a décrit que durant le développement une propriété tardive peut être déterminée de manière autonome par un facteur précoce (Wolfram et al., 2012). Sur cette base, j'ai déterminé si le facteur de transcription Gcm, exprimé précocement pour déterminer le destin glial des cellules neurales pluripotentes, contrôle également le processus tardif de la migration collective. Comme pour *fra*, j'ai découvert que Gcm affecte la première étape de l'initiation de la migration gliale. Effectivement, un niveau élevé de Gcm (GOF) accélère le début de la migration et un niveau bas (LOF) retarde cette étape.

. Le potentiel de transactivation de Gcm suggère un contrôle direct de gènes impliqués dans la migration de la glie. En utilisant un rapporteur GFP de l'expression de *fra* dans des cellules de drosophile, j'ai confirmé que l'expression de *fra* est directement induite par Gcm (Figure 4). J'ai ensuite montré que la surexpression de *fra* sauve le défaut de migration gliale observé chez les animaux *gcm* LOF

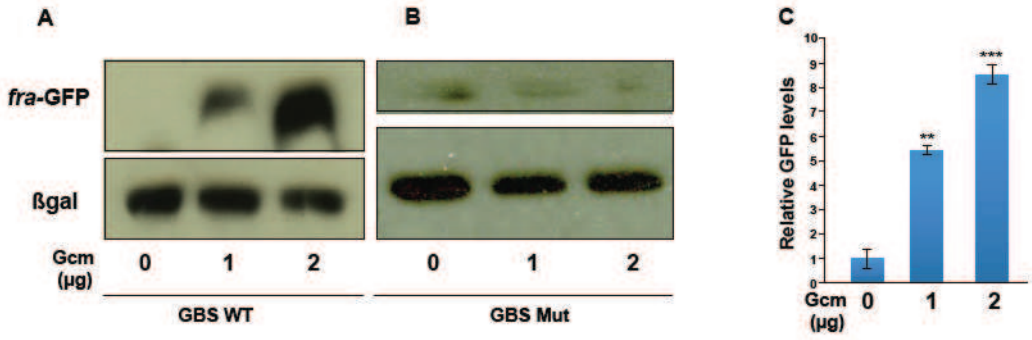


Figure 4: Fra est une cible directe de Gcm (A, B) Analyse par Western-blot d'un rapporteur GFP de Fra contenant le site de fixation de Gcm (GBS) sauvage **(A)** ou muté **(B)** (Blot représentatif d'un ensemble de triplicata). Le niveau de GFP augmente avec le niveau de Gcm pour le sauvage tandis que le niveau reste stable pour le rapporteur muté.

(C) Quantification des western blot du rapporteur sauvage. L'axe des ordonnées indique le niveau relatif de GFP, l'axe des abscisses indiquent la quantité de vecteurs Gcm utilisé pour la transfection.

Ces données indiquent qu'un déterminant précoce du destin glial contrôle également un évènement tardif en induisant l'expression d'un récepteur d'un chimioattractant.

Ensuite, pour déterminer quels ligands de Fra sont impliqués dans la migration de la glie, nous avons analysé les deux Netrines NetA et NetB, qui appartiennent à une classe de protéines secrétées guidant les axones en formation (Brankatschk and Dickson, 2006; Harris et al., 1996; Mitchell et al., 1996). Les deux Netrines agissent comme ligands pour le récepteur chimioattractant Fra et le récepteur chimiorépulsif Unc5 (Chan et al., 1996; Keino-Masu et al., 1996; Keleman and Dickson, 2001; Kolodziej et al., 1996; Lai Wing Sun et al., 2011; Timofeev et al., 2012; von Hilchen et al., 2010). J'ai étudié l'impact des deux Netrines sur la migration en utilisant les mutants nuls $NetA^{\Delta}$ et $NetB^{\Delta}$. La migration n'est pas altérée dans la glie mutante pour $NetA^{\Delta}$ mais la glie mutante pour $NetB^{\Delta}$ montre un retard significatif de migration (Figure 5C). En analysant ces animaux, j'ai découvert que la NetB est exprimée dans la partie proximale de l'aile en développement, vers laquelle la glie migre (Figure 5A et 5B). Enfin, j'ai analysé Unc5 et ai trouvé qu'à l'opposé de Fra, il ralenti la migration. La surexpression de Unc5 dans la glie retarde la migration, cependant l'inhibition de Unc5 n'a aucun effet sur la migration. Pour conclure, bien que Unc5 agisse comme répulsif pendant la migration, il n'est pas

suffisant pour affecter la migration, tandis que l'interaction NetB/Fra joue un rôle majeur dans la migration collective de la glie de la drosophile.

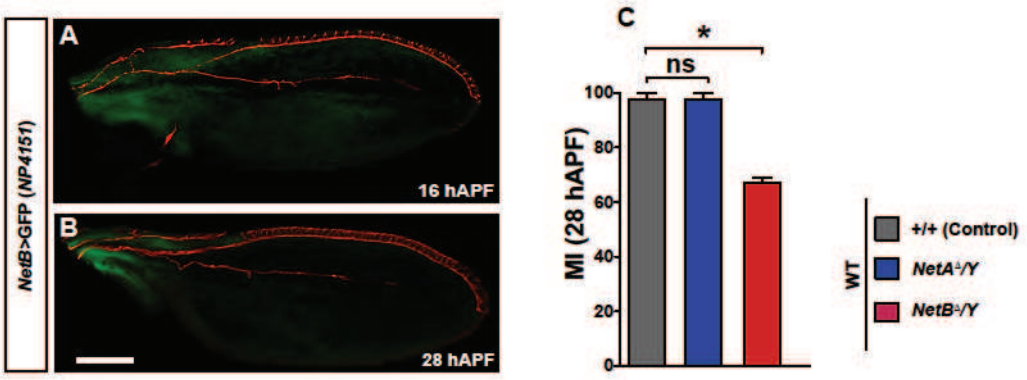


Figure 5: expression et role des Netrines. (A,B) Expression de NetB dans des ailes de Drosophile à 16 h et 28 h après formation de la pupe. NetB (GFP, vert) s'accumule en position proximale. Les neurones sont indiqués en rouge (anti-22C10). **(C)** Indice migratoire des génotypes indiqués. Le graphique représente la quantification sur des ailes de control et mutants $NetA^{\Delta}$, et $NetB^{\Delta}$.

Dans l'ensemble, ces données montrent une cascade de signalisation dynamique entre NetrinB et son récepteur Frazzled régulé par Gcm. Ce dernier agit en tant qu'initiateur de la migration de la chaine gliale dans l'aile de la drosophile.

Conclusion

Au sein du système nerveux, les cellules gliales représentent le type cellulaire le plus mobile. Elles migrent non seulement pendant le développement (depuis leurs lieux de naissance jusqu'à leurs positions finales), mais aussi durant leurs fonctionnements (Klambt, 2009). Les astrocytes dans le cerveau mammifère migrent sur les sites de

lésions ou pendant la neurodégénération. Ce processus est connu sous le nom d'astrogliose (Sofroniew et Vinters, 2010). De plus, les cellules gliales tumorales peuvent migrer extensivement dans le système nerveux conduisant à la formation de gliomes (Cayre et al., 2009). Comment ces cellules migrent et quels sont les facteurs qui initient et contrôlent la migration restent à découvrir.

Le développement de l'aile de Drosophile constitue un outil efficace pour comprendre les mécanismes en place qui contrôlent les mouvements collectifs de cellules gliales pendant lesquels ces cellules sont physiquement et fonctionnellement connecter entre elles (Aigouy et al., 2008; Aigouy et al., 2004). L'adhésion cellulaire joue un rôle important en apportant la base de l'organisation tissulaire statique (elle définit notamment l'arrangement cellulaire dans les épithélium polarisés) et aussi en formant le tissus en permettant la formation de connections modulable entre les cellules. Lors de la migration collective, un niveau plus faible de la molécule d'adhésion cellulaire cadhérine régule la vitesse et le comportement invasif des cellules (Kumar et al., 2015; Shih and Yamada, 2012; Silies and Klambt, 2010).

Dans la première partie de cette étude, nous montrons que la molécule d'adhésion cellulaire N-cadhérine est exprimée dans la chaine gliale qui migre lors du développement de l'aile de Drosophila, contrairement à ce qui a été montré précédemment. La modulation du niveau d'expression de la N-cadherin par surexpression ou repression dans la glie modifie l'efficacité de la migration. Notre analyse *in vivo* suggère qu'une augmentation du niveau du complexe cadhérine/caténine dans la membrane cytoplasmique diminue la motilité de la membrane tandis que la diminutuion de ce niveau augmente la motilité en controlant la dynamique du cytosquelette d'actine

(Benjamin and Nelson, 2008). Dans notre modèle de migration des cellules gliales lors du développement de l'aile de Drosophile, N-cad pourrait réguler le minutage de la migration. Lors de la première phase de migration, un niveau bas de N-cad permet l'initiation du mouvement cellulaire tandis que lors de la dernière phase de migration, N-cad s'accumule dans la membrane cytoplasmique et ralenti le mouvement cellulaire. Nous proposons que N-cad inhibe le processus de migration des cellules gliales en affectant l'initiation de la migration et la vitesse de migration et en remodelant le cytosquelette d'actine. Puisque nous n'avons jamais observé de blocage de la migration en altérant le niveau de N-cad dans la glie, nous pensons que N-cad joue un rôle permissif dans le mouvement de cellules gliales. Similairement, nous n'avons jamais observé de cellules migrant de manière isolée dans les ailes en bloquant N-cad. Pour conclure, le contrôle du niveau de N-cad dans la glie est important pour l'efficacité de la migration.

Ensuite, j'ai analyse le role de facteurs chemotropiques dans la migration de la glie L1. Plusieurs facteurs ont été étudiés pour leurs rôles dans la motilité cellulaire, cependant les molécules régulant ces facteurs chimiotropiques sont très peu connues (Chen et al., 2010; Kinrade et al., 2001; Liu et al., 2012; Spassky et al., 2002; von Hilchen et al., 2010). Au cours de cette étude, j'ai pu définir une voie de régulation qui joue un rôle instructif pour les cellules gliales. J'ai montré que le récepteur chimioattractif Frazzled (Fra) est exprimé dans les cellules gliales migrant dans le système nerveux périphérique. Fra agit comme facteur intrinsèque guidant la migration cellulaire contrairement à N-cad qui est un facteur permissif. J'ai aussi montré que le facteur de transcription Glide/Gcm (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996) régule l'expression de Fra d'une

manière dosage dépendante. L'expression du récepteur Fra déclenche l'initiation de la migration de la glie vers le chimioattractant Netrin B. Ainsi, les déterminants du destin cellulaire induisent non seulement l'expression en aval de facteur de transcription mais adjoignent aussi au programme de différentiation spécifique l'expression de gènes effecteurs.

Pour résumé, mes données révèlent les bases de la machinerie conduisant à la migration collective. 1) N-cad est nécessaire au mouvement des cellules gliales et 2) le facteur de transcription Glide/Gcm contrôle la migration en régulant l'expression de Fra. Pour la suite, je propose de combiner nos connaissances sur les facteurs intrinsèques et extrinsèques de la migration des cellules gliales avec celles des voies de régulation de la mobilité cellulaires. Ceci nous permettra d'avoir une vue globale de la coordination de la migration cellulaire. De plus, de plus ample investigations sur la migration gliale pourront nous aider à développer de nouvelles approches thérapeutiques pour la régénération nerveuses après une blessure ou lors de maladies neurodégénératives.

Summary

The remarkable ability of neurons and glia to undergo long distance and collective migration ensures the final architecture and function of the brain. This is an extremely dynamic process that not only depends on cell interactions, but also on the presence of specific transcription factors in the migrating cells. Adhesion molecules such as classic cadherins and chemoattractants/repellants are known to regulate directional migration, however, how are these pathways regulated is largely unknown. While the role of these molecules controlling cell interactions has been extensively investigated, the signaling cascades that trigger chemotropism are not understood. During the course of my PhD I have analyzed the role of an adhesion molecule and the impact of a chemoattractant regulated by an early transcription factor in the process. The glial chain in a developing *Drosophila* wing provides an excellent tool to study the molecular pathway underlying collective migration.

Objectives

(I) One of the main property that cells display during collective migration, is the ability to remain functionally connected to each other and never isolate themselves from the chain. This suggests an important role-played by adhesion molecules in process requiring cell interactions. Cadherins are transmembrane receptors and are widely known to mediate cell-cell adhesion during development. In the first aspect of studying collective glia migration I have investigated the role of N-cadherin (N-cad) adhesion molecule in the collective movement.

(II) Second and the main objective of my thesis was to investigated the role of chemotropic guidance cues in collective glia migration. Collective migration is a highly coordinated process involving dynamic cell interactions that requires chemoattraction. The transcription factors that regulate the timely and threshold expression of chemoattractant receptors remain unknown. For this purpose, I have analyzed: 1) the impact of Frazzled (Fra), a chemoattractant netrin receptor in glial migration. 2) I asked whether the transcription factor Glide/Gcm may affect glia migration by regulating the expression of the chemoattractant receptor Frazzled.

My data demonstrate for the first time the direct role of a fate determinant on a late and collective behavior. Hence, the integration of autonomous (Gcm) and regulatory (Fra) pathways ensures that glial migration occurs in a timely and efficient manner.

Results

(I) One of the main features of glia cells during migration is that of adhesion, as glia stick to each other while moving collectively. Based on this we decided to investigate the role of a cell adhesion molecule, N-cad in collective glia migration. We showed that N-cad is expressed in wing glia for the first time and playing with its dosage can alter the fate of the migratory glia. We used N-cad overexpression (gain-of-function or GOF) and RNAi (Knock Down or KD) alleles to show that N-cad negatively regulates migration. The migration was severely delayed in N-cad GOF animals and vice versa in the LOF animals. This suggests that N-cad plays a role in regulating the timely and efficient migration of the glial chain. We also showed that in N-cad GOF conditions, glial cells recruited Armadillo and α-catenin that controls the dynamics of the actin cytoskeleton.

Our in vivo analysis confirmed the same, as we found shorter and fewer actin filaments in the migrating glial cells. Furthermore, we found that the effects of N-cad GOF on glial migration and on actin dynamics were counteracted by CYFIP, a member of the WAVE/SCAR actin nucleation complex.

This study has already been accepted in a peer reviewed scientific journal (Kumar et al., 2015). My contribution in this study was to characterize the mutant phenotype of N-cad and other molecules involved in the pathway by using MARCM and other genetic tools.

(II) The L1 glial chain starts moving around 17 hours after puparium formation (hAPF) and completes migration by 28hAPF. I used the *repo Gal4 with a UAS mCD8 GFP reporter (repo>GFP) and gcm Gal4 UAS CD8 GFP (gcm>GFP)* transgenic line to check the expression profile and to manipulate gene expression precisely in glial cells. Firstly, I found that Fra accumulates in glia by 15 hAPF, earlier than the stage at which these cells begin to move as well as in the underlying axons. I then analyzed the percentage of 28hAPF wings displaying complete migration and found that glia move less efficiently upon downregulating Fra (LOF). We demonstrated this in mutant combinations and in a conditional knock down allele (*UAS fra RNAi*).

Accordingly, overexpressing *fra* (*UAS fra*, GOF) increases migration efficiency, with glial cells starting migrating earlier than in control wings. Importantly, when I co-express the *UAS fra* and the *UAS fra RNAi* transgenes, the migration efficiency of the glial chain is restored to control levels, confirming that *fra* plays a regulatory role in migration and that the RNAi effects are specific.

In conclusion, the Fra receptor is expressed in glial cells and is necessary for the migration efficiency.

Further the analysis of the *fra* mutant wings revealed that the phenotype is much stronger in animals that also carry the *gcm* Gal4 driver. So I decided to check whether Gcm and Fra interact genetically. I observed that delayed glial migratory phenotype was further enhanced in double heterozygous conditions for *fra* and *gcm*.

The electrical properties of a specific class of neurons have been recently shown to depend on a LIM-homeodomain protein, demonstrating that late features can be dictated autonomously by early cues, however, homeodomain proteins are stably expressed and electrical properties may not require cell interactions. Based on this data, I then asked whether the transiently expressed Gcm transcription factor, which triggers the fate choice between glia and neurons, also controls collective migration. Similarly as *fra*, I found that Gcm in accordance with its early and transient expression, seems to affects the first step, initiation of migration: high Gcm levels accelerate and low levels delay this step.

The transactivation potential of Gcm suggests that it directly controls the expression of genes triggering glial migration. By performing co-transfection assays in a *Drosophila* cell line followed by western blot analysis I confirmed that *fra* is a direct Gcm target. Additionally, *fra* was also found as a downstream Gcm target based on the presence in a screen for direct targets and published microarray data. Further experiments confirmed that *fra* constitutes an important Gcm target, as its overexpression rescues the *gcm* LOF phenotype and that it is epistatic to Gcm.

The above data indicate that an early expressed fate determinant also controls a late event in glial development by inducing the expression of a chemoattractant receptor.

The next question was to determine the ligand for Fra. To this purpose we investigated the two Netrins NetA and NetB, which are a class of secreted proteins and serve as guidance cues for navigating axons. They act as ligands with the help of the attractant receptor Frazzled (Fra) and the repulsive receptor Unc5. I investigated the impact of the two Netrin genes and analyzed the null mutant $NetA^{\Delta}$ and $NetB^{\Delta}$ animals. Migration was not altered in the glia lacking $NetA^{\Delta}$ whereas glia lacking $NetB^{\Delta}$ show significantly delayed migration. With the help of transgenic lines I found that NetB is expressed in the proximal part of a developing wing. Last but not the least, I analyzed Unc5 and found that it affects migration in an opposite manner than Fra. Overexpressing Unc5 in glia delayed migration, however, downregulation had no effect on the glia migration. Overall, I conclude that although Unc5 can act as a repellant during glia migration, it is not sufficient on its own to affect migration efficiency, whereas NetB-Fra signaling seem to play a key role in L1 glia migration.

All together my findings displays a dynamic signaling cascade between NetrinB and Frazzled regulated by Gcm that controls the migration of L1 glial chain by acting as an instructive cue.

In summary, my data reveals the basic intrinsic and extrinsic machinery underlying collective glia migration: (1) N-cadherin is necessary for the proper movement of the glial collective; (2) I show that the transcription factor Glide/Gcm (Gcm) controls glia migration by regulating the expression of Fra.

Introducing cell migration

Cell migration is a highly conserved and integrated process that involves multiple steps and is required from development to homeostasis (Gupta and Giangrande, 2014; Horwitz and Webb, 2003). Being fundamental to life, this process can be studied in non-living environments (soil), or in common *in vitro* setups (glass/plastic) or within complex multicellular organisms. (Bonner, 1998; Bretschneider et al., 1999; Parent and Devreotes, 1999).

Cells can migrate alone or collectively (**Fig. 1A,B**) (Horwitz and Parsons, 1999). Current understanding on how single cell movement is regulated on molecular and morphological level is quite well extended. In single cell migration, cells self-process information to translocate and use environment as a substrate (**Fig. 1A**). Collective cell migration, in contrast is a more complex process, as cells remain firmly connected to each other while communicating through homeostatic interactions (**Fig. 1B**) (Friedl and Gilmour, 2009; Gupta and Giangrande, 2014). It is currently accepted that the same set of effector components regulates both types of migration; nevertheless, collective migration contains additional features that allow coordination (Cantor et al., 2008; Insall and Machesky, 2009). Recent advancements in the field have shed light on several molecules involved in the process of collective cell migration but there still are many missing links. The questions that I am going to address are the following: What kind of adhesion molecules the collectively migrating cells require? What are the guidance cues? What regulates the molecules that are involved in cell migration?

Since we have accumulated a plethora of knowledge on single cell behavior (Biname et al., 2010; Charras and Sahai, 2014; Friedl et al., 2012; Friedl and Wolf, 2003;

Friedl and Wolf, 2009; Gupta and Giangrande, 2014; Krause and Gautreau, 2014; Petrie et al., 2012; Ridley et al., 2003), the complexity underlying collective cell migration makes it all the more interesting to investigate. First, I will present some of the common features underlying cell migration. After, I will focus on the collective cell migration.

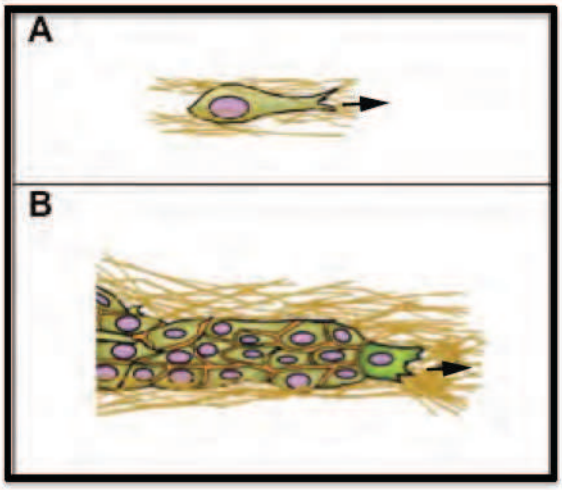


Figure 1: Modes of cell migration. (A) A cell displaying an individual pattern of migration based on the directed sensing. **(B)** Cells migrating as a large group. The black arrows indicate the direction of migration. (Friedl and Wolf, 2010).

How do cells move?

Our current understanding of cell migration comes from composite studies on different environments and cell types (Friedl and Wolf, 2003; Friedl and Wolf, 2009; Lauffenburger and Horwitz, 1996). Cell migration entails dynamic events such as cytoskeletal rearrangement, cell adhesion to the substratum and chemotaxis. The morphological and molecular characteristics of these events are discussed below.

Cytoskeleton rearrangement

In order to initiate migration, cells that typically contain leading and lagging regions have to communicate with the environment (**Fig. 2**). They do so by protrusion,

referring to the cells ability to extend cytoplasmic processes at the leading edge in the direction of migration (Aigouy et al., 2008; Kumar et al., 2015). Actin filaments are known to be the driving force behind protrusion, which can vary depending on the cell type (Gupta and Giangrande, 2014; Kumar et al., 2015; Rotty et al., 2013; Sepp and Auld, 2003). There can be flat, thin cytoplasmic sheet-like structures known as lamellipodia or long cylindrical finger-like projections called filopodia (**Fig. 2**) (Hall, 1998; Ridley et al., 2003). The increased understanding of the function of various actin-associated proteins during the initiation and elongation of actin filaments has provided new information on the mechanisms underlying cell migration.

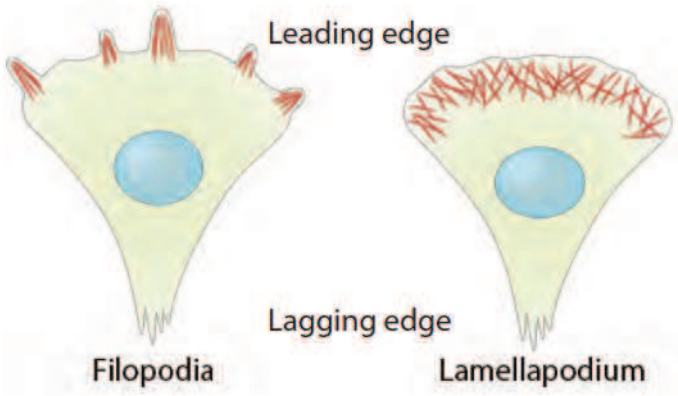


Figure 2: Actin filaments. Different types of actin filaments in cells and tissues.

Rho family small guanosine triphosphate (GTP) binding proteins are the key regulators of actin, adhesion complexes and manage the formation of lamellipodia and filopodia. Among the Rho GTPases Rac, Cdc42 and RhoG are pivotal for the formation of lamellipodia and filopodia. The Arp2/3 complex, which is activated by the members of the WASP/WAVE family, is the major target of Rac and Cdc42 to trigger actin polymerization in protrusion (Cory and Ridley, 2002; Pollard and Borisy, 2003; Ridley et

al., 2003).

While Rac stimulates actin polymerization in lamellipodia by activating WAVE proteins, Cdc42 is a key regulator in the emergence of filopodia with the help of WASP proteins. Thus, the Arp2/3 complex, which binds to the existing filaments and gives rise to the new daughter filaments, is the main regulator of lamellipodia, which in turn sense the surrounding guidance cues to promote cell motility. In the case of filopodia, additional proteins like Ena/VASP or the actin-binding proteins like alpha-actinin, filamins, organize the filaments into bundles (Ridley et al., 2003). The main function of filopodia is exploratory, allowing the cell to sense its local environment, while lamellipodia allows a cell to push through the plasma membrane and move in a particular direction.

In sum, actin polymerization leads to an active protrusion that allows a cell for directional extension. Cell adhesion, which is the next major step in cell motility, not only leads to traction during protrusion but also provides the basis for nucleation and regulation of the main components involved in actin reorganization.

Cell-cell adhesion

Cell adhesion is the process during which cells physically interact with their surroundings and stick to a surface or substratum. Adhesion is necessary for cells to produce traction by linking the extracellular substratum to actomyosin filaments. In addition, it organizes the signaling cascade that mediates migration and other cellular processes including proliferation, gene expression, and cell survival (Huttenlocher and Horwitz, 2011). Adherens junction proteins such as cadherins and other immunoglobulin

superfamily members and integrins, which are typically involved in cell matrix interactions, mediate cell-cell adhesion. All these proteins directly or indirectly connect to the actin cytoskeleton and thereby provide mechanically robust but dynamic coupling (Gumbiner, 1996).

Many types of migrating collectives are associated to epithelia and thus display classic cadherin-based interactions (Friedl and Gilmour, 2009). Epithelial or neural cadherins (E-cadherin or N-cadherin), two main types of Cadherins are transmembrane Ca2-dependent homophilic adhesion receptors that mediate coupling to the actin cytoskeleton (Friedl and Gilmour, 2009; Harris and Tepass, 2010). The cytoplasmic domain of cadherins consists of a juxtamembrane domain and a C-terminal domain. The cadherin cytoplasmic domain mediates key structural and signaling activities required for adhesion through its association with proteins known as catenins. β-catenin/Armadillo (Arm) binds directly to the C-terminal domain of the cadherin cytoplasmic tail and interacts with α-catenin, which associates with actin filaments (Jou et al., 1995; Kemler, 1993). The juxtamembrane region of cadherins interacts with the members of the p120-cat family, thereby modulating their dimerization and adhesive function (**Fig. 3**). In both morphogenesis and cancer models, the loss of E-cadherin results in weakened cell junctions followed by cell detachment and the onset of a single-cell mode of migration, termed the epithelial–mesenchymal transition (EMT). This effect implicates E-cadherin as the dominant mediator of collective cell interactions, the loss of which may or may not be compensated for by other cell–cell adhesion pathways (Gavert et al., 2008; Grunert et al., 2003; Lee et al., 2006; Thompson and Williams, 2008).

In *Drosophila* border cells, E-cadherin is also necessary to get proper adhesion to the cellular substratum (Niewiadomska et al., 1999). On the other hand, using a three-dimensional (3D) matrix based approach, the importance of N-cadherin mediated cell-cell adhesion in collective migration was recently demonstrated in epithelial cells (Shih and Yamada, 2012).

Functional differences between E and N cadherins could be assigned to their different expression profiles, physical properties and the migration strategies used by different collectives (Kumar et al., 2015). Basically the type of adhesion decides the presence of cadherins, other receptors, components and cell types. Thus, complicated signaling network controls cell migration in addition to the other cell type specific functions.

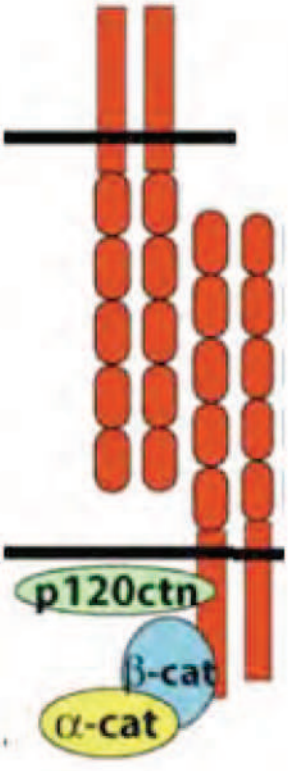


Figure 3: Structure of cadherin-catenin complex. The transmembrane cadherin associates with catenins via cytoplasmic domains (Niessen and Gottardi, 2008).

Chemotaxis

The movement of the cells is typically not random, but directed by extracellular guidance cues (chemotaxis) (Berzat and Hall, 2010). Cells at the leading edge are often responsible to sense the extracellular guidance cues. The interaction of these cues with plasma membrane receptors elicits spatially restricted intracellular signal transduction pathways, thus, influencing the assembly, disassembly, and arrangement of the actin cytoskeleton and the timing of migration in distinct ways to drive directional migration. Guidance mechanisms are evolutionarily conserved and have been widely studied in vertebrates (both *in vitro* and *in vivo*), *Caenorhabditis elegans* and *Drosophila*, as well as in the single cell organism, *Dictyostelium discoideum* (Berzat and Hall, 2010; Janetopoulos and Firtel, 2008; Montell, 1999; Weijer, 2009). The guidance cues required during cell migration were first demonstrated to play major role in axon navigation (Brankatschk and Dickson, 2006; Harris et al., 1996; Lai Wing Sun et al., 2011). These guidance cues comprises of wide variety of soluble or cell-bound ligands and matrix-coated surfaces. Among the many guidance molecules, Netrins, Semaphorins, Slits and Ephrins belong to the most studies class (O'Donnell et al., 2009).

As the main project of my thesis, I studied the role of Netrins and their receptors in collective glial cell migration and I will provide an overview on them.

Netrins family and receptors

Netrins are a family of secreted laminin related extracellular guidance cues, capable of eliciting either attraction or repulsion. There are two Netrin genes in *Drosophila*, Netrin A (NetA) and Netrin B (NetB), both of which are homologous to the

two vertebrate Netrins (netrin1 and netrin2) and to the *C. elegans* Uncoordinated 6 (Unc6) protein (Lai Wing Sun et al., 2011; Moore et al., 2007; Rajasekharan and Kennedy, 2009).

Netrin mediated attraction requires the receptor Frazzled (Fra), the *Drosophila* homolog of Unc40 in *C. elegans* and deleted in colorectal cancer (DCC) in vertebrates (**Fig. 4**) (Chan et al., 1996; Lai Wing Sun et al., 2011; Moore et al., 2007; Rajasekharan and Kennedy, 2009) (Kolodziej et al., 1996). In contrast, repulsion is carried out in response to its receptor Unc5 (**Fig. 4**), with coexpression of DCC in some cases. All Netrin receptors belong to the immunoglobulin (Ig) superfamily and are single pass type I transmembrane proteins (**Fig. 4**).

Netrins are known to function either as short-range or long-range cues (Hong et al., 1999; Keleman and Dickson, 2001; Lai Wing Sun et al., 2011). 'Short-range' refers to a role when Netrin is in the immediate vicinity of its cellular source, either close to the secreting cell or attached to its surface. By contrast, 'long-range' secreted cues function at a distance from the cell secreting the factor. Studies in different model systems suggest that long-range Netrin-induced repulsion requires the cooexpression of DCC and Unc5, but that Unc5 alone is sufficient for short-range repulsion (Hong et al., 1999; Keleman and Dickson, 2001; Lai Wing Sun et al., 2011). Although it is not clear as to what causes such differences, one possible explanation may be that together DCC and Unc5 form a more sensitive Netrin receptor complex, which is able to respond to lower concentrations of protein found at a greater distance from a source of Netrin secretion.

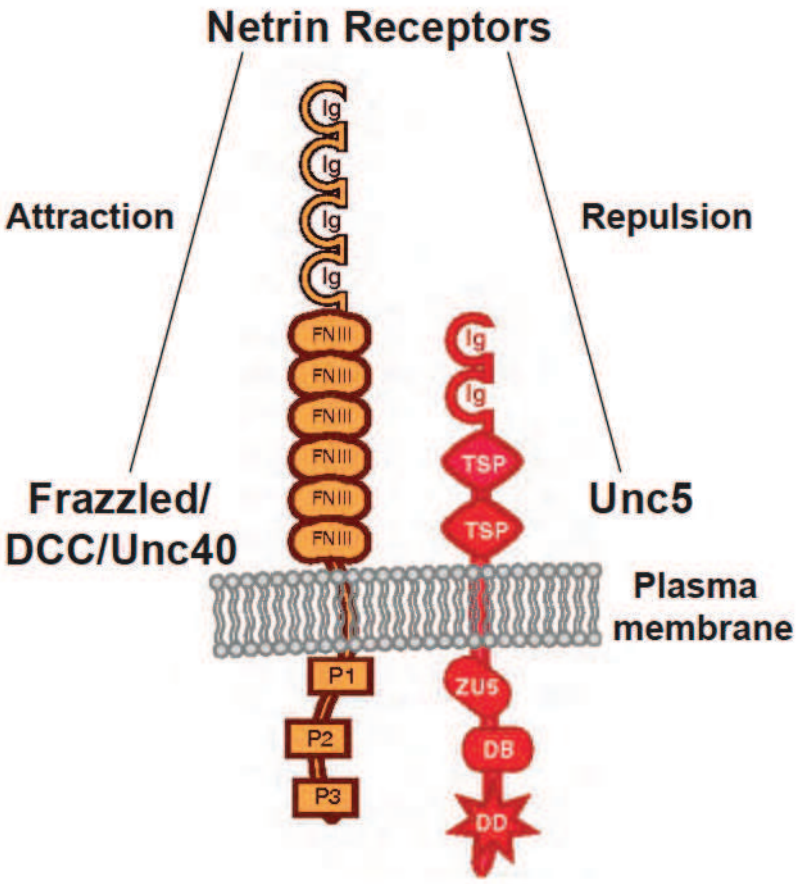


Figure 4: Netrin receptors. Netrins mediate attraction via its receptor Fra/DCC/Unc40 and repulsion through Unc5 receptors. Structurally, the extracellular domains of all members of the Fra/DCC family are composed of six-fibronectin type III repeats (FNIII) and four IG domains and a cytoplasmic domain with three conserved P motifs: P1, P2 and P3. Studies have suggested that Netrin 1 binds to the fourth and fifth FNIII repeats of DCC. The extracellular domains of Unc5 consist of two Ig repeats that binds Netrin and two thrombospondin type-I modules. The intracellular domain of Unc5 is composed of ZU-5 domain of undetermined function, homologous to a sequence in the scaffolding protein Zona Occludens-1 found at tight junctions, a DCC binding domain (DB) and a death domain (Lai Wing Sun et al., 2011).

Netrins and their receptors in cell migration

Netrins are best known for their phylogenetically conserved role in orchestrating axon guidance, dendritic growth and glial cell migration (Harris et al., 1996; Kennedy et al., 1994; Lai Wing Sun et al., 2011; Mitchell et al., 1996; Serafini et al., 1994; von

Hilchen et al., 2010). These functions of Netrins are studied primarily in the chick, mouse, *C.elegans* and in *Drosophila melanogaster*. The cells at the CNS midline in all these animals express Netrins. Furthermore, their function extend to the regulation of axonal pathfinding in the optic nerve head. Multiple CNS axonal projections are disrupted in Netrin 1 deficient mice, including corpus callosum and hippocampal commissure (Serafini et al., 1996). Such drastic phenotypes indicate the important role played by Netrins in mediating axonal navigation from one side of the CNS to the other. Similar, phenotypes are displayed by mice lacking DCC, highlighting the important role of DCC as a Netrin receptor (Fazeli et al., 1997).

In addition to its role in axonal guidance Netrins also influence glia migration. In *Drosophila*, Netrins are expressed by the midline glial cells (Mitchell et al., 1996), and guide the migration of two different populations of glial cells. Glia in Netrin mutant embryos fail to cross the midline (von Hilchen et al., 2010). In vertebrates, Netrin 1 released from the ventricular zone of the third ventricle repels migrating glial precursor cells, which have the capacity to differentiate into oligodendrocytes or astrocytes (Sugimoto et al., 2001), however, a direct role in the development of astrocytes has not been identified. In the embryonic spinal cord the gradient of Netrin 1 expressed in the floor plate is sufficient to repel the migrating oligodendrocyte precursor cells that express both DCC and Unc5 (Jarjour et al., 2003). In migrating OPCs, Netrin 1 mediates chemoreplusion by activating the small GTPAses via DCC and ROCK. In the adult CNS Netrin 1 expressed by Schwann Cells in the PNS (Madison et al., 2000) has been implicated in directing the migration of adult neural stem cells at sites of injury in the mature CNS (Petit et al., 2007).

Netrins act as important guidance cues for the migrating cells and axons during development. Despite of the fact that the majority of the studies have focused on their role during embryonic development, it is now apparent that Netrins and their receptors function in a much broader context both throughout development and in the adulthood. Significant insight has been gained into Netrin's function since their discovery. In view of this, I have tried to understand how Netrin and their receptors function together to regulate cell motility and what are the pathways that are regulating these receptors. Focusing on Netrins and their signaling mechanism in future studies serves as promising targets for developing therapeutics against cancer, neurodevelopmental disorders and vascular diseases.

Models for collective cell migration

Collective migration is a process conserved throughout the animal kingdom. Different possibilities may account for cells choosing to move collectively when they can migrate individually. At least three non-mutually exclusive reasons for collective migration include: 1) Collective migration ensures the integrity of tissue or structure while remodeling it. 2) Allows mobile cells to carry other non-motile cells with them in a cluster. 3) Finally, the coordinated behavior of cells can allow for more resilience in the system, thus the collective can be more resistant to disturbance (Friedl and Gilmour, 2009; Gupta and Giangrande, 2014; Rorth, 2009). Depending on the contexts, collective cell migration can occur on a two-dimensional surface or a 3D tissue scaffold. Most cells in mammals migrate along the three dimensional (3D) interstitial or cellular tissues (Friedl and Wolf, 2009; Ilina and Friedl, 2009). Researchers in the late 90s reckoned that the same principle must apply to the cells migrating in 2D and 3D surfaces. However, recent advances in the field made it quite evident that cells undergoing 3D migration entail somewhat different characteristics. For instance, in contrast to 2D, a 3D migrating cell bears a bipolar shape with a flexible cytoskeletal organization, lacking focal adhesion and stress fibers (Friedl and Brocker, 2000; Friedl and Wolf, 2009). Another interesting difference is the diverse range of protrusive structures (filopodia, blebs, lobopodia and pseudopods) used by the cells migrating on a 3D surface rather than on a 2D surface, where most cells use lamellipodia to migrate (Friedl et al., 2012).

Recent advances in high-resolution microscopy and powerful genetic tools have allowed us to accumulate significant knowledge about collective cell migration. In spite of this knowledge, the mechanisms by which collectively migrating cells integrate their

movements, differentiate and proliferate are widely unknown. Unraveling these mechanisms may add valuable information in a number of conserved processes such as organogenesis, tissue remodeling, and wound healing as well as to trigger new therapeutic treatments for cancer (Weijer, 2009).

The following sections will describe several examples of collectively migrating cells in different tissues and model organisms based on *in vitro* and *in vivo* studies.

In vitro models

The most popular method to study cell migration *in vitro* includes the scratch wound assay, due to its economical and straightforward approach. In this method, the confluent cell monolayer is disrupted upon creation of an artificial line shaped gap, so called "scratch". The cells on the edge will subsequently move in order to heal the gap creating new cell-cell interactions. This behavior resembles the process occurring during wound healing in an injured skin (Liang et al., 2007).

Most of the information comes from *in vitro* studies on migration across a 2D substrate. Nevertheless, it is also believed that the same types of receptors might be required for cell interactions *in vivo* in 3D and that the surrounding environment plays a major role as substrate in both cases. Despite this knowledge, the exact mechanisms underlying cell migration cannot be determined using such experiments because migration starts only after creating an artificial injury.

Though the *in vitro* scratch assay is compatible with the advanced imaging techniques such as live cell imaging allowing the analysis of cell interactions, it still does not completely mimic the physiological properties underlying a moving cell cohort *in*

vivo. In addition *in vitro* assays take relatively longer period of time in contrast to *in vivo* analysis, which makes it unsuitable to determine the intrinsic mechanisms underlying cell migration (Friedl and Gilmour, 2009). Thus, *in vitro* models are better suited to perform molecular screens upon high-resolution molecular and cellular imaging.

In vivo models

With the advent of more sophisticated molecular and cellular approaches it is now feasible to study collective cell migration *in vivo*. In the past years most mechanistic insights into collective cell migration have been obtained from model systems such as mouse, Zebrafish and *Drosophila melanogaster* since they allow us to combine genetics with *in vivo* imaging approaches that generally provide the highest fidelity (Berzsenyi et al., 2011; Friedl and Gilmour, 2009; Gupta and Giangrande, 2014; Kumar et al., 2015; Rorth, 2009).

In the following sections, I have described an example in each of these model systems (mouse, zebrafish and *Drosophila*), in which we have already gained a considerable amount of knowledge regarding collective cell migration.

Mouse model

Rostral migratory stream

A well-established example of collective migration is the rostral migratory stream (RMS) in the adult rodents. Neural progenitor cells migrate from the telencephalic subventricular zone (SVZ) of the forebrain to the olfactory bulb (OB) as a stream (Murase and Horwitz, 2004). RMS cells are not guided by radial glial or axonal fibers as a substrate but navigate on the surface of the neighboring neuronal precursor (Lois et al., 1996), thus forming a chain, which is flanked by adjacent astrocytes that serve as a tunnel for the collective (**Fig. 5**). Blood vessels have also been suggested to provide a structural scaffold for the migrating neuroblasts within the astrocyte tunnel (Snapyan et al., 2009; Whitman et al., 2009). A number of cell adhesion and chemotropic factors have been shown to affect the mode, speed, directionality and the coordinated movement in the RMS (Friedl, 2004; Sun et al., 2010). Neuronal Cell Adhesion Molecule (NCAM) is a major cell adhesion molecule expressed in the migrating neuroblasts. The importance of NCAM can be seen decisively in NCAM-deficient mice that display smaller OB and neuroblast clustering (Hu et al., 1996). Migration defects are more profound during embryogenesis, suggesting that NCAM-dependent chain formation is important for RMS migration. It has also been shown that the polysialylated form of NCAM (PSA-NCAM), influences the formation of the RMS. Upon removal of PSA from NCAM, the neuroblasts disperse from the chain and start moving individually (Battista and Rutishauser, 2010; Chazal et al., 2000). While NCAM deficiency results in RMS migration failure, non-polysialylated NCAM leads to ectopic migration of neuroblasts to the surrounding brain regions, suggesting an important role played by polysislylation in

the maintenance of neuroblasts in chains. Furthermore, N-cadherin was also shown to play an important role in forming cell cluster and in regulating the differentiation of RMS cells (Yagita et al., 2009).

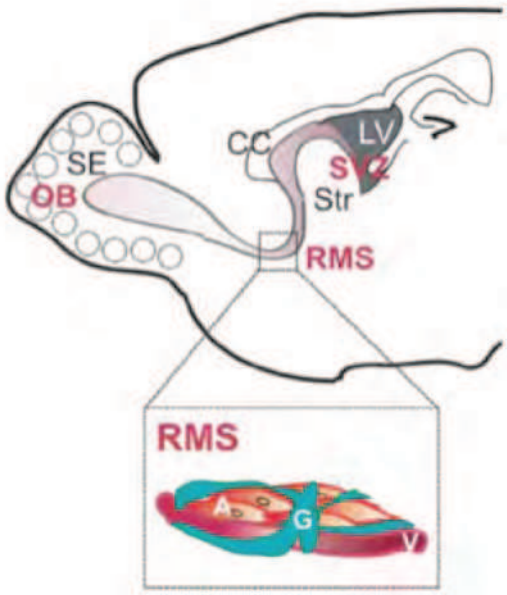


Figure 5: Rostral migratory stream. A schematic representation of RMS, neuroblasts (orange) born in the adult SVZ migrate towards the OB, forming an astrocytes (green) flanked chain, that serve as a tunnel for the collective (shown in the inset). Blood vessels (red) providing substrate to the migrating cells (A- type A neuroblasts; G- glial tube; V- blood vessels) (Sun et al., 2010).

Additionally, many chemoattractive or repulsive signals that are mainly implicated in axon guidance are also involved in the regulation of the RMS migration. For instance, the Netrin chemoattractant is expressed in the mitral cells present in the OB and RMS astrocytes during development and its receptor DCC is expressed in the migrating neuroblasts. Blocking DCC signaling alters the direction of protrusions on migrating cells, indicating the significance of Netrin-DCC signaling in the RMS migration (Murase and Horwitz, 2002). Among the chemorepellents, Slit-Robo signaling has been shown to play an important role for the directional RMS migration. Slits are expressed by the migrating neuroblasts and modulate astrocyte networks in the RMS (Eom et al., 2010; Kaneko et al., 2010; Nguyen-Ba-Charvet et al., 2004).

Fish model

Progress in transgenic tools, *in vivo* imaging and the availability of a large collection of mutants make the zebrafish (*Danio rerio*) an attractive model to study vertebrate development. Cells in zebrafish are transparent and hence highly traceable, in addition transplantation can be used to disrupt cell interactions *in vivo*.

Migrating lateral line primordium

The lateral line provides an interesting model to study collective migration/proliferation. It consists of mechanosensory hair like organs, neuromasts, deposited along the body surface in a species-specific manner. The neuromasts on the head form the so-called anterior lateral line system (ALL), the ganglion of which is located between the ear and the eye, while the neuromasts on the body and tail, including those on the caudal fin, form the posterior lateral line system (PLL), a cohesive cohort of more than 100 migrating cells (Ghysen and Dambly-Chaudiere, 2004). The neuromasts of the PLL migrate along the side of the animal to the tip of the tail and are deposited at regular interval by the primordium. This directional morphogenetic movement depends entirely on the expression of two receptors CXCR4 and CXCR7, both of which recognize the chemokine ligand stromal-derived factor 1 (SDF1) (Dambly-Chaudiere et al., 2007; David et al., 2002; Haas and Gilmour, 2006; Valentin et al., 2007) (**Fig. 6**). The migratory primordium follows a path that is defined by the expression of SDF1, which the primordium detects through the expression of its receptor Cxcr4: inactive SDF1 leads to a defective primordium with an absent PLL or defective migration (David et al., 2002; Haas and Gilmour, 2006). The PLL cell group can be divided into two parts depending

on the function and the expression of the receptor. The cells at the leading edge of the moving cohort are highly motile and express Cxcr4 whereas, the cells at the trailing edge strictly express SDF1 receptor CXCR7, which subsequently arrests migration and triggers the differentiation of cells into neuromasts (Valentin et al., 2007) (**Fig. 6**). Elegant studies have proved that the activity of CXCR4 is indispensable in the leading cells to direct cell strand polarity of the entire migrating PLL (Haas and Gilmour, 2006). The precise function of CXCR7 is still largely debated, however some studies have suggested that it may act as 'sink' for the SDF1 ligand and thereby creating a chemoattractant gradient along the PLL (Dambly-Chaudiere et al., 2007). It would be of great importance to determine whether the cellular activities of these two receptors are similar or different. On the whole, these studies indicate that such motile cells, while remaining physically and functionally in contact to each other, not only contribute to the overall collective movement, but also respond directly to tissue intrinsic mechanisms (cell proliferation) and extrinsic guidance cues perceived by different cells (leaders vs. followers) expressing distinct receptors (CXCR4 or 7).

