**Drosophila** integrin adhesion complexes are essential for hemocyte migration in vivo

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**Summary**

Cell migration is an important biological process which has been intensively studied in the past decades. Numerous techniques, mainly involving two-dimensional cell culture systems, have contributed to dissecting the essential mechanisms underlying this process. However, the development of three-dimensional cell culture and in vivo systems has shown some differences with what was previously believed to be well-established cell migration mechanisms, suggesting that two-dimensional cell motility would be a poor predictor of in vivo behaviour. *Drosophila* is a widely recognized model organism to study developmental and homeostatic processes and has been widely used to investigate cell migration. Here, we focus on the migration of small groups of pupal hemocytes that accumulate during larval stages in dorsal patches. We show that integrins, and other known nascent adhesion-related proteins such as Rhea and Fermitin 1, are crucial for this process and that their depletion does not affect polarization in response to environmental cues. We also present evidence for the importance of adhesion maturation-related proteins in hemocyte migration, namely Zyxin. Zyxin depletion in hemocytes leads to a significant increase of cell speed without affecting their response to a chemotactic cue. This is the first report of a systematic analysis using *Drosophila melanogaster* hemocytes to study adhesion-related proteins and their function in cell migration in vivo. Our data point to mechanisms of cell migration similar to those described in three-dimensional in vitro systems and other in vivo model organisms.

**Key words:** *Drosophila*, Hemocyte, Integrin, Migration

**Introduction**

Cell migration is a key mechanism that occurs during developmental and homeostatic processes such as tissue repair, immune surveillance and morphogenesis. The basic mechanisms underlying cell migration have been intensively studied in the past decades using different two-dimensional (2D) in vitro methods. These have allowed researchers to establish a model for cell migration that shows that an intermediate strength of adhesion is required for maximal cell speed (DiMilla et al., 1991; Palecek et al., 1997). This suggested that weak cell-adhesive interactions with the substratum would not provide enough traction and at strong adhesion, the cell would be too attached to the substratum to move efficiently. Interestingly, recent findings using three-dimensional (3D) in vitro and recently developed in vivo techniques have highlighted new features and intriguing differences in cell behaviour in comparison to what was previously described using 2D in vitro systems, namely in terms of cell morphology and signalling pathways controlling the process of cell migration (Baker and Chen, 2012; Beerling et al., 2011; Toetsch et al., 2009). As such, more and more researchers are turning to in vivo model systems (believed to be better mimicked by 3D in vitro systems) to clarify important aspects regarding cell migration.

*Drosophila melanogaster* has been a model organism of choice to look at cell migration in vivo. Border cell migration or epithelial cell migration, are some of best studied examples of collective cell migration (Montell et al., 2012; Weijer, 2009). To study single cell migration, immune circulatory cells, also known as hemocytes, which are equivalent to mammalian leukocytes in terms of functions and features, have emerged as a very useful model (Tirouvanziam et al., 2004; Wood and Jacinto, 2007).

Integrins are well-known transmembrane adhesion receptors involved in cell–extracellular matrix (ECM) interactions and play critical roles in cell signalling. They are one of the core components of adhesion sites where the ECM connects to the cellular cytoskeleton (Huttenlocher and Horwitz, 2011; Zaidel-Bar et al., 2007). The first step in adhesion establishment is the formation of nascent adhesions (NA), also referred to as focal complexes, which can then either disassemble or mature into focal adhesions (FA). The latter can undergo an additional maturation step which will give rise to fibrillar adhesions. In a study by Zaidel-Bar et al., the dynamics of the formation and maturation of these adhesion structures has been reported and some central differences in their composition have been shown (Zaidel-Bar et al., 2003). Although adhesion structures have been...
intensively studied in both 2D and more recently 3D in vitro systems (Frale et al., 2010a; Toleda et al., 2012a), very little is known about their existence, formation and roles in an in vivo context. In Drosophila, the integrin family consists of five βPS subunits (βPS1 to 5) and two β subunits, β3 and β5, the latter also known as myospheroid (Brown et al., 2000). The only previously reported links between hemocytes and integrins in Drosophila demonstrated that integrins act downstream of the GEF Dizzy and the Rap1 small GTPase to control both hemocyte shape changes and allow invasive migration during early embryonic development (Huelmsmann et al., 2006a; Siekhaus et al., 2010).

