

A dual role for the β PS integrin *myspheroid* in mediating *Drosophila* embryonic macrophage migration

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Summary

Throughout embryonic development, macrophages not only act as the first line of defence against infection but also help to sculpt organs and tissues of the embryo by removing dead cells and secreting extracellular matrix components. Key to their function is the ability of embryonic macrophages to migrate and disperse throughout the embryo. Despite these important developmental functions, little is known about the molecular mechanisms underlying embryonic macrophage migration *in vivo*. Integrins are key regulators of many of the adult macrophage responses, but their role in embryonic macrophages remains poorly characterized. Here, we have used *Drosophila* macrophages (haemocytes) as a model system to address the role of integrins during embryonic macrophage dispersal *in vivo*. We show that the main β PS integrin, *myspheroid*, affects haemocyte migration in two ways; by shaping the three-dimensional environment in which haemocytes migrate and by regulating the migration of haemocytes themselves. Live imaging revealed a requirement for *myspheroid* within haemocytes to coordinate the microtubule and actin dynamics, and to enable haemocyte developmental dispersal, contact repulsion and inflammatory migration towards wounds.

Key words: Integrins, Macrophage, Migration

Introduction

Integrins are $\alpha\beta$ heterodimeric cell-surface receptors that bind specific extracellular matrix (ECM) proteins or counter receptors on other cells (Hynes, 2002; Humphries et al., 2006). Besides their role in regulating cell adhesion, integrins transduce signals inside the cell that regulate actin cytoskeletal rearrangements, cell migration, cell shape, gene expression, cell proliferation and survival (Legate et al., 2009). These signals integrate with signals transduced from growth factors, cytokines and other transmembrane receptors to regulate numerous anchorage-dependent cellular properties (Hood and Cheresh, 2002).

Adult mature macrophages are strategically located throughout the body tissues where they play a central role in protecting the host and maintaining tissue homeostasis. Over the last few years, it has been appreciated that integrins are essential in regulating many of these macrophage responses, such as transmigration into the inflammatory site, migration across tissues, cytokine secretion and phagocytosis (Abram and Lowell, 2009). However, recent work has revealed strong discrepancies in the role that integrins play during leukocyte migration in two-dimensional (2D) versus three-dimensional (3D)

environments. Live imaging of leukocytes migrating *in vivo* as well as artificial 3D matrices of fibrin and collagen has shown that while integrins are essential to overcome tissue barriers, they are dispensable for interstitial leukocyte migration. These studies highlight the need to study leukocyte migration in the natural context of a living organism (Lämmermann et al., 2008).

Embryonic macrophages play important roles throughout embryonic development, sculpting and promoting organogenesis by clearing apoptotic cells and depositing ECM molecules. Central to their role in embryogenesis, embryonic macrophages must migrate and disseminate throughout the embryo. Yet despite their pivotal role during embryonic development, little is known about how embryonic macrophage migration is regulated. Recently the use of fluorescently labelled transgenic animals and real-time *in vivo* confocal microscopy has facilitated the study of embryonic macrophages *in vivo* within the complex 3D environment of embryos (Grabher et al., 2007). In this respect, *Drosophila* embryonic macrophages (haemocytes) have emerged as a powerful system to study embryonic macrophage biology *in vivo* (Evans and Wood, 2011). Deriving from the head mesoderm, haemocytes are highly migratory cells, achieving an even distribution throughout the embryo by the end of embryogenesis (Tepass et al., 1994). During their stereotypical migrations *Drosophila* embryonic macrophages contact several diverse

tissues surrounded by components of the ECM. Yet despite the fact that integrins are key regulators of cell migration over ECM substrates, and regardless of their central role in adult macrophages, further characterization of their requirement during embryonic macrophage migration is needed.

Here, we use *Drosophila* to analyse the function of integrins in embryonic macrophages. Eight β - and 18 α -subunits have been characterized in mammals, while the *Drosophila* genome encodes two β -subunits (β PS and β v) and five α -subunits (α PS1–5). β PS is widely expressed and forms heterodimers with all α -subunits, while the β v integrin subunit is predominantly expressed in the midgut endoderm (Brown, 2000). The α PS2 integrin subunit, *inflated*, is required for the invasive movement of embryonic macrophages from the head region into the tail (Siekhaus et al., 2010). In addition, integrins appear to act downstream of the Rap1 guanine nucleotide exchange factor (GEF) *dizzy* during haemocyte migration (Huelsmann et al., 2006), since removal of β PS integrin function can rescue haemocyte migration defects due to overexpression of *dizzy*. However, while haemocytes from *dizzy* mutant embryos displayed migration defects, a clear function for β PS integrins in this process has not yet been clearly demonstrated.

By using live imaging we have dissected cell autonomous and non-autonomous roles for integrins during haemocyte dispersal within the embryo; the β PS integrin *mysospheroid* is required for correct development of the 3D environment in which the haemocytes are migrating and also within the haemocytes themselves for their migration. In addition we show that the α PS1 and α PS3 subunits act redundantly to mediate *mysospheroid* function during haemocyte migration. Using high resolution, live imaging, we discover a requirement for *mysospheroid* in the coordination of the actin and microtubule dynamics within migrating haemocytes and that a loss of integrin function results in defective inflammatory migrations to wounds as well as a failure in contact repulsion between migratory haemocytes.