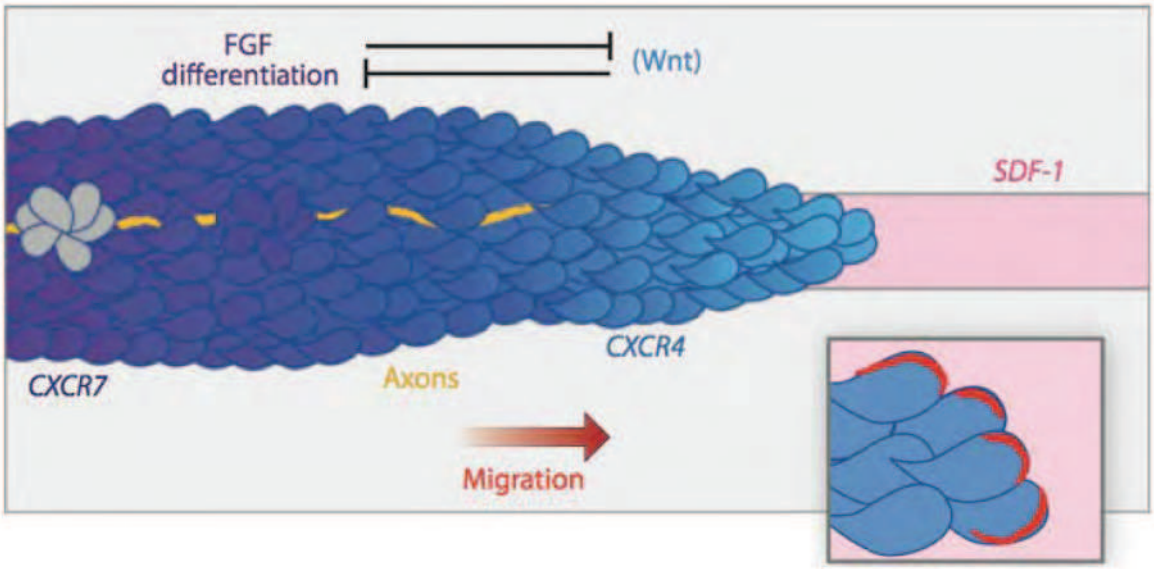


Figure 6: The posterior lateral line of zebrafish. The lateral line primordium of zebrafish consists of hundreds of migrating cell along the axonal bundle (yellow). These cells differentiate into clusters (grey) at the back and stops migration in a fibroblast growth factor (FGF)-dependent way; Migratory path is defined by the expression of ligand stromal-derived factor 1 (SDF1). Cells at the front express SDF1 receptor CXCR4 for the guided movement, whereas cells at the rear exclusively express CXCR7. Inset shows how each cells become polarized as defined by the intrinsic polarity of the slug; together with a permissive strip of SDF-1 expression, which can lead to the precise directed movement (Rorth, 2009).

Drosophila model

The simple body tissue organization and the genetic tools make *Drosophil*a a remarkable model for developmental studies. Its short life cycle combined with genome wide analyses and the advanced imaging platforms have made it comparatively easy to study collective cell migration *in vivo*.

Border cell cluster

Border cells are a group of six/eight cells that undertake well-defined and simple, directional, migration during oogenesis. These cells contribute to the formation of the specialized chamber micropyle that allows the sperm to enter during fertilization. Failure of migration or the complete absence of border cells leads to a poorly developed micropyle and is also a prime cause of female sterility (Montell, 2003; Rorth, 2009). Initially, border cells detach themselves from the epithelium and migrate as a free group. Border cells display a remarkable ability to invade the germ line cells, and use these giant cells (nurse cells) as a substrate to migrate (**Fig. 7**).

Much has been learned about the molecules mediating the cell-cell interactions that occur during border cell migration. Unpaired (UPD), a cytokine-like ligand expressed by the polar cells, signals through the signal transducer and activator of transcription (STAT) factor and specifies the migratory border cell cluster before migration onset (**Fig. 7**). Border cells do not start to migrate until stage 9 and so they require a developmental timing cue. This purpose is served by the insect steroid hormone ecdysone, the levels of which dramatically increase at the onset of migration. Further, STAT signaling is known to be indispensible throughout border cell migration as

demonstrated by the use of temperature-sensitive allele (Silver et al., 2005). These experiments demonstrated that egg chambers exhibited incomplete border cell migration when subjected to changing temperatures in a very short span of time (Silver et al., 2005). In addition, border cells at non-permissive temperature began to express a marker of another cell fate, suggesting that *stat* function is required throughout border cell migration, both to maintain their fate and to promote migration.

Once the cluster is formed and ready to migrate, directional migration is controlled by the localized attractive guidance cues coming from the environment. These cues are mainly perceived by the epidermal growth factor receptor (EGFR) and PVR (**Fig. 8**), which are required in a functionally redundant manner and loss of both genes triggers severe long distance migratory defects. Loss of PVF1 or PVR, which is the receptor of PVF1, leads to incomplete migration, whereas border cells lacking EGFR both fail to turn and to move dorsally (Duchek and Rorth, 2001; Duchek et al., 2001; McDonald et al., 2003; Montell, 2006). Thus, the combined effects of EGFR/PVR guidance cues may help in controlling directionality during border cell migration. Nevertheless, some or most border cells at least migrate a little suggesting the possibility of other guidance cues being at work.

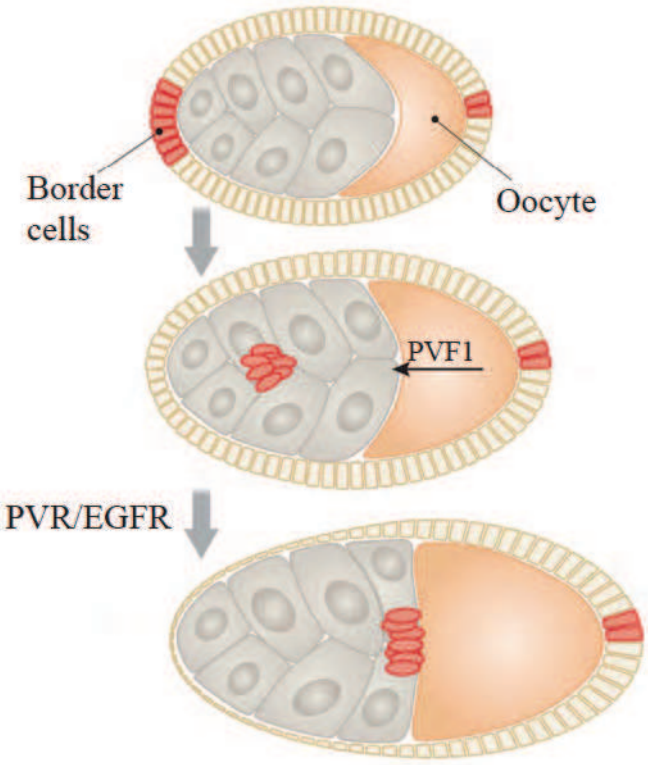


Figure 7: Border cell migration and signaling. In the *Drosophila* ovary border cells (yellow) detach from the follicular epithelium (brown) and migrate as a free group along the nurse cells (white/grey) towards the oocyte. PVR and EGFR receptors in the border cells recognize a gradient of extracellular cues (PVF1) and activate the downstream signaling to induce forward facing protrusion in cells at the front of the group. Adapted from (Berzat and Hall, 2010).

Since border cells migrate upon the surface of nurse cells, they must have a precise regulation of adhesion to gain traction at the front while simultaneously detaching at the rear. The homophilic cell adhesion molecule E-cadherin appears to act as a critical adhesion molecule for border cell migration. Loss of E-cadherin either from the border or the nurse cells completely disrupts migration (Niewiadomska et al., 1999). E-cadherin accumulates at higher levels in the BCs than in other follicle cells before the onset of migration (Niewiadomska et al., 1999). Interestingly, E-cadherin does not seem to be required for the formation of the BC cluster, since BCs lacking E-cadherin can delaminate and generate a cell cluster (Niewiadomska et al., 1999). A recent study also demonstrated that border cells with low amount of E-cadherin display less protrusion than the wild type cells and so fails to follow their migratory pathway (Cai et al., 2014).

Thus, E-cad mediated adhesion acts positively on border cell migration and on directional sensing and also mediates the cell-cell communication.

Glial cell biology

Neurons and glia, the two major cell types of the Central and Peripheral Nervous System (CNS and PNS), migrate extensively during development from the birth-place to their final destination in a very stereotyped pattern. Such long-distance migration is often controlled by the relationship existing between glia-glia as well as between neurons and glia. CNS glia migrate as single cells whereas, glia in the PNS often migrate as cohorts of cells. In the following sections, I will talk about glial cells in general, focusing on the collective migration in *Drosophila* PNS glia.

Drosophila glial cells

Glial cells have emerged as crucial regulators for the development, maintenance and function of the nervous system. Initially, glial cells were thought only to provide support or to nurture neurons; however, research over the years has made it obvious that glial cells have much broader role with their active participation in virtually all aspects of nervous system development/function (Aigouy et al., 2008; Altenhein et al., 2016; Berzsenyi et al., 2011; Chotard and Salecker, 2004; Freeman and Doherty, 2006; Gupta and Giangrande, 2014; Kumar et al., 2015). Glial cells are captivating and important because of their structural diversity, functional versatility and the fact that they can change the behavior of firing neurons even though they cannot discharge electrical impulses of their own. They guide early brain development and keep their fellow brain cells healthy throughout life, are metabolically coupled to neurons and provide them with lactate as a source of energy on demand (Chotard and Salecker, 2004; Freeman and Doherty, 2006; Herculano-Houzel, 2014; Lee et al., 2012). Thus, glia are not mere

structural fillers, as their name seem to suggest (Greek for glue) (Barres, 2008). Although it has been generally thought that glia outnumber neurons in the mammalian brain, recent evidence suggest that there might be equal number of glia and neuron (Klambt, 2009). In *Drosophila*, only 10% of the cell population belongs to glia (Klambt, 2009). Regardless of the true glia to neuron ratio, scientists have already shown that glia are, functionally, the brain's other half.

Based on the morphology and gene expression, vertebrate glia has been classified into: astrocytes and oligodendrocytes in the CNS and Schwann cells in the PNS, whereas *Drosophila* glia has been mainly classified according to their position (**Fig. 8**). This includes surface-associated glia (surface), cell-body-associated glia (cortex) and neuropil-associated glia (neuropil) in the CNS, as well as peripheral or nerve-associated glia in the PNS, which are very close to their mammalian counterpart in terms of functionality and morphology (**Fig. 8**) (Chotard and Salecker, 2004; Freeman and Doherty, 2006; Klambt, 2009).

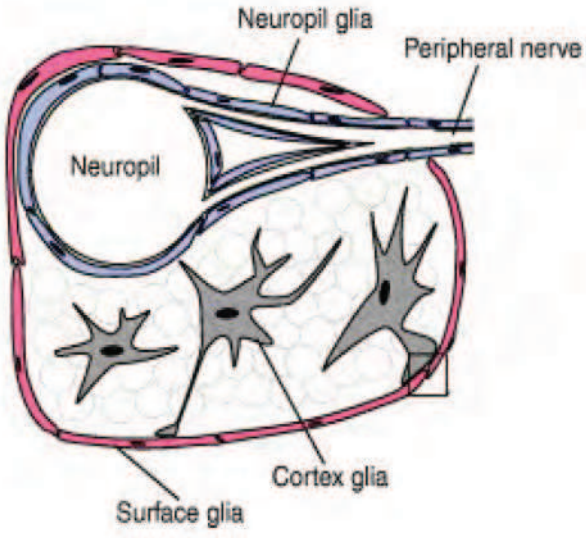


Figure 8: Glial subtypes in *Drosophila*. Cross-section of an embryonic *Drosophila* CNS hemisegment. Cortex glia are fixed within the neuronal cortex, also extend their cellular processes to the blood–brain barrier (BBB). Neuropil glia enwrap axons and dendrites and also covers the surface of the neuropil; peripheral glia ensheath the peripheral nerves in the PNS (not shown). Surface glia form a sheath around the CNS and constitutes the BBB. Cortex glia and certain components of the BBB are probably cellular conduits for transfer of nutrients from trachea or hemolymph to neurons. Adapted from (Freeman and Doherty, 2006).

Lateral glia, derived from the neurogenic region of the ectoderm, include the peripheral and the central glial cells with the exception of midline glia. The differentiation of these cells relies on the transcription factor encoded by the gene Glial cell missing/Glial cell deficient (Glide/Gcm, for the sake of simplicity Gcm in the rest of the text) (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Gcm is expressed very early and transiently during embryogenesis and acts as a master regulatory gene for glial cell development: removal of *gcm* converts all presumptive glia into neurons, whereas ectopic Gcm leads to supernumerary glia (at the expense of neurons) (**Fig. 9)** (Van de Bor and Giangrande, 2002). In the embryos, the expression of Gcm can be seen at early stage 9 and it declines after stage 14 (Laneve et al., 2013). To promote glial cell differentiation, Gcm acts by activating its downstream genes *repo* and *pointed* (*pnt*) (Granderath et al., 2000), and it represses the neuronal fate through the activation of *tramtrack* (*ttk)* (Giesen et al., 1997; Yuasa et al., 2003). Repo is a direct target of Gcm and is active throughout its life in glia and positively autoregulates, so its expression progressively becomes independent of Gcm (Cattenoz and Giangrande, 2013). Another study proposed that Gcm is a direct target of Polycomb group (PcG), which was initially identified as critical regulators of HOX transcription factors that are maintained in a silent or active state (Schuettengruber et al., 2011; Schwartz and Pirrotta, 2007). PcG proteins bind both *gcm* and *repo* and counteract the activity of Gcm (Popkova et al., 2012). Thus, Pc ensures the maintenance of a transiently expressed determinant, a process that ensures threshold levels of the determinant and is necessary for the correct fate choice.

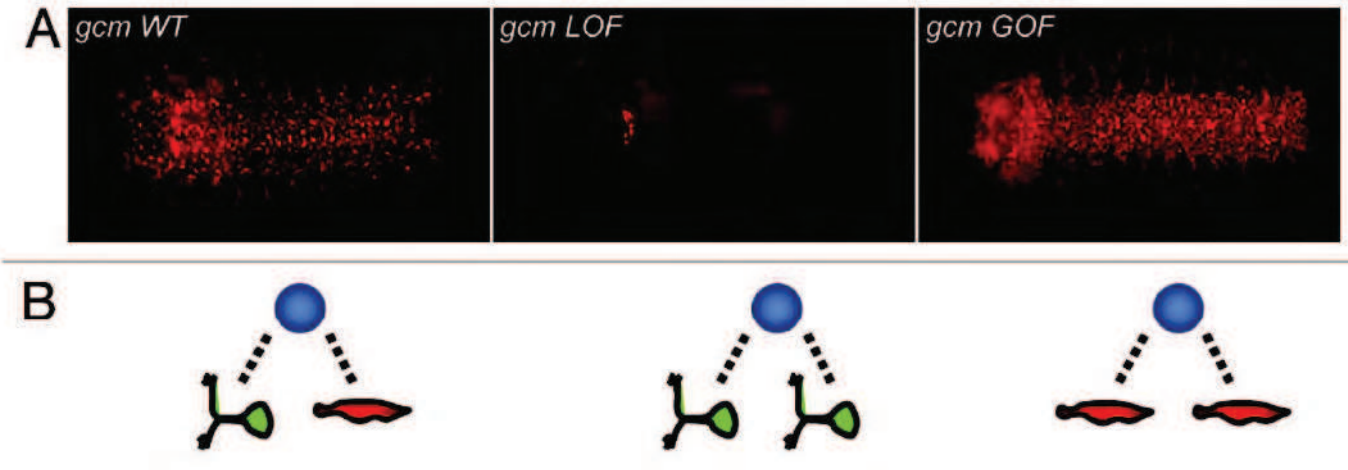


Figure 9: Gcm is indispensible for gliogenesis. **(A)** A WT *Drosophila* embryo immunolabeled for glial cells using a pan glial marker (anti Repo, left panel), LOF *gcm* with almost no glia (middle panel), and GOF *gcm* with ectopic glia (right panel). (**B**) A schematic representation of *gcm* determining the fate of glial cells. Neural stem cell-like precursors (blue), glia (red), neurons (green). Adapted from (Cattenoz and Giangrande, 2013).

One of the remarkable features displayed by *Drosophila* PNS glia is their ability to perform long distance migration, which is very similar to what is seen in the vertebrate Schwann cells (SC). Neural crest (NC) cells derived SCs display collective migration throughout their journey. One such example of SC migration is within the lateral line primordium of zebrafish, which I have discussed in the earlier section. It has been demonstrated via *in vivo* imaging of zebrafish embryos that the collectively migrating SC precursors use the underlying axons as a substrate in the lateral line to reach their final position. Substantiated by a well-known notion that glia are very loyal to their partner axons and will never exceed a stalled axon (Gilmour et al., 2002).

Peripheral glial chain migration has been analyzed in the *Drosophila* embryo and in the pupal wing, which is described in the following sections.

Peripheral glial migration

In the embryo

In the *Drosophila* embryo there are 12 glial cells per abdominal hemisegment that can be classified as peripheral glial cells (PG). Most of the embryonic PGs are derived from neural stem cells (neuroblasts) of the CNS. All PGs are associated with the nerves of sensory and motor neurons (von Hilchen et al., 2008). The axons of the sensory and motor neurons fasciculate and form the three main peripheral nerves: the anterior transverse nerve (TN), the intersegmental nerve (ISN) and the segmental nerve (SN). During embryogenesis, the peripheral nerve is ensheathed upon glial cell migration. By late stage 12/early stage 13 (ePG9) glial cells start moving towards the periphery and by late stage 16, the entire peripheral nerve appears enwrapped by the glial cells (**Fig. 10**) (von Hilchen et al., 2008). *In vivo* and its corresponding fixed preparations have revealed cell-specific dynamics and some variations of the migratory behavior of peripheral glial cells, though the order and final position of most of these cells is fixed. The ePG9 is the first cell that leaves the CNS and migrate along the ISN into the periphery followed by ePG7. This cell expresses filopodia-like structures, while the following cells do not (Edenfeld et al., 2007; Sepp and Auld, 2003). Interestingly, like vertebrate glia, *Drosophila* PNS glia never overtake the axonal growth cones.

The precision of chain migration is in part controlled by Notch signalling. Mutations in Notch and its antagonist Numb were identified in genetic screens for abnormal glial migration, and both genes act in a cell-autonomous manner to instruct peripheral glial cell migration along the peripheral nerves (Edenfeld et al., 2007). Notch mutant cells leading the migratory glial chain show an increase in the number of

filopodia, suggesting that Notch (directly or indirectly) influences the dynamics of the cytoskeleton. In addition, it has been shown that the glia require the activity of RhoA and Rac1 to restructure their cytoskeleton on the way to their target areas (Sepp and Auld, 2003).

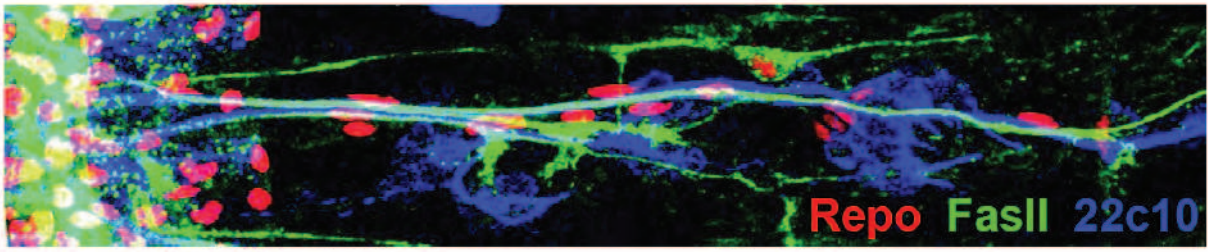


Figure 10: The embryonic PNS. Triple immunolabeling of a peripheral nerve at the late stage 16. Glial nuclei (red), motorneurons (green) and sensory neurons (blue). (von Hilchen et al., 2008)

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Another unusual and specialized mode of glial chain migration has been described during *Drosophila* eye development, when glial cells populate the eye imaginal disc by migrating between a type of subperineurial cell known as carpet cells and a thick layer of ECM (Rangarajan et al., 1999; Silies et al., 2007; Yuva-Aydemir and Klambt, 2011). Please refer to the chapter 1 for details regarding this form of glial migration.

In the pupae

The developing *Drosophila* wing provides an excellent means to study chain migration. A mature wing comprises of five longitudinal veins, two of which (L1 and L3) are innervated by the sensory nerves and are lined by the glial cells (**Fig. 11A**) (Giangrande et al., 1993; Murray et al., 1984; Van de Bor and Giangrande, 2002). In addition to the five veins, two other structures serve as a reference points for orientation in the wing: the costa (proximal anterior edge of the wing) and the radius (the proximal continuation of the L1 vein) (**Fig. 11A**).

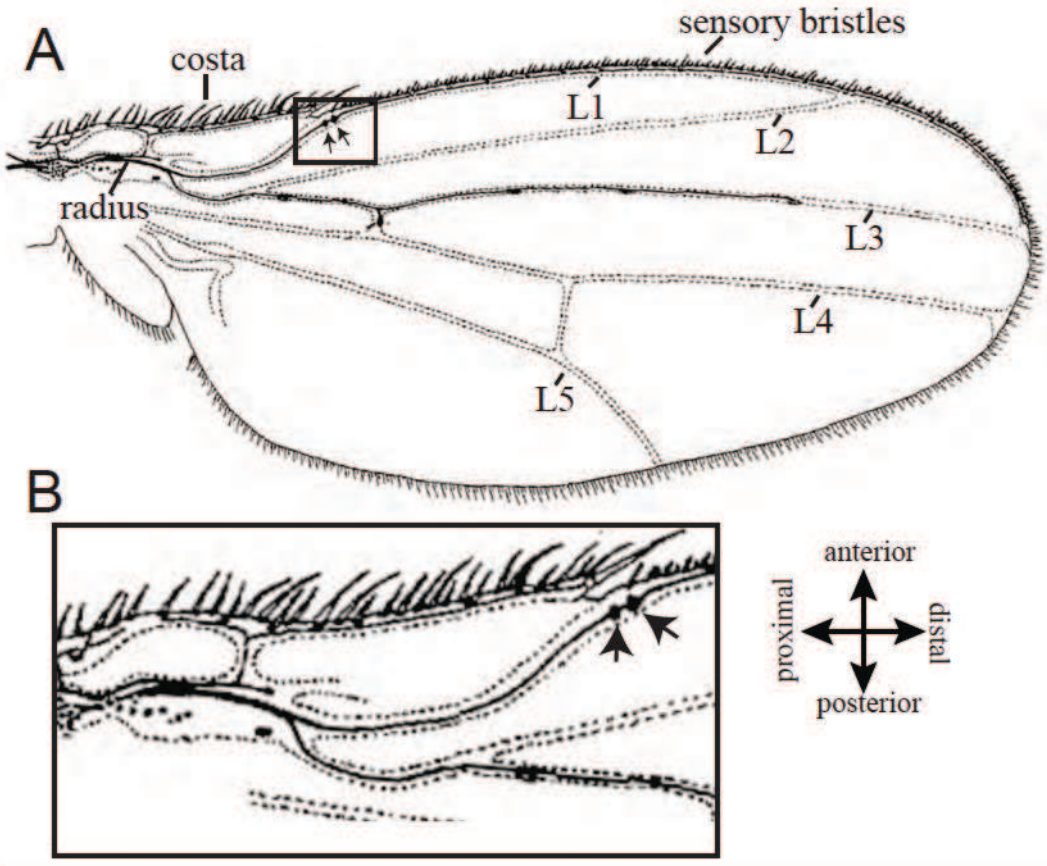

Figure 11: Adult *Drosophila* wing. **(A)** Picture of an adult wing indicating five longitudinal veins, along with costa and radius. Black box highlights the region enlarged in the panel (B). **(B)** Two black arrows indicate the position of early and late developing TSM.

Two types of sensory organ (SO) are present along the L1 and L3 veins at the anterior wing margin and in the wing blade, respectively. These are mechanosensory organs (bristles and campaniform sensilla) and chemosensory bristles (Hartenstein and Posakony, 1989). Five mechanosensory campaniform sensilla are found along the L3 vein, whereas numerous sensory organs are present at the wing margin. Twin Sensilla of the Margin (TSM) denominates one early and one late developing campaniform sensillum at the proximal most part of the anterior margin (**Fig. 11B**). The sensory axons present at the wing margin on L1 vein are clustered together in a nerve bundle called the L1 nerve, whereas the axons present in the L3 vein form the L3 nerve. The L1 and L3 nerves navigate towards the CNS upon joining the proximally located radius (Murray et al., 1984).

The early born TSM neuron gives rise to the pioneer axons, which start elongating proximally at around 3 hAPF, whereas the late TSM neuron appears around 9-10 hAPF (Murray et al., 1984; Palka et al., 1986). By 12 hAPF, the other neurons on the L1 nerve also starts to send their axons proximally, eventually forming a continuous bundle. The L1 and L3 neurons can be visualized by immunolabeling with the anti-Elav antibody (nuclei) (Campos et al., 1987), with the anti-HRP (Horseradish Peroxidase) antibody (membrane) (Jan and Jan, 1982) and with anti-22c10 antibody (microtubule-associated protein Futsch) (Hummel et al., 2000).

Glial cells arise from specific sensory organ precursors (SOP) upon expression of the glial cell specific protein Gcm (Giangrande, 1994; Van de Bor and Giangrande, 2002). Gcm precedes Repo expression in the wing glia and can be detected at as early as 9h after pupa formation (hAPF) (Van de Bor and Giangrande, unpublished data). Gcm

transcripts are visible in a WT wing at 19 hAPF by *in situ* hybridization with a *gcm*-specific probe, however, by 24 hAPF *gcm* transcripts are no more visible (**Fig. 12**) (Popkova et al., 2012). The glia present along the wing margin undergo differentiation in a way that the cells at the distal end of the chain appears earlier than the proximal ones.

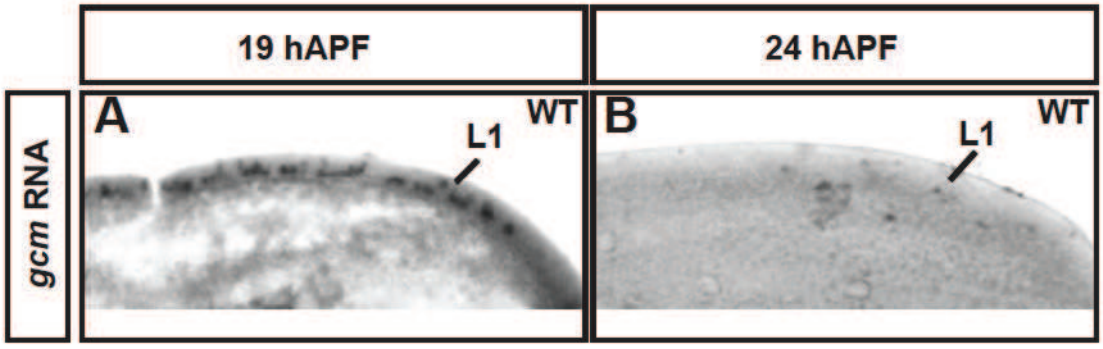

Figure 12: *gcm* RNA in WT wings. (A) *gcm* expression can be seen at the L1 nerve in a WT wing at 19 hAPF. **(B)** The expression of *gcm* disappears by 24 hAPF in a WT wing (Popkova et al., 2012).

Interestingly, glial cells along the anterior margin do not remain at the place where they are produced, but undergo collective migration up to the proximal region during development along the established axonal tracks. This process of migration proceeds in a coordinated manner and is well characterized in our lab with the help of time-lapse confocal microscopy (**Fig. 13**). The axons serve as navigational substrate for the migrating glial cells: in the absence of neurons in the developing wing, glia cells maintain their ability to migrate in chains but fail to migrate in the correct direction (Aigouy et al., 2004). These latter observations indicate that the axons do not control glial motility, but they affect the direction of the glial migration. The glial cells initiate/start to migrate along the L1 nerve at around 18h APF and completes migration upon joining the

so-called radius glia between 26 and 29h APF (**Fig. 14**) (Aigouy et al., 2004).

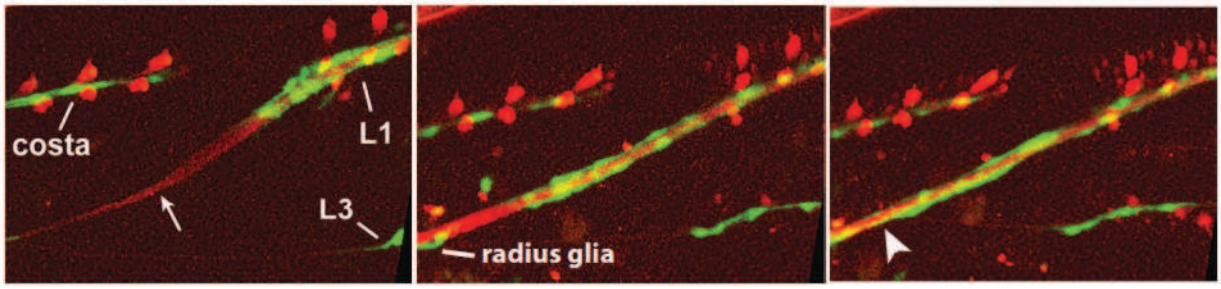

Figure 13: Glial cell migration along the axonal bundle. Snapshots from the time-lapse movie performed on UASGFP/+; repoGal4/elav-dsRed wing. Neurons are labeled in red and glia in green. The movie starts at 19 hAPF when glia movement has already started and completes by joining the proximally located radius glia (arrowhead). Adapted from (Aigouy et al., 2004).

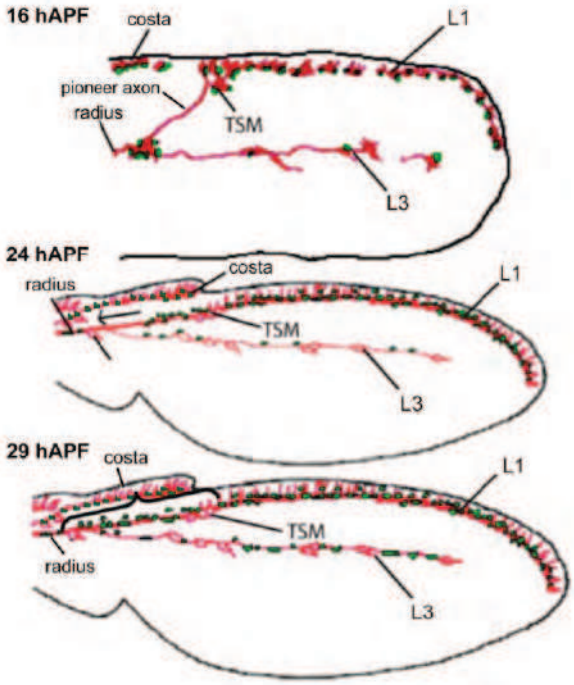

Figure 14: Glial cell migration. Schematic representation of the developing *Drosophila* wing. Glia is in green and neurons are in red, TSM: Twin sensilla of the Margin. Glia do not move at 16 hAPF and are closely located to their place of birth, however, L1 and L3 neurons have already form a continuous nerve. By 24 hAPF glia has already moved along both L1 and L3 nerves. Glia completes migration by 28-29 hAPF

Leaders and Followers

Before migration starts the most proximal (5-6) glia of the L1 nerve display a very dynamic morphology by extending long, exploratory cytoplasmic extensions in many directions (**Fig. 15**, white arrowheads) (Aigouy et al., 2004). Interestingly, the other cells in the chain do not seem to display such morphology, instead they are just aligned along the axons and send cytoplasmic processes parallel to them.

Moreover, the presence of the numerous actin rich protrusions at the migration front seems to be a common feature in the cell motility world. 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 cells at the tip of the chain causes a strong delay in the migratory process (Aigouy et al., 2008). Similarly, by separating group of cells at the migratory front it has been demonstrated that large collectives move more efficiently than the small collectives. Alterations in the cellular composition at the migratory front in the glial chain lead to a delayed and defective cell migration, indicating the importance of group size and cellular interactions in the migrating community (Berzsenyi et al., 2011).

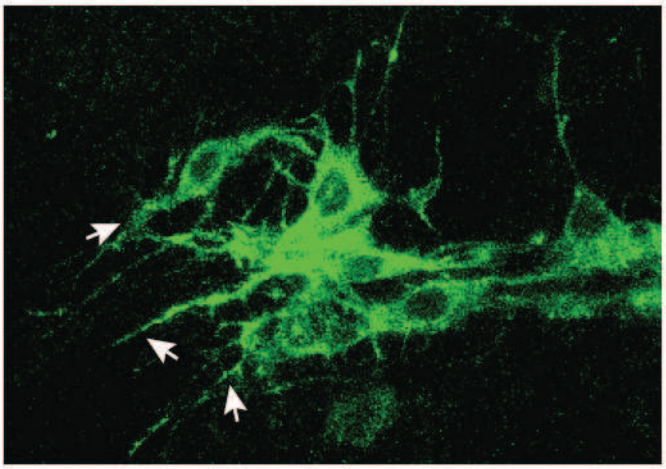

Figure 15: Pioneer cells displaying actin rich protrusions. Snapshot from a confocal time-lapse movie showing dynamic organization of the long glial cytoplasmic extensions at the chain front in the following genotype: *gcm Gal4; UAS Actin42A GFP*.

The questions that I have addressed in my thesis are the following:

1) What is the role of cell adhesion molecule N-cadherin on collective glial cell migration in the developing *Drosophila* wing? **[Chapter 2]**
2) How chemotropic guidance cues control collective glial cell migration and how are these pathways regulated in the timely manner? **[Chapter 3]**
3) What is the role of the novel cell population TSM-G in collective glial cell migration? **[Chapter 4]**
4) What are the pathways regulating the glial cell fate determinant Gcm that controls collective glial cell migration? **[Chapter 5]**

The *Drosophila* toolbox

This part introduces the most important tools and techniques that I have used to study the process of collective glial cell migration in the developing *Drosophila* wing.

The Drosophila life cycle

It takes around 10-12 days at 25°C to transform an embryo into an adult *Drosophila*. The embryonic stage lasts around twenty-four hours and is followed by three successive larval stages that last for 4-5 days. To undergo metamorphosis, the larva then hatches into a pupa. This pupal stage lasts for around five days, ultimately giving rise to an adult (**Fig. 16**).

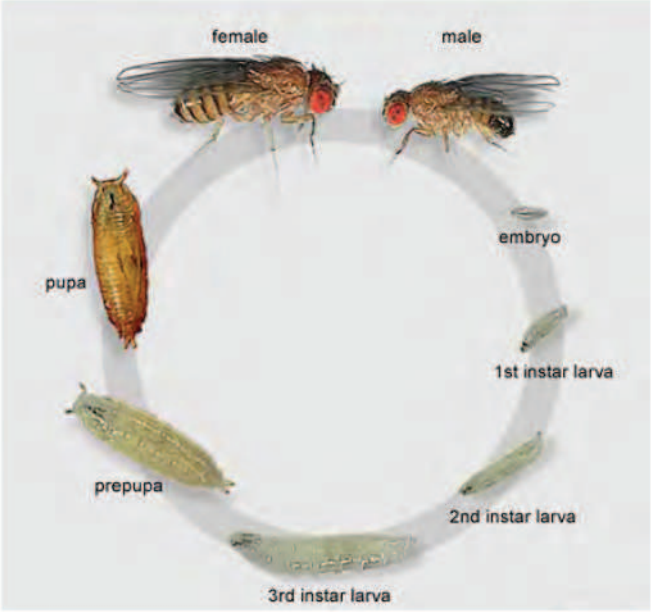

Figure 16: Life cycle of *Drosophila*.

The Gal4-UAS system

The development of the *Gal4-UAS* system allows the conditional expression of any cloned gene in a wide variety of tissue and cell specific patterns (Brand and Perrimon, 1993). One of the key aspects of this system is that the *Gal4* protein can be kept in isolation from its target gene in distinct transgenic lines, ensuring that the target gene is silent until the introduction of the *Gal4*.

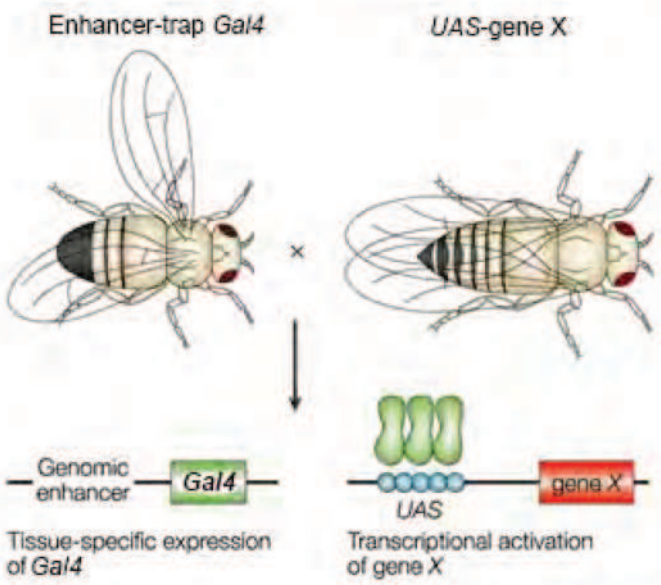

Figure 17: Targeted gene expression. (St Johnston, 2002)

In this system, the yeast transcriptional activator *Gal4* binds to its target sequences called *UAS* (Upstream Activating Sequences), which in turn activates the transcription of the fused downstream transgene (gene X) (Brand and Perrimon, 1993). Thus, by crossing a transgenic line carrying the *Gal4* driver of interest with a fly containing the *UAS* reporter, we can express different genes in a tissue specific manner. We can downregulate (loss-of-function) genes by means of "*UAS*-geneX-RNAi" constructs or overexpress them (gain-of-function) by using "*UAS*-gene X" transgenes (**Fig. 17**). Recent modifications and adaptations of the *Gal4* system have expanded the scope of this system, allowing a better temporal control over the activity of *Gal4*.

Time-lapse imaging of the developing Drosophila wing

The development of time-lapse confocal microscopy has allowed us to assess the cellular behavior in real-time. In this method, live cell imaging can be extended from a single observation in time to the observation of cellular dynamics over long periods of time. This approach was established years ago in our lab and has allowed us to visualize the collective migration in the developing *Drosophila* wing (Aigouy et al., 2008; Aigouy et al., 2004). To facilitate dissection, the living pupae are placed on a scotch on their left side and then the puparium case is removed in order to expose the wing (**Fig. 18A**). The exposed wing is covered with a tiny drop of 10S halocarbon oil (Voltalef) and the animal is taped facing down on a 35 mm glass base dish (**Fig. 18B**). These wings are then subjected to a time-lapse analysis in 4D by confocal microscopy equipped with a heating stage to maintain a constant temperature (25±2°C) (**Fig. 18C**). This non-invasive technique allows us to study cell migration in the whole animal under physiological asset (Aigouy et al., 2004).

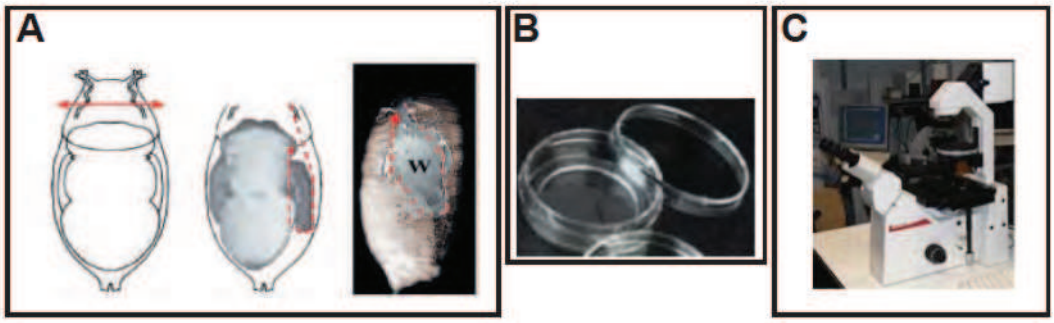

Figure 18: Time-lapse of the *Drosophila* pupal wing. (A) Red lines indicate the path of puparium case dissection. For time-lapse the puparium case over the wing is removed. Left and mid panels are dorsal views; right panel is a lateral view. Anterior is to the top. W-wing. **(B-C)** The exposed wing is taped on a glass base dish and live imaged using the confocal microscope. Adapted from (Aigouy et al., 2004)

Materials and Methods

Genetics and fly stocks

I used the conventional *Drosophila* Gal4-UAS system for most of my experiments (see introduction). Briefly, this system allow us to achieve targeted gene expression by crossing a transgenic fly carrying a Gal4 transposon to another fly carrying a UAS (Upstream Activating Sequences) sequence, which leads to the transcriptional activation of the fused downstream transgene.

All the fly stocks used for this study were maintained at 25°C in a standard fly medium. The following list describes the fly stocks I used during the course of my PhD. The Roman numbers indicate the chromosome on which the transgene is inserted.

Genotypes	Abbreviated as	Origin	Remarks about the transgene
UASENCGFP;repo Gal4/Tb (III)	*repo*>GFP	V. Auld	Drives glial specific expression of UAS ncGFP, (n,c indicates nuclear and cytoplasm)
repogal4,UASPHG FP/Tb (III)	*repo*>PH GFP	A. Zelhof	Fusion protein between the pleckstrin homology domain of PLC-d and the GFP coding sequence
repogal4 (II);UASmCD8GFP (III)	*repo*>GFP	(Kumar et al., 2015)	
UAS repo (III)	*repo* GOF	(Yuasa et al., 2003)	
repo RNAi	*repo* KD	Bloomington/*VDRC	
gcmGal4,UASmCD 8GFP/cyo;UASmc D8GFP	*gcm*>GFP	(Jacques et al., 2009)	Hypomorphic *gcm* allele used as an early glial specific driver, CD8GFP labels the membrane
gcmGal4,UASmCD 8GFP/cyo;repoGal8 0	*TSM-G*>GFP		

gcm^{rA87}/cyo action GFP	*gcm* LOF	(Vincent et al., 1996)	Enhancer trap carrying a LacZ transposon into the *gcm* promoter
gcm^{N7-4}/cyo action GFP	*gcm*$^{N7-4}$		Null allele
gcm^{34}/cyo action GFP	*gcm*^{34}		Imprecise excision
gcm^{26}/cyo action GFP	*gcm*^{26}		
gcm>GFP/gcm>GFP	*gcm* (homo)	(Popkova et al., 2012)	Homozygous mutant of *gcm*
gcm 6KB Gal4	*gcm* 6KB>	(Flici et al., 2014)	
UAS gcm (F18A)	*gcm* GOF	(Bernardoni et al., 1998)	
UAS gcm RNAi	*gcm* KD	Bloomington - 31519, 31518, 28913	
UAS string	string GOF	(Inaba et al., 2011)	
UAS dacapo	dacapo GOF	(Lane et al., 1996)	
fra^{3}/cyo actin GFP	*fra*^{3}	Benjamin Altenhein	Amorphic *fra* allele
UAS fra RNAi	*fra* KD	Bloomington - 31469, 40826	
UAS fra$^{\Delta}$C	*UAS fra*$^{\Delta}$*C*	Benjamin Altenhein	Dominant negative form
UAS-fraΔP1ΔP2 myc ; fra 3 / CyOWg	*UAS fra*$^{\Delta P1\Delta P2}$	Wesley Gruber	
3. fra 3 ; UAS-fraΔP3myc/ CyOWgLacZ	*UAS fra*$^{\Delta P3}$	Wesley Gruber	
UAS fra	*fra* GOF	Bloomington - 8814	
unc5^{8}	*unc5* LOF	Benjamin Altenhein	Null mutant
UAS unc5 RNAi;	*unc5* KD	Bloomington - 33756	
UAS unc5	*unc5* GOF	Benjamin Altenhein	
NetAB$^{\Delta}$		Benjamin Altenhein/Wesley Gruber	Double null mutant of *NetA* and *NetB*
NetB$^{\Delta}$		Benjamin Altenhein/Thomas Kidd	Null mutant of *NetB*
NetA$^{\Delta}$		Benjamin Altenhein/Thomas Kidd	Null mutant of *NetA*
NetA$^{\Delta}$ NetB^{TM}		Thomas Kidd	Membrane tethered form of NetB
NetA$^{\Delta}$ NetB^{myc}		Thomas Kidd	Myc tagged secreted

			form of NetB
UAS NetB RNAi	NetB KD	Bloomington - 34698,25861	
UAS NetB	NetB GOF	Iris Salecker	
UAS NetA RNAi	NetA KD	Bloomington - 31288,31665	
UAS NetA	NetA GOF	Thomas Kidd	
NP4151 Gal4		DGRC	Drives expression of NetB
NP4012 Gal4		DGRC	Drives expression of NetA
slimb,ago/TM3 ser GFP	DM	C. T. chein	Double mutant of *slimb* and *ago*
$slimb^{P1493}$	SM		Single mutant of *slimb*
ago^{1}	SM		Single mutant of ago
UAS slimb	*slimb* GOF		
UAS ago	*ago* GOF		
engrailedGal4	*en>*	Bloomington stock center	
GMR 29F05-Gal4	*GMR 29F05>*	Bloomington stock center	
collagen Gal4	*collagen Gal4*		

*VDRC: Vienna Drosophila Stock Center; Bloomington: Bloomington Drosophila Stock Center at Indiana University

Immunolabeling and antibodies

The anterior and posterior ends of staged pupae were cut off and the rest of the pupae were fixed in 4% PFA PBS (paraformaldehyde in phosphate buffer saline) overnight at 4ºC. 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 (PBS Triton-X100, 0.3%). Following this the wings were given 4 quick washes of 10 minutes in PBT and were incubated in the blocking reagent PBT-NGS (5% normal goat serum in PBT) for 60

minutes at room temperature on planar shaker. Samples were then incubated overnight in primary antibodies (diluted in PBT-NGS): mouse-anti-Repo labels glia (1:10) and mouse-anti-22c10 labels neurons (1:1000) (*DSHB), chicken-anti-GFP (1:1000) (Abcam), rat-anti-Elav labels neurons (1:1000) and rat-anti-Cadherin-N (1:50) (DSHB), rabbit-anti-Unc5 and rabbit-anti-Fra (1:500) were gifts from Benjamin Altenhein. After 4 washes in PBT, wings were incubated for 2 hours at room temperature in secondary antibodies (1:500) raised in mouse, rat, rabbit or chicken and coupled to Cy3, Cy5 or FITC fluorescent dyes diluted in PBT-NGS. Following a final wash in PBT, wings were mounted on slides in Aqua- Poly/Mount medium (Polysciences Inc.).