In this paper, we show that hemocytes that are located in dorsal patches, and are sessile during larval stages, depend on integrins and other known adhesion-related proteins to migrate after pupal formation. Whereas depletion of proteins described to be involved in NA formation leads to a decrease in hemocyte speed without affecting cell polarization, depletion of a recognized adhesion maturation-related protein increases cell speed. These findings suggest that the degree of maturation of the adhesions alters cell migration speed in a biphasic manner thus providing new molecular insights to further understand the biphasic model proposed by DiMilla et al., where an optimal adhesion strength maximises cell speed (DiMilla et al., 1991).

Results
 Mutations in the Drosophila βPS integrin subunit, myospheroid, lead to hemocyte migration defects
 During late 3rd instar larvae and early white pre-pupal stages of Drosophila melanogaster development, a dorsal population of hemocytes, hereupon referred to as dorsal patch-hemocytes, can be found attached to the dorsal epithelium aligned in periodic patches along the dorsal vessel (Lanot et al., 2001; Makhijani et al., 2011; Stolfano et al., 2008; Zettervall et al., 2004). During this period, hemocytes maintain a round morphology, a low migration speed and are unresponsive upon wounding of the epithelium (Babcock et al., 2008) in contrast to the hemocytes in embryonic (Stramer et al., 2005) or late pupal stages (Moreira et al., 2011). However, by 2 hours after pupa formation (APF), the dorsal sub-epidermal hemocytes have acquired a spread morphology with filopodium and lamellipodium-like structures and collectively initiate random single cell migration (supplementary material Movie 1).

Based on the MARCM (Mosaic Analysis with a Repressible Cell Marker) system described by Wu and Luo (Wu and Luo, 2007), we established a protocol (DEMON – Deleted in Hemocytes Only) that allows to test the effects of homzygous lethal mutations in hemocytes only, in an otherwise phenotypic wild-type heterozygote background (Fig. 1A,B). Using this method we were able to establish a protocol (DEMON – Myospheroid Knockdown) to specifically knockdown the genes of interest in hemocytes in vitro systems, other known adhesion components besides integrins, could play a role in dorsal patch-hemocyte migration upon pupal formation.

To test this hypothesis we decided to use the RNAi methodology combined with the UAS/GAL4 system (Duffy, 2002) to specifically knockdown the genes of interest in hemocytes. Initially, we established that knockdown of myospheroid led to a significant decrease in cell speed (mean cell speed 1.54±0.11 μm.min⁻¹) when compared to wild-type (mean cell speed 2.77±0.60 μm.min⁻¹) in a similar manner as observed in the mys1 hemocytes (Fig. 1E). This ensured that the RNAi based approach was suitable and sufficient for identifying calculated mean cell speed of 0.86±0.28 μm.min⁻¹. The movement in the mys1 hemocytes is likely to be a consequence of the constant wiggling and cell shape changes that lead to permanent adjustments in the midpoint of the cell (supplementary material Movie 2). This result led us to hypothesize that, like in in vitro systems, other known adhesion components besides integrins, could play a role in dorsal patch-hemocyte migration upon pupal formation.