Results

β PS integrins regulate haemocyte migration

In *Drosophila* embryonic haemocytes, α PS2 is specifically required for haemocyte movement into the tail (Siekhaus et al., 2010), indicating that either integrins are not required for other haemocyte developmental migrations or that other α PS integrins are involved. We sought to investigate the role of integrins in haemocyte migrations more broadly by removing the main β -integrin subunit, encoded by *mysospheroid* (*mys*). As integrin subunits must form $\alpha\beta$ heterodimers in order to be transported to the cell surface (Leptin et al., 1989), removing the main β -integrin subunit disrupts most integrin function within the embryo. As β PS is maternally deposited within the embryo, we generated maternal zygotic mutants to ensure complete elimination of integrin function (subsequently referred to as *mys^{M/Z}*). Initial dispersal of haemocytes from the head mesoderm, where they are specified, towards the cypeolabrum, the gnathal buds and the anterior ventral nerve cord (VNC) were unaffected by loss of *mys* (Fig. 1A,E). At this stage, ventrally located haemocytes undertake *pvf*-directed migration along the developing VNC of the embryo to occupy the length of the midline by stage 13 (Wood et al., 2006) (Fig. 1B). In *mys^{M/Z}* mutant embryos haemocyte migration at this stage is severely disrupted, with haemocytes limited to the anterior and posterior ends of the VNC (Fig. 1F). This disruption persists through to

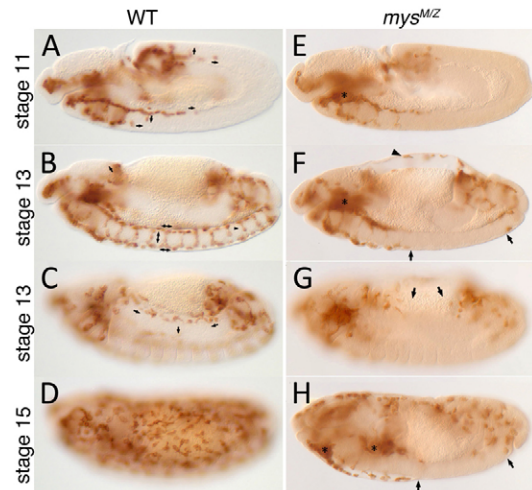


Fig. 1. Haemocytes in embryos mutant for *mysospheroid* show defects in developmental dispersal. Lateral view of fixed WT (A–D) and *mys* maternal and zygotic (*mys^{M/Z}*) mutant embryos (E–H). Haemocytes were visualized by expression of the heterologous cell membrane marker CD2 driven by the *srph-GAL4* driver and detected with an anti-CD2 antibody. (A,E) The initial phases of haemocyte migration from head mesoderm along the ventral nerve cord (VNC) are normal in *mys* mutant embryos (E; compare with A). (B,F) Mutant haemocytes fail to migrate along the length of the VNC by stage 13 of development (arrows in F). Mutant haemocytes can also be seen accumulating in the anterior region of the embryo (asterisk) and between the amnioserosa and yolk (arrowhead). (C,G) Haemocyte migration along the dorsal edge of the epidermis is also disrupted (arrows). (D,H) The migration phenotype is also observed at stage 15 (arrows in H).

stage 15, indicating that haemocyte migration is not slowed but inhibited (Fig. 1H). Removal of *mys* also affected haemocyte migration along the dorsal edge of the epidermis and the visceral mesoderm (Fig. 1C,G and data not shown), and resulted in accumulation of haemocytes between the amnioserosa and yolk regions (Fig. 1F, arrowhead). These haemocyte migration defects were phenocopied in embryos lacking both maternal and zygotic Talin, a key component of integrin mediated adhesion (supplementary material Fig. S1B). In addition, removal of zygotic and maternal β v integrin function did not enhance the *mys* phenotypes, indicating that β v integrin plays no role in haemocyte dispersal (data not shown).

mysospheroid is able to form heterodimers with all five α -subunits present in *Drosophila* (Brown, 2000). In order to identify which α -subunits are involved in regulating haemocyte migration, we analysed haemocyte migration along the VNC in embryos lacking the different α PS subunits. Integrin α -subunit genes show no maternal contribution (Brower et al., 1995; Stark et al., 1997), therefore zygotic mutants were used. Analysis of embryos mutant for α PS1 and α PS3 revealed a slight disruption of haemocyte migration along the VNC, with embryos showing midline segments devoid of haemocytes (supplementary material Fig. S2B,C), and in contrast to *mys* mutant embryos, this phenotype was not observed in stage 15 embryos (data not shown). These results show that migration was delayed but not inhibited in the absence of either of these α subunits. Only with removal of both α PS1 and α PS3 function did the posterior half of the VNC remain free of haemocytes until the end of embryogenesis (supplementary material Fig. S2D), indicating

that these two α subunits function redundantly to mediate *mys* function during haemocyte migration along the VNC in the developing embryo.

Integrins have been previously shown to regulate cell survival in different cell types in a variety of species (reviewed in Vachon, 2011). Furthermore loss of trophic support in *pvr* mutants contributes to defective developmental dispersal of haemocytes (Bruckner et al., 2004). To rule out increased haemocyte death in causing the reduced number of haemocytes found on the midline, we induced haemocyte-specific expression of the apoptotic inhibitor p35 in *mys* mutant embryos. Blocking apoptosis failed to rescue the strong defect in developmental dispersal of these embryonic haemocytes (data not shown), indicating that *mys* function plays no significant role in regulating the survival of these cells and that the dispersal defects seen are not the result of reduced haemocyte numbers.