*DSHB: Developmental Studies Hybridoma Bank

Confocal and in vivo imaging

Leica SP2 and SP8 inverted-based microscopes with 20, 40 and 63X objectives were used to obtain confocal fluorescent images. GFP/FITC was excited at 488nm; the emission filters 498-551 were used to collect the signal. Cy3 was excited at 568nm; emission filters 648-701 were used to collect the signal and Cy5 was excited at 633nm; emission signal was collected at 729-800nm. A step size between 0.2 and 1.5µm was used to collect the Z- series of images.

Time-lapse analyses were performed using the standard procedure as described elsewhere (see *Drosophila* toolbox) (Aigouy et al., 2008; Aigouy et al., 2004; Kumar et al., 2015; Soustelle et al., 2008). Photo bleaching was avoided by using a low magnification and reduced exposure time. Maximum projections for time-lapse and

confocal images were obtained by using the ImageJ software. Images were annotated by using Adobe Photoshop and Illustrator.

Statistical analysis

The number of wings dissected for each experiment were more than or equal to 30. The migratory index (MI) was calculated as described in (Kumar et al., 2015). Briefly MI defines the percentage of wings in which glial cells have completed migration at a given time point. The MI is calculated at 28 hAPF unless otherwise specified. Graphs were made using Prism software and the Student's *t* test method was used for the comparison between two different experimental sets. Bars indicate the standard error mean (s.e.m).

Molecular Cloning

For the *fra* gene, oligonucleotides surrounding the GBS's were designed with flanking restriction sites for KpnI at the 5' extremity and NHeI at the 3' extremity. Each pair of oligonucleotides was used to amplify the genomic region encompassing the GBSs using the Expand High fidelity polymerase (Roche). The amplicons were digested with 20 U of KpnI (NEB # R3142S) and 20 U of NheI (NEB # R3131S) in Cutsmart buffer (NEB # B7204S) for 2 hours min at 37ºC. The digested amplicons were then cleaned using the PCR clean-up kit (MN # 740609) according to manufacturer's instructions.

For ligation, 50 ng of the digested probe were used and cloned into the pGreen Pelican vector overnight at 18°C. 1µl of the ligated product was used for transformation of electro competent DH5α bacterial cells. Bacteria were then kept for 1 hour at 37°C and

plated on ampicillin containing medium. After overnight incubation at 37°C several colonies were picked up in separate tubes containing the LB and then were incubated overnight at 37°C. The following day, mini preps were performed using the kit; positive colonies were identified by gel electrophoresis and sent for sequencing for final confirmation.

Same procedure was conducted to build the mutated *fra* reporter plasmids. Following oligonucleotides were used:

fra WT forward:

5'GAGAGGTACCGTGTCCAAAAATGCGGGTCTGTTTCTCG3'

fra WT reverse:

5'GAGAGCTAGCGTTAAGACAAACATGCAGGCATAAAGACATG3'

fra Mutant forward:

5'GAGAGGTACCGTGTCCAAAAAAAAAAACTGTTTCTCGAAATTGAGTT3'

fra Mutant reverse:

5'GAGAGCTAGCGTTAAGACAAACAAAAAAAAATAAAGACATGAAATGGA TG3'

Co-transfection and Western blot assays

Co-transfections in S2 cells were carried out using Lipofectamine (Invitrogen). 6 x 10^6 cells were cultured in 6-well plates containing Schneider medium. In each well, cells were transfected with 1µg of *fra* WT or mutant reporter plasmid, 1µg of *pPAC-lacZ* as a transfection control, 0.5µg or 1µg or 2µg of *pPac gcm* expression vector and *pPac* 'empty' to make up the volume up to 4µg. Cells were collected 48 hours after

transfection, first washed in cold PBS and then resuspended in lysis buffer. Total protein extract was obtained by 4 freezing-thawing cycles in liquid nitrogen and centrifugation at 4 °C at 13000g. Protein expression was detected as per standard Western blot procedures. Primary antibodies used were as follows: mouse anti-β-Gal (1/2000, Sigma), rabbit anti-GFP (1/5000, Molecular Probes); mouse anti-HRP and rabbit anti-HRP (1/5000, Jackson ImmunoResearch) were used as secondary antibodies. Note that each experiment was performed in triplicate.

βgal assays were performed to measure the levels of LacZ for each replicate. 20µl of protein extract mixed with 50µl of βgal assay buffer containing ONPG was incubated at 37°C. Reaction was stopped by adding 50µl of 1M Na_2CO_3 once the solution turned yellow, DO was analyzed at 415nm. The levels of GFP were normalized to the LacZ value in each blot and were quantified by using ImageJ software. The background was subtracted from each band value and then the average was calculated.

The method and buffers used for co-transfection, Western Blot and qRT-PCR experiment are the same as in (Flici et al., 2014).

Reverse Transcription and qRT-PCR

Total RNA was extracted from S2 cells using Trizol (Invitrogen), 1µg of purified RNA was reverse transcribed by SuperScript II. qPCR was performed with the machine Roche LightCycler 480 and Sybr Green Master mix (Roche) using the following oligonucleotides:

fra WT forward:

5' GAGAGGTACCGTGTCCAAAAATGCGGGTCTGTTTCTCG 3'

fra WT reverse:

5'GAGAGCTAGCGTTAAGACAAACATGCAGGCATAAAGACATG 3'

fra Mutant forward:

5'GAGAGGTACCGTGTCCAAAAAAAAAAACTGTTTCTCGAAATTGAGTT 3'

fra Mutant reverse:

5'GAGAGCTAGCGTTAAGACAAACAAAAAAAAATAAAGACATGAAATGGA TG 3'

GFP forward: **ACATGAAGCAGCACGACTTCT**

GFP reverse: **TTCAGCTCGATGCGGTTCA**

Gcm WT forward: **5'GAGAGATCTTATCCCGATCCCCTAGC3'**

Gcm WT reverse: **5'CTACTACTACAGCAATACGGG3'**

LacZ forward: **TGTGCCGAAATGGTCCATCA**

LacZ reverse: **GTATCGCCAAAATCACCGCC**

For each gene, the expression levels were automatically calculated (LightCycler480 Software, release 1.5.0) by calibration to gene-specific standard curves generated on input cDNAs. Collected values, derived from three amplification reactions, each performed in three independent experiments, were normalized to βgal mRNA amounts.

Chapter 1

Review: Collective Cell Migration

The following chapter is based on a review article published in the Journal of Neurogenetics entitled **"Collective Cell Migration: "All for One and One for All" (Gupta and Giangrande, 2014)**.

Cell migration is an essential and highly regulated process for animal development and physiology. Cells migrate in a well-coordinated manner for tissue formation, vascularization, wound healing, and as part of the immune response. Cell migration is also an integral part of cancer metastasis, as tumorous cells become migratory and invade surrounding tissues. Cells can migrate as an individual or collectively in tightly or loosely associated groups (Friedl and Gilmour, 2009; Klambt, 2009; Rorth, 2009). Examples of collective cell migration include invasive tumor cells, neural crest cells and many types of epithelial cells. This review discusses examples of different types of collective cell migration in animal models and highlights recent results that provide insight into cell organization and behavior using *Drosophila* wing glia as a model. The physical attributes associated with cells migrating in a group are similar to that of independently migrating cells, however collectively migrating cells are subject to additional regulation and constraints as they affect one another mechanically and via signaling. This regulation and constraints eventually contribute to shaping, guiding, and ultimately ensuring tissue function.

I have already elaborated on some of the examples discussed in this chapter (see introduction). This review is the first publication I earned during the course of my PhD.

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Review

Collective Cell Migration: "All for One and One for All"

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Abstract: Cell migration is a key mechanism during neural development, as it allows cells to reach their final destination from their birthplace. In some cases, cells migrate in isolation, whereas in others they migrate in collectives, as chains, streams, clusters, or sheets. The coordinated and timely process of collective migration eventually ensures the proper organization of the nervous system and its misregulation leads to severe diseases, including neurological disorders. This review impinges upon the cellular and molecular interactions underlying collective cell migration in animal models, and highlights the recent advances made through in vivo analyses of the *Drosophila* wing glia.

Keywords: cell ablation, collective migration, Drosophila, glia, time lapse, transcription factor

AN INTRODUCTION TO CELL MIGRATION

Migration refers to the life process in which the cell translocates from one place to another, a widespread event in unicellular organisms and in metazoans. This conserved process is essential for development and homeostasis (Horwitz & Webb, 2003) and comes in different f avors. Cells can move in isolation, as prokaryotes that migrate using cilia and f agella, *Dictyostelium* cells that migrate towards food using directional sensing (Parent & Devreotes, 1999), and rapidly migrating neutrophils. Cells can also move as collectives, a process that allows the harmonious building of tissues and organs. In contrast to single-cell migration, collective migration involves a further level of regulation, as cells reach their f nal destination upon coordinated and directional movement. Whereas cells migrating in isolation self-process the information necessary for motility, the members of a collective integrate signaling pathways and communicate to each other through homeostatic interactions (Petrie et al., 2009; Friedl & Gilmour, 2009). In addition, cells of the migratory unit may perform different tasks depending on their position. For example, cells within a migratory chain abut moving cells on both sides, whereas cells at the extremities of the chain, the front and the rear edges, face members of the collective only on one side (Insall & Machesky, 2009). The cells at the front may hence receive attracting or repelling signals from cellular/noncellular substrates that control the migratory state of the collective.

Although we have reached a remarkable wealth of knowledge on the molecular pathways affecting adhesion, chemotaxis, and directional sensing (Cantor et al., 2008; Insall & Machesky, 2009; Horwitz & Parsons, 1999; Ridley et al., 2003), what controls the collective behavior, that is, what makes the collective perform as a supracellular unit, is still poorly understood. The main limitation is technical, due to the dynamic and complex nature of the process and to the need for in vivo approaches in the whole animal. The advent of confocal time-lapse microscopy and f uorescent dyes, combined with genetic and cellular manipulations, has made it possible to study cell migration in vivo. Leaning on specif c examples, we here present the recent progress made on the molecular and cellular processes involved in collective migration and discuss future challenges and questions.

THE ROSTRAL MIGRATORY STREAM IN THE RODENT NERVOUS SYSTEM

The nervous system represents the most complex tissue and is composed mainly of neurons and glia. The remarkable ability of these cells to migrate over long distances contributes extensively to the f nal architecture

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and function of the brain. Understanding the mechanisms that initiate, maintain, and appropriately terminate the migration of newly generated cells will provide insight into how alterations in the process contribute to neurodevelopmental disorders (Francis et al., 2006; Manzini & Walsh, 2011).

The rostral migratory stream (RMS) of adult rodents, composed of neuroblasts that originate in the subventricular zone (SVZ) of the forebrain and move towards the olfactory bulb (OB), is an excellent system for studying collective migration. RMS cells are not guided by radial glial or axonal fibers, but navigate on the surface of the neighboring neuronal precursor cells (Lois et al., 1996), forming a chain that is flanked by astrocytes (Figure 1a) (Murase & Horwitz, 2004). Several molecules have been implicated in RMS migration. ADAM2 (a disintegrin and metalloprotease 2) is expressed and required in the migrating neuroblasts: ADAM2 knockout mice appear to have a reduced OB and reduced migration, along with the loss of directionality (Murase et al., 2008). Many chemoattractive or repulsive signals such as Netrins, Slits, and Ephrins are also involved in the regulation of RMS migration.

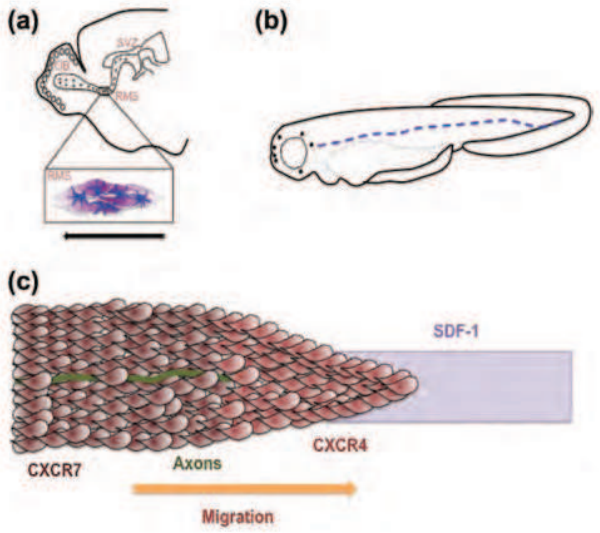

Figure 1. Collective cell migration in vertebrates. (**a**) In the rostral migratory stream (RMS), neuroblasts (*purple*) born in the adult subventricular zone (SVZ) migrate towards the olfactory bulb (OB), forming a chain that is flanked by astrocytes (*blue*) serving as a tunnel for the collective (shown in the inset) (modified from Woong et al., 2010). (**b**) The zebrafish posterior lateral line system. In a 2-day-old embryo, the neuromasts of the posterior lateral line (PLL) (*large black dots*) are deposited by a primordium that migrates along a stereotyped path (*blue dashed line*). (**c**) The lateral line primordium contains hundreds of cells moving to the right, towing, or directing along a bundle of axons (*green*). Collective movement of these cells depends largely on the activity of SDF1 and its receptor CXCR4. SDF1 defines a migratory path for the cells expressing CXCR4 mostly at the front, whereas the cells at the rear express CXCR7. Without CXCR4, cells are motile, but the PLL does not move forward (modified from Rorth, 2009).

For instance, mitral cells in the OB and RMS astrocytes express the Netrin family of chemoattractants and their receptor, DCC, is expressed in the migrating neuroblasts. Blocking DCC signaling alters the direction of protrusions on migrating cells (Murase & Horwitz, 2002). Slit proteins, on the other side, are secreted from the posterior brain structures such as the septum and their receptor Robo is expressed in the SVZ and RMS neuroblasts. In the absence of Slit proteins, a subset of neuroblasts migrates caudally, suggesting that Slit-Robo signaling acts as a repulsive signal (Nguyen-Ba-Charvet et al., 2004). Thus, chain migration requires dynamic cell interactions and signaling pathways that control directionality.

CELL MIGRATION IN THE ZEBRAFISH LATERAL LINE

The lateral line of the zebrafish consists of mechanosensory hair-like organs or neuromasts, deposited at regular interval by the lateral line primordium during development (Figure 1b). The neuromasts on the body and on the tail form the posterior lateral line system (PLL), a cohesive cohort of more than 100 cells migrating along the body surface (Ghysen & Dambly-Chaudière, 2004). This directional morphogenetic movement depends entirely on the expression of two receptors, CXCR4 and CXCR7, which both recognize SDF1, a fish ortholog of the chemokine stromal-derived factor 1 (David et al., 2002; Haas & Gilmour, 2006; Dambly-Chaudière et al., 2007; Valentin et al., 2007) (Figure 1c). SDF1 expression defines a path that is followed by the migratory primordium and SDF1 inactivation results in defective migration or in disruption of the primordium, with consequent absence of the PLL (David et al., 2002; Haas & Gilmour, 2006). The expression of CXCR4 is more prominent in the cells at the leading edge than in those at the trailing edge, whereas the latter strictly express CXCR7 (Valentin et al., 2007). Interestingly, CXCR7 arrests migration while triggering the differentiation of cells into neuromasts, and it may act as an SDF1 "sink" suppressing CXCR4 activity (Dambly-Chaudière et al., 2007). Thus, SDF1 serves as one of the major cues that determines and guides the migration of the primordium. Notably, SDF1-CXCR4 signaling was first characterized in leukocyte homing (Peled et al., 1999) and in the migration of neurons (Tran & Miller, 2003), germ cells (Doitsidou et al., 2002; Knaut et al., 2003), neural crest cells (Belmadani et al., 2005), and metastasis in both fish and mouse.

DROSOPHILA AS A MODEL TO STUDY COLLECTIVE CELL MIGRATION

Drosophila melanogaster provides a unique genetic model and resolution at single-cell level. Moreover, the

same signaling cascades are at work in vertebrates and invertebrates. Glial cells of the fly embryonic central nervous system (CNS) move during development and peripheral nervous system (PNS) glia migrate even more extensively (von Hilchen et al., 2008, 2013) to match the exponential growth of motor and sensory nerves occurring during the larval molts (Figure 2a). As in vertebrates, peripheral glia (PG) follow the axon bundles and do not move ahead of them. Most of these cells originate in the CNS and migrate towards the periphery as a chain of nine cells (Sepp et al., 2001; Sepp & Auld, 2003). Different signaling pathways contribute to PG migration. Mutations in *Notch* and its antagonist *numb* were identified in a genetic screen for abnormal glial migration; subsequent studies revealed that both genes act in a cell-autonomous manner to instruct PG migration along the peripheral nerves (Edenfeld et al., 2007). *Notch* mutant cells leading the migratory glial chain have an increased number of filopodia, suggesting that Notch influences cytoskeleton dynamics. In addition, PG require small guanosine triphosphatases (GTPases) Rho and Rac1 to remodel their actin cytoskeleton (Sepp & Auld, 2003). Altering the activity of RhoA and Rac1 triggers PG stalling at the CNS-PNS transition zone, leading to ensheathment defects. Finally, Netrins and their receptors represent another set of ligand-receptor signaling molecules involved in the migration of two glial subpopulations: longitudinal glia (LG) and PG (Hilchen et al., 2010). The cells of the ventral midline, a structure comparable to the vertebrate floor plate, express *Netrin* genes. Mutations in these genes or their attractant receptor Frazzled prevent the medially oriented migration of the LG within the ventral cord. Later in development, Netrin B provided by cells of the ventral midline repels two PG that stall at, or close to, their place of birth in the CNS via its repulsive Unc5 receptor (Hilchen et al., 2010). It has been reported that nearly all PG transiently express Unc5 (Keleman & Dickson, 2001; Freeman et al., 2003).

The *Drosophila* eye originates from the corresponding imaginal disc and is composed of almost 800 units called ommatidia; each unit contains eight photoreceptors that send axons towards the CNS. During early larval development, different subtypes of glial cells differentiate in the CNS and accumulate in the optic stalk, a structure that connects the CNS to the eye disc (Rangarajan et al., 1999; Silies et al., 2007) (Figure 2b). The outermost perineurial glia migrate over the so-called carpet cells, two very large and flat subperineurial glial cells. The nuclei of the carpet glia are generally located at the junction between the optic stalk and the eye imaginal disc, whereas their anterior end grows along the eye disc until the morphogenetic furrow, hence covering the entire eye field (Yuva-Aydemir & Klambt, 2011). The migration of perineurial glia within the eye disc requires fibroblast growth factor (FGF) signaling. FGF8-like ligand Pyramus, expressed by the carpet glia, initially activates FGF receptor Heartless (Htl), expressed broadly in the rest of the eye disc glia. The activation of Htl leads to perineurial glial cell motility. Once these glial cells reach the nascent photoreceptor axons expressing Thisbe, another FGF8-like ligand, they start expressing the Sprouty receptor to negatively regulate the Heartless/FGFR signaling pathway (Kramer et al., 1999; Hacohen et al., 1998). This results in the reduction of Heartless/FGFR activity, which halts migration and triggers glial differentiation (Franzdóttir et al., 2009). Thus, FGF growth factor pathway controls glial development and migration in a stepwise manner.

The above sections highlight the importance of molecular pathways controlling cell-cell and cell-substrate interactions, including the extracellular matrix, growth factors, chemokines, chemoattractant/repellants, and the Notch cascade. These pathways transmit the migratory signal into the cell, which interprets and responds to it using multiple pathways, including cycling small GTPases and fast actin cytoskeleton remodeling. The current challenge is to understand in vivo role and molecular nature of the different cell interactions controlling collective migration.

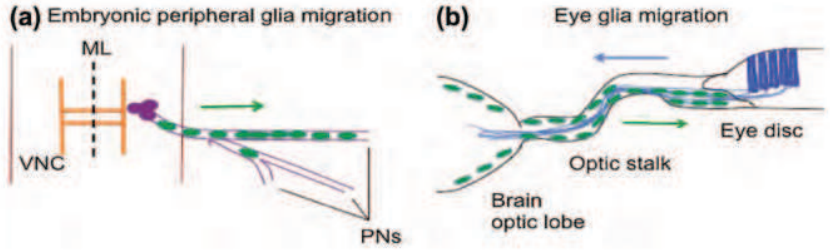

Figure 2. Collective glial migration in the *Drosophila* embryo and in the eye disc. (**a**) Embryonic peripheral glia migration: schematic frontal view of a neuromere of a mature embryo. VNC indicates the ventral nerve cord; ML, the midline; neurons are indicated in *purple*; longitudinal connectives and commissure in *orange*. Peripheral glial cells (*green*) born within the CNS move along the peripheral nerves (PNs) towards the periphery (*arrow*). (**b**) Schematic drawing showing glial cell migration in the eye imaginal disc: a monolayer of glial cells (*green*) born in the optic lobe migrates within the optic stalk towards the disc (see *green arrow*). Glial cells use growing axons (*blue arrow*) from the photoreceptors (*blue*) as a substrate for collective migration within the stalk (modified from Klambt, 2009, and Reddy & Irvine, 2011).

DROSOPHILA WING GLIAL MIGRATION

The accessible wing of *Drosophila* allows for genetic as well as cellular manipulations, and the glial cells present in this tissue form a large migratory collective. The wing is made of a double-layered epithelium that contains five longitudinal "veins" filled with hemolymph and running between the dorsal and the ventral epithelial sheets. Two veins, L1 and L3, are innervated by sensory nerves navigating proximally and connecting to the CNS (Murray et al., 1984) (Figure 3). Both nerves are lined by chains of glial cells that differentiate within the wing from the same sensory organ precursors that give rise to neurons (Giangrande et al., 1993; Van de Bor et al., 2000). After differentiation, the glial chains migrate proximally onto the sensory nerves and uniformly ensheath them (Aigouy et al., 2004) (Figure 3a–c).

The L1 glial chain consists of approximately 80 cells (Giangrande et al., 1993). Since the cells at the extremities (front and rear of the chain) are not in direct contact with each other, several questions arise concerning their coordinated and directional migration. Are L1 glia of different types, for example, do cells at the front act as pioneers that lead the rest of the collective? If so, are the glial cells behind the pioneers simple followers or do they contribute to the homeostasis of the collective? And, if homeostatic interactions are required, what aspect(s) of migration do they control: extent, direction, efficiency, or collective integrity? Last but not the least, how do such homotypic interactions compare with heterotypic interactions occurring with the neuronal substrate?

Pioneers and Followers

The role of cell interactions occurring in wing glia can be followed in situ by time lapse upon targeted single-cell ablation (Aigouy et al., 2004; Soustelle et al., 2008). A common feature in the cell motility world consists in the rapid extension and retraction of actin-rich processes exploring the environment. L1 glia at the front of migration are more dynamic than follower cells and their ablation impairs the migratory process, indicating that these cells act as pioneers (Figures 3d, e, 4a–d), in some cases, however, the remaining glial cells still reach their final destination, albeit at later stages (Aigouy et al., 2004, 2008). Thus, rather than playing an instructive role, pioneers seem to ensure a robust migratory phenotype, much like pioneer axons sustain the efficient navigation of later, follower axonal bundles (Lin et al., 1995). The distinct properties of pioneer and follower glia suggest that specific molecules are expressed in the different glial populations. Although numerous markers identify subsets of glia, none of them seems to specifically label pioneer cells (Gupta & Giangrande, unpublished).

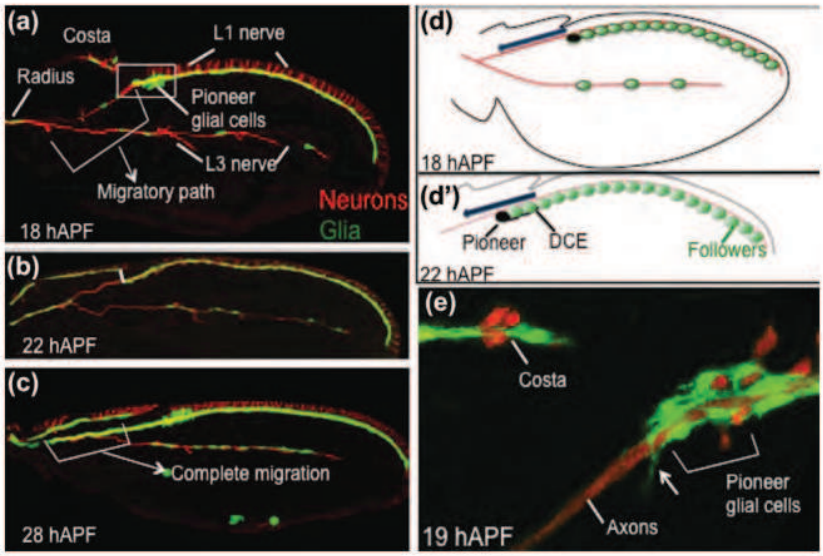

Figure 3. Glial chain migration in a developing *Drosophila* wing: (**a–c, e**) Immunolabeling showing wing glia (*green*) and neurons (*red*) at different hours after pupa formation (hAPF). Two proximal structures serve as additional reference in the migratory process: the costal nerve (proximal anterior edge of the wing) and the nerve on the radius. Inset in **a** shows the region magnified in **e**. (**a**) Initiation of migration; (**b**) reaching the level of the costal nerve (Costa); (**c**) completion of migration upon reaching the nerve on the radius (Radius). (**d, d'**) Schematic representation of a developing *Drosophila* wing. (**d**) By 18 hAPF, the glial cells (*green*, except for the pioneer in *black*) form a chain to migrate along the L1 nerve (*red*) towards the CNS (*blue arrow*). (**d'**) By 22 hAPF, one or more pioneer cells display a DCE (distal cellular extension) towards the follower glia. (**e**) Front of migration at around 19 hAPF showing glial cells (*green*), neurons, and their axons (*red*). Pioneer glia display more numerous and elaborate filopodia (*arrow*) than follower glia (modified from Berzsenyi et al., 2011).

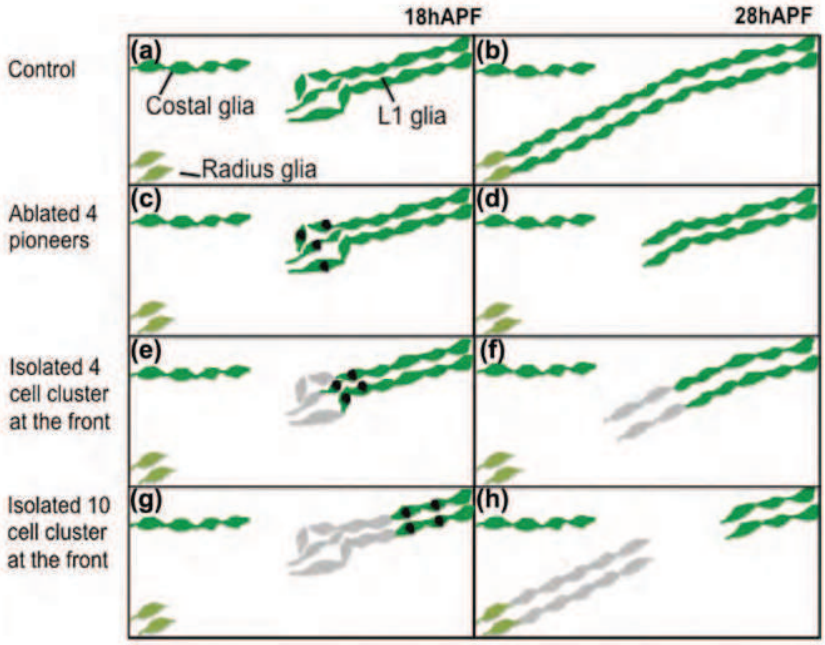

Figure 4. Homeostatic interactions in the migratory glial chain. (**a**) Chain front at around 18 hAPF. (**b**) Completion of migration by 28 hAPF, a different color labels the glia on the radius. (**c, d**) Ablating four cells at the chain front (*black dots*) leads to a migratory delay. (**e, f**) Isolating four glial cells (*gray*) at the chain front upon ablation of more distal cells also induces a migratory delay. (**g, h**) Isolating a cluster of 10 glial cells (*gray*) upon the ablation of further cells reconstitutes an eff cient migratory chain (modif ed from Berzsenyi et al., 2011).

Although we cannot exclude that the pioneer-specif c molecules have not been identif ed yet, it is possible that posttranscriptional modif cations dynamically affect pioneer cells in response to signals present in the environment. Interestingly, mutations altering the activity of growth factors or small GTPases signif cantly affect L1 glia migration eff ciency (Aigouy & Giangrande, unpublished results).

Random single-cell labeling depending on mitotic clones has shown that pioneer cells produce a very long distal cytoplasmic extension (DCE) that spans over several follower cells (Figure 3d') (Aigouy et al., 2008). In contact with the followers, the DCE may serve as a substrate for the followers to migrate along the L1 nerve. In principle, the DCE may also serve for bidirectional interactions within the glial chain, so that followers feed back to the pioneers. This is in line with the observation that pioneer cells in isolation do not migrate and in some cases fail to survive (Berzsenyi et al., 2011). The crosstalk between pioneers and followers likely provides a homeostatic control that f nely tunes collective migration and also guarantees that the pioneers do not leave the collective. The next challenge will be to develop markers to follow individual pioneer dynamics. Single-cell photoactivation approaches or the use of inducible promoters may help labeling specif c pioneer cells.

Quantitative Regulation

One of the most fascinating aspects of collective processes is their eff ciency and coordination. The robustness of these processes implies tight homeostatic regulation within the collective, a phenomenon that just starts being appreciated (see above, Berzsenyi et al., 2011; Theveneau & Mayor, 2013). Coordinated, directional migration seems not an all-or-none process and its eff ciency depends on the number of cells. Ablating one pioneer has a more modest effect than ablating four of them (weaker migratory delay and lower penetrance of the phenotype) (Aigouy et al., 2008), perhaps because only multiple pioneers provide enough forces to drag a large collective of cells.

Similarly, separating groups of cells at the front of the chain from the rest of the glia shows that migration eff ciency increases with the number of cells in the reconstituted collective (Figure 4e–h) (Berzsenyi et al., 2011). Again, large collectives may migrate more eff ciently than small ones because they provide stronger forces to breach the extracellular matrix. Also, small collectives may be unable to interpret shallow gradients of guidance cues that require integration across a tissue's length. Regardless of the molecular processes involved, these f ndings point to the importance of quantitative regulation and that of glial-glial interactions for eff cient migration. The recent development of quantitative approaches and the emerging

photonic technologies will certainly help addressing this important issue in collective cell migration.

Homotypic interactions are necessary all along the collective and glial ablation away from the front affects migration extent. Although glial cells are in contact with each other, removing few glial cells within the chain allows the remaining ones to cover larger distances, suggesting that adhesion between glial cells may quantitatively control the extent of migration as well (Berzsenyi et al., 2011; Aigouy et al., 2004). The importance of N-cadherin–mediated cell-cell adhesion in migration was recently shown in epithelial cells (Shih & Yamada, 2012), although the role and mode of action of these molecule is not fully understood. Cadherins seem to affect wing glial migration as well (Kumar & Giangrande, unpublished results). Time-lapse analyses of conditional mutant animals specif cally affecting cadherins in glia will help clarify the role of these calcium-dependent cell-cell adhesion molecules in collective migration.

Interpreting the Environment

Although homotypic interactions control migration eff - ciency and extent as well as collective integrity, they do not seem to affect the direction of glial migration. During development, glia migrate over the axons navigating towards the CNS, which provide a continuous cellular substrate. Axons do affect directionality in such a way that glia stop moving if the axons can no longer navigate and accumulate at the tip of the stalled nerve bundle (Giangrande et al., 1993). In addition, glia change the direction of migration to follow misrouted axons, con- f rming that the nerve bundle serves as a guiding substrate for glia (Aigouy et al., 2004). Embryonic PG seem to read an axonal gradient of the cell adhesion molecule Fasciclin II (Fas II) (Silies & Klambt, 2010). A similar directional process may apply to wing glia, as Fas II is expressed in wing sensory axons as well (Whitlock et al., 1993). Finally, glia-neuron interactions do not seem to control cell motility per se, as glial cells still move in wings where all the sensory organ cells, including the neurons, have been transformed into glia. In this case, axons never differentiate and glia lay in the vein forming a chain that lacks directionality. Thus, motility seems an intrinsic feature of glial cells (Aigouy et al., 2004).

Future Perspectives

Drosophila wing glia have proven to be a potent tool to characterize the cellular interactions involved in collective migration. We learned that during collective migration, the cells present at the chain front drag the followers and have distinct characteristics (Figures 4a–d, 5a, b). We now also know that the cells migrating in a community affect each other's behavior (Figure 4e–h), and that different types of cell interactions control distinct aspects of migration, thus highlighting the complexity of such collective process. With these data at hand, the analyses of conditional mutants for signaling pathways known to affect collective migration will clarify the relative role of these pathways in specif c steps.

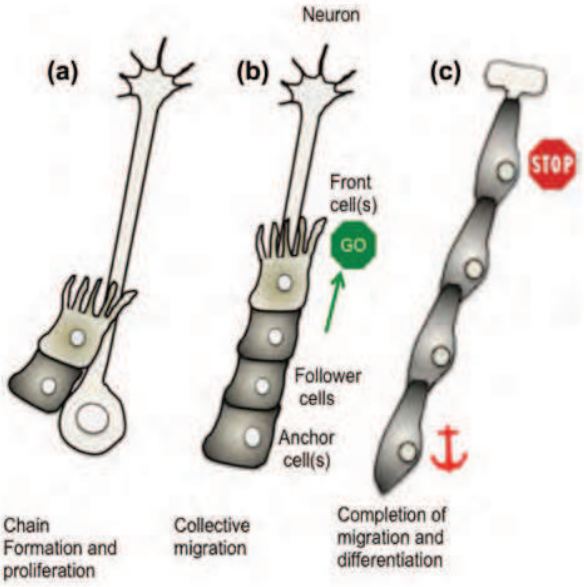

Figure 5. Schematics of glial chain migration. (**a**) The cell present at the chain front contains numerous f lopodia that are continuously exploring the environment. Note that cells at the front and at the rear of the chain contact other migratory cells only on one side, which may trigger asymmetric behaviors. (**b**) The pioneer cell drags the followers and promotes eff cient migration (GO sign and the arrow in green). (**c**) Upon reaching their f nal destination, cells stop migrating (STOP sign in red). The stop signal is unknown as is the mechanism that prevents the rear cell from moving away (ANCHOR sign in red) and leaving the axonal substrate naked (modif ed from Klambt, 2009).

Also, although we start grasping the cues controlling migration initiation, we still do not know what prevents overmigratory phenotypes. Typically, what controls the end of glial chain migration? How do glial cells at the distal edge remain onto that segment of the nerve rather than following more proximal cells? Do these regulatory steps depend on active stop/anchoring signals (Figure 5b, c)? Controlling migration initiation and arrest are equally important events, as leaving the nerve naked is harmful for neuronal function and survival. Future work on the underlying molecular mechanisms in combination to our knowledge on cellular interactions and homeostasis will enable us to have a global view on coordinated cell migration.

Finally, since migration is intimately linked to glial identity, it will be important to assess whether the glial fate determinant coded by the transiently expressed

transcription factor Glide/Gcm (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996) directly impinges onto migration. Microarray data have shown that Glide/Gcm controls the expression of molecules that affect migration (Egger et al., 2002; Freeman et al., 2003, Altenhein et al., 2006); however, this may be an indirect consequence of the fate alteration dependent on Glide/Gcm. Transcription factors Repo, Pointed, and Tramtrack, which are induced by Glide/Gcm, may control the expression of those molecules and hence affect glial migration (Xiong et al., 1993; Halter et al., 1995; Klambt, 1993; Giesen et al., 1997; Yuasa et al., 2003). Future genome-wide studies will assess whether Glide/Gcm directly controls signaling pathways involved in migration such as cell adhesion molecules, chemoattractants/repellants, or growth factors. It is indeed likely that late phenotypes are predetermined as part of cell-intrinsic developmental mechanisms. Relevant to this issue is the finding that the Islet protein, a member of the conserved LIM-homeodomain transcription factor family that contributes to cell specification in the nervous system, regulates potassium channel expression in flies (Wolfram et al., 2012). This elegant work shows that the neuronal electrical properties, which represent late features of a cell population, are dictated autonomously by early cues.

CONCLUSIVE REMARKS

Much progress has been made in understanding the mechanisms regulating cell migration over the past years. Particularly, many guidance cues have been identified for different classes of neurons and glial cells, which in turn has greatly expanded our knowledge of the cell biology of migration. There are, however, old and new questions that remain unanswered. Typically, we still do not know how the various signaling pathways integrate to promote efficient migration.

Also, different cell types have specific migratory potentials and adopt unique migratory strategies; therefore, it is likely that cell type–specific genes will be discovered that integrate positional and migratory cues.

Furthermore, collectives display distinct topologies, chains, sheets, streams, or clusters, depending on the cell type. This likely imposes specific constraints during migration. Sheets, for example, have a very large front of migration compared with chains and the role of pioneers cells may be different, as may also be that of follower cells. Whether distinct organizations involve specific forces and signaling pathways or specific integration of the same forces and pathways awaits the dissection of the homeostatic interactions in the different types of collective migration.

Finally, cells also move as collectives in pathological conditions and this process is highly prevalent in metastases; therefore, understanding cell migration in the context of neural development will significantly enhance our understanding of the molecular prerequisites for collective invasion and their contribution to cancer.

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Chapter 2

Published article: N-cadherin and collective glial migration

This section of my thesis is based on a research article published in the Journal of Cell Science entitled **"N-cadherin negatively regulates collective *Drosophila* glial migration through actin cytoskeleton remodeling" (Kumar et al., 2015)**.

Collective cell migration is a key process during development of the nervous system and drives the formation of many complex networks. Typically, neurons and glia migrate collectively from their birthplace to their final destination. Defects in this process have been associated with many diseases such as cancer and neurological disorders (Friedl and Gilmour, 2009; Klambt, 2009; Rorth, 2009). One of the main features of the collectively migrating cells is that of adhesion, which helps to maintain cell-cell interactions and coordination. Previous studies have shown that Cadherin mediated cell-cell adhesion is implicated in cell migration during morphogenesis and cancer metastasis (Berx and van Roy, 2009; Stepniak et al., 2009). The glial cells in the developing *Drosophila* wing provide a potent tool to study Cadherin-mediated adhesion, as they are closely associated with each other while migrating collectively (Aigouy et al., 2004; Berzsenyi et al., 2011). Hence, we decided to investigate the role of the most abundant classic cadherin molecule, Neural-cadherin (N-cad) in collective glia migration (Fung et al., 2008; Stepniak et al., 2009). We for the first time show that N-cad is expressed in wing glia and that it negatively affects the migration of glial cells by regulating actin cytoskeleton.

This work was carried out in collaboration with a former PhD student. My contribution in this study was to characterize the mutant phenotype of N-cad by performing MARCM and to also analyze the CYFIP mutants in glia migration.

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RESEARCH ARTICLE

N-cadherin negatively regulates collective *Drosophila* glial migration through actin cytoskeleton remodeling

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ABSTRACT

Cell migration is an essential and highly regulated process. During development, glia cells and neurons migrate over long distances – in most cases collectively – to reach their final destination and build the sophisticated architecture of the nervous system, the most complex tissue of the body. Collective migration is highly stereotyped and efficient, defects in the process leading to severe human diseases that include mental retardation. This dynamic process entails extensive cell communication and coordination, hence, the real challenge is to analyze it in the entire organism and at cellular resolution. We here investigate the impact of the N-cadherin adhesion molecule on collective glial migration, by using the *Drosophila* developing wing and cell-type specific manipulation of gene expression. We show that N-cadherin timely accumulates in glial cells and that its levels affect migration efficiency. N-cadherin works as a molecular brake in a dosage-dependent manner, by negatively controlling actin nucleation and cytoskeleton remodeling through α/β catenins. This is the first *in vivo* evidence for N-cadherin negatively and cell autonomously controlling collective migration.

KEY WORDS: Collective migration, glial cells, N-cadherin, actin cytoskeleton, *Drosophila*

INTRODUCTION

Collective migration of neurons and glia cells (clusters, chains, streams and sheets: (Berzsenyi and Giangrande, 2010; Gilmour et al., 2002; Gupta and Giangrande, 2014; Klämbt, 2009; Lemke, 2001; Marín et al., 2010; Rørth, 2003; Valiente and Marín, 2010) implies complex and dynamic cell interactions.

Adhesion molecules play an important role in collective events (Schwabe et al., 2009; Silies and Klämbt, 2010a; Togashi et al., 2009). The cadherin family of Ca^{2+}dependent cell adhesion molecules are primarily involved in homophilic interactions (Arikkath and Reichardt, 2008; Giagtzoglou et al., 2009; Kiryushko et al., 2004) and are required for cell polarity, adhesion and motility (Harris and Tepass, 2010). 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), which promotes the formation of rather small adherens junctions (AJs) and is thought to provide the mechanical basis for static tissue organization (e.g. defined cell arrangement in polarized epithelium) as well as for plastic connections between cells. N-cad has been extensively studied in the context of individual and collective cell migration; however, its precise role is still debated (Asano et al., 1997; Asano et al., 2000; Foty and Steinberg, 2004; Hegedüs et al., 2006; Rappl et al., 2008; Utsuki et al., 2002). Typically, N-cad plays a pro-migratory or an inhibiting role, depending on cell type, on approach (*in vivo* versus *in vitro*, 2D versus 3D assays) and on type of migration. N-cad is necessary in the developing cerebellum of the zebrafish, where it seems to promote differentiation as well as migration of the granule cells (Rieger et al., 2009). However, N-cad expression negatively controls the invasive behavior of gliomas (Péglion and Etienne-Manneville, 2012). The best conditions to understand the relevance of this pleiotropic molecule are the *in vivo* analyses of conditional mutants. For these reasons, we decided to work on a specific type of collective migration called chain migration by using the simple *Drosophila melanogaster* model. Glia cells in the developing wing migrate over the sensory nerve in a chain-like manner. Each member of this cell community is in contact with the neighboring cells, and glia–glia interactions tightly control migration extent, efficiency and coordination (Aigouy et al., 2004; Aigouy et al., 2008; (Berzsenyi et al., 2011). In *Drosophila*, N-cad controls growth cone guidance and axon bundle fasciculation (Iwai et al., 1997); however, it had been suggested that N-cad is not expressed in glia cells (Fung et al., 2008; Iwai et al., 1997). Here, we show that N-cad is dynamically and uniformly expressed in wing glial cells, and that it is necessary for the timely and efficient migration of the chain along the axon bundle. Glial-specific N-cad overexpression severely slows down the initiation of chain migration without affecting the number of AJs. Accordingly, N-cad downregulation triggers the opposite phenotype. Increased N-cad in glial cells enhances the accumulation of β-catenin (β-cat) and α-catenin (α-cat), which has been shown to control actin dynamics by preventing actin nucleation (Shapiro and Weis, 2009). As a consequence, fewer and less-dynamic filopodia are formed in the glial chain. Furthermore, increasing the levels of CYFIP (officially known as Sra-1), a member of the WAVE/SCAR actin nucleation complex, counteracts the effects of N-cad overexpression on glial migration and on actin dynamics. Our *in vivo* study here clarifies the role of N-cad in collective migration and helps to understand the impact of this molecular pathway in morphogenesis and cancer metastasis (Berx and van Roy, 2009; Stepniak et al., 2009).

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

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and move towards the central nervous system (CNS) (Fig. 1) (Giangrande et al., 1993; Murray et al., 1984; Van De Bor et al., 2000). The chain starts moving at ~18h after puparium formation (hAPF) and completes migration by 29 hAPF, upon reaching the proximally located radius glia (Berzsenyi et al., 2011). These glia are in contact with each other and need to interact, suggesting a role for cell adhesion molecules. We, therefore, analyzed the developmental profile of N-cad expression in wing glia (Fig. 1A-M) by using the *repoGal4*glial driver and a UAS GFP reporter (hereafter referred to as *repo >GFP*). N-cad starts being expressed in glial cells at around 17 hAPF (Fig. 1A,B) – just before glia start moving from distal to proximal regions (Fig. 1I) (Aigouy et al., 2004) – and continues to be expressed until the end of migration (29–30 hAPF) (Fig. 1A-H, see white arrows and arrowheads, and red arrowheads in the color-coded panels B′,D′,F′,H; supplementary material Fig. S1A). N-cad seems evenly distributed in the glial cells and is present all along the L1 chain (Fig. 1; supplementary material Fig. S1B,C). In addition, it also accumulates in the axons throughout development (Fig. 1, white and black asterisks).