Fig. 1. Myospheroid is required for proper hemocyte migration.
 (A) Outline of the MARCM protocol in hemocytes. Cross between DEMON males and FRT19a control or mys1 mutant virgin female flies for MARCM analysis. (B) Crosses are placed at 25°C for 24 hours. The progeny is submitted to three 1 hour heat-shocks (indicated by arrows) at 37°C before selection of 3rd instar females containing GFP-expressing hemocytes. (C) Movement of wild-type and mys1 GFP-expressing hemocytes in 3 to 4 hour APF flies, tracked for 12 minutes (1 min time-lapse interval). Arrowheads indicate filopodium-like protrusions produced by both wild-type and mys1 mutant hemocytes. Scale bars: 10 μm. (D) Graph showing individual mean cell speed for wild-type and mys1 clones (2 to 4 hours APF). (E) Graph showing average mean cell speeds for yv control and mys (valium 20) RNAi-expressing flies (between 3 to 4 hours APF). Mann–Whitney tests for non-Gaussian populations were used. Black lines indicate the samples’ means; Error bars = standard deviation; P-values shown above tested groups.
anomalies in hemocyte migratory behaviour upon knockdown of genes of interest (supplementary material Movie 4). It is important to notice that in the case of RNAi knockdown some migration was still detected, compared to mys mutant hemocytes, probably because this method does not result in a complete mys loss of function.

Knockdown of integrins, integrin-activating and other adhesion-associated proteins affects hemocyte migration

To understand the role of other integrin-related proteins known to be involved in the formation of NA (Choi et al., 2008; Zaidel-Bar et al., 2003) we decided to follow a candidate gene approach based protocol for specifically knocking down the genes of interest. Between 3 and 4 hours APF, whereas control hemocytes are already widely scattered cells, myospheroid deficient hemocytes remain closely clustered together forming a densely packed group of single cells (Fig. 2A; supplementary material Movies 3, 4). Two independent mys RNAi fly lines were used for comparison: a significant decrease in cell migration speed was observed with both (1.54±0.11 µm.min⁻¹ and 1.52±0.56 µm.min⁻¹ for the valium 20 and the valium 10 lines, respectively) (Fig. 2B). It is also interesting to notice that the patches form independently of the integrin knockdown.

Depletion of defined integrin-activating proteins such as Rhea (Talin homologue) or Fermitin 1 (Kindlin 1 homologue) (Anthis et al., 2009; Larjava et al., 2008) led to significant decreases in the mean cell speed (Fig. 2B; supplementary material Movies 5, 6). rhea knockdown reduces hemocyte cell speed to values comparable to the ones observed with the mys knockdown hemocytes (1.80±0.52 µm.min⁻¹ and 1.59±0.21 µm.min⁻¹ for the 33913 and the 32999 lines, respectively). fermitin 1 knockdown, although to a lesser extent than mys and rhea, also causes hemocyte migratory defects, with the latter reaching average speeds of 2.14±0.25 µm.min⁻¹.

Depletion of either Vinculin or Focal Adhesion Kinase (FAK), both described to be involved in early adhesion formation (Derramaudt et al., 2011; Zaidel-Bar et al., 2003), has no significant effect on hemocyte migration in vivo (Fig. 2B; supplementary material Movies 6, 7). These results correlate with previous findings which suggested that either molecule is non-essential for Drosophila’s viability (Alatortsev et al., 1997; Grabbe et al., 2004).

Following the stabilization of a NA, which in part involves the organized recruitment and assembly of the above discussed proteins, adhesion structures can develop into a FA, a process that relies on several factors, including tension which depends on myosin II activity (Parsons et al., 2010). This maturation process is accompanied by an increase in the number and diversity of proteins that assemble at adhesion sites, like zyxin. As such, we decided to investigate the effect of knocking down zyxin expression during hemocyte migration. Interestingly, zyxin knocked down hemocytes showed a significant increase in the mean cell speed (3.75±0.36 µm.min⁻¹) when compared to controls (Fig. 2B; supplementary material Movie 7), a phenotype which has been previously described in in vitro cultured cells (Fraleys et al., 2010; Hoffman et al., 2006).

Polarization in response to wounds is not affected by the knockdown of NA related proteins

Integrins also appear as key molecules in the establishment and maintenance of a polarity axis in directionally migrating cells; therefore we decided to analyse their role in hemocyte polarization and migration upon epithelial wounding.