Non-autonomous and autonomous roles for *mysospheroid* in haemocyte developmental dispersal

In the *Drosophila* embryo, *mys* genetically interacts with the growth cone repellent Slit to mediate axonal guidance within the developing CNS (Stevens and Jacobs, 2002). As *slit* mutant embryos exhibit disrupted haemocyte migration due to a failure in separation of the VNC from the overlying epithelium (Evans et al., 2010), we wondered whether this non-autonomous defect occurred in and contributed to haemocyte migration defects seen in *mys* mutant embryos. To examine this possibility, we used dye injections to visualise the spatial constraints encountered by haemocytes on the ventral side of the embryo (Evans et al., 2010). Dye injections of stage 15 WT embryos indicate separation of the epithelium from the VNC along the entire length of the midline (Fig. 2A, $n=17$). In contrast, in *mys* mutant embryos the dye fails to permeate along the length of the embryo, indicating areas where the epithelium has failed to detach from the VNC (Fig. 2B, $n=14$). In 64% of *mys* mutant embryos injected, presence of the dye coincides with areas occupied by haemocytes (Fig. 2Bi), suggesting that haemocyte migration defects in these embryos may be the result of spatial constraints within the embryo. However, in the remaining 36% of *mys* mutant embryos, haemocyte progression along the VNC is halted before the dye becomes more restricted, indicating that in some embryos, other reasons may underlie haemocyte migration failure (Fig. 2Bii).

In order to investigate a cell-autonomous requirement for integrins within haemocytes we expressed RNAi constructs specifically within the haemocytes using the *srp-HemoGAL4* driver (Bruckner et al., 2004). Expression of RNAi transgenes for either *mys* or *talin* was sufficient to mimic haemocyte migration defects seen with loss of *mys* function (Fig. 2D; supplementary material Fig. S1C), demonstrating an additional requirement for integrins within the migrating haemocytes. Integrins have roles in both adhesion and in promoting intracellular signalling in response to environmental cues (Huttenlocher and Horwitz, 2011). In order to determine the primary requirement for integrins in migrating haemocytes we utilised the $\text{Tor}^{\text{D}}/\beta_{\text{cyt}}$ fusion protein. $\text{Tor}^{\text{D}}/\beta_{\text{cyt}}$ consists of the cytoplasmic tail of *mys* fused to the extracellular and transmembrane domains of a dominant gain-of-function allele of the Torso receptor tyrosine kinase, and has been shown to block ECM adhesion mediated by endogenous integrins while activating signalling even in the absence of adhesion (Martin-Bermudo and Brown, 1999;

Narasimha and Brown, 2004; Tanentzapf et al., 2006). Ectopic expression of $\text{Tor}^{\text{D}}/\beta_{\text{cyt}}$ within wild-type haemocytes using the *srp-HemoGAL4* driver also phenocopied loss of integrin function (Fig. 2E). These findings suggest that one of the critical functions for integrins in haemocyte developmental dispersal appears to be adhesion. To investigate in more detail the contribution of integrin requirement in both the haemocytes and the surrounding tissue during their migration along the ventral midline, we used the GAL4 system to express the βPS subunit in either haemocytes, or in their migratory substratum, or both in embryos that lack *mys* function. To quantify the rescue effects we defined four distinct phenotypic classes according to the number of neuromeres of the VNC devoid of macrophages and determined for each genotype the distribution among these classes (Fig. 2F–L). Expression of *mys* solely in the haemocytes of *mys*^{MZ} mutant embryos resulted in a substantial rescue of the migration phenotype (Fig. 2L, *srp* >). Rescue of haemocyte migration was further improved when *mys* was coexpressed in the ventral midline, consistent with a requirement for this integrin subunit in the tissues surrounding the haemocyte at this stage of development (Fig. 2L, *sim* > *srp* >). In contrast, mutant embryos expressing *mys* only in the midline showed a very limited rescue of macrophage migration (Fig. 2L, *sim* >). Expression of *mys* in the midline glia cells, using *slit-GAL4*, was unable to rescue the migration defect (Fig. 2L, *slit* >) and did not enhance the rescue effect seen with expression of *mys* in the haemocytes (Fig. 2L, *slit* > *srp* >, compared to *srp* >). Taken together these results demonstrate that the βPS integrin subunit is required for migration of haemocytes along the ventral midline via a specific requirement in the haemocytes to regulate their adhesions as well as a role in the ventral nerve cord in regulating VNC–epithelial separation.

Haemocytes require *mysospheroid* for both random and directed migrations

To investigate in more detail the cell autonomous role for integrins in haemocytes we used live imaging of *mys* mutant haemocytes as they undergo their normal developmental dispersal in the embryo. From stage 13 wild-type haemocytes undergo a segmented and highly directional lateral migration away from the ventral midline (Fig. 3A,C) (Wood et al., 2006). Even using *mys* zygotic mutants, in which a low level of maternal protein is present, this developmental migration was almost completely abolished (Fig. 3B,D). Tracking individual cells revealed that only a small fraction of haemocytes migrated laterally in the absence of *mys* function and the few that did moved at significantly slower speeds than wild-type cells (Fig. 3E,F). This is in contrast to *slit* mutant embryos in which laterally migrating haemocytes migrated at speeds comparable to those observed in wild-type embryos (Evans et al., 2010). Therefore, although environmental constraints may account for the reduction in numbers migrating laterally, the reduction in migration speed of those that do is likely due to a haemocyte-specific requirement for *mys*. Similarly, *mys* mutant haemocytes moved at almost half the speed of wild-type cells when migrating randomly at stage 15 (Fig. 3G). However, we did not detect differences in the directionality of migration between wild-type and *mys* mutant haemocytes during lateral migration or random migration, suggesting that the polarity machinery and the ability to decipher migratory cues remained intact in these cells (data not shown). To analyse cell migration velocity in *mys* mutant