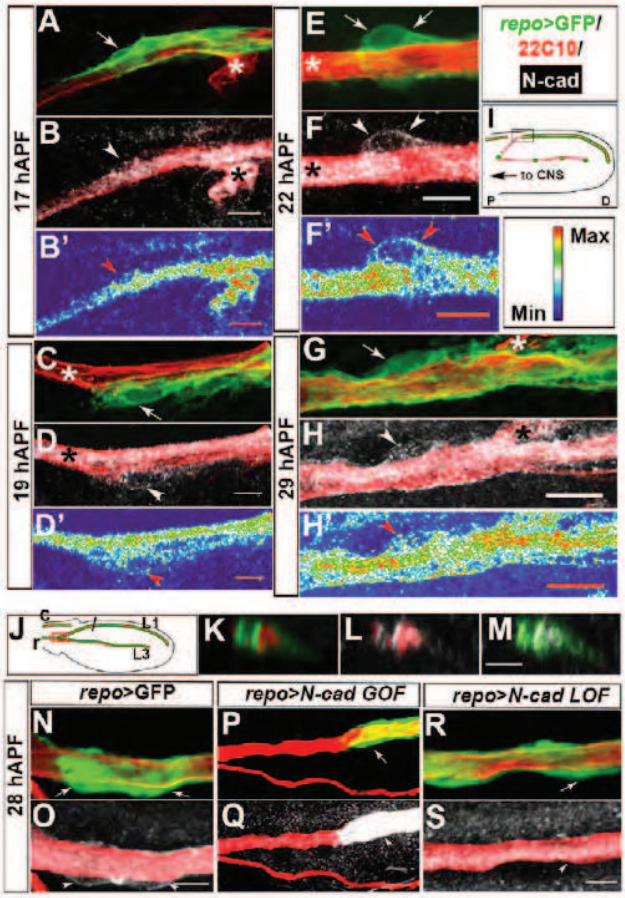

Fig. 1. Expression of N-cad in wing glia during development. (A-H′) Wings at different stages, immunolabeled with anti-22c10 to stain neurons (red), anti-N-cad (white) and anti-GFP (glial cells, green) in the transgenic line *repo >UAS PHGFP* (*repo >GFP*). Maximum confocal projections are shown in all figures, unless otherwise specified. Arrows indicate the glial cells, arrowheads (white and red) the glial N-cad labeling. White asterisks indicate neurons, black asterisks the N-cad signal in those cells. Panels B′,D′,F′ and H′ are the heat maps showing the N-cad expression levels from minimum (dark blue) to maximum (red). By 17 hAPF N-cad starts to accumulate in glia cells, which becomes more prominent at later stages (I,J) Schematic drawings of wings at 19 hAPF (I) and 29 hAPF (J). Neurons are in red, glia in green. c, costa; r, radius nerve; L1 and L3, L1 and L3 nerves (Murray et al.,1984); P and D, proximal and distal regions, respectively. (I) Inset indicates the region shown in panels (A-F′). Inset in J indicates the region shown in G-H′. (K-M) Single optical *z*-cross section of the L1 nerve at 29 hAPF, taken at the position shown in J, immunolabeling and genotypes as above. (N-S) N-cad expression (white arrowheads) in 29 hAPF wing glia (white arrows) of the following genotypes: (N,O) *repo >GFP* (control), (P,Q) *repo >N-cad GOF* and (R,S) *repo >N-cad LOF*. Note the elevated glial N-cad levels in *repo >N-cad GOF* and the reduced N-cad glial levels in *repo >N-cad LOF* compared to those found in *repo >GFP*. Scale bars: 5 µm (A-D′, K-M), 10 µm (E-H′), 5 µm (N-S).

Further, we found that the other abundant AJ-forming cadherin, E-cadherin, is neither expressed by wing glia or neurons (supplementary material Fig. S1D-F) nor does it affect glia migration upon downregulation (supplementary material Fig. S1G). Absence of E-cad and abundance of N-cad suggests that N-cad plays a crucial role in migratory wing glia.

The levels of N-cad affect migration efficiency

Because the expression of N-cad is not restricted to glia, we specifically modified N-cad levels in these cells by using the *repo* >driver and established a protocol in which we to compare glial migration efficiency in different genetic backgrounds. For each genotype, we dissected $n \geq 40$ pupae and compared the percentage of wings that displayed completed glial migration to that observed in control wings (*repo* >GFP). This value, which we define as the migratory index, provides a quantitative estimation of migration efficiency. To score for increased and decreased migration efficiency upon altering N-cad levels, we analyzed a stage at which migration is not fully achieved in control animals and calculated the migratory index by 25 hAPF (Fig. 2A, light grey column).

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 of N-cad levels in glia (Fig. 1N-Q). N-cad overexpression significantly decreases glial migration efficiency compared to that observed in control wings (Fig. 2B-G,A, light grey and dark grey columns).

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 in glial cells but not in neurons, which also confirms the efficiency of the used *RNAi* transgene (Fig. 1R,S). By counting the number of *repo >N-cad LOF* wings showing completed glial migration, we observed a significant increase in migration efficiency compared to that observed in control wings (Fig. 2A, red column). Importantly, when we co-expressed the *UAS N-cad* and the *UAS N-cad RNAi* transgenes (*repo >N-cad GOF & LOF*), migration efficiency was restored to control levels, confirming that N-cad overexpression has a regulatory role and that the RNAi effects are specific (Fig. 2A, grey/red striped column).

Glial cells proliferate as they move; therefore, their number increases over the migratory period and cell death occurs only occasionally (Aigouy et al., 2004). To assess whether *N-cad* overexpression also affects the number of glia – which may, in turn, indirectly affect glial migration – we used the panglial nuclear marker Repo (Halter et al., 1995; Xiong et al., 1994) and counted the cells present along the L1 nerve of control and *repo >N-cad GOF* wings at 29 hAPF. N-cad overexpression does not alter the number of glia (control: 90±6 cells; *repo >N-cad GOF*: 85±10 cells, $n=5$, $P=0.42$), indicating that the migratory phenotype

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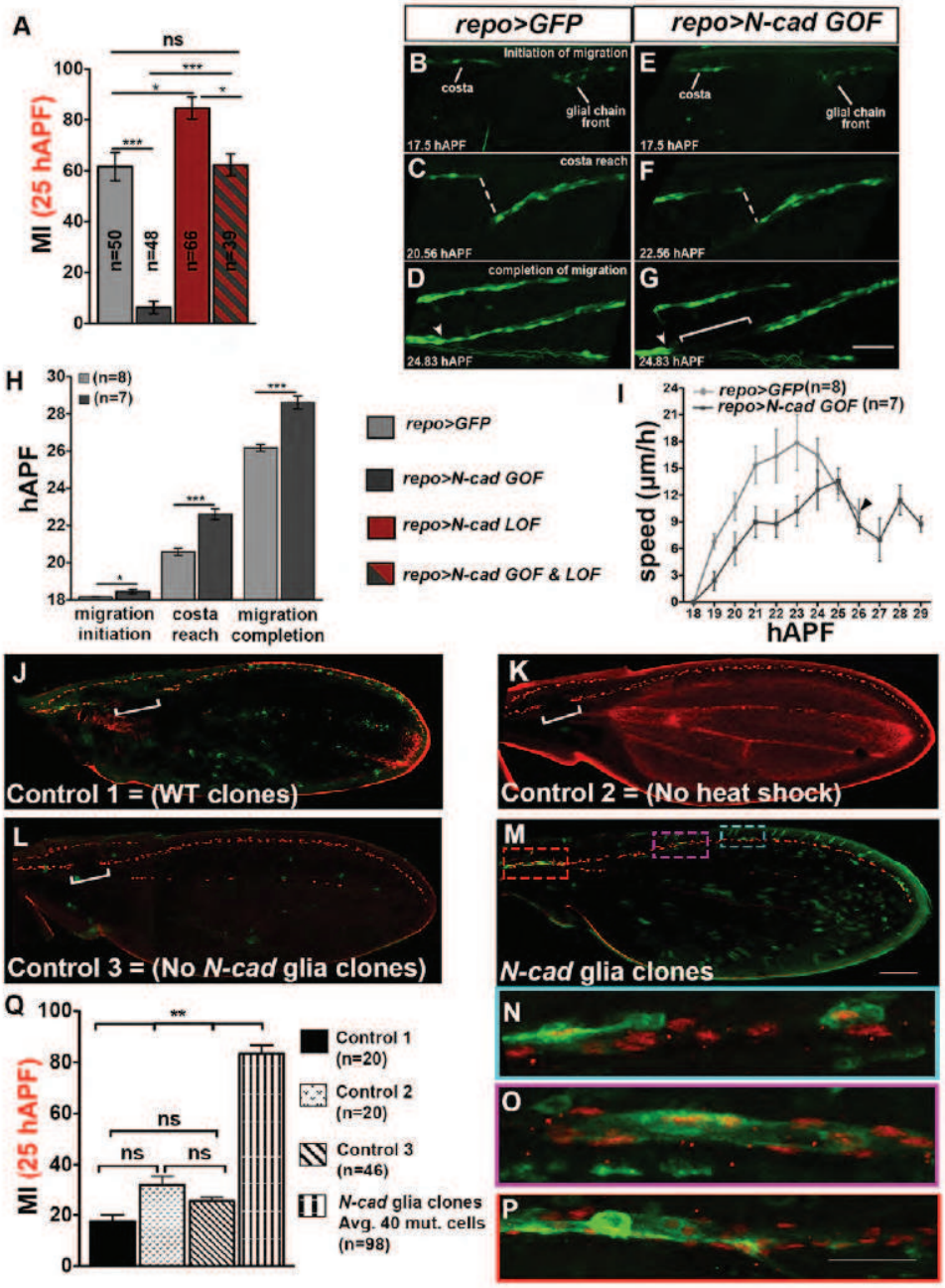

Fig. 2. See next page for legend.

observed in *N-cad GOF* wings is not due to changes in the chain size. We next asked whether N-cad acts cell autonomously in glial migration by analyzing the morphology of the axons in *repo >N-cad GOF* and *repo >N-cad LOF* wings. Since the axons grow in close proximity to glial cells, the excess or reduction of *N-cad* in glia might influence the navigation of the neuronal processes, which might trigger indirect glial migration defects. We compared the advancement of axon growth in *GOF*, *LOF* as well as in control wings and observed no detectable difference in axon/bundle morphology and organization (supplementary material Fig. S2A-D).

Finally, to further validate the migratory phenotype obtained in the conditional GOF and LOF experiments, we generated 'mosaic analysis with a repressible cell marker' (MARCM) clones (Lee and Luo, 2001) in the developing wing by using a *N-cad*-null allele (Iwai et al., 1997). By 25hAPF, wings carrying *N-cad* mutant clones clearly showed an increase in glial migration efficiency, as observed in *repo >N-cad LOF* wings (Fig. 2J-P, the clones carried on average 40 *N-cad* mutant glial cells on the L1 nerve). This phenotype is not observed in control wings that do not carry mutant clones or that carry clones that do not contain mutant glial cells. The results are quantified in (Fig. 2Q). These observations clearly demonstrate that the glial levels of N-cad specifically affect the efficiency of chain migration.

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Fig. 2. Role of N-cad in wing glia during development. (A) Glial cell migration phenotype upon N-cad overexpression (*GOF*) and downregulation (*LOF*) in glia using the *repo >GFP* line; see color coding in panel (H). The migratory index (MI) was calculated by counting the number of wings displaying completed migration (i.e. glial chain reaching the proximally located glia on the radius nerve) by 25 hAPF. (B-G) Snapshots at different stages from time-lapse analyses on *repo >GFP* and *repo >N-cad GOF* wings: (B,E) initiation of migration, (C,F) reaching the level of the costa and, (D,G) completion of migration. Note the gap in the wing overexpressing N-cad (bracket), between the L1 glia and the glia on the radius (arrowhead). In these and in the following panels, the nuclear and cytoplasmic GFP transgenic line (*UAS ncGFP*) was used, unless otherwise specified. (H) Quantification of the migratory behavior of control (light grey) and N-cad-overexpressing glia (dark grey) at the three phases highlighted in (B-G). (I) Graph shows the speed of the most-proximal glial cell of the chain (μm/h, *y*-axis; hAPF, *x*-axis) in control and in N-cad-overexpressing glia. The distance covered by the front cell is measured by analyzing the position of the glial soma. Arrowhead points to the completion of migration in control wings, which takes place slightly earlier *in vivo* than in fixed wings. (J-P). Low and high magnification confocal acquisitions of wings obtained for MARCM analysis. (J) Control 1: wild-type wing carrying wild-type clones in glia and showing incomplete migration at 25 hAPF (here, green cells are wild type like the rest of the wing tissue). Animals for control 1 were obtained by crossing *FRT40A* flies with *y,w,hsFLP; FRT40A,tubPGal80/(CyO,ActGFP); tubPGal4,UASmCD8::GFP/TM6,Tb,Hu* flies. (K) Control 2: *N-cad/+* wing without heat shock (i.e. no clones), which shows incomplete migration at 25 hAPF. (L) Control 3: Control 3 is a composite image to cover the whole wing. *N-cad/+* wing with no mutant clones in glia, also showing incomplete migration at 25 hAPF. (M) *N-cad/+* wing carrying mutant clones in glia (*N-cad* mutant glia are in green). Animals for control 2, control 3 and the *N-cad* glia clones were obtained by crossing *N-Cad^{M19}FRT40A* flies with *y,w,hsFLP;FRT40A,tubPGal80/(CyO,ActGFP);tubPGal4,UASmCD8::GFP/TM6,Tb,Hu* flies. The dashed rectangles (cyan, magenta and blue) indicate the regions enlarged in panels N, O and P, respectively. (Q) Quantitative analysis of the indicated genotypes. The white bracket in panels J-L indicates the gap between the L1 and the radius glia. ***P<0.0001; **P<0.001; *P<0.05; ns, not significant. Bars indicate the +s.e.m.; *n*, the number of samples. Scale bars: 50 μm (B-G, J-M), 80 μm (N-P).

High N-cad levels slow down the migration of the glial chain

To gain insights into the kinetics of glial migration in control wings and in N-cad-overexpressing wings, we followed the 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 costa; the latest phase refers to migration completion, upon connection of the chain with the proximal glia located on the radius nerve (Fig. 2B-D). N-cad-overexpressing glia start migrating later than control glia and seem to accumulate a delay in the early migratory phases (see Fig. 2H, initiation of migration and reaching the costa). This delay remains detectable until the late phases of migration. On average, the L1 chain reaches the level of the costa and completes migration 1.3 hours later than the control glia (Fig. 2E-G,H). To further quantify migration efficiency, we determined the average distance covered each hour by the first cell soma at the front of the chain. In control animals, the speed of migration increases progressively until 23 hAPF and, then, slows down until migration completion (Fig. 2I). This late slow phase, suggests that the chain front is able to sense some kind of stop signal(s).

When compared to control wings, glial cells overexpressing *N-cad* move at a lower speed when they begin to migrate but accelerate over time. Interestingly, a late slow phase is still detectable, although it is delayed (25 hAPF, Fig. 2I), supporting the view that the chain front may sense the proximity of target cells and that this process is not affected by N-cad. In sum, these data suggest that appropriate levels of N-cad in glial cells control the initiation and speed of the chain.

Ultrastructure of the wing nerve and glial cells

Cadherins constitute structural components of the AJs (Niessen and Gottardi, 2008), stable structures that ensure cell adhesion. It is, therefore, possible that the number of 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 analyzed whether *N-cad* overexpression produces morphological alterations at the ultrastructural level and compared the number of AJs that are present in control, *repo >N-cad GOF* and *repo >N-cad LOF* nerves. The fly wing is composed of two juxtaposed layers of epithelial cells, which are separated at the position of five hemolymph filled 'veins', two of which – L1 and L3 – are innervated (Murray et al., 1984) (Figs 1, 3A′). The L1 nerve bundle is composed of several hundred axons and is wrapped by the glial cytoplasmic processes (Fig. 3A). We analyzed eight control wings and eight N-cad-overexpressing wings (60 sections per genotype), as well as seven control wings and N-cad-downregulated wings (25 sections per genotype), then we calculated the percentage of the sections possessing zero to five AJ(s) between L1 glial cells (Fig. 3B). Overall, N-cad-overexpressing wings show a comparable number of AJs to those of control wings. In addition, 50% of the sections display 1–3 AJs between glial cells upon N-cad downregulation, indicating that AJs are still formed, which is in agreement with the observation that, in this background, the L1 chain does not seem loosened compared to that of wild-type wings. Using the same approach, we counted the number of AJs present between glia and axons, and found no significant difference upon changing N-cad levels either (supplementary material Fig. S3A,B). In sum, the manipulation of N-cad levels affects migration efficiency in the absence of significant AJ defects.

N-cad affects migration through catenins

The above data prompted us to analyze the impact of N-cad overexpression on intracellular signaling. One of the key factors associated with classic cadherins in *Drosophila* are β-catenin (β-cat) or Armadillo (Arm) (Yap et al., 1997). First, we immunolabeled *repo >GFP* control animals with an anti-Arm antibody (Fig. 4A-F). As expected, Arm is expressed in the wing epithelium (Fig. 4B, asterisks). Glial Arm labeling is relatively weak, compared to that of N-cad and could be best visualized at the glial membrane that faces the vein lumen (Fig. 4B,C; double headed arrows and arrowheads). We next analyzed the effects of *N-cad GOF* on Arm in the glial cells. To compare the Arm expression profile and levels in control and *repo >N-cad GOF* glial cells (Fig. 4G-L), we ran the experiments in parallel and used the same confocal parameters (which explains why Arm and N-cad levels seem lower in Fig. 4H,I than in Fig. 4B,C, respectively). Arm accumulates at high levels at the cell membrane in N-cad-overexpressing glial cells (Fig. 4K, double-headed arrows). 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). The levels of Arm depend on the N-cad doses, which we verified by using a weaker *repo* >driver (supplementary material Fig. S2E-H, red in E,F indicates high

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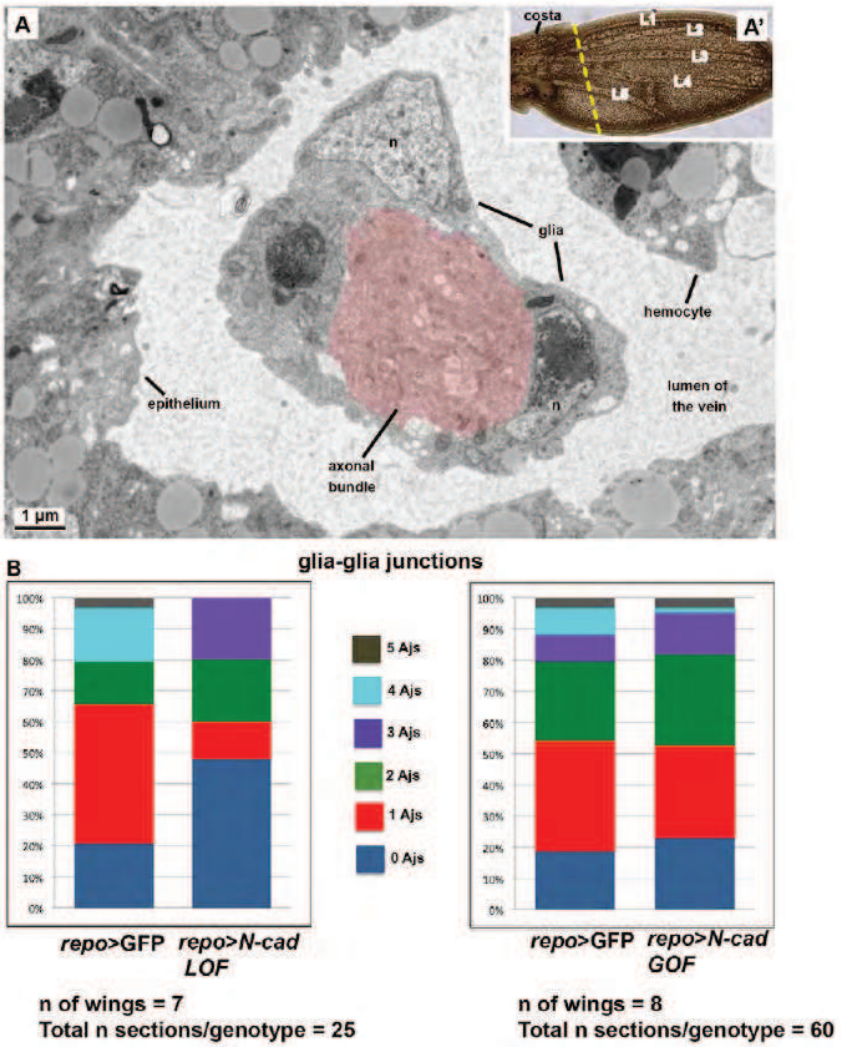

Fig. 3. Ultra-structural analysis of the L1 wing vein. (A) Ultra-thin cross-section of the L1 vein at 29 hAPF at the level of the yellow dashed line indicated in the inset A′. The L1 axonal bundle (pink shading) is surrounded by glial cells. *n*, nuclei of the glial cells. (B) Quantification of the glia–glia AJs in *repo >GFP* (control), *repo >N-cad LOF* and *repo >N-cad GOF* L1 cross-sections. L1 to L5 indicate the five veins present on the wing blade.

levels of Arm). These data strongly suggest that the N-cad-mediated phenotypes involve Arm.

Arm also acts in Wnt/Wg signaling and can be detected at the cell membrane as well as in the cytoplasm and in the nucleus, where it functions as a transcription cofactor (Pai et al., 1997; Peifer et al., 1994). Its actual localization and concentration within the cell eventually defines which function of β-cat/Arm dominates, because cell membrane localization links Arm to the cadherins, whereas cytoplasmic and nuclear localizations links it to Wnt/Wg signaling. The fact that glial cells do not show nuclear accumulation of Arm (Fig. 4A-F and data not shown), suggests that Wg signaling is not very prominent upon *N-cad* overexpression. Nevertheless, to clarify the mode of action of Arm in collective migration, we overexpressed it in glial cells, which leads to its strong accumulation in the nucleus (compare Fig. 4M,N with Fig. 4J,K). Interestingly, under these conditions, glial migration efficiency is similar to that of control wings both at early and late stages (Fig. 4O,P). Thus, the migratory effects of N-cad overexpression seem not associated to Wg signaling.

Drosophila N-cad is a multi-domain transmembrane protein whose extracellular region contains 16 cadherin repeats and whose 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*) (Yonekura et al., 2007) (Fig. 5A) under the control of the *repo* >driver. Whereas overexpression of the full-length *N-cad* transgene triggers high levels of N-cad and Arm (Fig. 5E-G, arrowhead and double-headed arrow, respectively), overexpression of the *N-cad ΔArm* transgene results in high levels of N-cad but normal levels of Arm (compare Fig. 5B-D with Fig. H-J). Moreover, overexpressing *N-cad ΔArm* in glia does not affect migration efficiency (Fig. 5K). We conclude that the cytoplasmic domain of N-cad participates in regulating glial cell migration via Arm.

β-cat binds α-cat, the main mediator between cadherins and actin that is able to bind both β-cat and actin molecules (Drees et al., 2005) (Rimm et al., 1995). As α-cat provides a bridge between the cell membrane and the cytoskeleton (Benjamin and Nelson, 2008), we analyzed the impact of this molecule onto migration and its genetic interaction with N-cad. Overexpression of α-catGFP fusion protein in glia (*UAS α-catGFP* transgene (Caussinus et al., 2008), Fig. 6A) delays migration (Fig. 6G, compare blue with light-grey column), which is rescued by downregulation of *N-cad* in the same background (*α-catGFP GOF*, *N-cad LOF*, Fig. 6C,D,G, red/blue-striped column). These findings are in line with the observation that *N-cad LOF* accelerates migration (Fig. 6G, red column). Furthermore,

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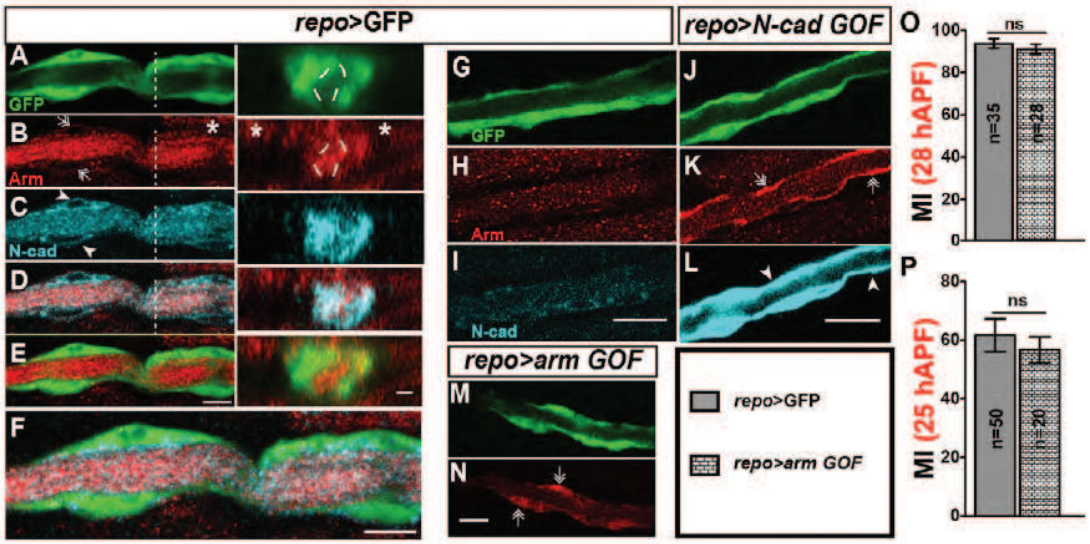

Fig. 4. Expression of Arm and its role in glia migration. (A-F) 29 hAPF *repo >GFP* wings labeled for GFP (green), Arm (red) and N-cad (cyan). Double-headed arrows and arrowheads show Arm (B) and N-cad (C) accumulation on glial membranes, respectively. Dashed line indicates the position of the cross-section shown in the right panels (A-E). (D) Merge of panels B and C. (E) Merge of panels A and B. (F) Merge of panels A,B and C. Arm is also localized in the wing epithelial cells (B, asterisks). (G-L) Comparison of Arm labeling in control and *N-cad GOF* glial cells. (J-L) Arm accumulates at high levels at glial membranes of a *N-cad GOF* wing (K). Note that the acquisition in these control and overexpressing wings was done under the same conditions and at much lower laser intensity compared to that used in panels A-F, which would have led to a saturated signal in N-cad-overexpressing wings. (M,N) *arm GOF* 25 hAPF wing showing nuclear localization of Arm. (O,P) Graphs show the migratory index (MI) upon Arm overexpression in glial cells compared to that observed in *repo >GFP* (control) animals at late and early stages. Note that in the following figures 5-7, the migratory index was analyzed at only 28 hAPF. Scale bars: 5 µm (A-F), cross-sections: 1 µm; 10 µm (G-L, M,N).

overexpressing *α-catGFP* in a *UAS N-cad* background (*α-catGFP GOF, N-cad GOF*, Fig. 6E-G grey/blue-striped column) aggravates the N-cad overexpression phenotype (*N-cad GOF*, Fig. 6G, dark-grey column). Finally, decreasing the levels of α-cat in a *UAS N-cad* background rescues the migratory delay induced by N-cad overexpression (*N-cad GOF, α-cat LOF*, Fig. 6G, grey/pink-striped column). Such genetic interactions confirm that α-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 α-cat are affected by N-cad, as shown by immunolabeling and western blot analyses. GFP was used to monitor α-cat expression in flies that carry the gene fusion *α-catGFP*. The levels of the GFP increase in those animals that also overexpress N-cad (*α-catGFP GOF N-cad GOF*) and decrease in animals that express low levels of N-cad (*α-catGFP GOF, N-cad LOF*) (Fig. 6A-F see arrowheads, S2J). Interestingly, we did not observe a significant difference in *α-cat* mRNA levels in *repo >α-catGFP GOF, N-cad GOF* or *N-cad LOF* backgrounds compared to those observed in the control *repo >α-catGFP GOF* animals (supplementary material Fig. S2I). The change in protein but not in RNA levels indicate that N-cad regulates α-cat post transcriptionally, perhaps by stabilizing the protein. Thus, the levels of α-cat change according to Arm in response to different N-cad levels through a post-transcriptional process.

Rescue of actin cytoskeleton remodeling restores glial migration

It has been proposed that, at high levels of cadherin, α-cat accumulates at the membrane, hence increasing the pool of α-cat molecules (Benjamin and Nelson, 2008). As a consequence, α-cat homodimers form and bind actin filaments, thereby preventing the activity of the ARP2/3 complex (Drees et al., 2005). The latter is known to nucleate the branched actin filaments at the cell leading edge and to promote migration (Rotty et al., 2013). Thus, the migratory phenotype induced by altered N-cad levels may be due to defects in the actin nucleation pathway.

Nucleating complexes such as WAVE/SCAR, of which the CYFIP/Sra1 (CYFIP) adaptor is an integral member, activate the ARP2/3 complex (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 counteracts the defects induced by high levels of N-cad. Indeed, glial migration efficiency is completely rescued in wings that overexpress both CYFIP and N-cad (Fig. 7A, compare green/grey-striped column with dark-grey and light-grey columns). This is also in line with CYFIP acting downstream of N-cad. In addition, downregulation of CYFIP in *repo >N-cad GOF* animals aggravates the migratory defect observed in wings that overexpress N-cad alone (Fig. 7A, compare grey/blue-striped column with dark-grey column). Downregulation of CYFIP in an otherwise wild-type background only slightly reduces migration efficiency (Fig. 7A, compare blue with light-grey column), suggesting that CYFIP is not present in limiting amounts, in agreement with the finding that overexpressing CYFIP in an otherwise wild-type background has no effect on glial cell migration (Fig. 7A, compare green with light-grey column).

To further validate our data, we analyzed the genetic relationship between CYFIP and N-cad using a mutant $CYFIP^{485.1}$-null allele (Schenck et al., 2003). Because CYFIP is widely expressed and required, its complete lack is lethal. However, previous data have demonstrated that heterozygous (*CYFIP het*) animals already show mutant phenotypes (Schenck

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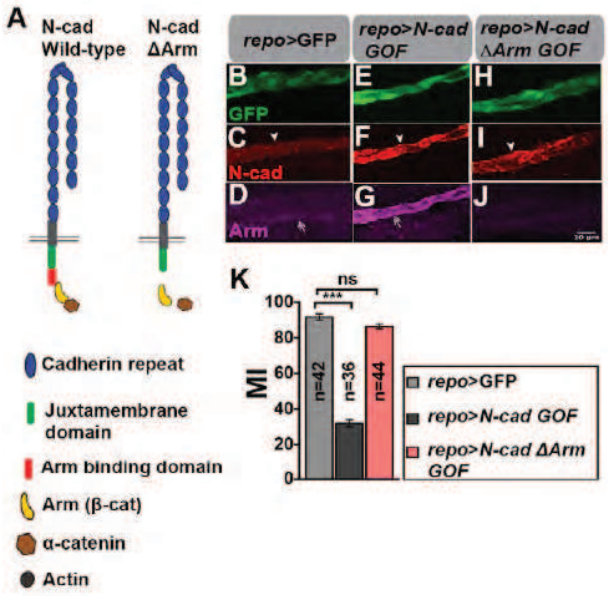

Fig. 5. Deletion of the Arm binding domain of N-cad and the effect of it. (A) Illustration of wild-type N-cad (left) and the *N-cad ΔArm* construct in which the Arm-binding domain is deleted. (B-J) Immunolabeling for GFP (green), N-cad (red) and Arm (magenta) in control wings and in wings overexpressing the *N-cad* transgenes (*N-cad* full-length and *N-cad ΔArm*) under the *repo* >driver. *N-cad GOF* glia display elevated N-cad and Arm levels (compare arrowhead and double-headed arrow in F,G and C,D), whereas in *N-cad ΔArm GOF* glia no Arm can be detected (J). (K) Glial migratory index (MI) in the indicated genotypes. Scale bar: 10 µm (for B-J).

et al., 2004) and we, indeed, found that *CYFIP het* reduces migration efficiency without reducing the number of glial cells (Fig. 7B, yellow column), allowing us to extend the genetic interaction analyses. We reasoned that, if downregulation of N-cad enhanced migration efficiency by enhancing actin nucleation, *CYFIP het* animals that express low levels of N-cad would show an attenuated phenotype. Indeed, in *CYFIP het*, *repo >N-cad LOF* wing glia migrate like in the control wings (Fig. 7B, compare red/yellow striped column with yellow column), although they still differ from glia that only express low levels of N-cad (Fig. 7B, compare red/yellow-striped column with red column), which may have several explanations. For example, CYFIP-independent pathways might also be active or removing one copy of *CYFIP* gene might not be enough to compensate for the vast excess of CYFIP available following knockdown of N-cad. The data that stem from using the *CYFIP*-null allele, nevertheless confirm the hypothesis that N-cad counteracts actin cytoskeleton remodeling, which was further validated by analyzing *CYFIP het*, *repo >N-cad GOF* wings. As predicted from the conditional mutant backgrounds, removing one copy of *CYFIP* aggravates the migratory phenotype triggered by increasing N-cad levels (Fig. 7B, compare grey/yellow-striped column with Fig. 7A dark-grey column). The phenotype is even stronger than that obtained with *CYFIP RNAi*, perhaps because in the latter case *CYFIP* is only downregulated in glia (Fig. 7B, compare grey/yellow-striped column with grey/blue-striped column). All together, these data show that CYFIP plays an important role in the N-cad cascade affecting collective migration.

Finally, we investigated the different migratory phases by using time-lapse confocal microscopy in order to analyze the kinetics of the *N-cad CYFIP* interactions *in vivo*. At all stages, migration efficiency is similar in wings overexpressing both N-cad and CYFIP compared with control wings (Fig. 7C, compare light-grey with green/grey-striped columns). In contrast, overexpressing N-cad alone results in delayed migration in all phases (Fig. 7C, compare dark-grey with light-grey columns). Overexpressing CFYIP progressively rescues N-cad overexpression: at the initiation phase, it mildly improves the migratory defect induced by N-cad overexpression and the rescue becomes more effective at later phases (Fig. 7C, compare dark-grey with green/grey-striped columns, initiation phase: $P=0.132$; reaching the costa nerve: $P=0.002$; completion of migration: $P=0.0003$). As expected, downregulation of N-cad accelerates all phases of migration (Fig. 7C, compare light-grey with red

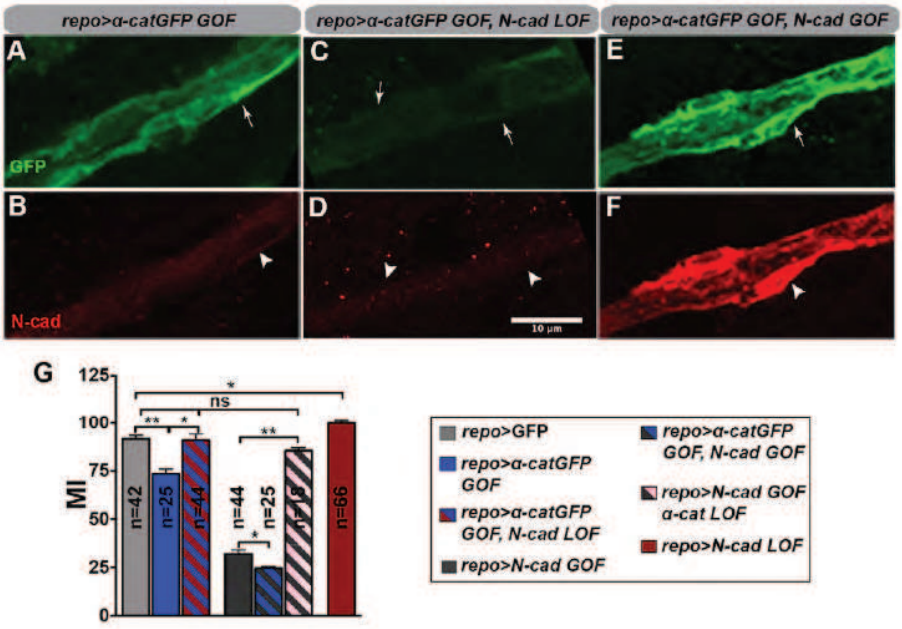

Fig. 6. Distribution and role of α-cat upon *N-cad* gain and loss of function. (A-F) 28 hAPF wings showing α-catGFP (green) and N-cad (red) expression upon *N-cad GOF* and *N-cad LOF* using the *repo >α-catGFP* transgene. Note the reduced amount of GFP *in repo >α-cat GFP; N-cad LOF/+* (C,D) and the elevated levels of GFP in *repo >α-catGFP; N-cad GOF/+* (E,F) glial cells compared to *repo >α-catGFP/+* control wing (A,B). (G) Migratory index (MI) in the indicated genotypes. Scale bar: 10 µm (for A-F).

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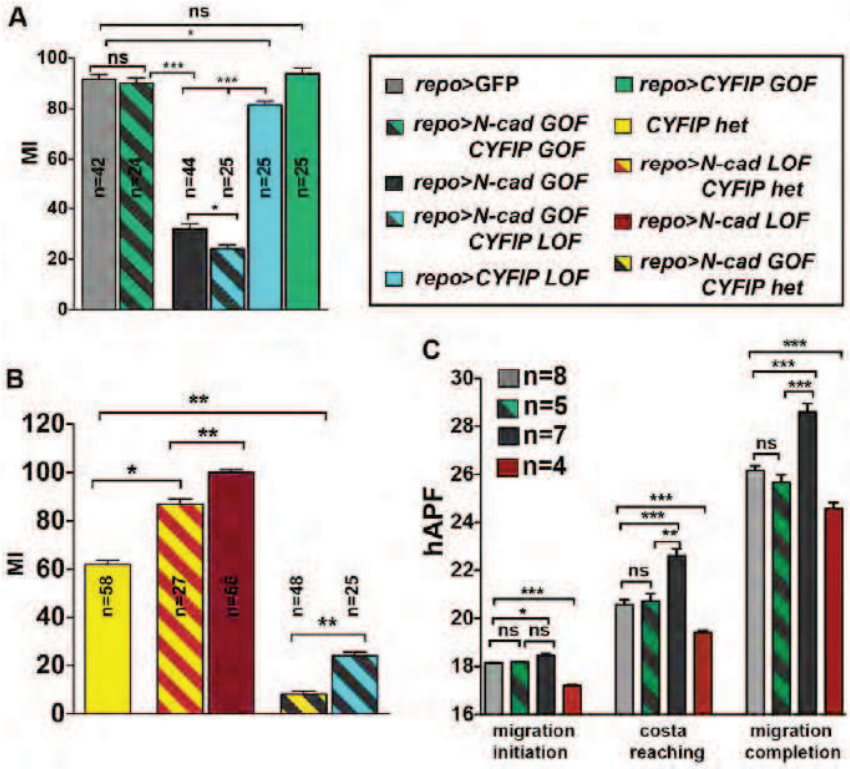

Fig. 7. CYFIP rescues N-cad-mediated migratory defects. (A,B) Migratory index (MI) in the indicated genotypes. (C) Quantification of the migratory behavior at the three phases in the indicated genotypes.

columns). In sum, promoting actin nucleation counteracts the effects of high N-cad levels during collective migration.

N-cad affects actin cytoskeleton remodeling

The above data highlight the role of N-cad in signaling to α-cat and indicate that the migratory phenotypes depend on actin cytoskeleton defects. To investigate these phenotypes *in vivo*, we followed the organization of the actin cytoskeleton at the stage at which cells start to migrate. We used the *UAS actin42AGFP* transgene under the control of the *repo* >driver and focused on the cells at the front of the chain, where the behavior of filopodia can be more easily scored and is more prominent. Using fast time-lapse confocal microscopy, we analyzed control wings (*repo >actin42AGFP*), N-cad-overexpressing wings (*repo >actin42AGFP; N-cad GOF*), wings that overexpress both N-cad and CYFIP (*repo >CYFIP GOF, N-cad GOF*) and wings that express low N-cad levels *(repo >actin42AGFP; N-cad LOF)*. Filopodia are more static in N-cad-overexpressing than in control glial cells, a phenotype that is rescued when CYFIP is reintroduced (Fig. 8A-C). The most dynamic filopodia are of glial cells that express low levels of N-cad (Fig. 8D).

Next, we quantified two parameters, the number of filopodia and their length, in the four genetic backgrounds. To achieve this, we analyzed the cell somata at the migration front and, for each cell soma, we measured the length of single filopodia as well as the number of filopodia (see graphs in Fig. 8E,F).

The number of filopodia significantly decreases in *N-cad GOF* compared to that observed in control glial cells and the cytoplasmic processes are also considerably shorter in *N-cad GOF* glia (Fig. 8A,B,E,F). Conversely, downregulation of N-cad in glia results in increased filopodia length (Fig. 8D,E). This difference is not due to the number of glia at the front of chain, which does not change in the four indicated genotypes (Fig. 8G).

Quantitative analyses in animals overexpressing both N-cad and CYFIP revealed that the number and the complexity of filopodia are significantly different from those observed upon overexpression of N-cad alone, thus, *CYFIP* rescues the N-cad-overexpression phenotype, at least in part (Fig. 8C,E,F). Panels H and I in Fig. 8 summarize the quantitative data on filopodia length and on the number of filopodia per soma in the indicated genotypes (Fig. 8, H and I).

Taken together, these results show that the levels of N-cad tightly regulate CYFIP-mediated actin cytoskeleton remodeling in migrating glial cells.

DISCUSSION

Cadherins act in many biological processes including collective migration, a dynamic event that involves three-dimensional constraints. To grasp the role and mode of action of cadherins in collective migration, the use of animal models and accurate genetic analyses are of paramount importance. We here show that N-cad is expressed in the glial cells present in the developing wing of *Drosophila* and that it negatively regulates chain migration efficiency by controlling actin dynamics.

N-cadherin and glial migration

Glial cells constitute a highly motile population. They migrate extensively during development and even some adult glial cells do so: astrocytes in the mammalian brain, for example, migrate to lesions caused by injury or neurodegeneration, a process commonly known as reactive astrogliosis (Sofroniew and Vinters, 2010). Tumor glial cells move through the nerve tissue, leading to formation of glioma, the most aggressive form of cancer in the nervous system (Cayre et al., 2009). In addition, glia of the peripheral nervous system (PNS), such as vertebrate perineurium and Schwann cells (Klämbt, 2009), as

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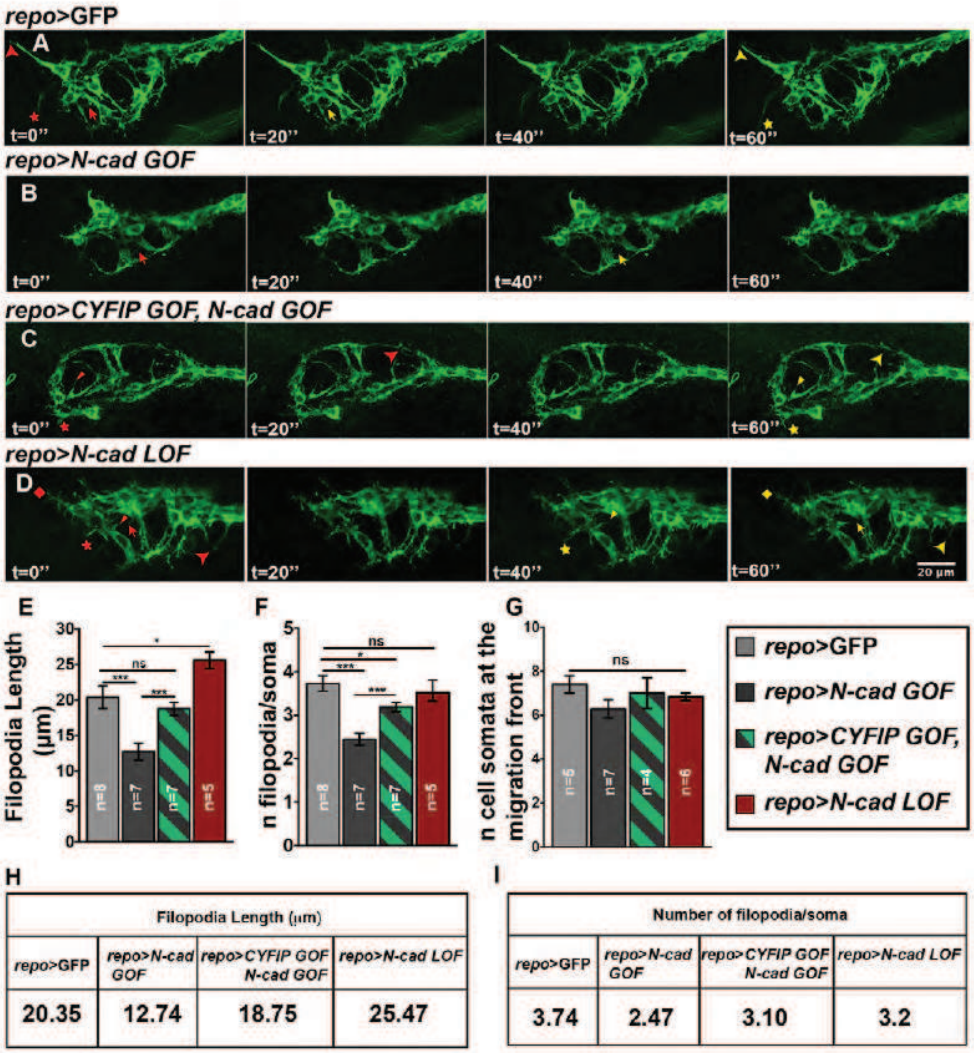

H

Filopodia Length (µm)			
repo>GFP	repo>N-cad GOF	repo>CYFIP GOF N-cad GOF	repo>N-cad LOF
20.35	12.74	18.75	25.47

I

Number of filopodia/soma			
repo>GFP	repo>N-cad GOF	repo>CYFIP GOF N-cad GOF	repo>N-cad LOF
3.74	2.47	3.10	3.2

Fig. 8. N-cad and actin cytoskeleton dynamics. (A-D) Confocal time-lapse images of *Drosophila* wings taken at 20-second intervals that show the organization of glial cytoplasmic processes at the chain front in the following genotypes: *repo >GFP* (control); *repo >N-cad GOF*; *repo >CYFIP GOF N-cad GOF* and *repo >N-cad LOF*. Red arrows, triangles, square and stars indicate the initial shape and position of the filopodium, symbols in yellow show the same filopodium upon reorganization. (E) Quantification of filopodia lengths (in µm) at initiation of migration in all genotypes. (F) Number of filopodia present per cell soma in the indicated genotypes. (G) Number of cell somata present at the migration front. (H,I) Summaries of the data shown in panels E and F. Scale bar: 20 µm (for A-D). Note that the *UAS Actin42A GFP* line was used in this figure to follow actin dynamics.

well as embryonic and pupal *Drosophila* and *Manduca* glia, move as collectives to reach their final destination (Cafferty and Auld, 2007; Klämbt, 2009; Koussa et al., 2010; Silies and Klambt, 2010a; von Hilchen et al., 2008; von Hilchen et al., 2013). This makes glia a valuable tool to analyze the role of cadherins in cell migration in the nervous system. In contrast to previous reports (Fung et al., 2008; Iwai et al., 1997), we found that N-cad is expressed in peripheral glia and is required for their migration.