Three to four hours APF, the hemocytes in the dorsal patches area show a random migratory pattern with a filopodial protrusion extending in the direction of the migration. Upon wounding, part of the nearby hemocyte population responds by extending the filopodial protrusion towards the wound site (Fig. 3A; supplementary material Movie 8). Quantification of the number of hemocytes at the wound site over time in a control situation suggests that within the first 30 minutes after wounding, a first wave of rapidly responding hemocytes reaches the wound site (Fig. 3B). Over the next few minutes, some cells still respond to the chemotactic cue although the rate at which they reach the area of interest is diminished compared to the initial response phase. This behaviour is enhanced in zyxin knockdown hemocytes thus suggesting that blocking adhesion maturation does not affect hemocyte chemotaxis. For myospheroid and rhea-depleted hemocytes the response to a wound was less perceptible since only a few cells ever reach the area of interest during the time of analysis. However, since blocking the initial formation of

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**Fig. 2.** NA and FA proteins are essential for optimal hemocyte migration.

(A) Example tracks of yv control and mys RNAi-expressing flies. Scale bars: 10 µm. (B) Graph showing average mean cell speeds for different genetic backgrounds in 3 to 4 hour APF flies. val 10 = TRIP valium 10; val 20 = TRIP valium 20. Mann–Whitney tests for non-Gaussian populations were used. Both rhea RNAi lines were compared to the y'/sc'/v' control line. All other lines were compared to the y'/v' control line. Black lines indicate the samples’ means; Error bars = standard deviation; P-values shown above tested groups.
NA leads to striking migratory defects, we decided to further characterize the hemocytes’ response to a chemotactic cue: for that, we analysed the mean angle of the hemocytes’ polarization arm relative to the wound before and after wounding (Fig. 3C). In the control population, we calculated a significant change in the mean angle of the polarization arm relative to the wound site. Interestingly, the knockdown of *myospheroid*, *rhea* or *fermitin 1* had no impact in the extension of the filopodial protrusion towards wounds: as for controls, in all situations, there was a shift to lower values of the mean angle of polarization, which is indicative of the arm pointing towards the wound site. This was observed independently of the capacity of hemocytes to migrate towards wounds (Fig. 3C; supplementary material Movies 9, 10, 11), thus suggesting that integrin-containing adhesions, although crucial for hemocyte migration, are not necessary for the establishment of polarity upon wounding.

In opposition to what happens with NA-related proteins, zyxin-depleted hemocytes showed no significant changes between before and after wounding in terms of the mean polarization angle (Fig. 3C; supplementary material Movie 12) which could suggest that *zyxin* knocked down hemocytes failed to reorient themselves towards the wound and/or were less sensitive to environmental cues. However, we have shown that the response of *zyxin*-depleted hemocytes occurs more rapidly than in a control situation thus probably making it a lot more difficult to detect shifts in the mean polarization angle as in all other analysed situations.

**Discussion**

In this paper, we have looked at cell migration in vivo using *Drosophila* dorsal patches-hemocytes as our model system. These patches of immune cells are found attached to the dorsal epithelium aligned along the dorsal vessel of the late 3rd instar and young white pre-pupas (Lanot et al., 2001; Stofank et al., 2008). The origin of these tightly packed groups of cells is partially unknown, but it has been suggested that these serve as a major hematopoietic compartment in the larva that can be activated upon an immune challenge (Márkus et al., 2009). How these cells remain attached to the epithelium prior to the onset of metamorphosis is also not clear. Our results suggest that integrins (more specifically *myospheroid*, *Drosophila*’s main beta integrin subunit) and integrin-containing adhesions play no role in the original attachment of these hemocytes to the dorsal patches areas, thus indicating that alternative adhesions systems may also be involved in hemocyte attachment to these locations.