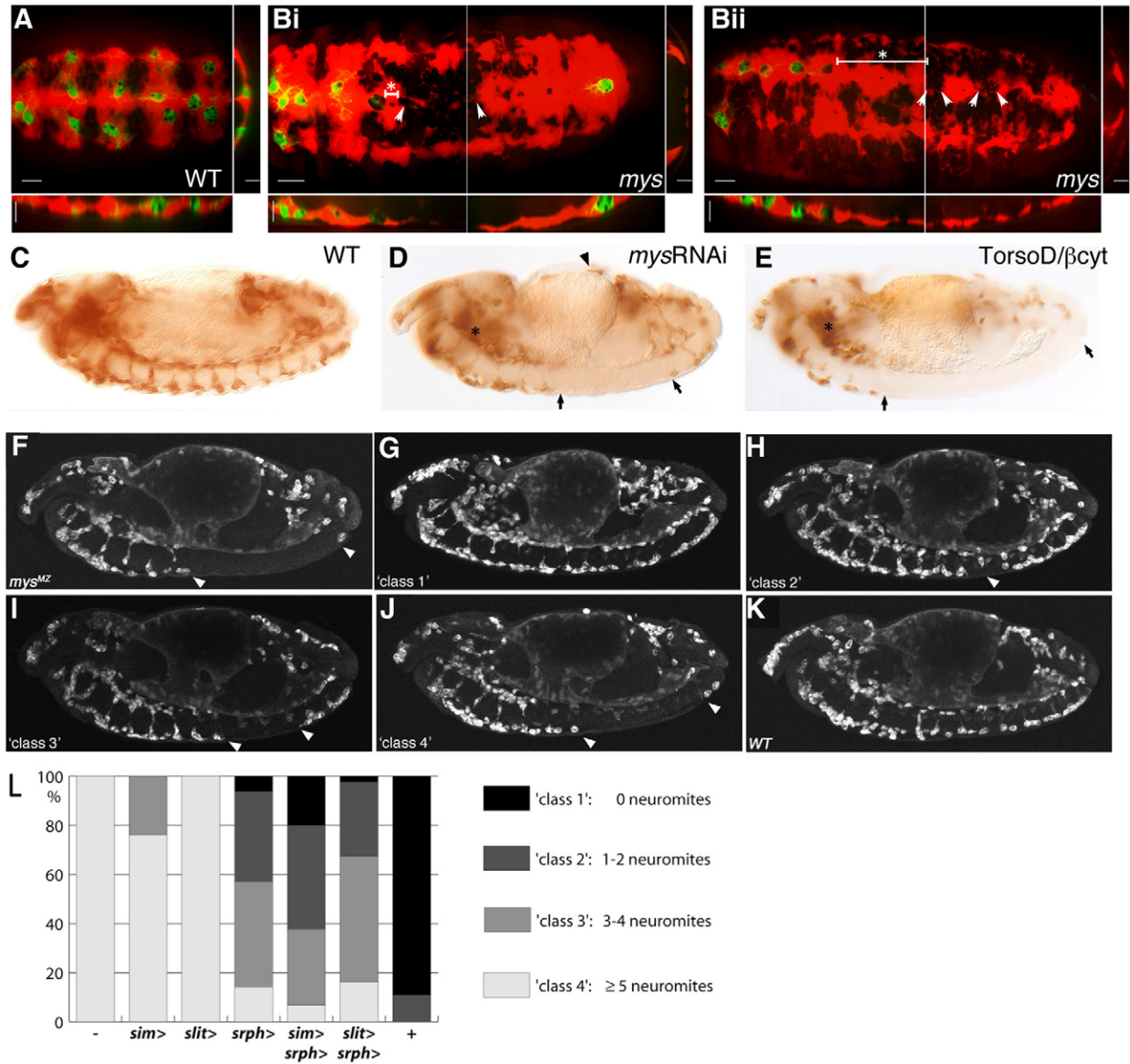


Fig. 2. Environmental and haemocyte specific requirements for β PS integrin. (A,B) Orthogonal projections of ventrally orientated stage 15 WT and *mys* mutant embryos with haemocyte-specific expression of GFP (green), injected with dextran dye (red) to reveal spatial constraints surrounding the haemocytes. Scale bars: 20 μ m (ventral view); 5 μ m (orthogonal projections). (A) Within injected WT embryos the dye permeates along the length of the embryo, indicating VNC–epithelial separation. (B) Within *mys* mutant embryos spreading of the dye becomes restricted, indicating incomplete VNC–epithelial separation (white arrowheads indicate restricted area of ventral midline). (i) In some embryos the absence of dye coincides with the distance reached by the lead haemocyte migrating from the anterior of the embryo along the ventral midline, whereas in others (ii) the lead haemocyte fails to reach the area where the dye becomes restricted (distance between lead haemocyte and spatial restriction indicated by line and asterisk), suggesting that spatial constraint is not the sole cause of disruption to haemocyte migration along the ventral midline. (C–E) Lateral view of fixed stage 13 embryos. Haemocyte *mysospheroid* requirements were assessed by coexpressing UAS-CD2 (C) and either UAS-*mys*RNAi (D) or a dominant-negative version of the *mys* subunit, UAS-TorsoD/ β cyt (E), under the control of the *srph*-*GAL4* driver, and staining with an anti-CD2 antibody. Expression of either UAS-*mys*RNAi or UAS-TorsoD/ β cyt phenocopies the haemocyte migration defects observed in *mys* mutant embryos. (F–K) Lateral views of fixed and stained stage 13 embryos. (F) *mys*^{M/Z} embryo; (G–J) grading of embryos into ‘classes’ based on level of haemocyte migration rescue; and (K) WT embryo. (L) Quantification of haemocyte migration rescue at stage 13 when UAS-*mys* is expressed in the midline under the control of the *sim* and *slit* GAL4 drivers, and/or in the haemocytes under the control of *srph*-*GAL4*.

embryos in the absence of spatial constraint, we used laser ablation to create epithelial wounds in the anterior trunk of the embryo, in areas populated with haemocytes. In *mys* mutant embryos haemocytes were competent to respond to an inflammatory cue (Fig. 3J,K), but there was a significant reduction in the number of haemocytes recruited to wounds at

the early stages of this inflammatory migration (Fig. 3L). At later time points the inflammatory response recovered to that of WT embryos. This lag in recruitment is likely a reflection of the significant reduction in migration speed exhibited by *mys* embryos (Fig. 3M), as the highly directional routes taken by haemocytes towards the wound are comparable to those seen in