N-cad negatively controls the glial migratory process, its levels modulating migration initiation and speed. In line with the findings that N-cad stays on after migration has ended and that its overexpression delays rather than stops migration, N-cad has a permissive rather than instructive role. We propose that timely expressed pro-migratory cues trigger the movement of the chain, and that N-cad fine-tunes the process by acting as a brake. N-cad is, therefore, part of the network that controls the efficiency of collective migration. Interestingly, N-cad is expressed in embryonic PNS (supplementary material Fig. S4A,B, arrowheads) but not in CNS glia cells (supplementary material Fig. S4C), which seem to move mostly as single cells (Klämbt, 2009).

In summary, whereas N-cad is known to be present in vertebrate PNS glia during development and in pathological conditions this is, to our knowledge, the first *in vivo* report on its cell-specific role in glial migration.

N-cadherin and collective migration

Border cells in the *Drosophila* ovary have been extensively studied as a model for collective migration and they depend on E-cad to migrate (Godt and Tepass, 2009; Montell, 2003; Rørth, 2009). Moreover, it has been recently shown that border cells that express low levels of E-cad protrude less than wild type cells and fail to follow their migratory pathway (Cai et al., 2014; Montell et al., 2012). Thus, E-cad acts positively on border cell migration and on directional sensing. We propose that the opposite roles of E-cad in border cells and N-cad in glial cells reflect the different organization and migratory strategies of the two 'collectives'. Border cells move as a compact cluster and present features of apico-basal polarity; cells in the glial chain, instead, show less-tight contacts with each other (supplementary material Fig. S4D,E) and do not display apico-basal polarity. Thus, whereas border cells move like a patch of epithelial cells, glia cells show a behavior that is more similar to that of mesenchymal cells. The size and shape of the collective significantly differs as well, and one or two border cells are sufficient to trigger the migration of the cluster containing 8–12 cells. In contrast, several pioneer glia

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cells are necessary to drag the long chain made of 60–70 cells, spanning over almost 800 µm. Moreover, the long glial chain relies on relay mechanisms and homeostatic interactions to migrate efficiently (Aigouy et al., 2004; Berzsenyi et al., 2011). As proposed by Montell and collaborators (Cai et al., 2014), various mechanisms can account for the diversity of morphogenetic movements; therefore, understanding the signaling cascades mediated by cadherins will require the analysis of different collectives (chains, clusters, streams, sheets and tubes, and large versus small migratory communities).

Finally, E-cad and N-cad seem to have different structural properties and are associated with AJs of different size (Chu et al., 2006; El Sayegh et al., 2007; Uchida et al., 1996) (Harris and Tepass, 2010; Tepass et al., 2000). Thus, E-cad may control the tension and asymmetry that are necessary in compact and small migratory groups, whereas N-cad regulates dynamic processes that have a stronger impact on loose and large collectives, actin nucleation providing the required relay mechanisms. It will be interesting to assess the precise role of N-cad in other migratory collectives that show loose contacts.

Cadherins and AJs

Cadherins are habitually regarded as components of the AJs; however, N-cad is widely distributed in wild-type glia cells and altering its protein level affects migration in the absence of significant AJ defects. The number of glia–glia AJs does not change significantly between control and N-cad-overexpressing glia, nor does the structure of the AJs seem to change, suggesting that excessive N-cad levels do not induce the formation of larger AJs with altered adhesive capacity (supplementary material Fig. S3C-F). Similarly, conditional N-cad knockdown does not eliminate AJs. Our results are in line with the finding that cadherin–catenin complexes can be observed all along the cell membrane in MDCK epithelial cells (Näthke et al., 1994). Moreover, *in vitro* assays in which CHO cells or fibroblastic L-cells were used have suggested that cadherins can mediate adhesion between cells that lack zonula adherens AJ structures (Brieher et al., 1996; Nose et al., 1988). These data suggest that cadherins can organize differently, i.e. junctional and non-junctional, hence providing distinct functions. Our data, obtained by using glia cells of the *Drosophila* wing, are in agreement with this hypothesis. The assembly of N-cad in glial AJs may occur at preferred cell contact points that are more stable or serve as anchor sites for cell translocation. The rest of diffusely distributed cadherin/catenin complexes are likely to trigger the formation of more-transient contacts that are important in the push-and-pull process at work in the glial chain.

Immunoprecipitation studies have shown that cis dimers can be formed by cadherins of the same cell (Brasch et al., 2012; Shan et al., 2000; Shapiro and Weis, 2009); however, it is unknown which role such cis interfaces play *in vivo* (Brasch et al., 2012). Since glial cells at the chain front are not in contact with other glial cells on their proximal side, N-cad lateral clustering may occur at that position. A non-mutually exclusive possibility is that N-cad at the chain front interacts with axonal N-cad. Finally, the extracellular matrix (ECM) constitutes another key player in cell migration and interacts with cadherins (for reviews, see Doyle et al., 2013; Weber et al., 2011). Also, integrins in glia have been recently shown to shape the larval *Drosophila* CNS (Meyer et al., 2014). It will be interesting to assess whether and how N-cad and ECM interact in the collective process of glial migration. Although additional work will be necessary to clarify these issues, the present study provides novel hints on the role of N-cad in collective migration.

Cadherin–catenin interaction controls actin dynamics

At their cytoplasmic domains, cadherins are associated with adapter molecules that link cell membrane to actin cytoskeleton dynamics. Cadherin activation by clustering can lead to a change in the arrangement of actin filaments at the cell membrane, which in turn regulates cell shape and migration (Nelson, 2008). A key organizer of actin assembly at the cell membrane is α-cat (Kobielak and Fuchs, 2004; Oda et al., 1993), which has been shown to either bind β-cat as a monomer or the actin filaments as homodimer (Drees et al., 2005; Yamada et al., 2005). Cadherin-dependent accumulation of α-cat below the membrane leads to its dissociation from the cadherin–catenin complex (Drees et al., 2005). This promotes actin bundling and suppresses the branching activity of the ARP2/3 actin nucleation complex, which is crucial for cell migration (Benjamin and Nelson, 2008). Thus, when the amount of cadherin is increased, the continuous assembly of the actin network by the ARP2/3 complex – to which CYFIP belongs – is reduced, and migration is less efficient. Conversely, depletion of cadherin causes a reduction of α-cat at the membrane, hence, enhancing actin branching and cell motility. The migratory phenotypes observed upon glial-specific manipulation of gene expression, the rescue experiments, the quantitative analysis of α-cat mRNA/protein and the *in vivo* analyses of actin dynamics all support this hypothesis. Finally, whereas cadherin overexpression may cause perturbation of Wnt/Wg signaling (Sanson et al., 1996), N-cad-dependent accumulation of Arm at the membrane and the fact that nuclear overaccumulation of Arm does not induce migratory defects, show the importance of N-cad and Arm in the control of actin dynamics. It is interesting to notice that, although cadherins have opposite effects on the migration of border and glia cells, Arm's sole function seems to link them to α-cat (border cells: Pacquelet and Rørth, 2005).

In sum, our *in vivo* investigation supports the hypothesis that classic cadherins control collective migration through remodeling of the actin cytoskeleton, hence, providing a new platform for understanding the molecular signaling cascade underlying this process in physiological and disease conditions.

MATERIALS AND METHODS

Fly stocks and genetics

Flies were raised at 25°C. *repoGal4* (indicated as *repo >*) (from V. Auld, University of British Columbia, Vancouver, Canada) was used to drive glial-specific expression of *UAS ncGFP* (nc indicating nuclear and cytoplasmic) (Aigouy et al., 2008), *UAS mCD8GFP* (m indicating membrane) (Aigouy et al., 2008), *UAS PHGFP* (fusion protein between the pleckstrin homology domain of PLC-δ and the GFP coding sequence) (was from A. Zelhof, Indiana University, Bloomington, Indiana, United States of America) (Zelhof and Hardy, 2004), *elav-DsRed* (Aigouy et al., 2008); *UAS N-cad* (was from T. Uemura, Kyoto University, Kyoto, Japan) (Iwai et al., 2002); *UAS N-cad RNAi* (VDRC stock center) (Cai et al., 2014); *UAS Arm* (Bloomington stock center), *UAS N-cad ΔArm* (was from S. Yonekura, Shinshu University, Nagano, Japan) (Yonekura et al., 2007), *UAS α-catGFP* (was from M. Affolter, Biozentrum, Basel, Switzerland) (Caussinus et al., 2008), *UAS α-cat RNAi* (Bloomington stock center), *UAS Actin42AGFP* (was from J. Casanova, IRB, Barcelona, Spain) (Gervais and Casanova, 2011), *UAS CYFIP* (Schenck et al., 2003), *UAS CYFIP RNAi* (Galy et al., 2011), $CYFIP^{\Delta 85.1}$ (Schenck et al., 2004); *y,w,hsFLP;FRT40A,tubPGal80/(CyO,ActGFP);tubPGal4,UASmCD8::GFP/TM6,Tb,Hu* (was from H. Reichert Biozentrum, Basel, Switzerland) (Bello et al., 2008), $N\text{-}Cad^{M19}FRT40A$ (was from M. Kurusu, National Institute of Genetics,

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Shizuoka, Japan) (Kurusu et al., 2012), *FRT40A* (Bloomington stock center). Clones were obtained after a 37℃ 20–30 minute heat-shock at early L3. Note that, in supplementary material Fig. S2I,K a different and weaker *repoGal4* line was used for comparison (B.W. Jones) (Lee and Jones, 2005).

In vivo Imaging

Dissection, time-lapse imaging and immunolabeling were performed as described in (Aigouy et al., 2008; Aigouy et al., 2004; Soustelle et al., 2008). Fast imaging of glial cells was performed using 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 20-second intervals. We avoided photo bleaching by using a low magnification and reducing the exposure time. Since the time defined to quantify filopodium length was short, no reduction in GFP intensity was noticed. Maximum projections for time-lapse movies were obtained using the ImageJ software. Images were annotated using Adobe Photoshop and Illustrator,movies were converted to QuickTime format using ImageJ. Statistical analysis was done using Student's *t*-test; bars indicate standard error of the mean (±s.e.m). For quantitative analyses, distances were calculated manually and then converted into micrometers upon considering the used magnification.

Measuring the length of filopodia

Filopodium length in each of the isolated soma was measured from the surface of the soma to the end of the filopodium by using the ImageJ software (http://imagej.nih.gov/ij/), and is represented in pixels (x). Since we know the size (s) of each pixel (in µm) from the confocal acquisition information panel, we can get the actual length in µm of each of these filopodia by multiplication (x×s).

Electron microscopy

High-pressure freezing

Wings were dissected in cold phosphate buffered saline (PBS) and transferred to flat carriers (200 µm deep, Leica) filled with 20% BSA in PBS (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℃ in 1% osmium tetroxide (OsO_4), 0.5% uranyl acetate, 0.5% glutaraldehyde and 2% water in pure acetone. Temperature was slowly raised to −30℃ at a 3℃/hour rate. After 6 hours at −30℃, samples were extensively rinsed with pure acetone and permeated by a graded concentration of epoxy resin Epon 812. When the concentration of the resin reached 70%, the temperature was gradually raised to 20℃. The permeation was then finished by three incubations in pure Epon (2 hours each). Blocks were left for 48 hours at 60℃ for polymerization. Ultra-thin sections (50–0 nm) were collected on carbon/formvar-coated copper slot grids, contrasted with uranyl acetate and lead citrate. Images were acquired using an Orius1000 CCD camera (Gatan) mounted on a Philips CM12 transmission electron microscope operated at 80 kV.

Chemical fixation

The wings were dissected in PBS buffer and immediately immersed in the fixative [2.5% glutaraldehyde and 4% formaldehyde in 0.1 M pH 7.4 phosphate buffer (PB)]. After a minimum fixation time of 2 hours, samples were rinsed with PB and post-fixed for 1 hour in 1% OsO_4 at 4℃. After several rinses in distilled water, samples were gradually dehydrated in acetone (50%, 75%, 90%, 95%, 100%) and permeated with epoxy resin in pure acetone (25, 50, 75 and 100%). The wings were flat-embedded between two sheets of aclar (EMS) as described (Kolotuev et al., 2010) and left to polymerize for 48 hours at 60℃. Targeted ultra-microtomy was used to systematically section the wings in the same region. Ultra-thin sections (60 nm) were collected on electron microscope grids.

Western blot assay

Wing extracts were produced from the following genotypes: (a) *repo >, UAS α-catGFP/+*; (2) *UAS N-cad/+; repo >, UAS α-catGFP/+* and (c) *UAS N-cad RNAi/+; repo >, UAS α-catGFP/+*. Protein lysates (30–40 µg) extracted by freeze-thawing cell pellets (in 400 mM KCl, 25 mM Tris HCl pH 7.9, 10% glycerol) were loaded on gradient acrylamide gels (Invitrogen), transferred onto nitrocellulose membrane after electrophoresis and probed with primary antibodies: chicken anti-GFP (ab13970, Abcam, 1:5000) and rabbit anti-actin (A2066 Sigma Aldrich, 1:5000). Signals were detected with Pierce ECL western blot substrate (Thermo Fisher Scientific, Waltham, MA) using horseradish peroxidase (HRP)-conjugated rabbit anti-chicken and goat anti-rabbit (Jackson ImmunoResearch) secondary antibodies (1:10,000). Note that for both western blot and quantitative reverse transcriptase PCR (qRT-PCR) assays ~200 wings were dissected for each mentioned genotype and each experiment. Wings were staged between 20 and 23 hAPF.

Reverse transcription and quantitative reverse transcriptase PCR

Total RNA was analyzed in control wings (*repo >GFP/+*) and in wings of the following genotypes: (a) *repo >, UAS α-catGFP/+*; (b) *UAS N-cad/+; repo >, UAS α-catGFP/+* and (c) *UAS N-cad RNAi/+; repo >, UAS α-catGFP/+*. RNA was purified with TriReagent (MRC), reverse transcribed with SuperScriptII reverse transcriptase (Invitrogen) by using a mix of random hexamers (6 µM) and oligodT primers (5 µM), and analyzed by quantitative PCR (qPCR) (Roche LightCycler480) with Syber Green (Roche) Master mix. For each gene, expression levels were automatically calculated (LightCycler480 Software, release 1.5.0) by calibration against gene-specific standard curves generated by input cDNAs. Obtained values, normalized to the against Actin5C, derived from three amplification reactions, each performed in three independent experiments. PCR primers are: *α-cat*(forward): 5′-TGACCAACGTGTA-GGAGCAG-3′, *α-cat*(reverse): 5′-ACTCCGTCGTAAACCAAACG-3′; *actin*(forward): 5′-TCCAGTCATTCCTTTCAAACC-3′, *actin*(reverse): 5′-GCAGCAACTTCTTCGTCACA-3′.

Immunolabeling and antibodies

Staged pupae were fixed in 4% PFA-PBS (paraformaldehyde in PBS) at 4℃ for 2 hours. Pupae were dissected in PBT (PBS + Triton-X100,0.3%). After quick washes in PBT, wings were incubated in PBT-NGS (5% normal goat serum in PBT) for 20 minutes at room temperature on a planar shaker. Then, the samples were incubated overnight with primary antibodies (diluted in PBT-NGS): the panglial mouse anti-Repo antibody (1:800), the neuron-specific mouse anti-22c10 antibody (1:1000) (DSHB7), chicken anti-GFP (1:1000) (Abcam), rat anti-N-cad (1:50-1:100) (DNEx8-DSHB) and mouse anti-Arm (1:50-1:100) (N27A1-DSHB). After three washes in PBT, wings were incubated for 2 hours at room temperature with secondary antibodies raised in mouse coupled to Cy3 and Cy5, in rat coupled to Cy5 and in chicken coupled to FITC, fluorescent dyes were purchased from Jackson Immuno Research Laboratories, diluted 1:500 in PBT-NGS. Following a final wash in PBT, wings were mounted on slides in Aqua-Poly/Mount medium (Polysciences Inc.).

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Competing interests

The authors declare no competing or financial interests.

Author contribution

A.K., S.B. and T.G. carried out all the experiments. A.G. created the model. A.K., S.B., T.G. and A.G. analyzed the data and wrote the manuscript.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.157974/-/DC1

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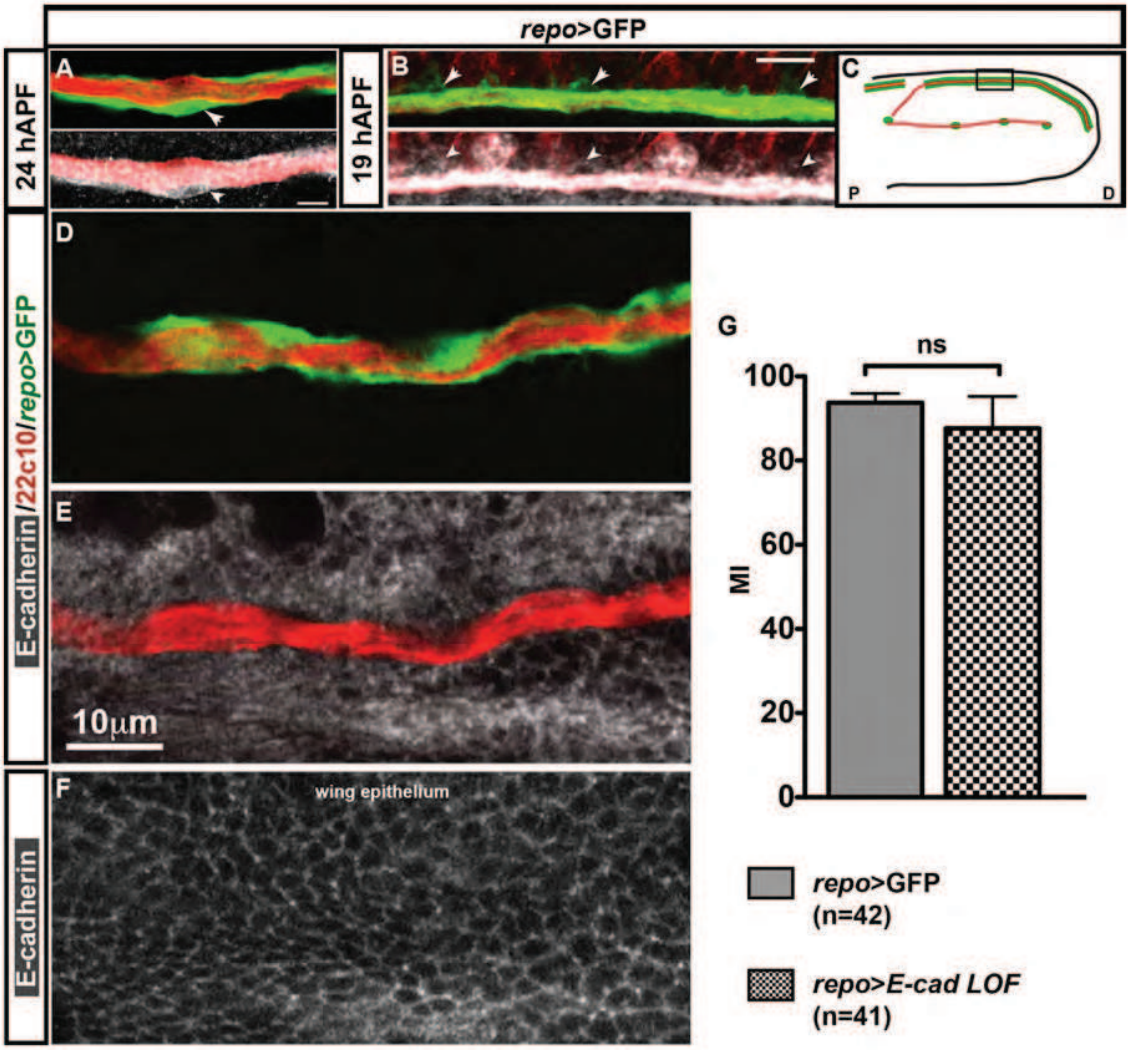

Figure S1: N- and E-cadherin expression profiles

(**A**) 24 hAPF control wing, immunolabeling with anti-22c10 (neurons, red), anti-N-cad (white) and glial cells (green) clearly shows the presence of N-cad in the glial soma (arrowhead). (**B**) Anti-22c10 (neurons, red), anti-N-cad (white) and glial cell (green) labeling on a 19 hAPF *repo>GFP* wing, L1 nerve at the position shown in inset in (**C**). (**D,E**) Anti-22c10 (neurons, red), anti-E-cad (white) and glial cell (green) labeling at 29 hAPF. E-cad is detectable in the wing epithelium but not in axons or in glial cells (**D,E**). (**F**) Shows a single optical section of the wing epithelium labeled with E-cad. (**G**) Graph showing the migratory index upon E-cad downregulation in glia as compared to the control. Scale bars: 10 μm

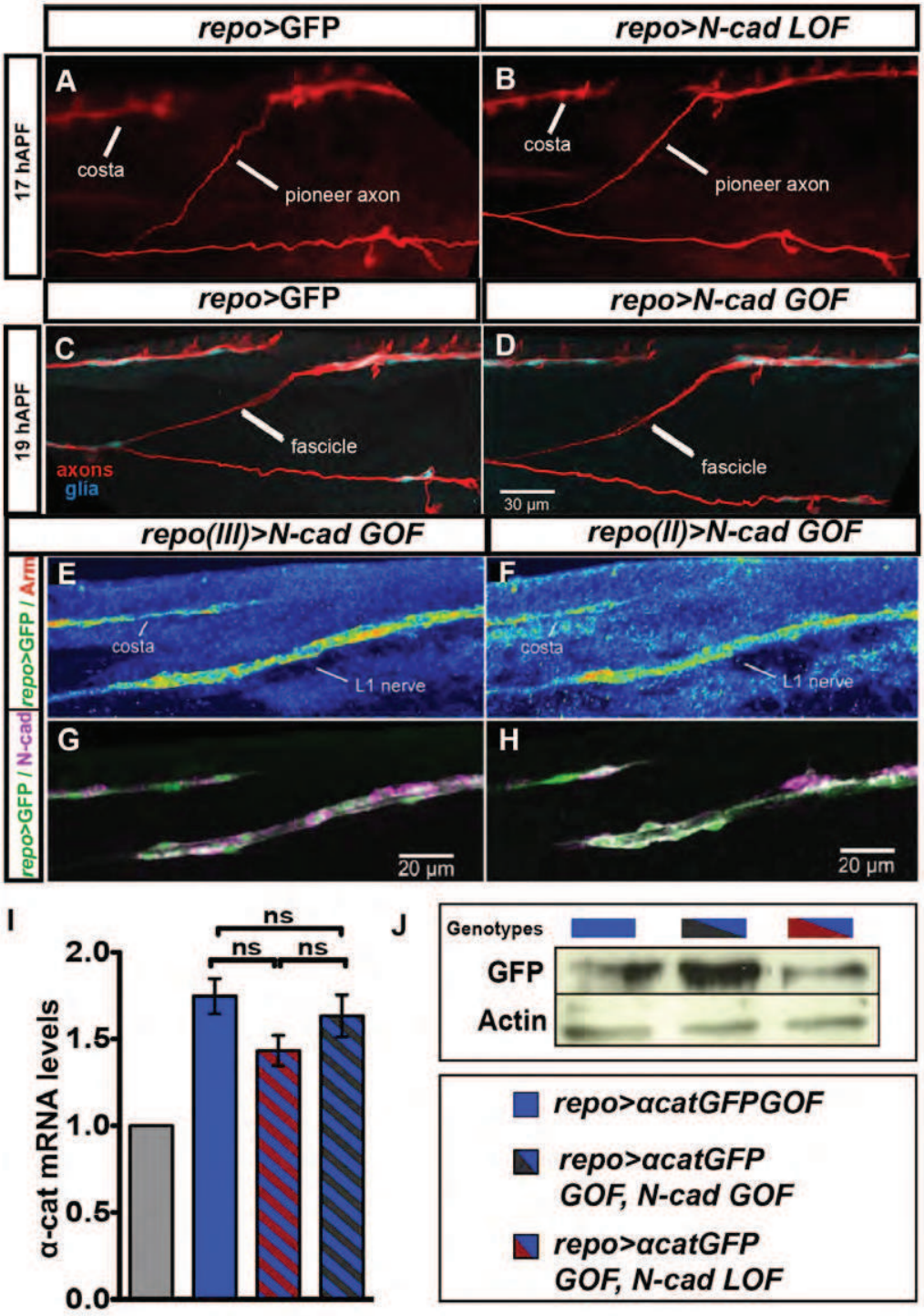

Figure S2: Expression profiles, axon morphology upon N-cad manipulation and quantitative analysis of *α-cat* mRNA and protein

(**A-D**) *repo>GFP* (control), *repo>N-cad GOF* and *repo>N-cad LOF* wings (at 17 and 19 hAPF) immunolabeled with anti-22c10 (neurons, red) and anti-GFP (glial cells, cyan). Note that in GOF and LOF wings, axons navigate similarly to those in control wings. (**E-H**) *repo*(III)*>N-cad GOF* and *repo*(II)*>N-cad GOF* wings immunolabeled with anti-Arm, which is color-coded (red being the highest intensity). Glia are shown in green (*UAS GFP*) and N-cad in magenta in panels (**G,H**). Note that the Arm signal is less intense in (**F**) than in (**E**). (**I**) qRT-PCR analysis (n = 3) of the relative expression of *α-cat* mRNA normalized to *actin* mRNA upon *N-cad GOF* and *LOF* in *repo>α-catGFP* background and in *repo>GFP* animals. The latters are considered as controls and given the arbitrary value = 1. (**J**) Immunoblot of *repo>α-catGFP* (control) as well as *repo>α-catGFP, N-cad GOF* and *N-cad LOF* wing protein extracts monitored for GFP expression (representative Western Blot out of a sample of 2). Actin was used as loading control. Scale bars: (**A-D**), 30 μm; (**E-H**), 20 μm.

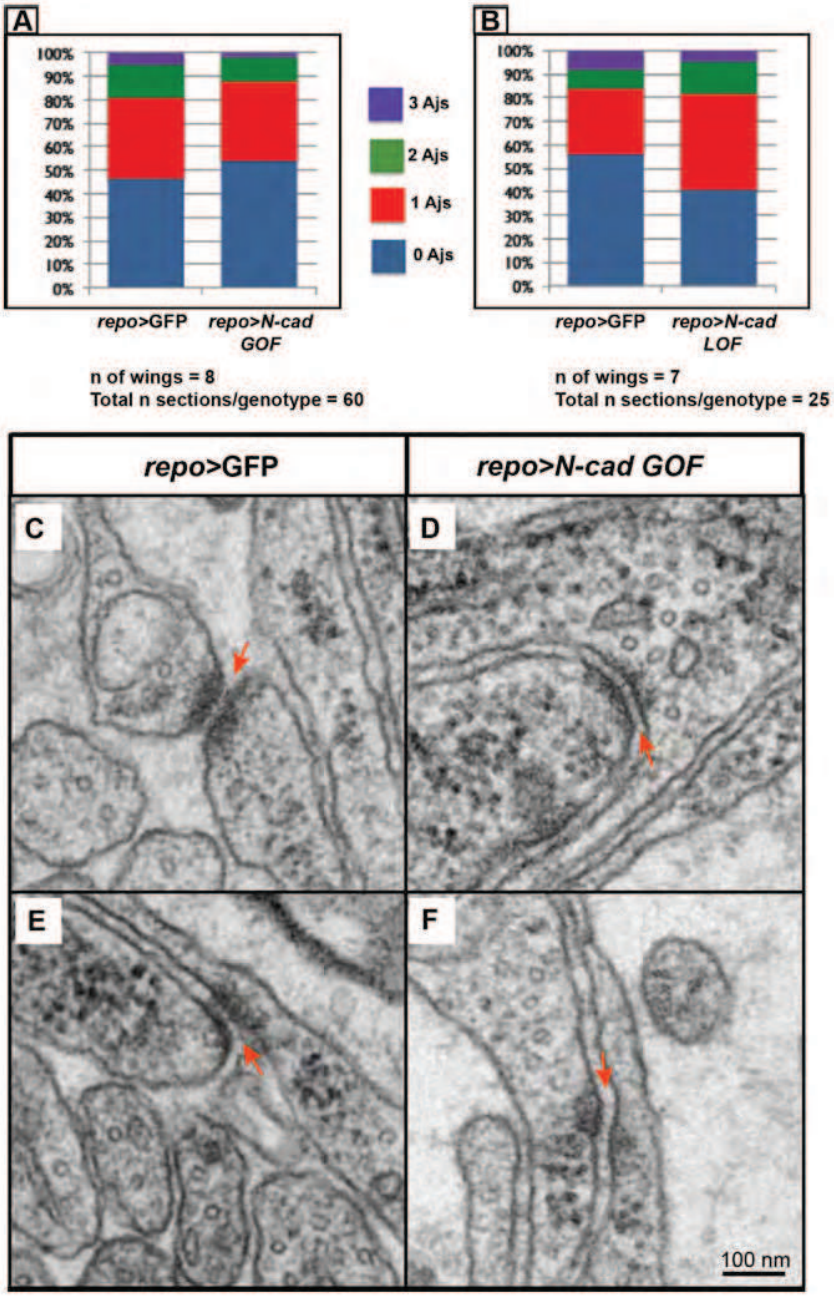

Figure S3: Adherens Junctions in wild type and mutant conditions

(**A**,**B**) Quantification of the neuron-glia AJs in *repo>GFP* (control), *repo>N-cad LOF* and *repo>N-cad GOF* L1 cross-sections. (**C-F**) Ultrastructural organization of control and *repo>N-cad GOF* AJs between glial cells, indicated by the red arrows. Scale bar: (**C**-F), 100 nm.

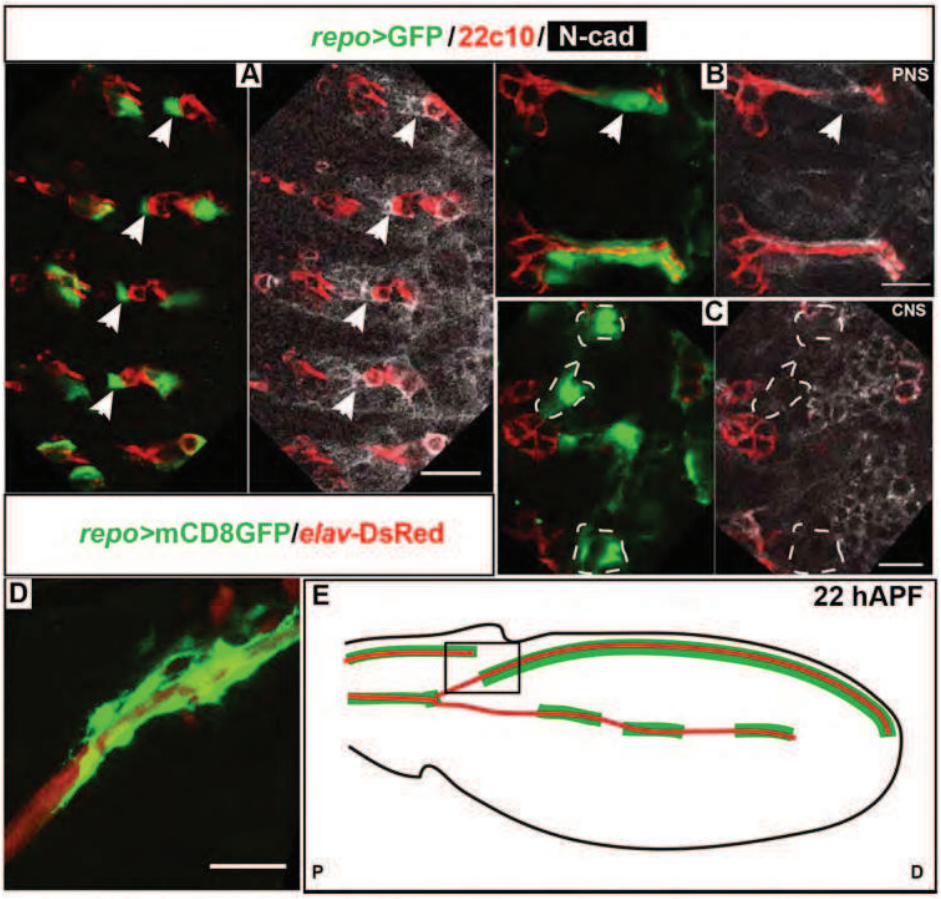

Figure S4: N-cad expression in the embryo and membrane labeling on the L1 glial chain

Single z section of late stage *repo>GFP* embryo immunolabeled with anti-22c10 (neurons, red), anti-N-cad (white) and anti-GFP (glial cells, green). Anterior to the top, dorsal to the left. (**A**) Arrowheads point to peripheral glial cells close to sensory organs and expressing high N-cad levels. (**B**) High magnification of two peripheral nerve fragments covered by glia expressing N-cad (arrows). (**C**) High magnification within the CNS. Glial cells are encircled by dashed white lines and display no N-cad expression. Yellow dashed line indicates the midline. (**D**) Single z section taken from a time-lapse on a double transgenic line labeling glia in green and neurons in red. Note that glial cells are loosely connected to each other while moving as a chain. To follow the glial membranes, we used the *UAS mCD8GFP* transgene. (**E**) Indicates the position of the moving glial chain in (**D**). Scale bars: (**A**), 20 µm; (**B**,**C**), 10 µm; (**D**), 30 µm.

Chapter 3

Collective cell migration is an extremely dynamic process that needs cell-cell interaction, however, its precise behavior calls for autonomous cues being at work as well.

To this purpose, I have analyzed the impact of an early transcription factor in the process. The glial chain in the developing *Drosophila* wing provides an excellent tool to study the molecular pathways underlying collective migration.

The following manuscript addresses the question of whether the transiently expressed Gcm transcription factor, which triggers the fate choice between glia and neurons, also controls collective migration. I could successfully demonstrate that Gcm affects migration in a dosage dependent manner by inducing the expression of Frazzled (Fra), a membrane receptor for the Netrin chemoattractant. My data demonstrate for the first time the role of a fate determinant on a late and collective behavior. Hence, the integration of autonomous (Gcm) and regulatory (Netrin) pathways ensures that glial migration occurs in a timely and efficient manner.

In the previous study (Kumar et al., 2015), we demonstrated a permissive role of N-cad in driving L1 glia migration. However, this study focuses on a specific aspect of L1 glia migration; initiation, and shows Fra as an instructive cue. The following chapter comprises a manuscript that is under preparation and will soon be submitted to a peer reviewed scientific journal. Following the is additional data related to the manuscript.

Manuscript in preparation

Title

The DCC/Frazzled chemoattractant receptor triggering collective glia migration is tightly regulated by the Gcm fate determinant

Author

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Keywords

Collective glia migration, Gcm, Frazzled

SUMMARY:

Collective migration is a complex process that contributes to build precise tissue and organ architecture. Several molecules involved in cell interaction control collective migration, but how is their expression finely tuned to orchestrate the different steps of the process is poorly understood. Here we show that in the *Drosophila* wing, the glial determinant Glide/Gcm induces the glial expression of the Netrin receptor Frazzled at appropriate levels and time. In so doing, a transiently expressed transcription factor regulates the efficiency of collective migration by triggering migration initiation in a dosage dependent manner. NetrinB serves as a chemoattractant and Unc5 as the repellant Netrin receptor for glial migration. Our model displays a right provision of localized ligand, a cell autonomously acting receptor and a fate determinant that coordinately act to direct glia towards their final destination. It also shows that a fate determinant has a much broader role than expected.

INTRODUCTION:

Neurons and glia show mutual reliance in many functional and developmental aspects of biology. Glia migrate collectively and extensively over long distances to establish an intricate relationship with neurons. Defective glial migration is associated with several human diseases including glial brain tumors and defective regeneration following injury in the nervous system (Klambt, 2009; Kocsis and Waxman, 2007; Oudega and Xu, 2006). Hence a thorough understanding of the molecules involved in the process may contribute to the development of therapeutics for these pathologies. Research progress in the recent years has shown the involvement of chemotropic

guidance cues in glia migration (Chen et al., 2010; Kinrade et al., 2001; Liu et al., 2012; Spassky et al., 2002; von Hilchen et al., 2010). Netrins provide a class of secreted laminin related extracellular proteins that function as chemotropic guidance cues for axons and migrating cells during neural development (Harris et al., 1996; Ishii et al., 1992; Kennedy et al., 1994; Lai Wing Sun et al., 2011; Mitchell et al., 1996; Serafini et al., 1994). Netrins acts as chemoattractants through the DCC/Frazzled family of receptors (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Lai Wing Sun et al., 2011; Timofeev et al., 2012; von Hilchen et al., 2010) and chemorepellant through the Unc5 receptor family (Keleman and Dickson, 2001; Labrador et al., 2005). In particular, Netrins secreted by the floor plate cells attract or repel the migratory oligodendrocytes precursor cells depending on the type of receptors these cells express (Jarjour et al., 2003; Spassky et al., 2002; Sugimoto et al., 2001; Tsai et al., 2003). In *Drosophila*, the two Netrins (NetA and NetB) and their receptor Frazzled (Fra) mediate the attraction of embryonic longitudinal glia towards the midline (von Hilchen et al., 2010). Despite the extensive knowledge on these ligands and receptors, the transcriptional control underlying chemoattraction and the precise role of the receptors remain largely unknown. For example, do the receptors affect a specific step of collective migration, initiation, maintenance, arrest or do they act on all phases of migration? Related to this issue, which transcription factors regulate the cell-specific and timely expression of the guidance receptors? We here investigate the chemoattraction cascade controlling cell migration using the chain of glial cells across the L1 nerve in the developing *Drosophila* wing (Aigouy et al., 2008; Aigouy et al., 2004; Berzsenyi et al., 2011; Kumar et al., 2015).

We show that only one of the two *Drosophila* Netrins (NetA and NetB) (Harris et al., 1996; Keleman and Dickson, 2001; Mitchell et al., 1996), NetB, serves as a chemoattractant for collective glial migration. Moreover, Fra triggers the timely initiation of glial migration in a dosage dependent manner and Unc5 acts as the repellant receptor that controls glial arrest. Finally, we identify the transcription factor that controls the expression of Fra at the appropriate time and levels: Glial cell deficient/Glial cell missing (Glide/Gcm or Gcm, for the sake of simplicity) (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Gcm is a fate determinant that is expressed early and transiently in the glial lineages. To our knowledge this is the first report showing that a fate determinant directly controls a late glial process. Thus, early genes regulate the expression of transcription factors that execute a specific cell fate and also contribute to the execution of that fate by regulating effector genes.

RESULTS

Frazzled expression in the glial cells of the developing *Drosophila* wing

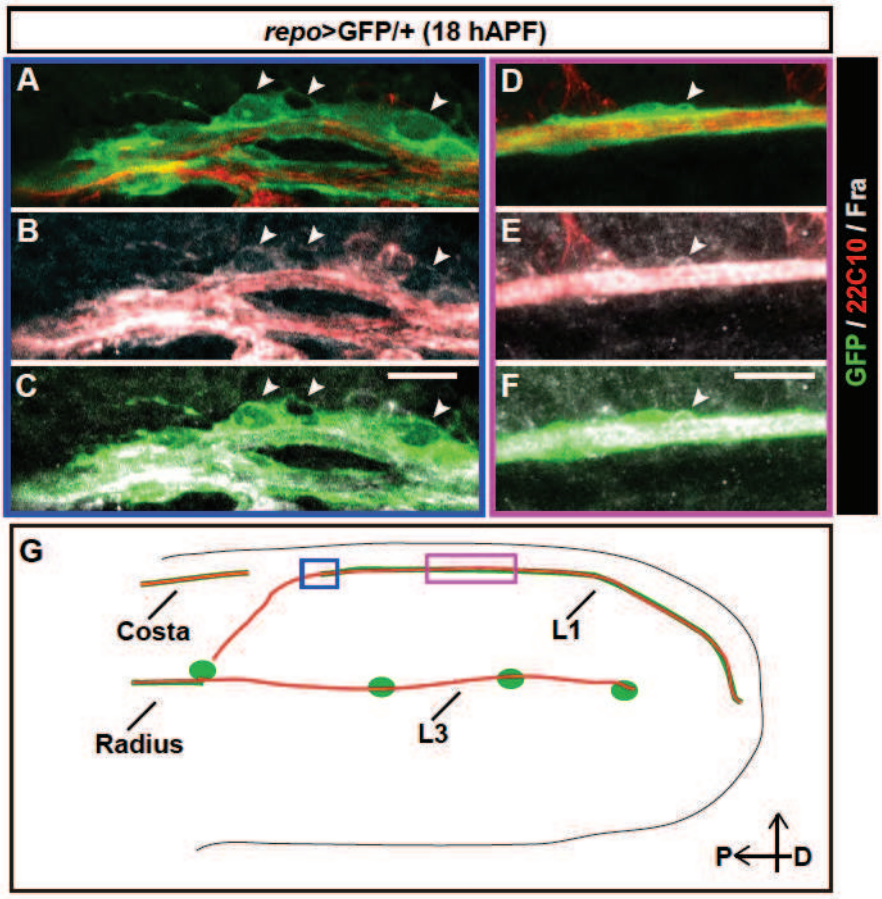
Fly wings are innervated by two major sensory nerves that navigate along the so-called L1 vein located at the anterior margin (L1 nerve) and along the L3 vein (L3 nerve) (**Figure 1A-D).** Glial cells originating from the sensory organ precursors (SOPs) present on the anterior margin migrate proximally i.e. towards the central nervous system (CNS) following the axon bundle and ensheathing it throughout its length. L1 glia start moving at around 18 hours After Puparium Formation (hAPF), reach the level of the Costal nerve at around 22–24 hAPF and join the glial cells on the Radius by 28 hAPF. The migratory

process has been accordingly subdivided into three steps: 'Initiation', 'Costa reach' and 'Complete migration' (**Figure 1A-1C**).

To gain insight into the molecular pathway triggering collective glia migration, we first examined the expression of Fra chemoattractant receptor by using the pan glial lines *repo Gal4 UAS* PH GFP (henceforth *repo*>GFP) or the *gcm Gal4 UAS* CD8 GFP (*gcm*>GFP), which label the glial membranes. Fra is detected in glia at the time these cells begin to move as well as in the underlying axons (**Figure 1E-1G, Figure S1A-S1C and S1G).** The Fra protein seems evenly distributed along the L1 glial chain (**Figure S1D-G**). Thus, migrating glial cells of the peripheral nervous system (PNS) express Fra.

Next we studied the impact of Fra on glial migration, we used the semi quantitative approach described in (Kumar et al., 2015). In short, we analyzed the percentage of wings displaying complete glia migration at 28 hAPF. This value, which we defined as the migratory index (MI), provides an estimation of migration efficiency (Kumar et al., 2015). For each genotype, we analyzed a large number of wings ($n \geq 30$). We first focused on the most characterized loss-of-function (LOF) allele fra^3 (Kolodziej et al., 1996). Since this mutation is embryonic lethal in homozygous conditions, we analyzed fra^3 heterozygous wings and found a significant delay in L1 glia migration, as shown by the position of the glial nuclei labeled by the panglial marker Repo (**Figure 1H,K,L**). The number of glial cells is not affected; hence this cannot be the cause of the migratory defect (**Figure 1I**). We reasoned that nuclei may not migrate properly but glial processes may still reach the final destination. We hence assessed the migratory index in flies that are fra^3 heterozygous mutant and carry *repo*>GFP transgene. These wings also show a migratory delay (**Figure 1J**).

In sum, the Fra receptor is expressed in glial cells and is necessary for the efficiency of their migration.



Supplementary Figure 1: Expression profile of Fra. (A-C) 18 hAPF *repo*>GFP/+ wing, immunolabeled with anti-22c10 (neurons, red), anti-Fra (grey) and glial cells (green) clearly shows the presence of Fra in the glial soma (white arrows). **(D-F)** Anti-22c10 (neurons, red), anti-Fra (grey) and glial cell (green) labeling on 18 hAPF *repo*>GFP wing, shows the accumulation of Fra protein along the L1 nerve. **(G)** Schematic of a wing displaying the region shown in panels **(A-F)**. Blue box indicates the region shown in panels **(A-C),** magenta box indicates the region shown in panels **(D-F).** Scale bars: **(A-F)**, 10 μ m.