Interestingly, in spite of the numerous similarities between *Drosophila* hemocytes and mammalian leukocytes, we seem to have found what appears to be a striking difference in terms of their migratory behaviour: whereas vertebrate immune cells can move in complex in vivo, ex vivo and 3D in vitro environments in a integrin-independent fashion, using the force of the actin-network expansion (Lämmermann et al., 2008), *Drosophila* dorsal patch-hemocytes are dependent on integrin-containing adhesions to migrate. Hemocytes seem to therefore fit a more
conventional model which describes integrin-containing adhesions as essential for cell migration (Huttenlocher and Horwitz, 2011). Besides myospheroid, we have also shown that rhea (talin homologue) and fermitin 1 (kindlin 1 homologue) depleted hemocytes suffer from similar migratory defects, both showing significant decreases in the mean cell speed, a phenotype that has been previously observed in HT-1080 talin-depleted cells in a 3D in vitro assay (Fraley et al., 2010). Also, both Talin and Kindlin 1 have been shown to bind directly to the β integrin cytoplasmic tail causing integrin conformational changes and promoting integrin activation (Harburger et al., 2009; Ye et al., 2011). Our data suggest that a similar integrin activation mechanism could occur in migrating hemocytes in vivo and that depletion of either molecule could impair the formation of NAs.

Vinculin is another core component of NAs (Zaidel-Bar et al., 2003). Vinculin binds directly to talin and the actin cytoskeleton (Humphries et al., 2007) thus acting both as a bridge and a force-transmitter (Grashoff et al., 2010). Disruption of vinculin in 2D cultured cells was shown to play a critical role in cell migration, leading to a significant increase of cell speed (Coll et al., 1995). However, more recently it was also shown that in a 3D environment, vinculin depletion decreases cell motility, a phenotype that was associated to a decrease in adhesion strength and lowering of the traction forces (Mierke et al., 2010). Our results in vivo show that vinculin depleted hemocytes suffer no significant changes in terms of cell speed when compared to controls, which is in line with previous findings by Alatortsev et al., who have shown that vinculin is non-essential for Drosophila’s survival and development (Alatortsev et al., 1997). As vinculin is one of the main force transmitters identified in integrin-containing adhesions, it has yet to be clarified whether other molecules could be playing this role in migrating hemocytes.

Focal Adhesion Kinase (FAK) is another molecule which plays a critical role in both NA formation and adhesion turnover, depending on the state of phosphorylation (Derramaud et al., 2011). In mice, FAK depletion causes numerous defects that ultimately lead to embryonic death (Ilić et al., 2004). In Drosophila, fak expression was shown to be non-essential, and the absence of FAK has no effect on integrin-dependent mechanisms nor does it seem to influence border cell migration (Grabbe et al., 2004). Similarly, we observe that fak knockdown had no effect on hemocyte migration. fak over-expression however, has been shown to act negatively on integrin adhesion in Drosophila, and is potentially critical for adhesion remodelling. It would therefore be interesting to see if any migration defects would also occur upon over-expression of fak in hemocytes.

It is known that upon stabilization, NAs can overcome several maturation steps which imply the arrival of adhesion maturation-related proteins such as Zyxin to the adhesion sites. Zyxin is a stretch-sensitive mechanosensor (Yoshigi et al., 2005) important for the maintenance and repair of actin stress fibers (Smith et al., 2010) and it has been described as a molecular hallmark of the transition from an NA to an FA (Zaidel-Bar et al., 2003). We have shown that zyxin depletion enhances hemocyte migration speed, a phenotype which has already been shown by others and was correlated with an enhanced adhesion capacity that did not affect integrin expression levels (Fraley et al., 2010; Hoffman et al., 2006). Further analysis should elucidate whether adhesion strength and/or myospheroid expression are affected in zyxin-depleted hemocytes. Our results therefore suggest that zyxin knockdown may prevent the transition of NAs into FAs as in cell culture studies. Zyxin may be involved in the maintenance of hemocyte integrin activity to regulate migration. This is similar to the biphasic model (DiMilla et al., 1991), where an intermediate strength of cell–substratum interaction is essential for maximal cell migration. It is possible that the disruption of initial NA formation decreases cell adhesion and leads to a decrease in cell speed due to a lack of the necessary traction for migration. On the other hand, disrupting the maturation of FAs (stronger adhesive structures than NAs) decreases what should be a stronger cell–substratum interaction, and therefore causes an increase in cell migration. This suggests that cell adhesion maturation may be an important factor for controlling cell speed.