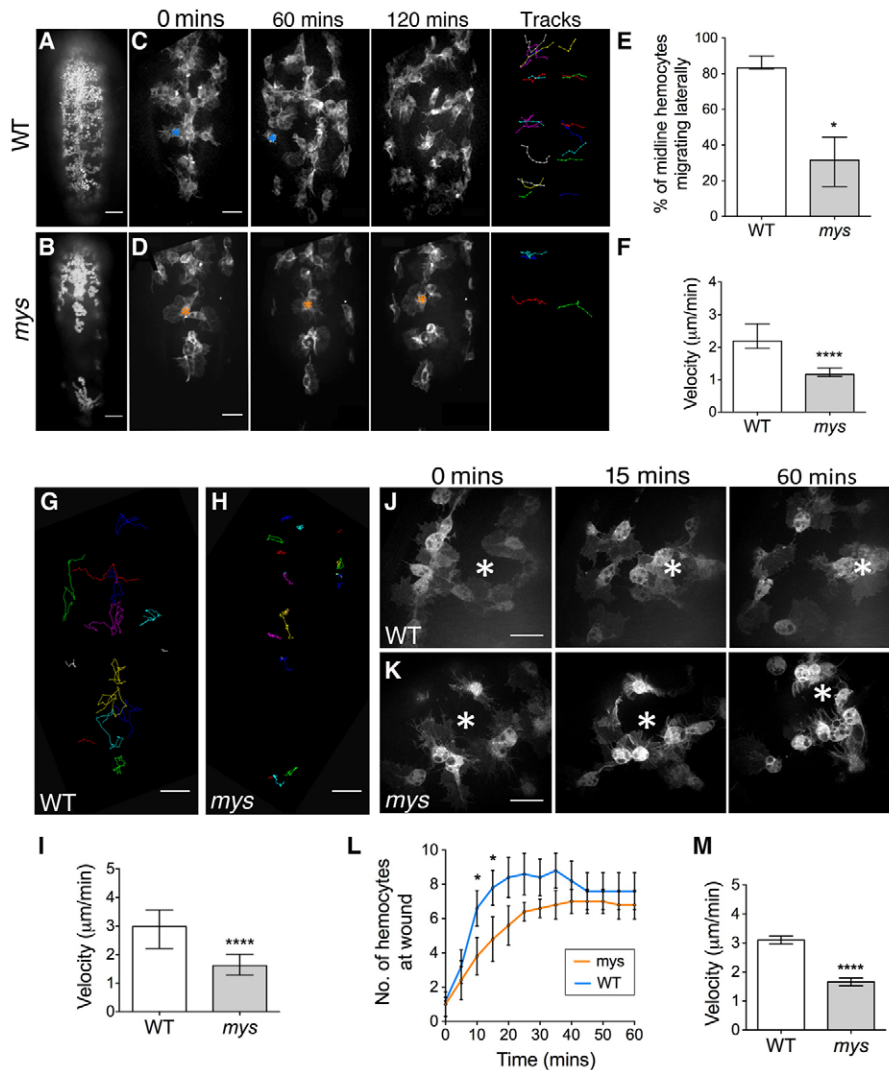


Fig. 3. *myspheroid* is important for lateral migration, random migration and inflammatory migration. (A,B) Single projection of live imaging of haemocytes expressing the nuclear marker red stinger under the control of the *srp-GAL4* promoter during lateral migration from the ventral midline at stage 13/14. (A) In WT embryos haemocytes migrate from the midline laterally along highly organised, segmental paths. (B) In the *mys* mutant these stereotyped lateral migrations are almost completely abolished. Scale bars: 25 μm . (C,D) Still images taken from live-cell imaging of haemocytes expressing mCherry–Moesin (to label F-actin) undergoing lateral migration. (C) In WT embryos haemocytes polarise, before rapidly migrating laterally (blue asterisk). (D) In *mys* integrin mutant embryos haemocytes often polarise but fail to migrate laterally (orange asterisk). Scale bars: 10 μm . (E) Tracking haemocytes undergoing lateral migration reveals that in *mys* mutant embryos a lower percentage of the haemocytes present on the midline at the start of imaging migrate laterally than in WT embryos (average decrease from 85.2% and 30.9%, $P < 0.05$, $n = 5$ embryos per genotype). (F) The velocity of the laterally migrating haemocytes was significantly lower in the mutant embryos (average velocity of WT and *mys* haemocytes were 2.21 ± 0.11 $\mu\text{m}/\text{minute}$ and 1.19 ± 0.13 $\mu\text{m}/\text{minute}$, respectively; $P < 0.0001$. Median and interquartile range (IQR) plotted for $n = 49$ (WT) and $n = 18$ (*mys*) haemocytes. (G–I) Tracking of haemocytes expressing red stinger undergoing random migration at stage 15 (G,H) reveals (I) slower random migration velocity of *mys* mutant haemocytes at this stage (WT and *mys* haemocytes migrated at 1.8 ± 0.57 $\mu\text{m}/\text{minute}$ and 2.7 ± 0.45 $\mu\text{m}/\text{minute}$, respectively). $P < 0.05$; values are means \pm s.e.m. for $n = 69$ (WT) and $n = 85$ (*mys*) haemocytes. Scale bars: 25 μm . (J,K) Stills taken from movies of haemocytes in a WT and *mys* mutant embryo, respectively, migrating to an epithelial wound (asterisk). Scale bars: 10 μm . (L) Monitoring the number of haemocytes at the wound every 5 minutes post wounding over a 60 minute time period indicates a small but significant reduction in the number of *mys* haemocytes present at early time points following wounding ($P < 0.05$ at 10 and 15 minutes post wounding). (M) Tracking reveals a reduction in the velocity of haemocytes in *mys* mutant embryos compared with WT when migrating towards a wound (1.8 ± 0.6 $\mu\text{m}/\text{minute}$ and 3.0 ± 0.3 $\mu\text{m}/\text{minute}$, respectively). $P < 0.01$; median and IQR plotted for $n = 31$ (WT) and $n = 20$ (*mys*) haemocytes.

wild type (data not shown), again highlighting a role in providing a driving force for haemocyte migration rather than a pathfinding role for integrins.