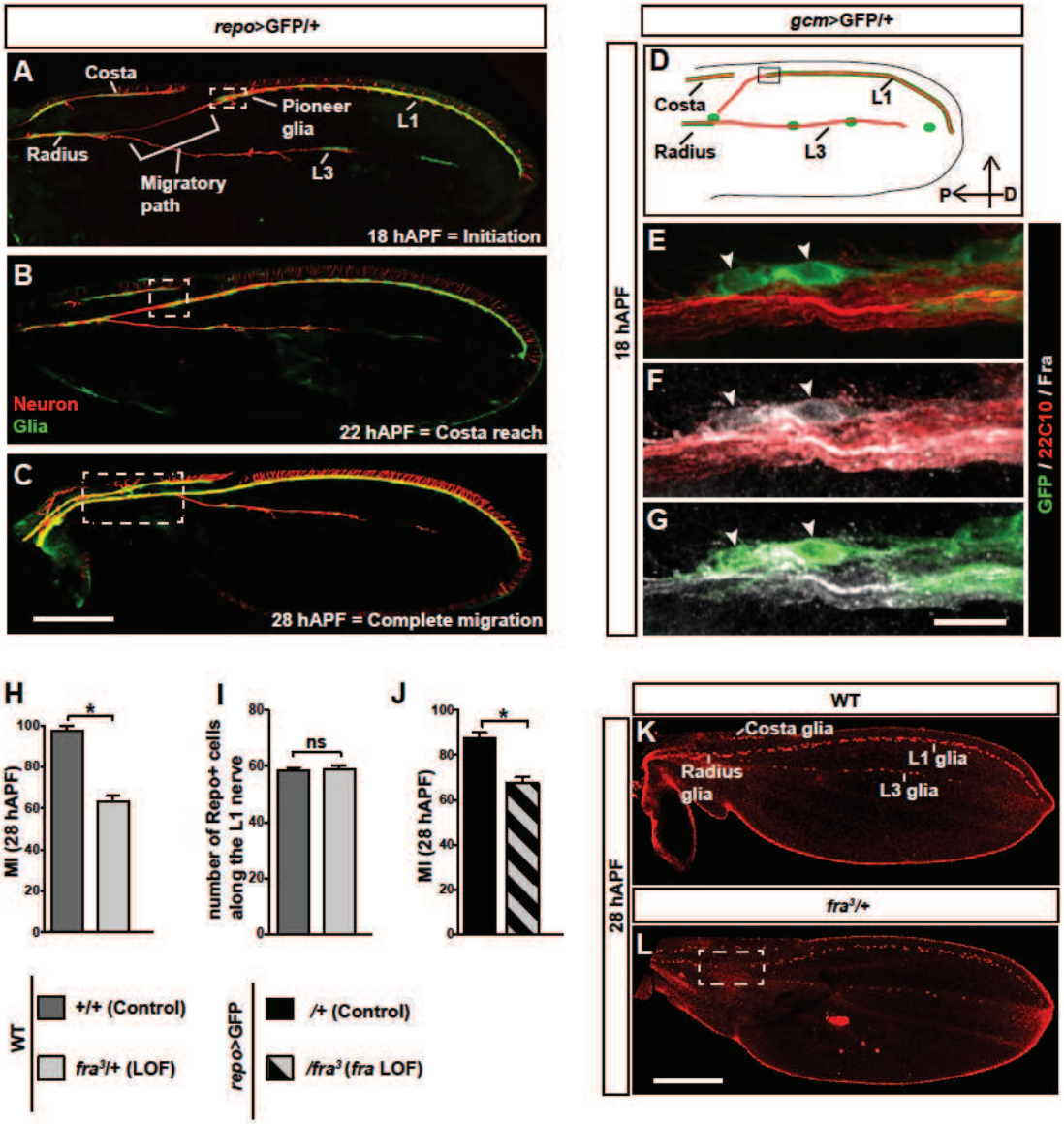


Figure 1: Expression and role of Fra in wing glia. (A-C) Immunolabeled wings with glia (green) and neurons (anti-22c10) at different stages. **(A)** Initiation of migration, **(B)** reaching the level of costa, **(C)** migration completion. **(D)** Schematic of 18 hAPF developing *Drosophila* wing, box indicates the region shown in panels **(E-G).** Neurons are in red, glia in green. L1 and L3, P and D indicate L1 and L3 nerves, proximal and distal regions, respectively. **(E-G)** 18 hAPF wing immunolabeled with anti-22c10 (neurons, red), anti-Fra (grey) and anti-GFP (glial cells, green) in the transgenic line *gcm>UAS* mCD8GFP, CD8 GFP labels the membrane (*gcm>GFP/+*). Maximum confocal projections are shown in all figures, unless otherwise specified. White arrows indicate the glial cells with Fra labeling. **(H)** Graph represents the migratory index (MI) of the indicated genotypes. Note that in this panel, the nuclear labeling was used to calculate the MI. **(I)** Graph represents the number of glial nuclei in the indicated

genotypes. **(J)** Graph represents the migratory index (MI) of the indicated genotypes. Note that in this panel, cytoplasmic GFP was used to calculate the MI. The migratory index (MI) was calculated by counting the number of wings displaying completed migration (i.e. glial chain reaching the proximally located glia on the radius nerve) by 28 hAPF unless otherwise specified. **(K)** 28 hAPF wing showing complete migration labeled with nuclear anti-repo in a control animal. **(L)** 28 hAPF wing showing incomplete migration (dashed box) labeled with nuclear anti-repo in a *fra* mutant animal. In this and in the following figures, stars indicate *P* values: ***$P<0.0001$; **$P<0.001$; *$P<0.05$. Bars indicate the s.e.m. In this and in all the following graphs number of n is equal to or greater than 30. Scale bars: **(A-C)**, **(K, L)**, 80 μ m; **(D-F)**, 10 μ m.

fra plays an instructive role in L1 glia migration

The lethality of the fra^3 homozygous animals and the expression of Fra in neurons prompted us to down regulate *fra* specifically in glial cells by means of the *UAS fra RNAi* line (Manhire-Heath et al., 2013). The knockdown of *fra* (*fra* KD) in glial cells using the *repo*>GFP or the *gcm*>GFP drivers reveals a significant decrease in migration efficiency as compared to what is observed in the control wings (**Figure 2A, compare blank and light blue columns, Figure S2A**). Because *gcm Gal4* is the earliest glial driver, the rest of the analyses were performed using this transgenic line. To exclude the possibility of off target effects, we analyzed wings that express the *UAS fra RNAi* together with the *UAS fra* transgene and found complete rescue of the migratory phenotype induced by the *fra* KD (**Figure 2A, patterned light blue column**). This indicates that the *RNAi* line induces a specific phenotype and that *fra* acts in a cell autonomous manner.

We then asked whether Fra has an instructive role in glia migration and we assessed whether migration is more efficient upon overexpressing *fra* in glia (*fra* GOF). We first checked the MI of *fra* GOF wings at 28 hAPF and found that the percentage of wings that show complete migration at this stage is higher as compared to that of control wings (**Figure 2B, compare blank and dark blue columns**). Since most control wings

show complete migration by 28h APF (90%), we also analyzed an earlier stage, when migration has been achieved only in few control wings (24h APF; 12.5%) and found that many more *fra* overexpressing wings show complete migration than control wings (**Figure 2C**). This strongly suggests that high doses of Fra significantly increase the efficiency of migration. We verified that Fra levels in *fra* KD and *fra* GOF conditions are reduced and increased, respectively, as compared to the control wings (**Figure 2D-I**).

To clarify why the migratory efficiency increases in *fra* GOF animals, we performed a time-lapse analysis and found that *fra* overexpressing glia start migrating earlier than control glia, indicating that the phenotype is due to precocious initiation rather than overall acceleration (**Figure 2J-L**) and this is associated with precocious Fra accumulation in *fra* GOF glial cells compared to control glia (**Figure 2M-P"**).

The cytoplasmic tail of Fra is known to play a major role in mediating Fra dependent attractive responses *in vivo* and in cell culture studies (Bashaw and Goodman, 1999; Hong et al., 1999; Ming et al., 1997). We therefore asked whether this region is important in mediating glial cell migration. A transgenic construct that lacks the Fra intracellular cytoplasmic domain was previously described acting as a dominant negative mutation (Garbe et al., 2007) and indeed the expression of this reporter significantly reduces migration efficiency (**Figure 2B, compare blank and moss columns**).

In sum, Fra expression in glial cells triggers the initiation of their migration through the Fra cytoplasmic domain.

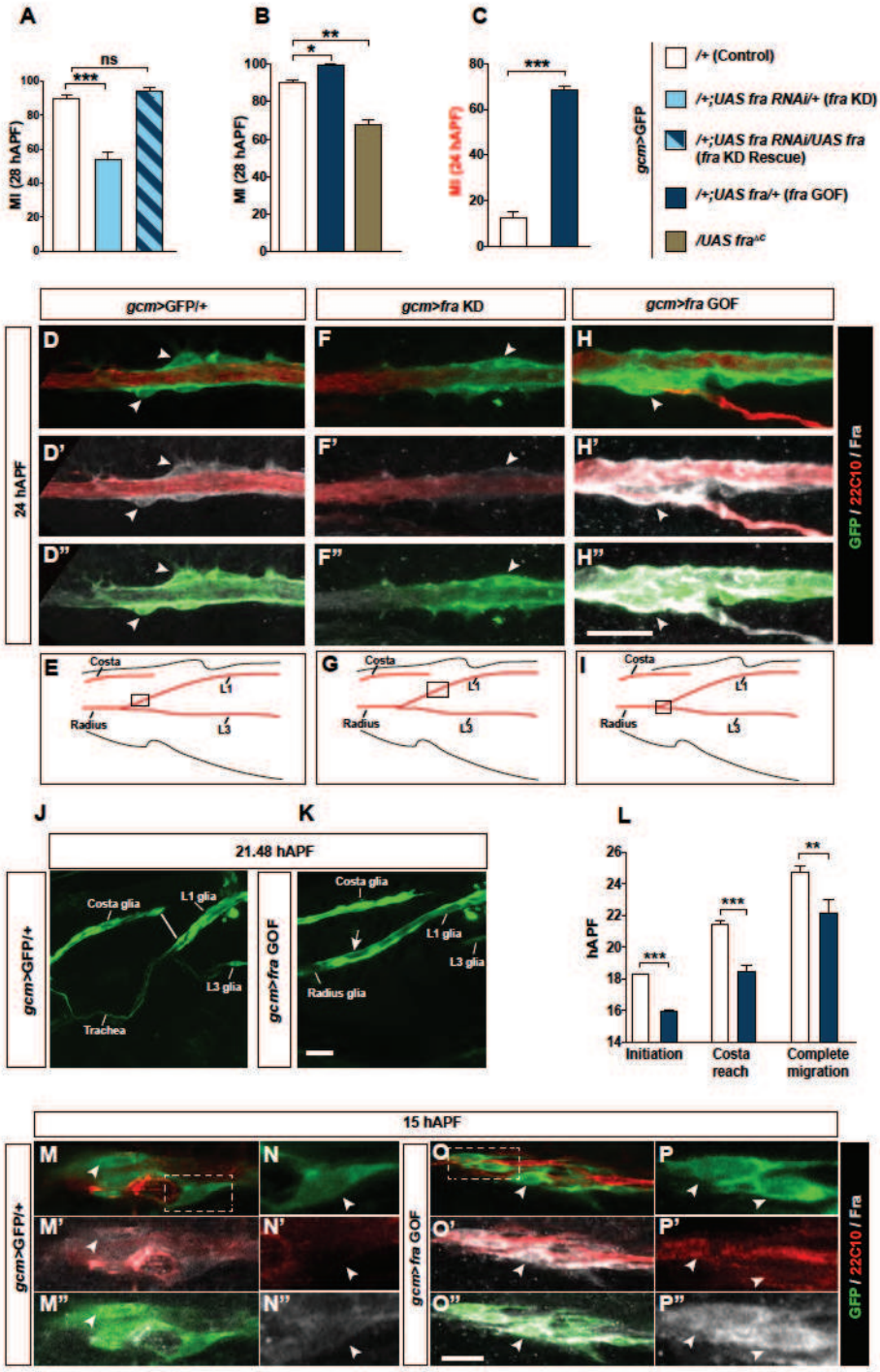
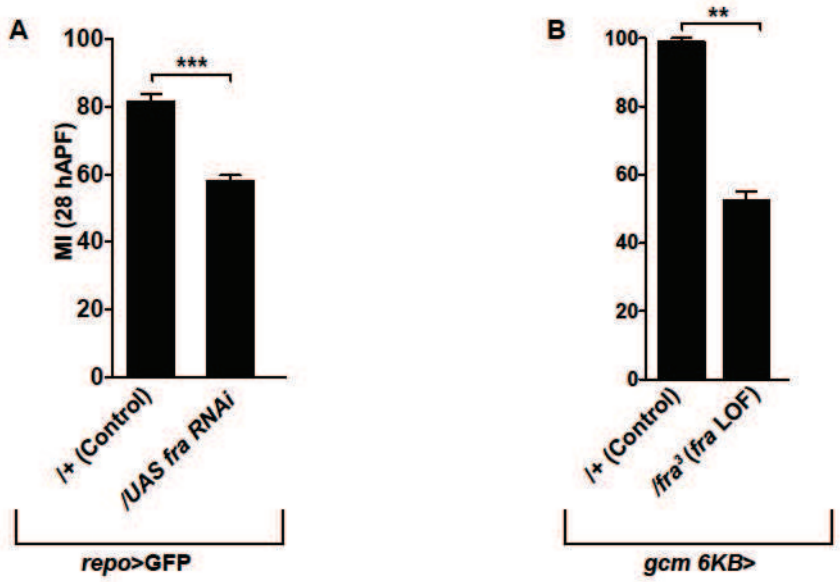


Figure 2: An instructive role of chemoattractant receptor Fra in collective glia migration. (A-C) Graph representing the MI upon Fra knockdown (*fra KD*) and overexpression (*fra GOF*) using the *gcm>GFP/+* line. Note that for the graph **(C)** MI was calculated at 24 hAPF. Note that in these and in the following panels, the cytoplasmic GFP transgenic line (*UAS* mCD8 GFP) was used, unless otherwise specified.

(D-D", F-F" and H-H") Expression profile of Fra in control, *gcm>fra* KD and *gcm>fra* GOF animals at 24 hAPF. See the reduced and enhanced protein levels in panels **(F-F")** and **(H-H")** as compared to **(D-D")**. **(E, G and I)** Wing schematic. **(E)** Box indicates the region shown in the panels **(D-D"). (G)** Box indicates the region shown in the panels **(F-F"). (I)** Box indicates the region shown in the panels **(H-H"). (J, K)** Snapshot of a 21:48 hAPF time-lapse analyses on *gcm*>GFP/+ and *gcm>fra* GOF wing**.** Note the glia position in **(J)** *gcm*>GFP/+ wing and the migration completion (white arrow) in **(K)** *gcm>fra* GOF wing**. (L)** Graphical representation of the migratory behavior of *gcm*>GFP/+ and *gcm>fra* GOF wings at three highlighted phases **(n=10)**. **(M-P")** Expression profile of Fra in control, and *gcm>fra* GOF animals at 15 hAPF. See the enhanced protein levels in panels **(O-P")** as compared to **(M-N")**. Region blown up in panels **(N-N")** is highlighted by a dashed white bracket in **(M),** whereas the one in **(P-P")** is highlighted in **(O)** Scale bars: **(D-D"), (F-F"), (H-H"), (J, K), (M-P"),** 10 μ m.



Supplementary Figure 2: Downregulation of *fra* in wing glia using different drivers. **(A, B)** Graphs represent the MI of indicated genotypes.

The efficiency of glia migration depends on the dose of Gcm

The migratory phenotype of *fra3/+* glia that also carries the *gcm>GFP* driver is much stronger than that of *fra3/+* glia and the phenotype is further enhanced in glia that express both the *gcm>GFP* and a *gcm RNAi* line (**Figure 3A**). Since the *gcm Gal4* driver is a hypomorphic *gcm* allele that is due to the insertion of a *Gal4* containing transposon

into the *gcm* promoter (Jacques et al., 2009), the above result raised the possibility that Gcm and Fra interact genetically. We hence analyzed the glia migration phenotype in double heterozygous conditions for *fra* and two other *gcm* hypomorphic alleles, including the gcm^{rA87} enhancer trap carrying a LacZ transposon into the *gcm* promoter and the imprecise excision line gcm^{34} (Jacques et al., 2009; Vincent et al., 1996). This confirms that lowering the dose of Gcm enhances the fra^{3}-mediated phenotype (**Figure S3A**). As a further approach to confirm the genetic interaction between *gcm* and *fra,* we crossed the *fra3* mutation with a transgenic line carrying 6Kb of the *gcm* promoter inserted on the third chromosome, which does not affect the *gcm* locus. In these wings, we did not observe the enhanced migratory phenotype present in the fra^{3}, *gcm Gal4* wings (**Figure S2B**).

Gcm is a transiently expressed transcription factor that acts very early in glial differentiation (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). To clarify the role of Gcm on glia migration, we analyzed wings that are only mutant for *gcm* and used hypomorphic alleles that allow bypassing the lethality of the null mutation. Migration is indeed less efficient when the amount of Gcm is reduced and the MI is restored to normal values upon reintroducing Gcm expression (**Figure 3B, patterned columns**). Three allelic conditions were tested: *gcm Gal4*, gcm^{rA87} hypomorphic alleles (**Figure 3B, light brown column,** MI = 63%, vs. the MI = 90% of the control line (*gcm>GFP/+*), *gcm Gal4* homozygous wings (**Figure 3B, orange column, MI =** 41%) and wings transheterozygous for gcm^{rA87} and for the null $gcm^{N7\text{-}4}$ mutation (Bernardoni et al., 1997) (**Figure S3A, last column, MI = 28%**). Finally, we used the *UAS gcm RNAi* line to reduce the amount of Gcm and also observed a migratory defect (**Figure 3B, dark**

brown column, MI = 25%). The rescue obtained upon co-expressing the *UAS gcm* and the *UAS gcm RNAi* transgenes indicates that *gcm* plays a regulatory role in migration and that the *RNAi* effects are specific. Finally, overexpressing Gcm using the *UAS gcm* transgene (*gcm* GOF) is sufficient to increase the migration efficiency of glial cells as the percentage of wings showing complete migration increases compared to that of control animals (**Figure 3C**).

To determine which migratory step is affected, we analyzed the *gcm* hypomorphic (*gcm*>GFP/*gcm*^{rA87}) and the *gcm* GOF (*gcm*>GFP/+;*UAS gcm*/+) wings by confocal time-lapse microscopy and found that migration starts later in those expressing low Gcm levels and earlier in *gcm* GOF wings, compared to what was observed in control wings, (**Figure 3D-G**). Thus, Gcm affects the initiation of glial cell migration, like Fra, and it does so in a dose dependent manner: high Gcm levels increase the efficiency of this step and low levels delay it.

In sum, Gcm interacts with Fra and its levels are critical for the initiation of glia migration.

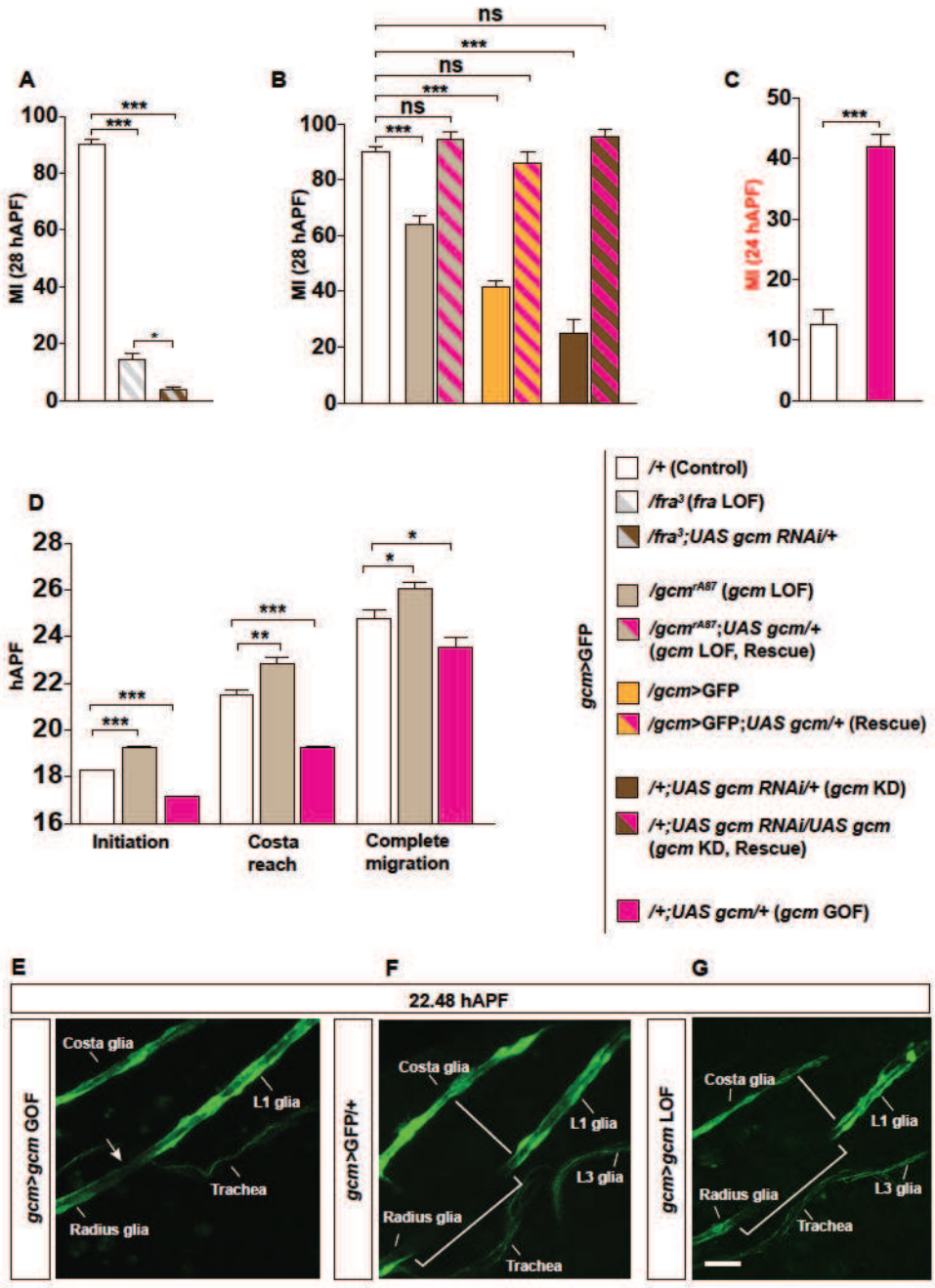
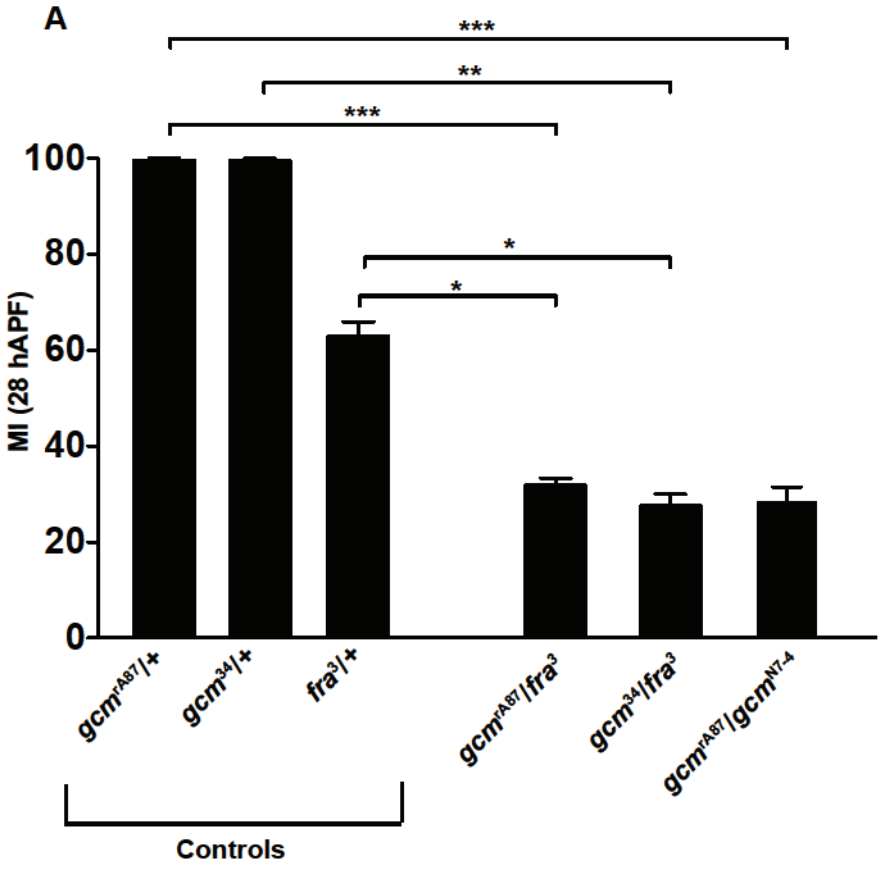


Figure 3: Gcm affects collective glia migration in a dosage dependent manner.
(A, B) MI of the indicated genotypes. Graph consists of the quantification on control, *fra* LOF, *gcm* LOF and *gcm* KD wings. **(C)** MI calculated at 24 hAPF *gcm* GOF wings. **(D)** Graphical quantification of control, *gcm* LOF and *gcm* GOF wings at three different migratory phases i.e. Initiation, costa reach and complete migration. **(E-G)** Snapshots

from a time-lapse movie of control, *gcm* LOF and *gcm* GOF wing at different time points. The time indicated at which these wings complete migration. Scale bars: **(E-G)**, 10 μ m.



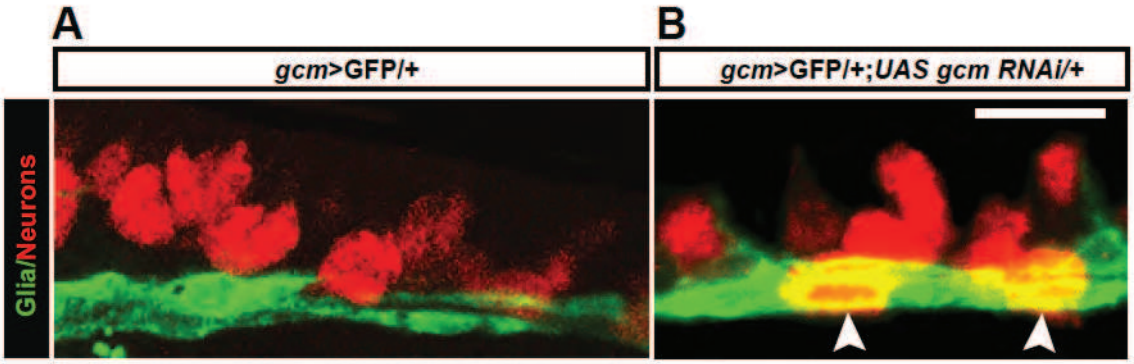
Supplementary Figure 3: Different dosage of *gcm* and *fra*^{3} mutants in glia migration. (A) Graph represents the migratory profile of different *gcm* and *fra*^{3} mutants. Genotypes as indicated. Note that, MI in this figure was calculated by using a nuclear labeling.

Gcm affects migration independent of its role as a fate determinant

The glial to neuron conversion described in the *gcm* mutant flies prompted us to ask whether this early defect could impact the glial migratory process indirectly (Bernardoni et al., 1997; Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). We inspected the rate of glia to neuron conversion in wings expressing low Gcm levels by

using the anti-Elav antibody, which specifically recognizes neurons at the analyzed stages (**Figure S4 *gcm* KD**,). Only a minor fraction of the wings contains converted cells and only few cells are converted, strongly suggesting that fate conversion is not the cause of the altered MI (10% of the *gcm* KD wings, 10 GFP/Elav double positive cells are present along the L1 nerve in average, ≥15 wings were analyzed per genotype). In addition, we restrained the analysis to wings that do not show fate conversion and still found a strong delay in migration (MI = 45%).

These data strongly suggest that *gcm* specifically affects glia migration.



Supplementary Figure 4: Fate conversion does not explain the Gcm migratory phenotype. (A, B) *gcm*>GFP/+ and *gcm*>GFP/+;*UAS gcm RNAi*/+ wing (at 17 hAPF) immunolabeled with anti-Elav and anti-GFP. Please note the converted glia to neuron cells (white arrows) in *gcm*>GFP/+;*UAS gcm RNAi*/+ wing. Scale bars: **(A, B)**, 10 μ m.

Gcm affects migration independent of glial cell number

Another cause for the migratory phenotype observed in the *gcm* mutant wings might be the control exerted by Gcm on the number of glial cells. It is indeed plausible that the number of cells in the collective somehow affects the mechanical forces that control migration efficiency, for example through the amount of chemoattractant receptor. The number of Repo positive cells in *gcm* LOF and KD backgrounds is indeed

lower than in the wild type glial chain and, accordingly, *gcm* GOF wings contain supernumerary glial cells (**Figure 4A, light brown, dark brown and pink columns, respectively)**.

To assess the impact of glial cell number in migration efficiency more directly, we analyzed wings that overexpress proteins promoting or repressing cell division. The exit from the cell cycle results from the timely inactivation of the Cyclin-dependent protein kinases (Cdk) and Cyclin E (Cyc E) complexes. String/Cdc25 encodes a phosphatase that triggers mitosis by activating the Cdc2 kinase, hence enabling cell proliferation (Edgar et al., 1994; Edgar and O'Farrell, 1989; Lasko, 2013). On the other hand, Dacapo functions as an inhibitor of the Cdk/Cyc E complex both *in vivo* and *in vitro*, ultimately leading to cell cycle arrest (de Nooij et al., 1996; Lane et al., 1996). First, we produced animals overexpressing String or Dacapo in glia (*gcm*>GFP) and verified that this induces a significant change in glial cell number compared to that present in control wings (**Figure 4A, compare blank with vertical and horizontal lines columns**). Then, we analyzed the migration efficiency in both backgrounds and found that it is not affected (**Figure 4B**), even though the glial number increase induced by String overexpression and the decrease induced by Dacapo overexpression are comparable to the changes observed in *gcm* GOF and LOF, respectively. Finally, we found defective glia migration even in *gcm* KD wings containing a wild type number of glial cells, in agreement with the above data (Data not shown).

Thus, the absolute number of glia does not affect migration efficiency, further corroborating the hypothesis that Gcm specifically affects this process.

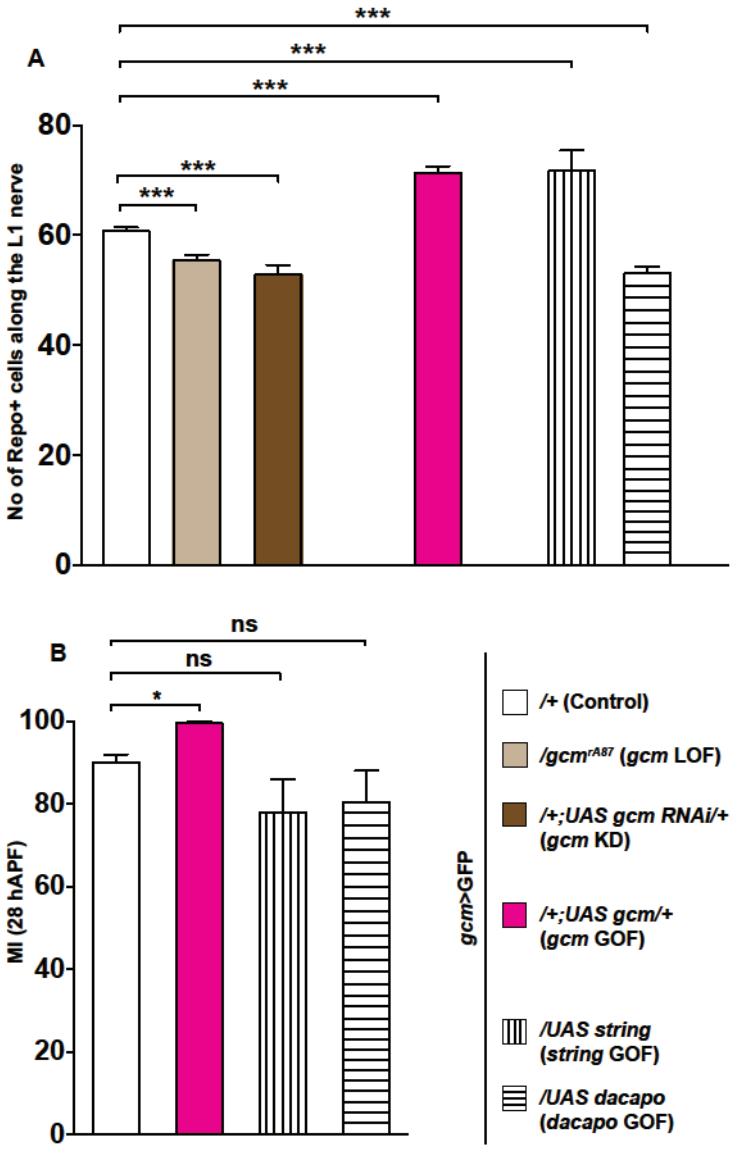


Figure 4: Effect of Gcm on collective glia migration is independent of any change in the number of repo+ nuclei. **(A)** Graph represents the counting of glial nuclei in the mentioned genotypes. **(B)** MI of the mentioned genotypes.

fra is a direct Gcm target

The gene expression profiles and the observed genetics interaction prompted us to assess whether Gcm acts on glia migration by inducing Fra expression. A DNA adenine methyltranferase identification (DAM ID) screen aiming at finding the direct targets of Gcm indeed suggests that this potent transcription factor may directly control the expression of genes involved in glia migration, including *fra* (Cattenoz et al., 2015).

There are three canonical Gcm binding sites (GBS) in the *fra* locus, two of which are located at the position of a strong DAM ID peak, which is indicative of Gcm binding (**Figure 5A**). To confirm that *fra* is a direct Gcm target we assessed whether *fra* expression can be activated by Gcm in *Drosophila* S2 cells. To do so, we amplified the region containing two GBSs and put it in front of a reporter plasmid that expresses the GFP (**Figure 5A,A'**). qRT PCR assays clearly show an increase in the levels of GFP upon co-transfection of the reporter vector with a Gcm expression vector (**Figure 5B, columns with a red color gradient**). GFP expression is induced in cells transfected with 1µg of Gcm expression vector and further increases upon transfecting with 2µg of the same vector. To demonstrate that the effect of Gcm on *fra* is direct (**Figure 5A''**), we showed that the Gcm dependent activation of the reporter is completely abolished upon mutagenesis of the two GBSs (**Figure 5B, columns with a yellow color gradient**). The levels of transfected Gcm were verified by qRT PCR (data not shown) and those of GFP were confirmed by Western blot assays (**Figure S5A-C).**

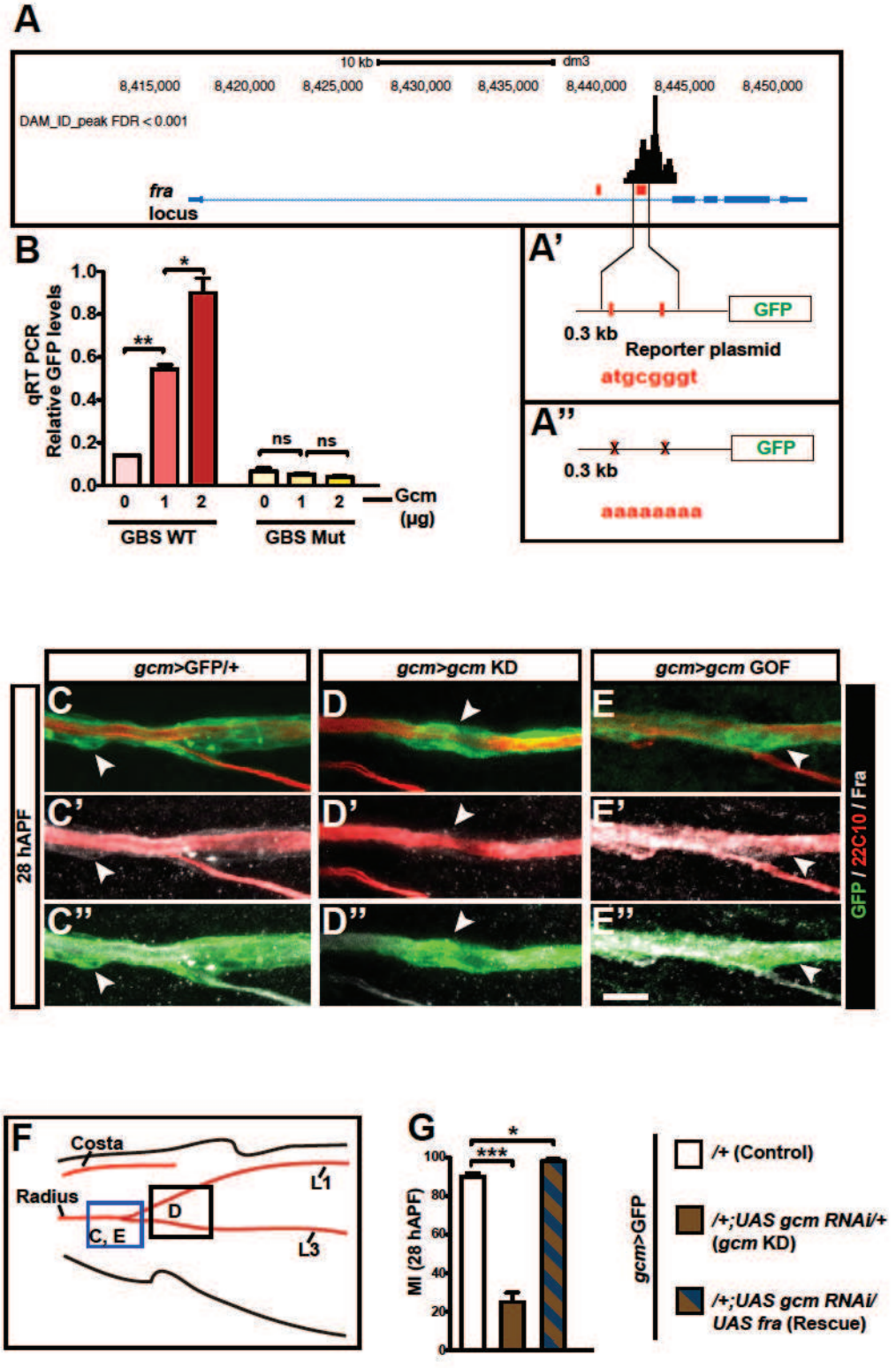
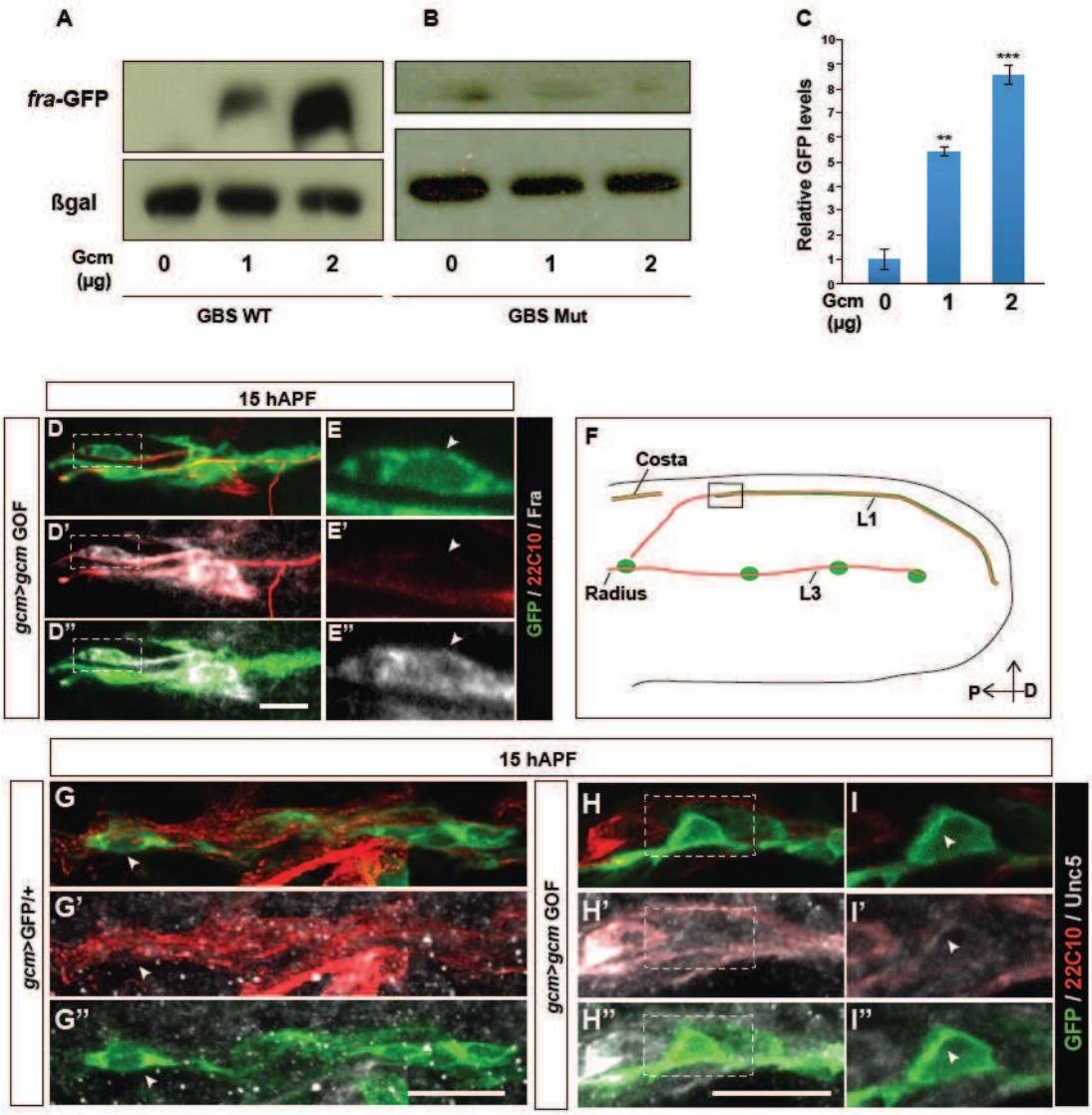


Figure 5: Fra is a direct Gcm target. **(A)** A schematic representation of the *fra* loci. The gene *fra* is indicated by the rectangle in blue. The thin ones indicate the untranslated

regions and the thick ones indicate the coding exons, pale blue arrowheads indicating the direction of transcription. GBS's are indicated in red, the black histogram above represent the *gcm* peak. **(A')** Represents the two GBS's that were amplified and put in front of the GFP reporter to generate a *fra* WT plasmid. **(A")** Implies that the same two GBS's were mutated in order to generate a *fra* Mut plasmid. **(B)** Quantification of the qRT PCR analysis of an increase in the level of *fra* GFP using a WT *fra* plasmid with the gradients of Gcm (gradient in red color). Similarly *fra* Mut plasmid shows no change in the GFP level with an increasing amount of Gcm (yellow color gradient). The amount of Gcm was used in μg. **(C-E")** Immunolabeling of the control *gcm*>GFP/+, *gcm*>*gcm* LOF and *gcm*>*gcm* GOF wings at 28 hAPF using anti-22c10 (neurons, red), anti-Fra (grey) and anti-GFP (glial cells, green) showing an *in vivo* loss and gain of Fra protein. Note the reduced Fra expression in *gcm*>*gcm* LOF and increased Fra expression in *gcm*>*gcm* GOF wings as compared to the control. **(F)** Schematic of a wing displaying the region shown in the panels **(C-E")**. Blue box indicates the region shown in the panels **(C-C")** and **(E-E")**, black box indicates the region shown in the panels **(D-D")**. **(G)** MI of the indicated genotypes. Scale bars: **(C-E")**, 10 μm.

Finally, we completmented the *in vitro* data with two *in vivo* assays. First, we showed that Fra levels are affected in opposite direction in *gcm* LOF and GOF wings **(Figure 5C-5C'', 5D-5D'', 5E-5E'', F).** In the *gcm* GOF wings, the levels of the Fra protein already increase by 15 hAPF, in agreement with the observed precocious initiation of migration **(Figure S5D-E" and F, compare with Figure 2M-N")**. Second, we hypothesized that Fra may constitute an important target of Gcm in L1 glia migration and overexpressed Fra in *gcm* KD wings. Fra overexpression is indeed sufficient to completely reverse the migratory phenotype due to *gcm* KD (**Figure 5G, patterned dark blue and brown column**).

In sum, Gcm activates the expression of Fra, which represents its major target in glia migration.



Supplementary Figure 5: Western blot and *gcm* GOF analysis confirms *fra* is a direct Gcm target and early Unc5 expression. (A) Immunoblot analysis of a WT Fra plasmid containing the GBS's monitored for an increase in the level of GFP (representative Western Blot out of a sample of 3). An increasing GFP level suggests that Fra is a direct target of Gcm. WB on total protein extracts from transfected S2 cells using anti GFP for Fra detection (top part) and anti β gal (lower part). No antibody was used for the detection of Gcm because of the use of WT Gcm plasmid. LacZ was used as a loading control. **(B)** Same WB was performed using a mutated Fra plasmid; detection of no GFP level upon using the gradients of Gcm confirms our analysis of Fra being a direct target of Gcm. **(C)** Quantification of the Western blot, Y-axis indicates the relative GFP levels of Fra upon S2 cells co-transfection assay. X-axis indicates the used gradients of Gcm in (μ g). **(D-E")** Expression profile of Fra in *gcm* GOF animals at 15 hAPF. Note that the blown up region in panels **(E-E")** is highlighted by a dashed white bracket in **(D-D").** See the enhanced protein levels in panels **(D-E")** as compared to the panels in

(Figure 2 M-N"). **(F)** Schematic of a wing displaying the region shown in panels **(D-E"and G-I")**. **(G-I")** Expression profile of Unc5 in control, and *gcm* GOF animals at 15 hAPF. Unc5 protein could not be detected at 15 hAPF in control wings **(G-G")**, whereas a slight Unc5 protein could be detected in *gcm* GOF wings at the same time **(H-I")**. Note that the blown up region in panels **(I-I")** is highlighted by a dashed white bracket in **(H-H")**. Scale bars: **(D-E")**, **(G-I")**, 10 μ m.