Immune cell migration, besides being a naturally occurring developmental process, can also be triggered by external environmental cues such as wounds or infections (Niethammer et al., 2009). Migration, a complex multi-step process, starts with the formation of an internal cellular asymmetry of molecules and structures that allows the establishment of a front–rear cell polarity that eventually translates itself in a directional movement towards the site of interest (Bornens, 2008; Li and Gundersen, 2008; Mellman and Nelson, 2008; Stramer et al., 2010). Amongst the many molecules involved in the establishment of this polarity axis are the integrins. Using a previously described migration assay (Moreira et al., 2011), we were able to conclude that, in hemocytes, integrins and other NA-related proteins are not essential for the establishment of a polarization axis towards a wound. This is not the first report of immune cells responding to environmental cues in an integrin-independent fashion (Lämmermann et al., 2008), which implies that other receptors apart from integrins are functioning in detecting environmental changes and in establishing a front–rear polarity axis in dorsal patch-hemocytes.

The polarization results obtained upon zyxin knockdown suggested that zyxin-depleted hemocytes could be less sensitive to environmental cues, similar to what was described for zyxin-depleted fibroblasts in a haptotatic migration assay towards different integrin ligands (Hoffman et al., 2006). However, we have shown that zyxin-depleted hemocytes still reached the wound area at a higher rate than control cells within the first 30 minutes post wounding. Altogether, the data suggest that zyxin depletion solely affects cell migration speed without affecting a cell’s response to a chemotactic cue.

Ultimately, our results clarify the importance of a tight regulation of adhesion formation and maturation for optimal cell migration in an in vivo context, which, similarly to the previously described biphasic model by DiMilla et al. based on cell adhesion (DiMilla et al., 1991), suggests that integrin-containing adhesions formation and maturation is the major force controlling hemocyte migration in vivo.

Materials and Methods
Fly stocks
w; hml-GAL4,UAS-GFP; (Sinenko and Mathey-Prevot, 2004) was used to visualize dorsal patches-hemocytes and to drive the expression of other UAS constructs in hemocytes specifically. The choice of a truncated version of the original hml driver by Goto et al. (Goto et al., 2001) relied on the hemocyte-specific pattern of expression that recovers all the wild-type hemocyte specific characteristics that had been lost in the original construct. To generate the DEMON stock the following stocks from the Bloomington Stock Center were
used: Invitrogen, Life Technologies, and a European Research Council Starting Grant and time lapses were analysed using Fiji software. Images were imaged during 1 hour. Pupas were imaged during 20 min (2 min interval) before wounding. Wounding was performed by a 40x/1.3 Plan Fluor PFS objective (Nikon Instruments) with a 488 nm Sapphire laser and a LP 505 nm emission filter. Live imaging. pupas were imaged in an Andor Revolution spinning disc confocal microscope (Andor Technology, UK) using a 40x/1.3 Plan Fluor PFS objective (Nikon Instruments) in conjunction with a 488 nm Sapphire laser and a LP 505 nm band-pass emission filter. For the polarization assay, pupas were imaged using a Zeiss LSM 5 Live line-scanning confocal microscope (Carl Zeiss, Jena) using a 40x/1.3 Plan-Neofluar oil immersion objective in conjunction with a 488 nm Sapphire laser and a LP 505 nm emission filter. Images and time lapses were analysed using Fiji software.

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Author Contributions

C.G.A.M., A.J. and S.P. conceived and designed the experiments. C.G.A.M. performed the experiments and analyzed the data. C.G.A.M. performed the experiments and S.P. wrote the paper.
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