Together these results indicate a haemocyte-specific requirement for integrins in both directed and random

migrations. In all types of migration analysed the migration speed but not the directionality of haemocytes was affected by loss of *mys* function. Consistent with the above data this suggests that integrin-mediated adhesion, rather than signalling, is the predominant functional requirement during haemocyte migration.

A role for *myspheroid* in enabling contact repulsion

Recent studies have shown that microtubule-mediated contact repulsion acts as a major driving force behind lateral migration of haemocytes from the midline and the subsequent maintenance of their even distribution (Stramer et al., 2010). In order to address whether integrins may play a role in this important process we used time-lapse imaging of haemocytes expressing cytoplasmic GFP to analyse the ability of *mys* mutant haemocytes to undergo contact repulsion. In wild-type embryos haemocyte cell–cell contact arrested migration and stimulated mutual repulsion (Fig. 4A) (see also Stramer et al., 2010). In contrast, *mys* mutant haemocytes remained in contact for prolonged periods; on average haemocytes spent six times longer in contact than wild-type equivalents (Fig. 4B,C). These results demonstrate a role for integrins in enabling haemocyte contact repulsion. Previous studies have shown that contact repulsion is driven by realignment of the polarized microtubule cytoskeleton in contacting cells. We therefore analysed microtubule dynamics in *mys* mutant haemocytes.

Loss of *myspheroid* in haemocytes results in disruption of the actin and microtubule cytoskeleton

In *Drosophila* the use of fluorescent probes to label actin (mCherry–Moesin) and microtubules (GFP–CLIP170), has shown that embryonic haemocytes assemble a polarised array of microtubules that extend into lamellipodia during migration. Coalescence of stabilized microtubules drives the formation of a ‘microtubule arm’ that orients the cell in the direction of migration (Stramer et al., 2010). Formation of the microtubule arm is pivotal in polarizing haemocytes in response to external cues that drive their developmental migrations and migration towards wounds as well as enabling haemocytes to undergo contact repulsion (Stramer et al., 2010). Interestingly, *in vitro* experiments have revealed a role for integrins in microtubule stabilisation downstream of the small GTPase Rho (Palazzo et al., 2004). To investigate the microtubule dynamics in *mys* mutant haemocytes we timelapse-imaged haemocytes expressing GFP–CLIP170. Visualization of wild-type haemocytes at stage 15 reveals bundling of microtubules into an arm and close co-ordination of microtubule arm disassembly and lamellipodial retraction (Fig. 5A; supplementary material Movie 1) (Stramer et al., 2010). Analysis of *mys* mutant haemocytes revealed that while

microtubules polarised and initially formed an arm, this structure was not maintained and rapidly collapsed within persisting lamellipodia, indicating that a loss of integrins reduces the stability of microtubule arms (Fig. 5B; supplementary material Movie 2). Despite this apparent instability there were a comparable number of microtubule arms formed by wild-type and *mys* mutant haemocytes (Fig. 5C), consistent with a role for integrin in maintaining a polarized microtubule arm. Closer analysis of microtubule dynamics revealed that, whereas in wild-type haemocytes microtubule arm disassembly was almost always triggered by contact with another haemocyte, in *mys* mutant embryos microtubule arm loss predominantly occurred independently of cell–cell contact (Fig. 5D). Microtubule arm alignment is essential for contact repulsion (Stramer et al., 2010). Therefore, this microtubule arm loss phenotype, coupled with the more general deficits in cell translocation, which would perturb movement of colliding haemocytes away from each other post-contact, presumably underlies the failures in contact inhibition also seen in *mys* mutant embryos.

Analysis of individual microtubule dynamics by high-speed microscopy and subsequent tracking of mCherry–CLIP to show movement of microtubule + tips towards the cell periphery showed that microtubules in *mys* mutant haemocytes protruded at slower speeds than in the wild type (Fig. 5E–G). In keratinocytes loss of integrin-linked kinase (ILK), a signalling protein that associates with the intracellular domain of integrins, results in a failure of peripheral microtubule to reach the cell cortex (Wickström et al., 2010). However, in haemocytes, loss of *mys* did not result in a significant reduction in the final distance microtubules polymerised into the lamellipodia (Fig. 5H), indicating that although slowed, individual polymerizing microtubules are as stable as in WT cells. Together this indicates a specific requirement for integrins in the stabilization of microtubules that are captured and bundled into the microtubule arm structure.