Role and Expression of Netrins

The above data indicate that a fate determinant controls a late event by inducing the expression of a chemoattractant receptor in a dose dependent manner. To dissect the molecular pathway controlling glia migration, we assessed the impact of the two Netrin ligands by using the null mutant $NetA^{\Delta}$ and $NetB^{\Delta}$ animals (Brankatschk and Dickson, 2006; Harris et al., 1996; Mitchell et al., 1996). $NetA^{\Delta}$ mutant glia do not display any migratory phenotype (**Figure 6A, compare grey and blue columns**), whereas migration is significantly delayed in $NetB^{\Delta}$ mutant glia (**Figure 6A, compare grey and red columns**). As it's long been known that Netrins can elicit short-range attraction at the *Drosophila* embryonic midline (Brankatschk and Dickson, 2006; Harris et al., 1996; Mitchell et al., 1996), we checked whether NetB could act in a similar manner in the migrating L1 glia. To this purpose, we used a transgenic line that does not express NetA and only expresses the membrane-tethered form of NetB (Brankatschk and Dickson, 2006). This lines $NetA^{\Delta}$ $NetB^{TM}$ and $NetA^{\Delta}$ *NetB* had been obtained through homologous recombination and hence expresses the modified or the wild type NetB protein at near endogenous levels (Brankatschk and Dickson, 2006; Harris et al., 1996; Mitchell et al., 1996). Glia migration was found to be comparable to that of wild type glia in $NetA^{\Delta}$ *NetB/Y* wings whereas it was delayed in $NetA^{\Delta}$ $NetB^{TM}/Y$ wings, suggesting the requirement of secreted *NetB* in glia migration (**Figure 6A, compare light and dark**

purple columns). These results suggest a major role of *NetB* as a ligand that functions as a long-range guiding cue for Fra expressing glial cells.

To define the source of NetB, we used a transgenic line that is routinely employed as a reporter of NetB expression (Hayashi et al., 2002; Timofeev et al., 2012). *NP4151>UAS GFP* shows *NetB* driven expression in the proximal region of the wing at the time L1 glial cells are about to migrate (16 hAPF), with a more restricted pattern at late stages (28 hAPF) (**Figure 6B,C**). This profile of expression fits well with the distal to proximal migration of L1 glia and we reasoned that if NetB were to act as a chemoattractant, its loss should cause glial migratory defects similar to those induced by the loss of Fra**.** We hence knocked down *NetB* upon crossing *NP4151>* to *UAS NetB RNAi (NetB* KD*)* flies and found a severe migration defect that could be rescued by simultaneously expressing *UAS NetB* and *UAS NetB RNAi,* which rules out the possibility of off target effects (**Figure 6D**). Moreover, overexpressing *NetB* in its territory of expression enhances the efficiency of glial cell migration (**Figure 6E**).

Finally, NetA is almost ubiquitously expressed in the epithelium as revealed by the use of the *Gal4* transgenic line *NP4012* (**Figure 6F,G**) (Hayashi et al., 2002; Timofeev et al., 2012) and knocking down or overexpressing NetA with that driver has no impact on glia migration (**Figure 6H,I).** Moreover, NetA overexpression in the NetB expression territory fails to enhance migration efficiency (**Figure S6A**) and to rescue the *NetB* KD phenotype (**Figure S6B**). Thus the two proteins have different potentials.

Altogether, our data strongly support the hypothesis that secreted NetB in the proximal wing provides a crucial guidance cue for Gcm-mediated Fra expression, hence controlling the efficiency of glia migration.

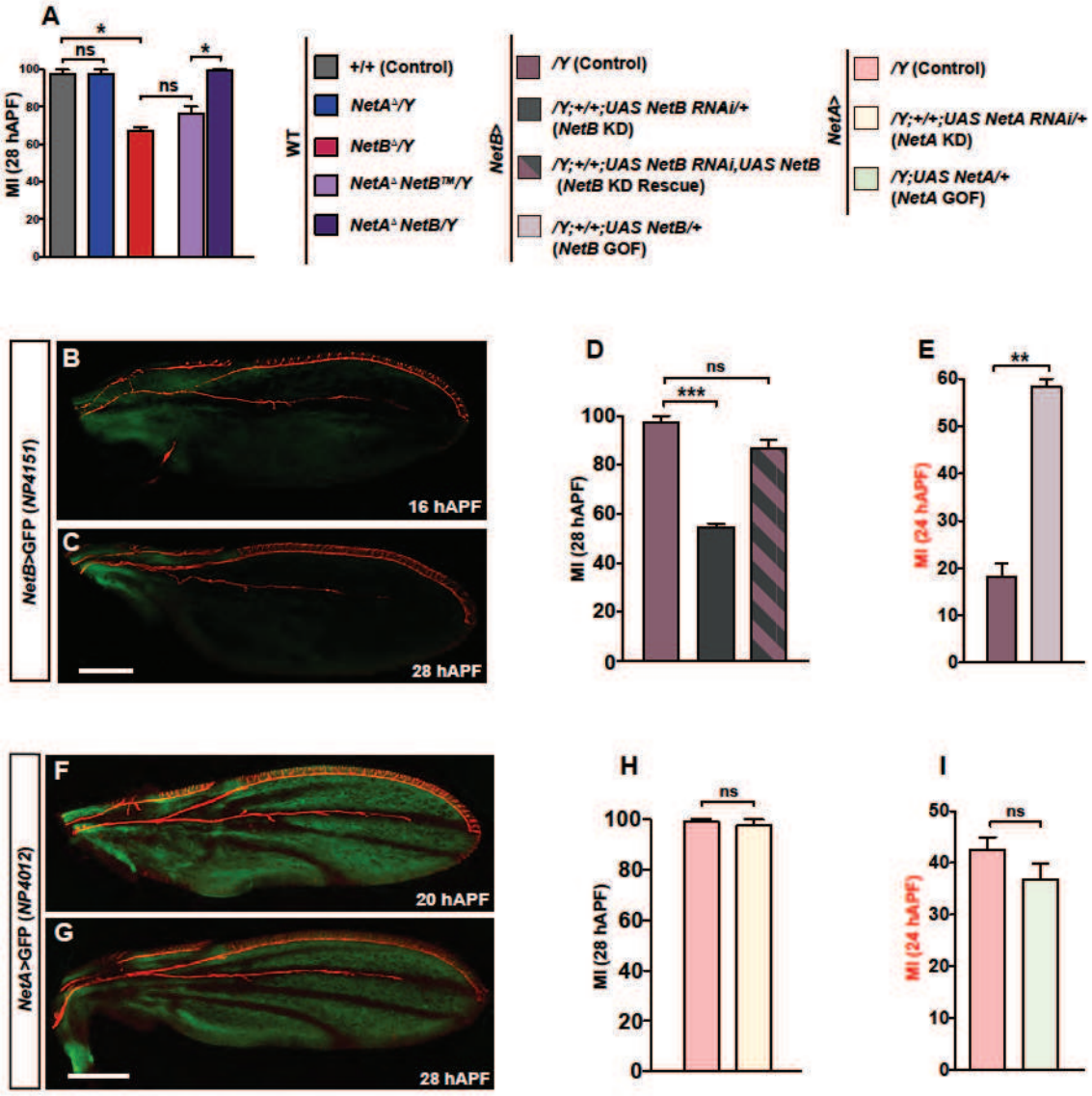
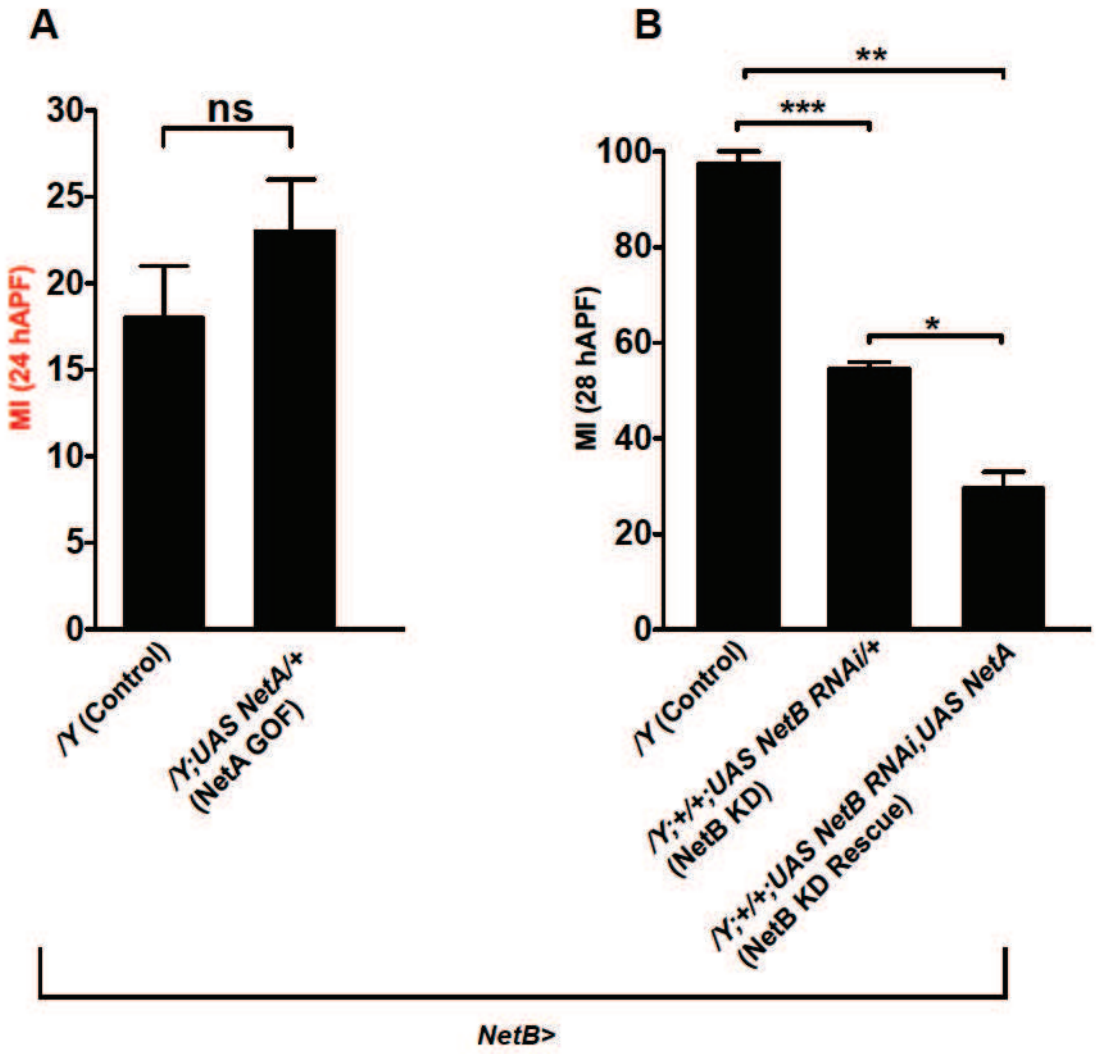


Figure 6: NetB may serve as a chemoattractant in collective glia migration. **(A)** MI of the indicated genotypes. Graph consists of the quantification on control, *NetA*$^{\Delta}$, *NetB*$^{\Delta}$ mutant wings and *NetA*$^{\Delta}$*NetB*^{TM}, *NetA*$^{\Delta}$*NetB*^{myc} wings. **(B, C)** *NP4151*-Gal4 driven GFP expression of *NetB* in a 16 and 28 hAPF wing. *NetB* seems to be proximally accumulated as revealed by the profile of GFP. Neurons are in red (anti-22C10) and GFP in green. **(D, E)** Graphs represent the MI of the indicated genotypes. **(F, G)** *NP4012*-Gal4 driven GFP expression of *NetA* in a 20 and 28 hAPF wing. *NetA* is expressed in the wing epithelium as revealed by the profile of GFP. Neurons are in red (anti-22C10) and GFP in green. **(H, I)** Graphs represent the MI of the indicated genotypes. Note that, MI of all graphs indicated in this figure was calculated by using a nuclear labeling. Scale bars: **(B-C)**, **(F, G)**, 80 μ m.



Supplementary Figure 6: Netrins in collective glia migration. **(A-B)** Graphs representing the MI of the indicated genotypes.

Unc5 acts as a repulsive migratory cue for L1 glia

unc5 provides a repellant receptor for Netrins and has been previously shown to be transiently expressed and required in the embryonic exit and peripheral glia (PG) associated with both the SN and ISN (Freeman et al., 2003; Keleman and Dickson, 2001; von Hilchen et al., 2010). We therefore asked whether *unc5* also controls glia migration in the wing and analyzed its expression profile. Unc5 is detected at as early as 18 hAPF;

expression is very weak and progressively decreases to fade completely by 29 hAPF (**Figure 7A-7D''**). Of note, Unc5 expression could not be detected at 15 hAPF (**Figure S5G-G", F**). However, slight Unc5 protein could be detected in *gcm* GOF glia at 15 hAPF (**Figure S5H-I", F**).

If *unc5* were to act as a repulsive receptor, the efficiency of glia migration would increase by lowering its expression, however, neither its *RNAi*-mediated KD nor the null $unc5^{8}$ mutation (Labrador et al., 2005) affect glia migration efficiency, and this was analyzed both at early and late stages (**Figure 7E, Figure S7A**). Thus, the loss of Unc5 does not enhance migration efficiency of L1 glia in the developing wing. We then asked whether Unc5 expression must be tightly regulated and found that overexpressing Unc5 affects the efficiency of glia migration in a manner that is opposite to that observed upon Fra overexpression (**Figure 7F, compare blank and green column**). Furthermore, the delayed migration phenotype was rescued by introducing the *unc5* KD construct or the mutant allele $unc5^{8}$ showing a direct effect of *unc5* on glia migration (**Figure 7F, patterned green and purple column, Figure S7B, third column**).

Thus, *fra* and *unc5* have opposite potential and role in glia migration, the first being necessary to trigger migration, the second being able to delay the migratory process. The two molecules seem to work in the same signaling pathway as the *unc5* GOF phenotype is further enhanced by lowering the levels of Fra (**Figure 7F, patterned green and blue column**). Also, the migratory phenotype induced by *unc5* overexpression is rescued by simultaneously overexpressing Fra (the rescue was analyzed at an early stage for a better understanding and quantification; **Figure 7G, patterned green and**

dark blue column). Finally, knocking down *unc5* rescues the *fra* KD phenotype (**Figure S7B, last two column**).

Overall, we conclude that Unc5 can act as a repellant but its expression is not sufficient to affect migration efficiency, which is mostly controlled by Net-Fra interaction.

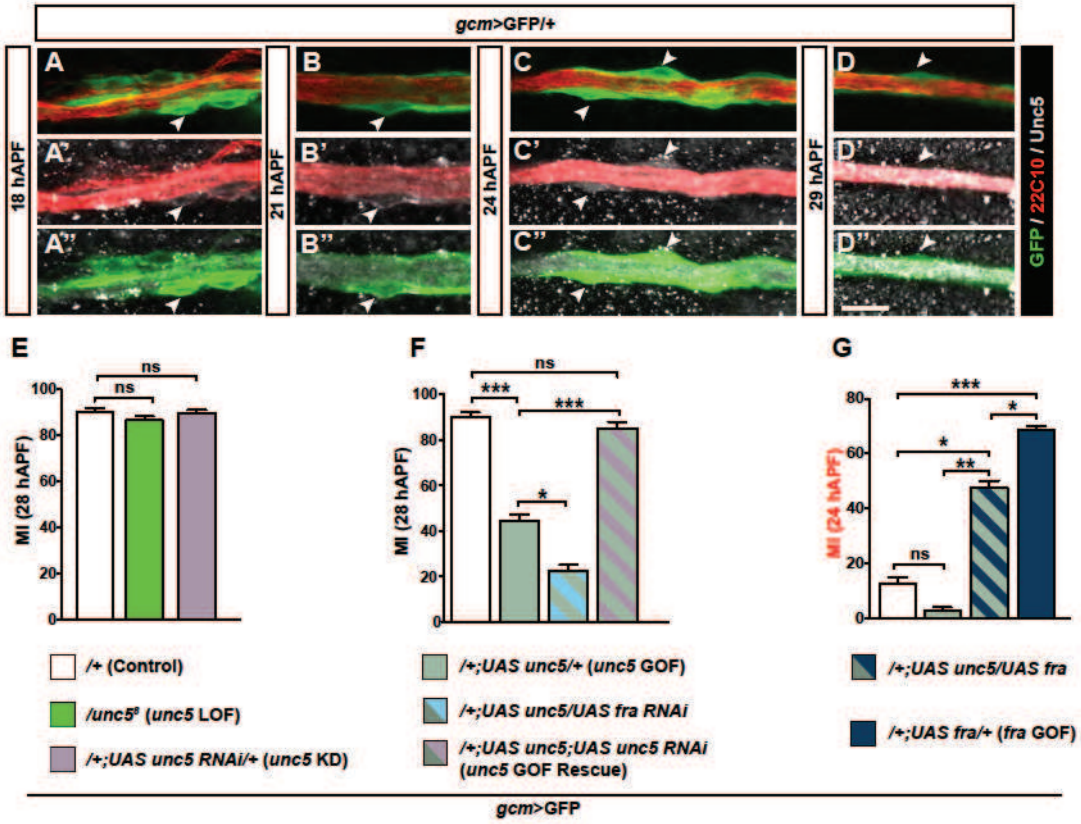
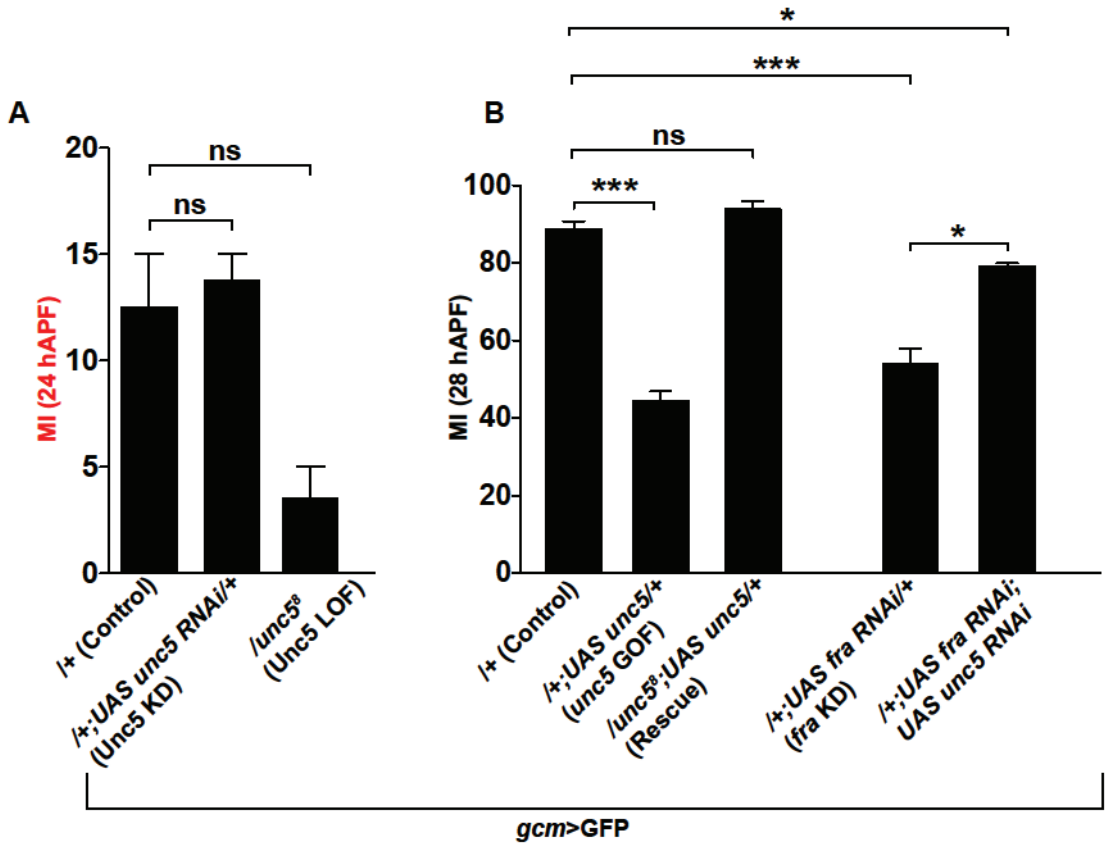


Figure 7: Unc5 acts as a repellant in glia migration. (A-D") Wing immunolabelled with anti-22c10 (neurons, red), anti-Unc5 (grey) and anti-GFP (glial cells, green) in the transgenic line *gcm*>GFP/+ at different migratory stages. Unc5 transiently starts to express both in glia and neurons at around 18 hAPF and fades away by 29 hAPF. **(E-G)** Graphs represent the MI of the indicated genotypes. Scale bars: **(A-D")**, 10 μ m.



Supplementary Figure 7: Unc5 in collective glia migration. (A, B) Graphs represent MI of the indicated genotypes.

DISCUSSION

Collective migration is a complex biological process allowing cells to leave their place of birth and reach their destination in a coordinated and timely manner (Friedl and Gilmour, 2009; Gupta and Giangrande, 2014; Petrie et al., 2009; Rorth, 2009). We here dissect the process of *Drosophila* wing glia collective migration and its underlying signaling pathway. We show that the Fra chemoattractant receptor controls glial migration in response to a long distance signal sent by the secreted Netrin B chemoattractant. Fra starts being expressed before glia migrate and its role is to trigger the initiation of glial migration. Finally, the time of migration initiation depends on the levels of Fra expression, which are directly controlled by the Gcm transcription factor. Thus, autonomous cues affect a specific step of collective migration, with the fate determinant directly triggering the expression of an effector gene.

Fra chemoreceptor controls migration initiation in a dosage dependent manner

Collective migration comes in different flavors, streams, chains, sheets and clusters which all imply tight coordination and cell-cell interactions (Gilmour et al., 2002; Gupta and Giangrande, 2014; Klambt, 2009; Marin et al., 2010; Rorth, 2003). The small cluster of *Drosophila* border cells migrate through nurse cells towards the border of the oocyte in response to growth factors. (Montell, 2003; Montell et al., 2012). The fish lateral line is composed of several hundred migrating and proliferating cells as a stream that maintains the intrinsic polarity by expressing different chemokine receptors at the front and back (Dambly-Chaudiere et al., 2007; David et al., 2002; Ghysen and Dambly-Chaudiere, 2004; Haas and Gilmour, 2006). While we have gain substantial knowledge

on the nature of the signaling cues, the precise role and regulation of those cues is still not understood. Typically, which steps are affected in the mutants and what controls the timely regulation of these molecules, which ultimately allows cells to reach their final destination and form the complex architecture of organs and tissues?

By focusing on the Netrin signaling pathway, which has been extensively studied in the context of axonal navigation and cell proliferation, we have addressed the above questions using the migrating wing glia of *Drosophila*. The analysis of the mutant phenotypes and the time-lapse approach show that the Fra receptor triggers migration initiation of the glial cells present along the L1 nerve. Reducing the amount of Fra delays migration whereas excessive Fra in the glial cells triggers their precocious migration. Thus, the Fra receptor is specifically required in the first step of collective migration. Importantly, the Fra phenotype is dosage dependent, highlighting the importance of quantitative regulation. Large cohorts of cells likely need strong forces to switch from an immotile to a motile phenotype, therefore only strong expression of the receptor allows migration towards the chemoattractant. Similarly, epithelial cells migrating in groups were shown to exert much stronger forces than an individual cell before and after epithelial-mesenchymal transition (du Roure et al., 2005).

The early fate determinant controls migration by inducing Fra expression

The timely regulation of the Fra chemoattractant receptor and its early role in collective migration allowed us to hypothesize that an early glial gene controls its expression. The similar migratory phenotypes of the Fra and Gcm mutation, the demonstration that Fra is a direct Gcm target and the fact that Fra expression rescues the

Gcm migratory phenotype indicate that Gcm affects migration through this receptor. Thus, early genes not only trigger the expression of transcription factors that in turn implement a specific developmental program but also directly contribute to the acquisition of specific phenotypes, such as the migratory potential. Three microarray screens were published identifying the genes acting downstream of *gcm* (Altenhein et al., 2006; Egger et al., 2002; Freeman et al., 2003), however, these screens did not allow the genome wide analysis of the direct targets of *gcm*. Following this, recently our lab published a high-throughout put DNA adenine methyltransferase identification (DAM ID) based screen for the direct targets of Gcm, which identified several genes involved in late gliogenesis/glial function, prompting us to revisit the concept of fate determinants as genes that only work at the very top of the developmental hierarchy (Cattenoz et al., 2015). Similarly, the Lim-homeodomain transcription factor Islet was shown to specify the electrical properties of motor neurons by repressing the expression of the ion channel Shaker during development, suggesting that such regulation of late genes by early transcription factors might as well be a conserved phenomenon (Wolfram et al., 2012).

While Gcm is only expressed at early stages, Fra expression stays on, suggesting that other transcription factors may replace Gcm at later stages. Our *in vivo* and cell transfection data are in line with this hypothesis and suggest that Repo may provide such factor (**Figure 8A**). The G-protein regulator *locomotion defects* (*loco*) is shown to be part of a similar regulatory network (Granderath et al., 1999). Loco that is necessary for the proper function of fully differentiated glial cells is initially activated by Gcm, which is then maintained by Pointed and Repo (Granderath et al., 2000; Yuasa et al., 2003). This suggests that the glial terminal differentiation program can also be regulated by the early

glial determinant. Likewise, Repo first depends on Gcm then becomes independent through autoregulation (Flici et al., 2014). This indicates a transition from early to late events in gliogenesis. Thus, a similar sequence of events may take place in Fra expression in wing glia, however, it is clear that the first step strictly depends on Gcm, as overexpression of Fra using the repo promoter does not induce migratory defects both at early and late stages, likely due to the later expression of such driver (**Figure 8B and data not shown**).

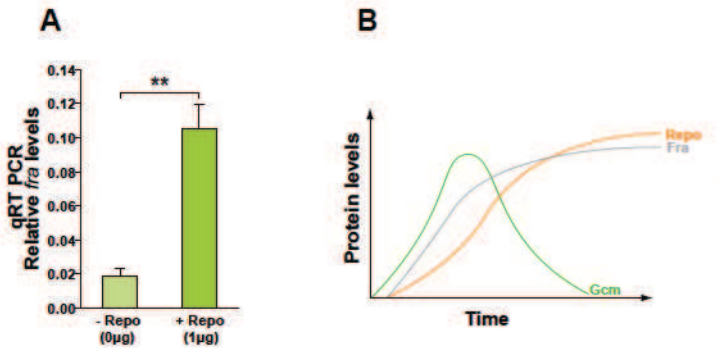


Figure 8-: Repo regulates Fra at later stages. (A) Histogram showing the endogenous expression of *fra* upon S2 cell transfection with a Repo expression vector and FACS sorting. The y-axis represents the relative expression levels in cells transfected with Repo compared to cells without Repo. **(B)** Schematic summarizing the regulatory network. **(C, D)** Graphs representing the MI of *fra* GOF animals with *repo* promoter at early and late stages.

The Ligand and the receptors in glia migration

Netrins secreted by the cells of the ventral midline are best known to guide commissure axons at either short or long -range (Brankatschk and Dickson, 2006; Lai Wing Sun et al., 2011). In addition to axon guidance, Netrin and their receptors also guide the migration of *Drosophila* embryonic glial cells (von Hilchen et al., 2010). It was proposed that both Netrin A and B act on embryonic longitudinal glia (LG) through Fra (Hilchen et al., 2010). Based on the mutant data, in contrast, NetB only serves as a chemoattractant ligand to guide L1 glia migration in signaling to Fra. While it is possible

that NetA may have a role in glial biology, the fact that NetA cannot rescue the NetB phenotype strongly suggests that the two Netrins have different potentials. By far, both Netrins are reported to be chemoattractants, except a study reported that NetB but not NetA mediates the dendritic targeting via Fra (Matthews and Grueber, 2011).

One explanation in our case could be assigned to their different expression profiles. The long-range attraction and the proximal localization of NetB, which also matches the distal to proximal migration of glial cells, provide a clue as to why only NetB and not NetA matter in this case. It will be of great interest to determine whether these functional differences are reflected in the binding specificity of these ligands to their receptors and whether its only NetB that works as a long-range guidance cue.

Furthermore, we analyzed the Unc5 repulsive Netrin receptor and propose that it may act as a stop signal. According to the previous report in mammals both DCC and Unc5 are expressed by the migrating oligodendrocyte precursor cells, however their exact role remains undetermined (Tsai et al., 2003). We here propose that both Fra and Unc5 are expressed by the L1 peripheral glia; however, they both act in a very defined manner. While Fra is expressed before migration initiation and act as an instructive cue, Unc5 starts to be prominently expressed at the late migratory phase and therefore may act in a manner that terminates migration. Our hypothesis that Unc5 acts as a break in L1 glia migration is also supported by time-lapse data showing that *unc5* overexpression delays migration but does not affect initiation (Data not shown). This suggests that Unc5 starts to act only at late migratory phases and supposedly converts the Netrin mediated signal from attraction to repulsion, a well known phenomenon (Hong et al., 1999), thereby terminates the migration of L1 glial cells. Previously, Unc5 has been identified as a Gcm

regulated gene within the embryonic nervous system and has been reported to regulate glia migration in the embryos as ectopic expression of *unc5* supposedly repelled LG away from the midline within the CNS (Freeman et al., 2003). Intriguingly, in our case downregulating Unc5 in L1 peripheral glial cells at late developmental stages did not cause any migratory phenotype, whereas ectopic expression of *unc5* delayed glia migration. One reason for this could be the low amount of protein present in the LOF condition, which is not sufficient to produce any phenotype. Another reason as also mentioned earlier could be because Unc5 as observed is active only at the late migratory phases; whereas, the decision to start the migration has already been made by Fra that is expressed at migration initiation. It is evident with this and previously published data that *unc5* acts differently when assessed in different set of cell population. It will be interesting to assess what precisely causes the change in behavior of this Netrin receptor when studied in different cell populations.

Overall, this paper displays a model in which the ligand (NetB) is expressed rather early during development whereas the receptors are expressed at later stages and cell autonomously control the different steps of collective migration. Fra expression instructs L1 glia to start moving whereas Unc5 conversts Netrin mediated attraction to repulsion further acting as a brake. The acquisition of the Fra levels that trigger migration is part of the cell-specification program dictated by the Gcm glial determinant

EXPERIMENTAL PROCEDURES

Fly stocks and genetics

Fly stocks were raised at 25°C in standard medium. *repo Gal4* (indicated as *repo>*) was used to detect glial-specific expression of *UAS* ncGFP (nc: nuclear and cytoplasmic) (Kumar et al., 2015) or *UAS* PHGFP (Kumar et al., 2015) (both display membrane localization). Note that in Fig 1J a weaker *repo Gal4* line was used (Kumar et al., 2015); *gcm Gal4 UAS* mCD8 GFP, CD8 GFP labels the membrane (*gcm*>GFP) and was used as an early glial specific driver (Jacques et al., 2009); *gcm 6KB>* (Flici et al., 2014); fra^{3} (von Hilchen et al., 2010); *UAS fra* (von Hilchen et al., 2010); *UAS fra RNAi* (Manhire-Heath et al., 2013); *UAS* $fra^{\Delta}C$ (Garbe et al., 2007); gcm^{rA87} (Vincent et al., 1996); $gcm^{N7\text{-}4}$ (Vincent et al., 1996); *UAS gcm* (*F18A*) (Bernardoni et al., 1998); *UAS gcm RNAi*; *gcm*>GFP/*gcm*>GFP (used as a homozygous mutant of *gcm*) (Popkova et al., 2012); *UAS string* (Inaba et al., 2011); *UAS dacapo* (Lane et al., 1996); gcm^{34} (Vincent et al., 1996); $NetA^{\Delta}$ (Newquist et al., 2013a; Newquist et al., 2013b; von Hilchen et al., 2010); $NetB^{\Delta}$ (Newquist et al., 2013a; Newquist et al., 2013b; von Hilchen et al., 2010); $NetA^{\Delta}$ $NetB^{TM}$ (Brankatschk and Dickson, 2006); $NetA^{\Delta}$ $NetB^{myc}$ (Brankatschk and Dickson, 2006): Please note that both $NetA^{\Delta}$ $NetB^{TM}$ and $NetA^{\Delta}$ $NetB^{myc}$ encode the c-myc epitope tags; *UAS NetB RNAi* (Manhire-Heath et al., 2013); *UAS NetB* (Timofeev et al., 2012); *NP4151 Gal4* and *NP4012 Gal4* (DGRC, Kyoto) (Hayashi et al., 2002; Timofeev et al., 2012); *UAS NetA RNAi* (Manhire-Heath et al., 2013); *UAS NetA* (Newquist et al., 2013a; Newquist et al., 2013b); $unc5^{8}$ (Labrador et al., 2005); *UAS unc5 RNAi*; *UAS unc5* (von Hilchen et al., 2010). Note that all the RNAi lines were obtained from Bloomington and/or VDRC stock center.

Molecular Cloning (Supplementary info)

For the *fra* gene, oligonucleotides surrounding the GBS's were designed with flanking restriction sites for KpnI at the 5' extremity and NHeI at the 3' extremity. Each pair of oligonucleotides was used to amplify the genomic region encompassing the GBSs using the Expand High fidelity polymerase (Roche). The amplicons were digested with 20 U of KpnI (NEB # R3142S) and 20 U of NheI (NEB # R3131S) in Cutsmart buffer

(NEB # B7204S) for 2 hours min at 37ºC. The digested amplicons were then cleaned using the PCR clean-up kit (MN # 740609) according to manufacturer's instructions.

For ligation, 50 ng of the digested probe were used and cloned into the pGreen Pelican vector overnight at 18 °C. 1 μ l of the ligated product was used for transformation of electro competent DH5 α bacterial cells. Bacteria were then kept for 1 hour at 37 °C and plated on ampicillin containing medium. After overnight incubation at 37 °C several colonies were picked up in separate tubes containing the LB and then were incubated overnight at 37 °C. The following day, mini preps were performed using the kit; positive colonies were identified by gel electrophoresis and sent for sequencing for final confirmation.

Same procedure was conducted to build the mutated *fra* reporter plasmid.

Following oligonucleotides were used:

fra WT forward:

5' GAGAGGTACCGTGTCCAAAAATGCGGGTCTGTTTCTCG 3'

fra WT reverse:

5'GAGAGCTAGCGTTAAGACAAACATGCAGGCATAAAGACATG 3'

fra Mutant forward:

5'GAGAGGTACCGTGTCCAAAAAAAAAAACTGTTTCTCGAAATTGAGTT 3'

fra Mutant reverse:

5'GAGAGCTAGCGTTAAGACAAACAAAAAAAAATAAAGACATGAAATGGA TG 3'

Co-transfection and Western blot assays

Co-transfections in S2 cells were carried out using Lipofectamine (Invitrogen). 6 x 10^6 cells were cultured in 6-well plates containing Schneider medium. In each well, cells were transfected with 1 μ g of *fra* WT or mutant reporter plasmid, 1 μ g of *pPAC-lacZ* as a transfection control, 0.5 μ g or 1 μ g or 2 μ g of *pPac gcm* expression vector and *pPac* 'empty' to make up the volume up to 4 μ g. Cells were collected 48 hours after transfection, first washed in cold PBS and then resuspended in lysis buffer. Total protein extract was obtained by 4 freezing-thawing cycles in liquid nitrogen and centrifugation at

4 °C at 13000g. Protein expression was detected as per standard Western blot procedures. Primary antibodies used were as follows: mouse anti-β-Gal (1/2000, Sigma), rabbit anti-GFP (1/5000, Molecular Probes); mouse anti-HRP and rabbit anti-HRP (1/5000, Jackson ImmunoResearch) were used as secondary antibodies. Note that each experiment was performed in triplicate.

β gal assays were performed to measure the levels of LacZ for each replicate. 20 μl of protein extract mixed with 50 μl of β gal assay buffer containing ONPG was incubated at 37 °C. Reaction was stopped by adding 50 μl of 1M Na_2CO_3 once the solution turned yellow, DO was analyzed at 415nm. The levels of GFP were normalized to the LacZ value in each blot and were quantified by using ImageJ software. The background was subtracted from each band value and then the average was calculated.

The method and buffers used for co-transfection, Western Blot and qRT-PCR experiment are the same as in (Flici et al., 2014).

Reverse Transcription and qRT-PCR

Total RNA was extracted from S2 cells using Trizol (Invitrogen), 1 μg of purified RNA was reverse transcribed by SuperScript II. qPCR was performed with the machine Roche LightCycler 480 and Sybr Green Master mix (Roche) using the following oligonucleotides:

fra WT forward:

5' GAGAGGTACCGTGTCCAAAAATGCGGGTCTGTTTCTCG 3'

fra WT reverse:

5'GAGAGCTAGCGTTAAGACAAACATGCAGGCATAAAGACATG 3'

fra Mutant forward:

5'GAGAGGTACCGTGTCCAAAAAAAAAAACTGTTTCTCGAAATTGAGTT 3'

fra Mutant reverse:

5'GAGAGCTAGCGTTAAGACAAACAAAAAAAAATAAAGACATGAAATGGATG 3'

GFP forward: **ACATGAAGCAGCACGACTTCT**

GFP reverse: **TTCAGCTCGATGCGGTTCA**

Gcm WT forward: **5'GAGAGATCTTATCCCGATCCCCTAGC3'**

Gcm WT reverse: **5'CTACTACTACAGCAATACGGG3'**

LacZ forward: **TGTGCCGAAATGGTCCATCA**

LacZ reverse: **GTATCGCCAAAATCACCGCC**

For each gene, the expression levels were automatically calculated (LightCycler480 Software, release 1.5.0) by calibration to gene-specific standard curves generated on input cDNAs. Collected values, derived from three amplification reactions, each performed in three independent experiments, were normalized to βgal mRNA amounts.

Immunolabeling and antibodies

Pupae of desired stage were collected and fixed in 4% PFA PBS (paraformaldehyde in phosphate buffer saline) overnight at 4°C. They were dissected in PBT (PBS Triton-X100, 0.3%) and wings were given 4 quick washes of 10 minutes in PBT and were incubated in the blocking reagent PBT-NGS (5% normal goat serum in PBT) for 60 minutes at room temperature on planar shaker. Samples were then incubated overnight in primary antibodies (diluted in PBT-NGS): mouse-anti-Repo labels glia (1:800) and mouse-anti-22c10 labels neurons (1:1000) (DSHB), chicken-anti-GFP (1:1000) (Abcam), rat anti-Elav labels neurons (1:1000) (DSHB), rabbit-anti-Unc5 and rabbit-anti-Fra (1:500) were gifts from Benjamin Altenhein. After 4 washes in PBT, wings were incubated for 2 hours at room temperature in secondary antibodies (1:500) raised in mouse, rat, rabbit or chicken and coupled to Cy3, Cy5 or FITC fluorescent dyes diluted in PBT-NGS. Following a final wash in PBT, wings were mounted on slides in Aqua- Poly/Mount medium (Polysciences Inc.).

***In vivo* Imaging**

Time-lapse analyses were performed using the standard procedure as described in (Aigouy et al., 2008; Aigouy et al., 2004; Kumar et al., 2015; Soustelle et al., 2008). Photo bleaching was avoided by using a low magnification and reduced exposure time. Maximum projections for time-lapse and confocal images were obtained by using the ImageJ software. Images were annotated by using Adobe Photoshop and Illustrator.

Statistical analysis

The number of wings dissected for each experiment were more than or equal to 30. The Migratory Index (Kumar et al., 2015) defines the percentage of wings in which glial cells have completed migration at a given time point (28 hAPF in most cases). Graphs were made using Prism software and the Student's *t* test method was used for the comparison between two different experimental sets. Bars indicate the standard error mean (s.e.m).

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Additional data related to the manuscript

Role and mode of action of Fra on glia migration

(A) *Non-autonomous requirement of Fra*

In the manuscript, I have shown that Fra single handedly is capable of initiating the migration of L1 glial cells as playing with its dosage is deleterious for the whole process. My analyses on the fixed material and in living animals show that threshold levels of Fra are crucial for timely chain migration and that glial migration is complete in only 14% of the *fra* mutant wings. Interestingly, reintroducing the UAS *fra* transgene in glial cells rescues the *fra* mutant phenotype partially (**Figure 19A, last column**). Given the high levels of Fra expression in the *gcm>fra* GOF wings it is unlikely that the partial rescue is due to suboptimal levels of Fra (**see Figure 2 in the manuscript**). Rather, the lack of total rescue may be ascribed to the neuronal requirement of Fra. **Figure 1 in the manuscript** shows that Fra is expressed in glia as well as in neurons and indeed around one third of fra^3/+ wings show an axonal navigation phenotype (**Figure 19B, C, D**). In these wings, axonal navigation is delayed, which may indirectly affect glial migration. Accordingly, I analyzed wings where Fra is specifically knockdown in neurons using an Elav driver and got a very strong phenotype (Data not shown). However, the available Elav drivers are not specific to neurons in wings as they are also expressed in glia and in epithelial cells. Thus in the wing, I was unable to use a driver that specifically activates or knockdown gene expression in neurons.

(B) *Loss of fra delays migration*

The delayed glial migratory phenotype in *fra* LOF wings led me to ask whether eliminating *fra* completely arrests migration or delays it. By analyzing different stages I found that migration is only delayed in *fra* LOF wings **(Figure 19E).**

(C) *Role of the cytoplasmic P3 domain of Fra in glia migration*

I have shown already that the cytoplasmic domain of Fra is important for L1 glia migration. The 278 amino acids long cytoplasmic domain of Fra consists of three highly conserved motifs, named P1, P2 and P3 (Hong et al., 1999; Keino-Masu et al., 1996; Kolodziej et al., 1996) and the cytoplasmic domain is thought to have important roles in the recruitment of cytoplasmic signal transduction molecules (Lai Wing Sun et al., 2011; Moore et al., 2007; Rajasekharan and Kennedy, 2009). Hence, I asked which among the three domains of cytoplasmic Fra is necessary for glia migration by overexpressing *fra* transgenes deleted in the P3 or in the P1 and P2 domains.

This migratory phenotype observed in the fra^{3}/+ wings is rescued by using full-length, and the *UAS* $fra^{\Delta P1\,\Delta P2}$ transgenes (Matthews and Grueber, 2011). In stark contrast, *UAS* $fra^{\Delta P3}$ is not able to rescue the migratory phenotype significantly (**Figure 19F**). These results indicate that the P3 domain, but not P1 and P2 is important for Fra mediated signaling in glia migration.

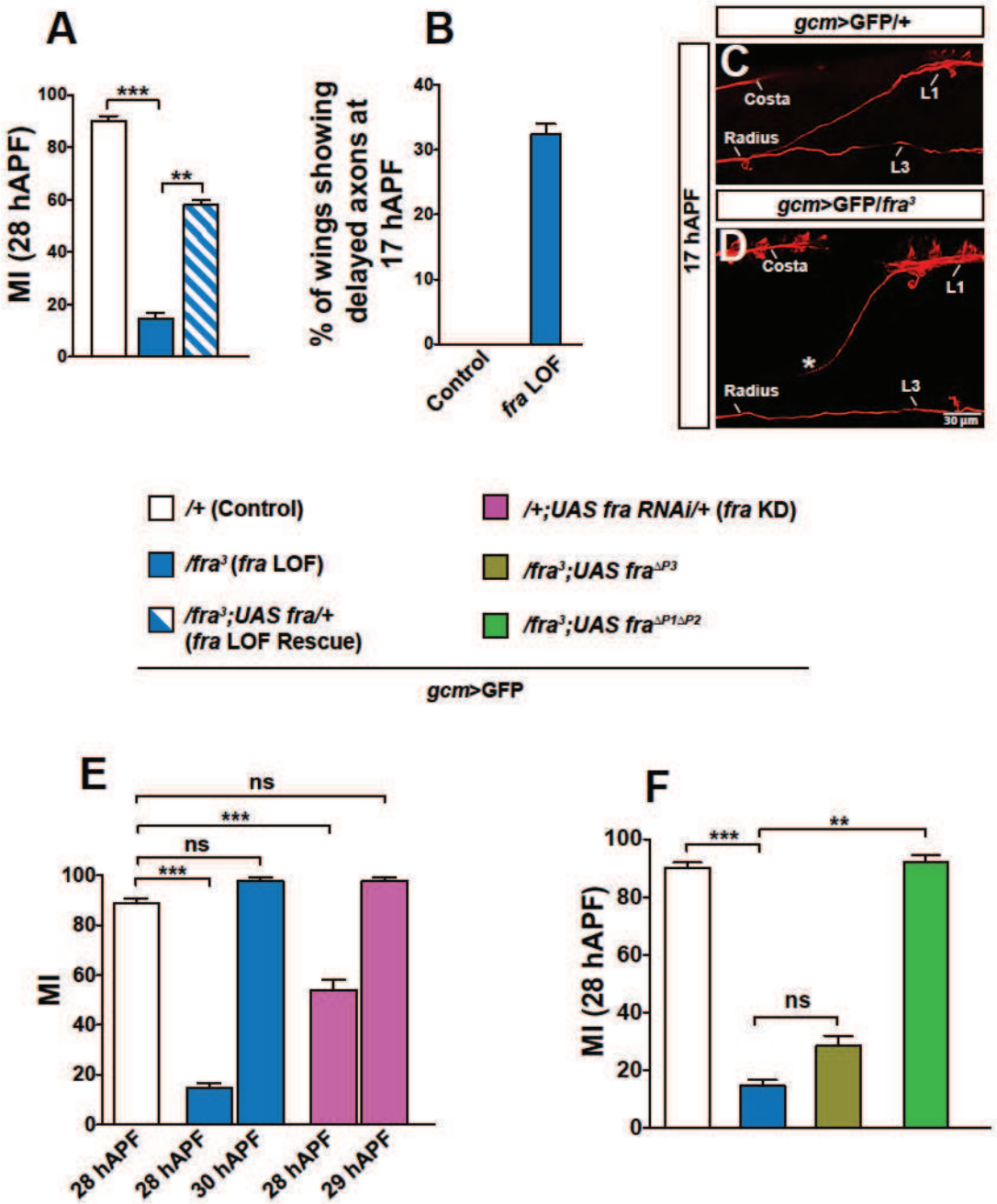

Figure 19: Fra in collective glia migration. (A, B, E, F) Graphs represent the MI of the indicated genotypes. **(E)** MI of *fra* LOF and KD wings at different time points as indicated on the X axis. **(C)** 17 hAPF control wing displaying normal axonal migration. **(D)** Delayed axon bundle in a *fra* LOF wing at 17 hAPF.