Cell migration is a highly orchestrated process, which requires co-ordination between the actin and microtubule cytoskeleton. Integrin engagement with the ECM recruits and activates numerous signalling molecules, which in turn can activate the actin polymerisation machinery, shown to be important in driving haemocyte migration (DeMali et al., 2003). To determine whether alterations in microtubule dynamics observed in *mys*

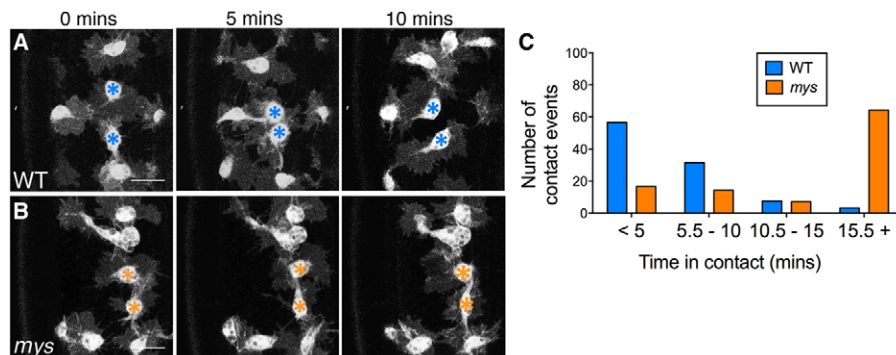


Fig. 4. *myspheroid* plays a role in contact repulsion. (A,B) Stills taken from live-cell imaging of GFP-expressing haemocytes in WT and *mys* mutant embryos undergoing random migration at stage 15. (A) In WT embryos contacting haemocytes (blue asterisk) demonstrate contact repulsion, rapidly repolarising and migrating away from one another. (B) In *mys* mutant embryos haemocytes remain in contact, unable to undergo contact repulsion (orange asterisks). Scale bars: 10 μ m. (C) Quantification of the time the lamellipodia of two haemocytes remain in contact. There is a dramatic increase in this time interval in *mys* mutant embryos (average time in contact for WT and *mys* haemocytes was 5.6 and 27.1 minutes, respectively), $P > 0.01$, $n = 89$ (WT) and $n = 43$ (*mys*) contact events.