Role and mode of action of NetB on glia migration

(A) *Specific role of NetB on glia migration*

The analysis of single Netrin mutants as described in the manuscript suggest that NetB expressed in the proximal wing compartment serves as a ligand for Fra mediated L1 glia migration. To complete the analysis I also investigated the glial phenotype of Netrin AB double mutant animals using the null allele $NetAB^{\Delta}$ (Matthews and Grueber, 2011) and found that animals lacking both *Netrin* genes display significantly delayed migration when analyzed in heterozygous condition (**Figure 20A, second column from the left)**, similar to the phenotype obtained with $fra^{3}/+$ mutant glia (**see Figure 1H in the manuscript**). Moreover, $NetAB^{\Delta}$ mutant glia display an even stronger migratory phenotype when analyzed in hemizygous condition (**Figure 20A, third column from the left)**. Interestingly, the latter phenotype is also significantly stronger than that observed in *NetB/Y* hemizygous animals (**see Figure 6 in the manuscript**). This suggests a possible background effect, due to the deletion of other genes in the $NetAB^{\Delta}$ double mutant flies. Additional rescue experiments of hemizygous $NetAB^{\Delta}$ animals by *NetB* and *NetA* overexpression will further confirm this hypothesis.

(B) *NetB has a permissive role on glia migration*

Since increasing the levels of NetB in its normal territory of expression enhances the efficiency of glial cell migration, I decided to ectopically express it and asked whether NetB is instructive for glia migration. NetB was expressed in the posterior wing compartment using the *engrailed Gal4* driver (*en>*) (Hidalgo, 1994; Lawrence and Morata, 1976) or in the distal part of the wing using the *GMR 29F05 Gal4* (*GMR*

29F05>) driver (Pfeiffer et al., 2008). If NetB were to have an instructive role, its mislocalization would likely attract glial cells to ectopic positions. However, we did not observe such phenotype nor did we detect migratory defects along the L1 nerve, the migratory efficiency of L1 glia remaining unaffected (**Figure 20B**). These experiments further suggest that NetB expressed in the proximal wing compartment acts as a ligand to Fra driven glia migration and has a permissive role on glial cells that move over a neuronal substrate.

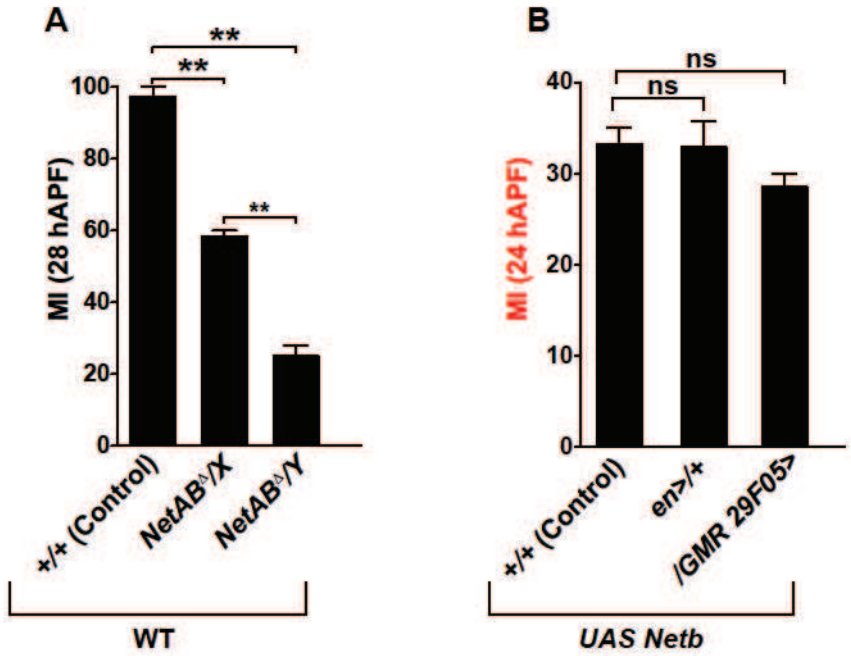

Figure 20: NetB in glia migration. (A, B) Graphs represent MI of the indicated genotypes.

Role of blood cells in glial migration

(A) *gcm expression in blood cells does not affect glial migration*

As I have already shown in the manuscript, Gcm affects the migration of L1 glial cells in a cell autonomous manner by regulating the expression of Fra. Another series of evidence supports this hypothesis. It is already known that *gcm* is also expressed in hemocytes (Bataille et al., 2005; Bernardoni et al., 1997; Waltzer et al., 2010). In view of this, I checked if the severe migratory delay is specific to reducing the amount of *gcm* in glia and not in hemocytes. For this purpose, I used an independent hemocyte drivers, *collagen Gal4*, which is expressed in the embryonic hemocytes (Asha et al., 2003). I first knocked down (KD) *gcm* in hemocytes (*collagen Gal4* crossed with the *UAS gcm RNAi*) and found no defect in glia migration (**Figure 21A**). Subsequently, I overexpressed *gcm* in those cells (*collagen Gal4* crossed with the *UAS gcm*) and found no acceleration of glial migration, as was instead observed upon overexpression of *gcm* in glia (**Figure 21B**). Intriguingly, the increase of Gcm levels in blood cells non-autonomously delays glial migration. While this dominant, gain of function effect remains at present unexplained, the loss of function data argues against a role of Gcm expression in blood in the control of glial migration.

(B) *Specific downregulation of Fra in glia cell autonomously affects migration*

Although Gcm is autonomously required in glia, it could still be possible that Fra expression in blood cells is required non-autonomously to control glial migration as we have detected the Fra protein in blood cells of the wing (Data not shown). However, the fact that *fra* KD using the glial specific driver *repo Gal4* cell autonomously affects glia

migration eliminates this possibility. Furthermore, I also knocked down Fra using a temperature sensitive *Gal80* construct of *gcm* driver that is not expressed in the blood and confirmed that in these conditions too glia migration is affected (**Figure 21C**). In sum, Fra and Gcm affect glial migration cell autonomously.

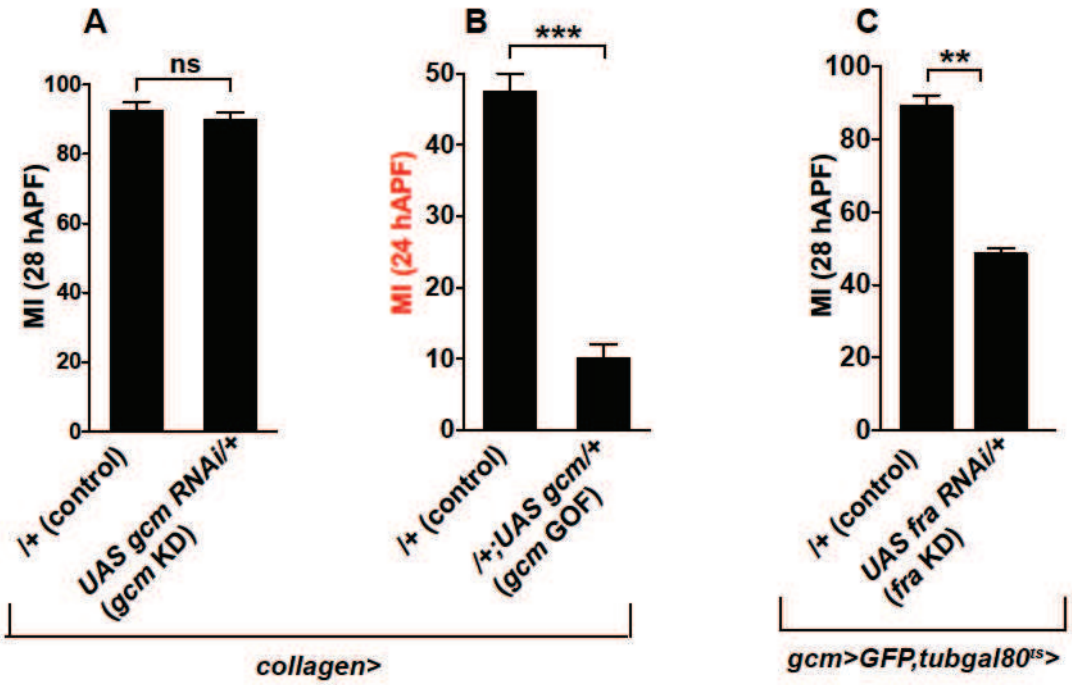

Figure 21: Role of hemocytes in glia migration. (A, B, C) MI of the indicated genotypes.

Discussion of the additional data

The developing *Drosophila* wing provides an excellent model to study the intrinsic factors underlying collective glial cell movement. Determining how signaling pathways regulate collective glia migration is important for understanding the circuitry formation in the nervous system and will undoubtedly shed light on neural developmental disorders as well.

DCC/Frazzled family members, attractive receptors for Netrins, play important roles in many developmental contexts, particularly in promoting midline crossing of commissural axons (Kolodziej et al., 1996; Lai Wing Sun et al., 2011). However, our understanding of the key factors that control receptor activation and signaling is still fragmented.

In the *Drosophila* embryo and in the visual system, as well as in vertebrates, Fra has been shown to be an important factor in axonal navigation and targeting (Keino-Masu et al., 1996; Kolodziej et al., 1996; Timofeev et al., 2012). My results also suggest that *fra* downregulation delays axonal navigation in *gcm*>GFP/*fra*^{3} mutant wings. Thus, Fra is expressed and required in neurons as well as in glia and the expression and role of Fra in neurons may account for a modest, indirect effect on glial migration. However, glial specific knockdown of *fra* and the fact that in these conditions axonal navigation proceeds as in wild type animals demonstrate the absolute requirement of Fra in glia (Data not shown).

It is also very important to note that the glial chain, both in *fra* GOF and LOF conditions, eventually completes migration and forms a continuous sheath around the axons. Moreover, I never observed an obstruction in migration upon altering Fra levels in

the glia. Time-lapse analysis of the Fra GOF glial cells also revealed a retarded time of migration initiation. Thus, these data suggest that Fra plays a direct, dosage dependent role that triggers efficient and timely migration of glial cells.

A number of studies provide evidence that the ability of receptors to attract or repel lies within their cytoplasmic domain (Bashaw and Goodman, 1999). Thus, I analyzed the role of cytoplasmic domain of Fra using an allele $fra^{\Delta}C$, in which the entire domain is deleted and found an impaired migratory phenotype. Further on, the deletion analyses of the different domains suggest that P3 plays an important role in controlling Fra mediated glia migration. This data is in agreement with studies performed in vertebrates suggesting a potential requirement of P3 for Fra related function (Stein et al., 2001). Interestingly, the requirement of the different domains for DCC/Fra/Unc-40 seems to be species-specific as in *C.elegans* P1 and P2 play an essential role (Gitai et al., 2003). Recent work in *Drosophila* has shown that the P3 domain is able to active transcription during axon guidance (Neuhaus-Follini and Bashaw, 2015). Future investigations will determine whether Fra's P3 domain also functions as an activation domain in glia migration and which might be its targets.

The analysis of the membrane-tethered NetB transgene along with its proximal most expression profile suggest that NetB serves as a ligand for Fra and acts as a long-range guidance cue in the developing wing.

Ectopic expression of NetB in distal and posterior compartments of the wing did not alter the migratory phenotype. One possible explanation to this finding is the fact that glia migrate along the axonal bundle and so ectopic NetB cannot make glia move in other

directions. Another possibility is that in our conditions, the endogenous source of NetB is still present.

The results presented in the following chapters are preliminary data. I initiated the projects described below but since I switched to the analysis of other aspects of L1 glia migration I did not further pursued them. Nevertheless, I consider the parts discussed here relevant to the global understanding of the collective migration of L1 glial cells.

Chapter 4

Role of a novel cell population in glia migration

Introduction and Results

The TSMs (Twin Sensilla of the Margin) are the two sensory organs located at the proximal region of the anterior wing margin. Thus, the TSM neurons are the most proximally positioned neurons along the wing margin (see Introduction). Next to the two TSM neurons, a very peculiar cell population is positioned at the front of the glial chain. Interestingly, this cell population is neither neuronal nor glial, as it does not express either Repo or Elav. These cells, however, constitutively express Gcm and I have therefore called them TSM-G, unlike glial cells that transiently express Gcm. The TSM-G cells surround the proximal tip of the glial chain before migration onset and do not belong to the assembly of glial cells. The TSM-G cells can be detected with gcm^{rA87} an enhancer trap line in which the lacZ containing P-element is inserted in the *gcm* gene (Giangrande et al., 1993; Klambt and Goodman, 1991) and, most importantly, by *in situ* hybridization with a *gcm* specific probe (**Fig. 22A)** (Popkova et al., 2012). These cells can be first spotted at 15 hAPF and are organized in three dorsal clusters. These Gcm+ clusters are composed of a variable number of cells going from 6 to10 per cluster (**Fig. 22C, D)**.

Given the close localization of the TSM-G cells to the proximal most glia, and their time of appearance, we hypothesized that these cells are involved in the regulation of glial cell migration. To determine the role of the TSM-G cluster in glia migration, I performed experiments in which I specifically manipulated the levels of Gcm in the

TSM-G cluster. To do so, I used the *gcm-Gal4* driver, which mimics the endogenous expression profile of *gcm*, and spatially restricted its action to the TSM-G cells upon combining it with a transgene that expresses the Gal4 inhibitor Gal80 in glial cells (*repo-Gal80*) (Lee and Luo, 1999). Introducing the *UAS-CD8GFP* transgene in the *gcm-Gal4, repo-Gal80* background confirms that the GFP is only detected in TSM-G cells but excluded from glial cells (**Fig. 22B)**. For the sake of simplicity, I will call the "*gcm-Gal4, repo-Gal80; UAS-CD8GFP"* driver as the *TSM-G*>GFP driver in the rest of the chapter. Note that all the genetic manipulations in TSM-G clusters were performed using the *TSM-G*>GFP flies.

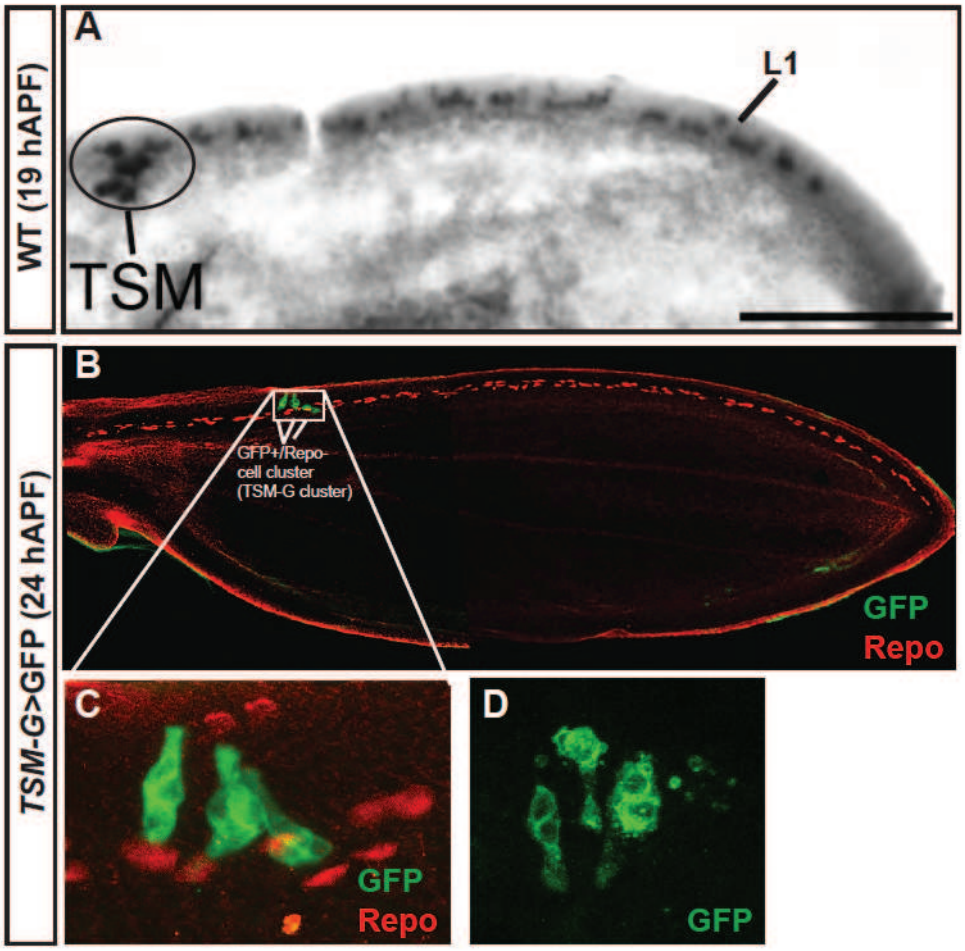

Figure 22: TSM-G cluster. (A) *In situ* hybridization with a *gcm*-specific probe showing the expression of Gcm in the TSM-G cells (black circle) of a WT wing at 19 hAPF (Popkova et al., 2012). **(B)** *gcm-Gal4, repo-Gal80; UAS-CD8GFP* wing at 24 hAPF**.** Double labeling with anti GFP (green) and anti Repo (red). **(C)** High magnification image of the region containing TSM-G cells shown in the panel **(B)**. **(D)** High magnification image of TSM-G clusters in another wing.

To test the role of *gcm* in the TSM-G clusters, I performed a conditional knockdown (KD) of *gcm* (*gcm* KD) by crossing the *UAS gcm RNAi* line to the TSM-G line and observed a delayed migration (**Fig. 23A, brown column**).

Moreover and in line with the above data, using the *UAS gcm* transgene to enhance the amount of Gcm in TSM-G clusters increases the percentage of wings in

which glia completes migration as compared to what observed in control animals (**Fig. 23A, grey column**). Thus, downregulating and overexpressing *gcm* in TSM-G clusters affects the migratory efficiency of glial cells in an opposite manner. Noteworthy, the gain and the loss of function phenotypes are milder in comparison to what is observed upon manipulating the levels of Gcm in glial cells (**see Figure 2A in the manuscript**).

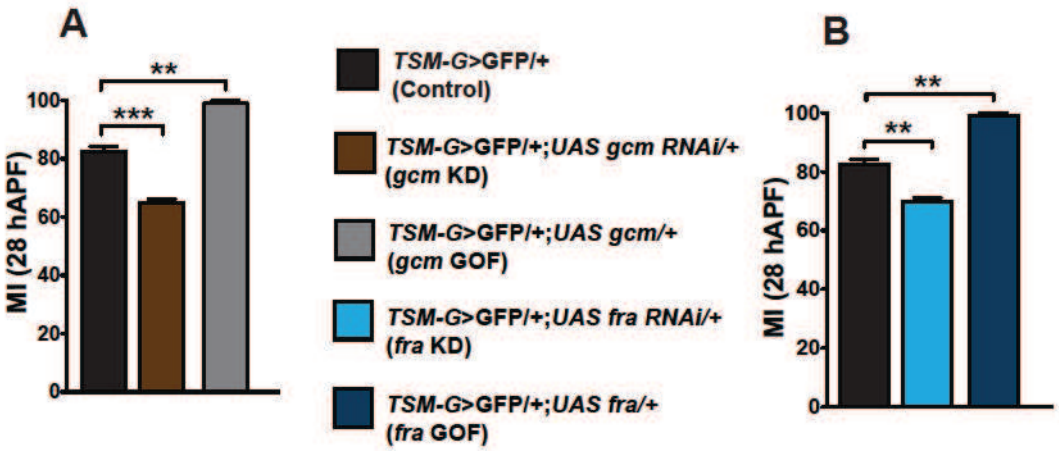

Figure 23: Gcm and Fra in TSM-G cluster. (A, B) Graphs represent the migratory index of the indicated genotypes.

These results prompted us to evaluate the possible role of Fra in the TSM-G clusters, I first assessed the expression of Fra in the TSM-G clusters and found it at as early as 15 hAPF (**Fig. 24A-C**). As in the case of *gcm*, I used a conditional approach and down regulated *fra* specifically in TSM-G clusters using the *UAS fra RNAi* line (Manhire-Heath et al., 2013). *fra* KD in the TSM-G clusters also leads to delayed glial migration as in TSM-G *gcm* KD animals (**Fig. 23B, blue column**). Overexpressing *fra* in the TSM-G clusters increases the percentage of wings I which glia migration is complete (**Fig. 23B, dark blue column**).

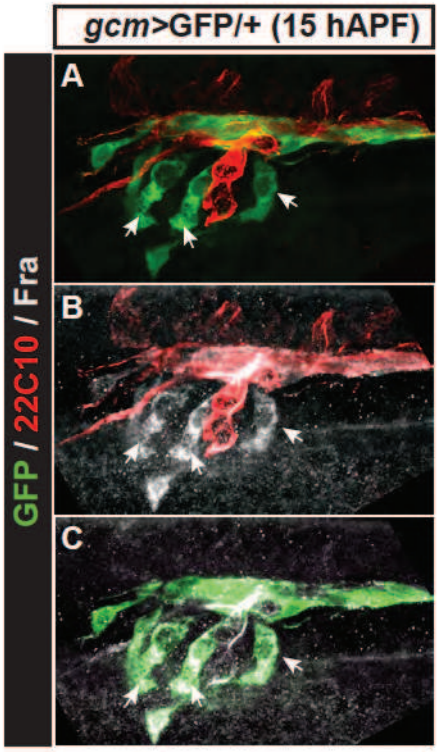

Figure 24: Expression of Fra in TSM-G clusters. **(A-C)** 15 hAPF wing immunolabeled with anti-22c10 (neurons, red), anti-Fra (grey) and anti-GFP (glial cells, green) in the transgenic line *gcm*>GFP. White arrows indicate the TSM-G cells with Fra labeling

Discussion

The results presented in this section suggest that the peculiar Gcm+/Repo- cell population called TSM-G+ might play a role in collective glia migration. One important point is that, even though increasing and decreasing the amount of Gcm and Fra in the TSM-G+ cluster affects the migration of glia cells, these phenotypes are not as strong as what is observed upon manipulating the levels of Gcm in glial cells (**see Figure 2 in the manuscript**). This suggests that Gcm and Fra affect the migration of glial cells primarily through its expression in glial cells.

An additional analysis revealed that downregulating *gcm* in TSM-G cells resulted into a neuronal conversion phenotype, as observed in *gcm*>GFP/+;*UAS gcm RNAi*/+ (**Fig 25A,B**). This could account for the delayed migration. It would be interesting to analyze

the overexpression phenotype, whether there is ectopic glia at the position of the TSM-G clusters in *gcm*>GFP/+;*UAS gcm* /+ wings. The identity and function of the TSM-G+ cell population is completely unknown in the wing. The TSM-G cell population might play similar roles as the so-called boundary cap cells in vertebrates, which are localized at the dorsal root entry zone and motor exit point of the embryonic spinal cord. Specific location of the boundary cap cells at the central nervous system (CNS) and peripheral nervous system (PNS) interface and genetic ablation showed that these cells are involved in the formation of PNS–CNS boundaries. The boundary cap cells prevent the soma migration of the motoneurons out of the spinal cord, thus these cells provide some constrain to keep the neurons in their right place (Zujovic et al., 2010; Zujovic et al., 2011).

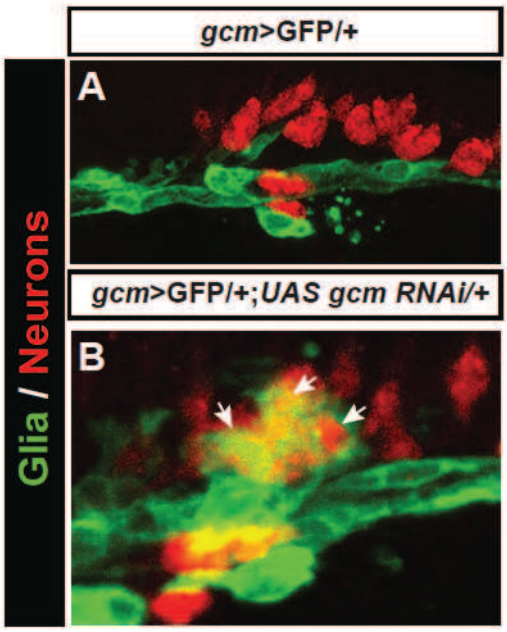

Figure 25: Conversion in TSM-G clusters. **(A, B)** *gcm*>GFP/+ and *gcm*>GFP/+;*UAS gcm RNAi*/+ wing (at 17 hAPF) immunolabeled with anti-Elav and anti-GFP. Please note the converted TSM-G cells to neurons (white arrows) in *gcm*>GFP/+;*UAS gcm RNAi*/+ wing.

Further experiments may help understand the exact role of TSM-G cluster in glia migration. 1) An important experiment will be to selectively ablate these cells; this will

provide a prerequisite to elucidate their putative role in glial chain migration. 2) Another possible experiment will be to analyze the transcriptome of these cells to characterize their properties. The combination of genetic and imaging tools will hopefully help us achieve this goal.

Chapter 5

Ubiquitin proteasomal degradation pathway

Introduction

Dynamic protein levels play an important role in maintaining the tight coordination between many inter-cellular activities, which can be highly controlled by processes such as synthesis and degradation under stringent temporal and spatial conditions. The process of protein synthesis has been studied in great detail, but in the past years ubiquitin proteasome degradation pathway has emerged as an important means for the destruction of proteins (Ho et al., 2006; Pickart, 2004; Pickart and Eddins, 2004). Proteins that are destined for degradation are covalently attached to a small molecule ubiquitin (Ub) a process called ubiquitination. Ubiquitination occurs through a series of events in the Ub-proteasomal degradation pathway. The process requires three major enzymes to carry out degradation namely: E1, E2, and E3 which are the activating, conjugating and ligase enzymes. The Ub moieties are activated by the E1 enzymes that utilize ATP to form high-energy thioester bonds and then are conjugated by the E2 enzymes respectively, whereas E3 ligases are responsible for transferring the Ub moieties from the E2 enzymes to the substrate for destruction by the 26S proteasome (Ho et al., 2006; Pickart, 2004). E3 ligases carry out this process either by HECT or RING domain. While Ring domain transfer the Ub moieties directly on to the substrate for degradation, HECT domain forms a thioester bond between the E3 ligases and the Ub, which is then transferred to the substrates (**Fig. 26**). The SCF (Skp–Cullin–F-box) complex is one of

the most characterized RING ligases and comprises of four major components: Skp1, Cul1/Cdc53, Roc1/Rbx1/Hrt1, and an F-box protein.

Ubiquitin proteasomal degradation pathway is also responsible for the degradation of Gcm (Ho et al., 2009). Gcm interacts with two members of the F-box protein, Supernumerary limbs (Slimb) and Archipelago (Ago) that leads to its destruction by 26S proteasome. Slimb is known to control the protein levels of Cubitus interruptus to mediate the transduction of Hedgehog signaling and the destruction of Period protein in the circadian clock (Grima et al., 2002; Ho et al., 2006; Jiang and Struhl, 1998; Ko et al., 2002). On the other hand Ago regulates the degradation of both Cyclin E during cell cycle progression and Dmyc in cell growth (Ho et al., 2006; Moberg et al., 2001; Moberg et al., 2004).

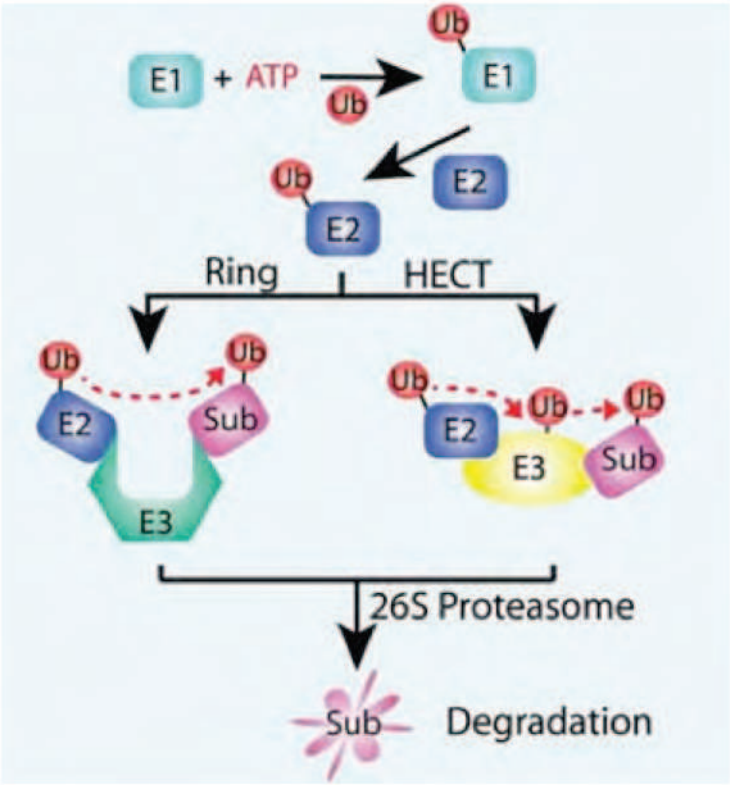

Figure 26: Ubiquitin mediated protein destruction. Elements of the ubiquitin proteasomal degradation pathway. Proteins are targeted for destruction by 26S proteasome (Ho et al., 2006).

In the *Drosophila* embryo, an increase in the glial cell number was observed in homozygous *slimb* and *ago* mutant animals suggesting a role of these F-box proteins in regulating gliogenesis by affecting the levels of Gcm (Ho et al., 2009).

The previous chapter has shown that Gcm affects the migration of L1 glial chain in a dosage dependent manner by regulating the expression of a chemoattractant receptor. One intriguing issue in this regard is to understand how the activity of such cell type-specific transcription factor is regulated. Typically, how its protein levels are downregulated once the migratory L1 glial chain has started to migrate. To this purpose, I decided to study the role of Slimb and Ago on collective glia migration.

Results

Starting from the data that Slimb and Ago degrade Gcm (Ho et al., 2009) and that Gcm affects the migration of L1 glial cells, I decided to investigate whether Slimb and Ago may degrade Gcm during L1 collective glia migration and affect the efficiency of glial migration. I used the already characterized *slimb ago* double mutant (DM) null allele in heterozygous condition since the strain is not viable in homozygous conditions at pupal stage. If Slimb and Ago were to degrade Gcm during glia migration, then one would expect to see an increase of migration efficiency in the double *slimb ago* mutant. Contrary to our expectations, however, *slimb ago* DM wings display a significant reduction in glial migration efficiency (**Fig. 27A, compare black and light grey columns**). This suggests Slimb and Ago may affect several targets and Gcm does not represent the major target. Given the broad range of activity of Slimb and Ago this interpretation seems plausible. Next, I wanted to check the genetic interaction between

Gcm, Slimb and Ago during L1 glia migration. To do so, I used a *gcm* null allele, gcm^{26}, which shows a delay in migration in heterozygous conditions and combined it to the *slimb ago* DM to generate triple mutants. The suppression of the migratory phenotype in the triple mutants further supports the hypothesis that Slimb Ago may regulate *gcm* during glia migration (**Fig. 27A, compare patterned grey column with black column**).

Finally, as a control, I wanted to see whether the number of glial cells are affected in double *slimb ago* mutants. If Slimb and Ago were responsible for the destruction of Gcm during L1 glia migration, then one would expect to see an increase in the number of L1 glia in a double *slimb ago* mutant background as in this case the levels of Gcm are enhanced. Indeed this is what I observed, an increase in the number of glial cells in *slimb ago* DM as compared to the wild type animals (**Fig. 27B**).

I also analyzed the single mutants of *slimb* and *ago* to score their specific role on glial migration. Heterozygous *ago* wings display a much stronger migratory phenotype (**Fig 27C, compare dark brown and black columns**) than heterozygous *slimb* wings (**Fig 27C, compare blue and black columns**). Interestingly, combining heterozygous *ago* wings with a *gcm* hypomorphic (henceforth *gcm>*) allele significantly enhances the migratory index, hence partially rescuing the heterozygous *ago* phenotype (**Fig 2CD, patterned dark brown column**).

In toto, these results suggest that the two F-box proteins behave in the same manner on Gcm in embryos and in pupa, however, Gcm seems not to be the primary target during glial migration. Between the two proteins, Ago plays a more important role than Slimb in regulating L1 glia migration.

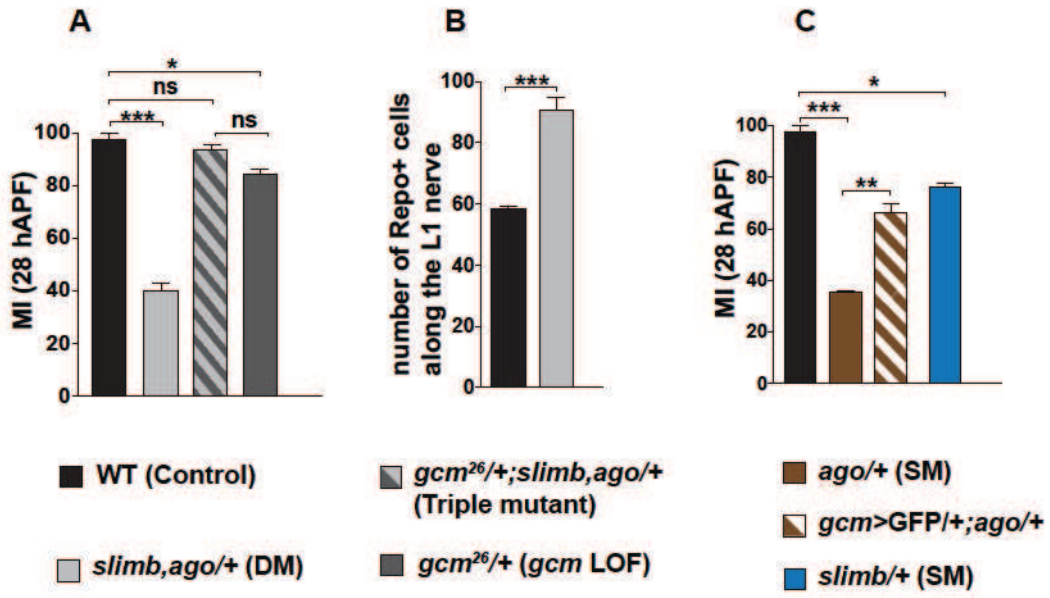

Figure 27: MI and Repo count in the indicated genotypes. (A, B, D) Graphs represent the migratory index of the indicated genotypes. **(C)** Count of the glial nuclei using repo marker in the indicated genotypes.

Discussion

The data presented in this chapter show that Slimb and Ago are required to control collective glia migration. These data are in line with what has already been described with respect to the ubiquitination of Gcm by Slimb and Ago during embryogenesis (Ho et al., 2009) but also show the importance of other Slimb Ago targets in pupal glial migration. The analyses of the single mutants suggest that Ago might play a more important role in regulating Gcm than Slimb during collective glial migration.

To further clarify the possible role of Slimb and Ago in regulating Gcm during collective glia migration it will be important to characterize whether the increase in the number of glial population in the double *slimb ago* mutant is due to the neuron to glia fate conversion. Another interesting idea would be to check the morphology of glia cells in the mutant conditions using time-lapse microscopy. It will also be interesting to study

the downstream signaling genes of Slimb and Ago that may regulate Gcm at late stages given the partial effects of these two F-box proteins.

Chapter 6

Discussion and Perspectives

Groups of cells organized in sheets, streams, chains and clusters of different size move through an ever-changing scenario, and we are just beginning to understand how they are specified and directed, what controls the timing of migration and what factors govern the coordinated cell movement. Such diversity makes it all the more interesting to study motility in many different cell types and in their natural settings. The combination of decades of genetic screenings and candidate gene testing with live imaging has significantly advanced our understanding of the mechanisms that govern collective cell movements. *In vivo* time-lapse microscopy has allowed us to discriminate among the different migratory steps such as initiation, maintenance, end of migration and has shed important insights on the presence of different cell types: pioneers at the tip of a migrating cohort and followers lagging behind (Aigouy et al., 2008; Ghabrial and Krasnow, 2006; Hellstrom et al., 2007). To date the mechanisms of pioneer vs. follower cell specification have also been characterized in tracheal and vascular branching/sprouting morphogenesis. In the *Drosophila* tracheal system, one-two motile tip cells trigger the outgrowth of tracheal branches (Affolter and Caussinus, 2008). Branched outgrowths are originally arranged in a head to tail manner. Interestingly, when growing branches are disconnected from the stack cells, the groups of separated cells keep moving efficiently (Caussinus et al., 2008). UV laser mediated ablation approaches complemented those analyses and allowed us to understand key events underlying collective migration. Typically, the first few cells (pioneers) at the migration front play a crucial role in promoting the movement of the rest of the collective. Moreover,

homeostatic events at the migratory front control collective integrity, efficiency, and coordination, hence emphasizing the importance of interactions and cell counting in fine-tuning collective processes (Aigouy et al., 2008; Aigouy et al., 2004; Berzsenyi et al., 2011).

Primarily, signaling pathways with intricate feedback loops specify the fate of the migratory cells, ensuring proper developmental timing. Expression of growth factor receptors, chemoattractant/chemorepellent receptors and/or appropriate cell adhesion molecules, such as FGF, Netrins or cadherins is then modulated so that the motile cells can respond to signals in the environment. The integration of attractive and inhibitory cues specifies the direction of a migrating collective and ensures that the cells stay together. Some of them may also define the hierarchy within the collective (Berzat and Hall, 2010; O'Donnell et al., 2009). Although the framework of collective cell migration has gained impetus in recent years, we still lack a mechanistic understanding of many underlying concepts.

Glial cells display extensive migratory abilities during development and functioning of the nervous system (Gupta and Giangrande, 2014; Klambt, 2009). For example, astrocytes in the mammalian brain migrate at the site of injury or during neurodegeneration, a process commonly known as reactive astrogliosis (Sofroniew and Vinters, 2010). Tumorous glial cells can migrate widely through the nervous system, leading to the formation of gliomas (Cayre et al., 2009). This makes glia an interesting model to study collective cell migration *in vivo*. The data presented here clearly demonstrate that glial cells in the developing *Drosophila* wing require the activation of at least two different signaling pathways to migrate collectively. 1) The DCC/Fra derived

guidance signaling cascade ensures that glia start migrating at the appropriate time and in the appropriate direction during development. 2) The cell adhesion molecule N-cadherin remodels the actin cytoskeleton assembly to secure efficient motility.

Chemotropism and glial cell migration

Several guidance factors have been studied with respect to their role in cell motility. Members of the Netrin family are essential chemotropic guidance cues that direct cell and axon migration in the nervous system during embryogenesis (Harris et al., 1996; Kennedy et al., 1994; Lai Wing Sun et al., 2011; Mitchell et al., 1996; Serafini et al., 1994; von Hilchen et al., 2010). While the control of commissure formation has been a major focus of the study of Netrin function, it is now clear that the Netrin family members also play key role in directing the formation of neural circuits other than guiding axons relative to the midline in the developing CNS. Studies of Netrin function in the nervous system and in non-neural tissues have also revealed its contributions in regulating cell-cell interactions and cell-ECM adhesion that are important in wound repair (Rajasekharan and Kennedy, 2009). Nevertheless, how these chemotropic guidance pathways controlling cell motility are regulated remains to be determined.

Netrins are thought to act either in a gradient at long range, as secreted molecules, or at short range, as membrane tethered molecules (Lai Wing Sun et al., 2011). In contrast with studies in the *Drosophila* embryo and visual system (Brankatschk and Dickson, 2006; Timofeev et al., 2012), I observed that NetB in the developing wing acts at a long range, as glia migration is delayed when solely membrane-tethered NetB is available at near-endogenous levels. Secreted NetB is converted into a long-range signal

because it is expressed at a distance from the migrating glial cells that express the receptor. Previous studies have shown that both Netrins (NetA and NetB) act on embryonic LG glia through Fra (von Hilchen et al., 2010). Clearly, in our case NetB alone is capable of attracting Fra-expressing peripheral glial cells. Although we cannot formally rule out a possible role of NetA in glial cell migration, the fact that downregulating and overexpressing NetA have no impact on the migrating glial cells and that NetA cannot rescue the NetB phenotype strongly suggests that the two Netrins do not share the same biological potentials.

Therefore, it would be worthwhile to analyze whether such differences lie within intrinsic potentials of the two ligands (e.g. affinity with which NetA and NetB bind to their receptors), whether they rely on extrinsic cues (e.g. cell-specific cofactors modulating the activity of NetA and/or NetB) and whether it's only NetB that works as a long-range guidance cue.

In the migrating L1 glia, the profile of expression of the ligand is compatible with a directional signal. Interestingly, in the zebrafish lateral line primordium, cells migrate, proliferate and differentiate over a narrow stripe of the chemokine ligand SDF-1. This trail of SDF-1 plays only a permissive role in the collective migration of the LLP, since in mutant fishes in which the extent of SDF-1 track is reduced to a small region of the anterior body wall, the LLP cells can move along the chemokine stripe in both directions. Thus, SDF-1 does not provide a guidance cue to the directional movement of the cell cohort, therefore the polarizing feature is probably determined by the migrating cells of the LLP per se.

Furthermore, the chemottractant receptor DCC/Fra and the chemorepellant

receptor Unc5 have been studied in different cell types, so it is unclear as to whether the same process and cells requires the counteracting activities of these molecules. In the case of mammalian migrating oligodendrocyte precursor cells, it has been shown that they express both DCC and Unc5, however the precise function of these receptors has not been assessed (Tsai et al., 2003). My data suggest that the two receptors Fra and Unc5 are expressed by the same set of PNS glial cells, where they appear in a timely manner and seem to regulate different steps of glial cell migration. While Fra cell autonomously control the migration of PNS glial cells by specifically regulating the initiation step in an instructive manner, Unc5 appears at late stages and may act as a stop signal for the migrating cells. This may explain how the same guidance cues can work so differently on various aspects of collective migratory behavior.

Together, these findings in the *Drosophila* wing system suggest that the dynamic and coordinated actions of chemotropic guidance cues contribute to the timely and efficient migration of the glial cells. A similar molecular mechanism relying on Netrins or other localized attractive guidance cues and their receptors may be used in other cases of collective migration.

Implication of cell adhesion molecule in glial cell migration

Cell adhesion plays an important role in providing the mechanical basis for static tissue organization (e.g. defined cell arrangement in polarized epithelium) and also in shaping the tissue by enabling plastic connections between cells. The adhesion between the cells need to be dynamic since the members of the group constantly move and change positions relative to each other. In the epithelium, the cell adhesion complexes are

localized to stereotyped regions in the cells (along the basolateral membrane), whereas in collectively migrating cells they are positioned to contact points according to the actual arrangement of the members in the group.

N-cadherin cell adhesion molecule is expressed uniformly in the migrating glial chain of the developing *Drosophila* wing, in contrast to previously published reports saying that N-cad is not expressed in glia (Fung et al., 2008; Iwai et al., 1997). Altering the amount of N-cad in glia affects the efficiency of the chain movement in a cell-autonomous manner. The *in vivo* analysis suggests that an increase in the level of cadherin/catenin complex in the cell membrane reduces membrane motility, while low cadherin/catenin levels increase it by controlling actin cytoskeleton dynamics. Hence, N-cad negatively regulates the migratory speed by limiting actin nucleation. Since we never observed a blockage in migration or saw cells moving in isolation upon altering N-cad levels in the glia, we believe that N-cad plays a permissive role in the glial chain movement.

In cancer cell biology, the invasive behavior of some tumor cells can be observed as a result of downregulation of cell adhesion molecules promoting cell contacts (Berx and van Roy, 2009) and several studies have shown the implication of N-cad in cancer cell migration. In breast cancer, for example, N-cad positively regulates metastasis and migration (Hazan et al., 2004). However, no clear conclusion can be drawn from these studies, as the results obtained by analyzing the expression level of N-cad in different gliomas are somewhat controversial (Barami et al., 2006). In the case of wing glia, it is clear that low levels of N-cad increase, while high levels of N-cad decrease the efficiency of the collective chain movement.

Gcm controls glial cell migration by regulating fra

A deterministic role of transcription factors in controlling early developmental events has been well studied, however, whether these same factors are sufficient to trigger late events remains poorly understood. Typically, *gcm* is known to act as a binary switch between neurons and glia during morphogenesis and also in determining the fate of hemocytes (Bataille et al., 2005; Bernardoni et al., 1997; Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996; Waltzer et al., 2010). My work shows that Gcm also plays a key role in glia migration. The dual role of transcription factors like Gcm indicates that late and early events may be determined by common developmental mechanisms, at least in part. Hence, fate determinants not only induce the expression of downstream transcription factors but also directly implement a specific developmental program by triggering the expression of effector genes. It would be of great interest to see whether Gcm also affects the migration of glial cells during embryogenesis and/or other late events in glia and hemocytes.

It is noteworthy that both Gcm and Fra mediated migratory phenotypes are dosage dependent. Through a detailed series of experiments, I demonstrate that changing the levels of Gcm or Fra modulates the efficiency of glial migration. More precisely, the correct levels of Gcm and Fra control the exact time of initiation, in response to the NetB ligand. This emphasizes the importance of quantitative regulation in collective events. In the future, it would be interesting to test whether this is more important for big collective of cells, which is the case for the numerous L1 glial cells, than for small groups of migratory cells.

The key element in this study is time, as Gcm progressively accumulates, it triggers the onset of glial migration by regulating the expression of *fra* and then subsequently declines. The fact that *fra* expression stays on till the end of migration suggests that another player, possibly a direct target of Gcm, maintains *fra* expression. Being a major Gcm target, *repo* represents a potential candidate. Some preliminary data do suggest that Repo may play a regulatory role in the maintenance of *fra* at late migratory stages, nonetheless further studies will be required.

Our data raise many questions on the mechanisms controlling other migratory collectives. Typically, the zebrafish lateral line primordium is made of cells that move directionally over two receptors CXCR4 and CXCR7 with different roles (leaders and followers) (Dambly-Chaudiere et al., 2007; David et al., 2002; Haas and Gilmour, 2006). Given the primary and cell autonomous role of these receptors in directional lateral line migration, it will be very important to clarify how are these receptors regulated. Typically, is their expression controlled by fate determinants, as in the case of wing glia, is quantitative regulation also crucial for their activities?

Conclusive remarks

It remains to be seen how general these mechanisms will prove to be in the control of other cell migrations during development and functioning of the nervous system. Future studies will reveal whether different modes of migration (clusters, sheets, chains, streams) require different signaling pathways or different cellular and molecular strategies. Time-lapse has already allowed us to dissect the migratory process *in vivo* in physiological assets and will continue to do so in future. I firmly believe that a major

breakthrough will come from characterizing and tracing the morphology and behavior of single, identified cells within the collective. Furthermore, the genetic or pharmacological manipulation of such targeted cells will provide important insight onto the impact of the cell-cell interaction in migrating collectives.

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