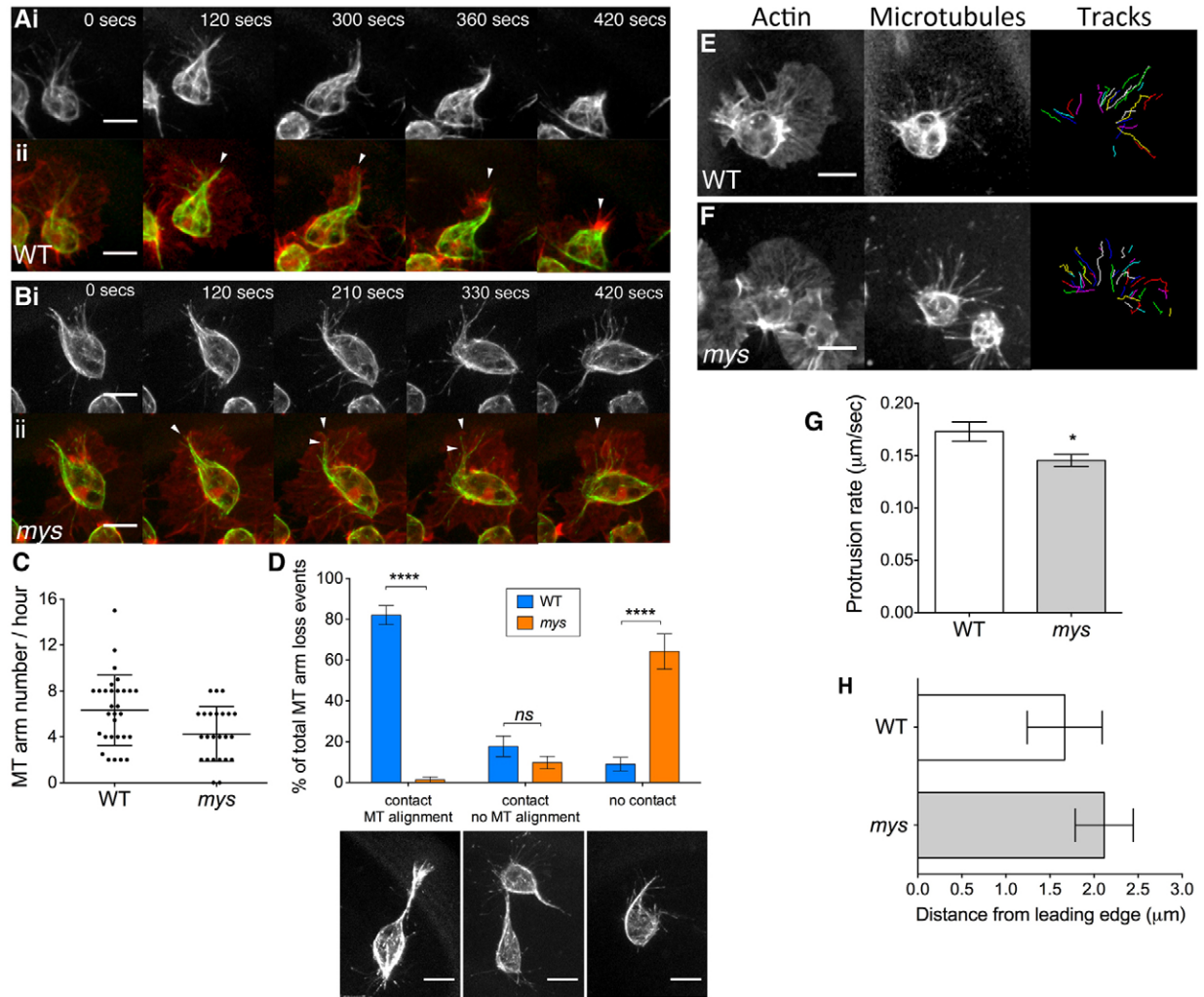


Fig. 5. *myospheroid* is important in maintaining haemocyte microtubule dynamics. (A–F) Stills taken from live-cell imaging of haemocytes expressing Clip170–GFP, to label microtubules (MTs), and mCherry–Moesin, to label F-actin, migrating randomly at stage 15. The imaging reveals disruption of MT dynamics in haemocytes lacking functional *myospheroid*. (A) In WT haemocytes, loss of the MT arm coincides with repolarisation of the actin cytoskeleton. (B) In *mys* mutant haemocytes the MT arm often collapses within the actin protrusions. (C) Quantification of the number of MT arms formed in a haemocyte per hour reveals no significant difference between WT and *mys* mutant haemocytes. Values are means \pm s.e.m. for $n=30$ (WT) and $n=24$ (*mys*) haemocytes. (D) Quantification of the interactions triggering MT arm loss upon cell–cell contact with MT arm alignment or with no MT arm alignment, or MT arm loss independent of cell–cell contact, in WT and *mys* haemocytes. Values are means \pm s.e.m. for $n=30$ (WT) and $n=24$ (*mys*) total MT arm loss events. (E,F) Stills taken from rapid live-cell imaging of haemocytes expressing mCherry–Clip and Moesin–GFP under the control of a single copy of the *srp-GAL4* driver to label only the MT + ends. (G) Tracking the MT tips revealed that in the absence of *myospheroid*, the MT protrusion rate was decreased (WT and *mys* protrusion rates were 0.17 and 0.15 $\mu\text{m}/\text{second}$, respectively, $P<0.05$). Values are means \pm s.e.m. for $n=9$ haemocytes for each genotype. (H) The distance to the leading edge reached by the MT tips in *mys* mutant haemocytes was not significantly different from that in WT (mean distance was 2.1 μm and 1.7 μm , respectively). Values are means \pm s.e.m. for $n=9$ haemocytes per genotype. Scale bars: 10 μm (A, B), 10 μm (E, F), 10 μm (D).

mutant haemocytes could be the result of disruption to the actin cytoskeleton, time-lapse imaging of haemocytes expressing GFP–Moesin was conducted. Analysis of lamellipodia area over time revealed that *mys* mutant haemocytes form dynamic actin-rich lamellipodial protrusions comparable to those observed in the wild type (Fig. 6A–C). Cell culture experiments have shown that integrin-dependent attachment to the ECM can control transition from a spheroid to a flattened morphology, which underlies cell-spreading events that occur during cell migration (reviewed in Holly et al., 2000). The fact that *mys* haemocytes are able to make lamellipodial protrusions as dynamic and of the same size (Fig. 6F) as wild-type cells demonstrates that a loss of βPS does not affect the ability of

haemocytes to spread *in vivo*. However, closer analysis of these protrusions revealed that the organization of the actin cytoskeleton within the *mys* haemocytes is altered with an increase in the number of microspikes (F-actin struts within lamellipodia) and filopodia (microspikes that extend beyond the lamellipodial leading edge) with respect to wild-type cells (Fig. 6D,E). Interestingly, this is in contrast to *in vitro* fibroblast experiments in which integrin binding triggers the formation of microspikes (Levy et al., 2003).

Discussion

Embryonic macrophages play essential roles throughout embryonic development, clearing apoptotic corpses and secreting ECM

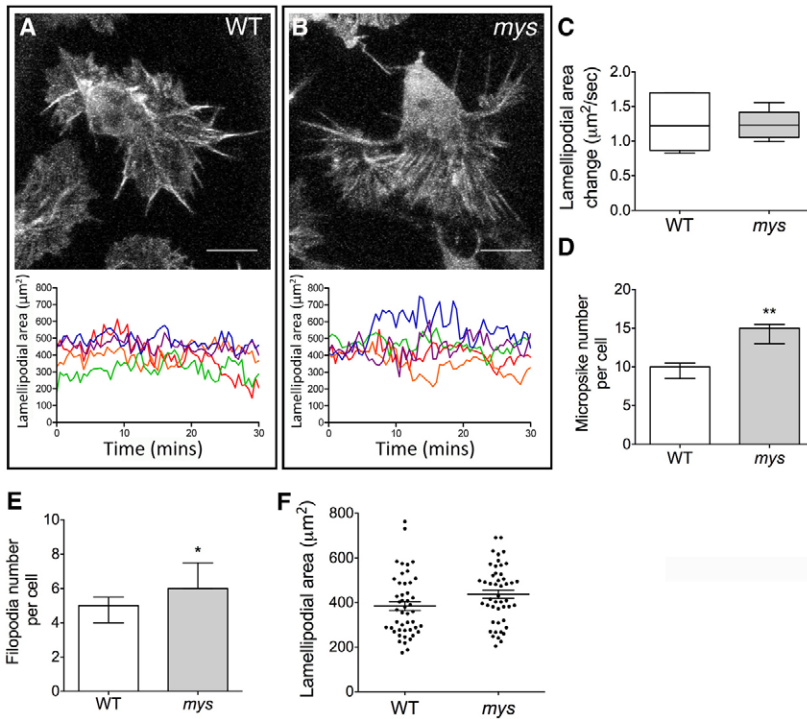


Fig. 6. Haemocytes lacking *myspheroid* show altered actin dynamics. (A,B) Stills taken from live-cell imaging of haemocytes expressing LifeAct under the control of *srp-GAL4* in WT and *mys* mutant embryos. Scale bars: 10 μm. The graphs beneath show the lamellipodial area of five haemocytes measured at 30 second intervals over a 30 minute time period. The large fluctuations in WT and *mys* mutant haemocytes indicate that the overall lamellipodial dynamics remain unchanged in the absence of integrin βPS. (C) This was confirmed when the average lamellipodial area change per haemocyte is compared with that in WT ($n=5$ haemocytes per genotype). (D,E) Other actin-dependent structures within the haemocytes are affected in the *mys* mutant, with (D) an increase in the number of microspikes compared to WT (average 4.8 and 6.6, respectively, $P<0.05$) and (E) the number of filopodia per haemocyte (average 9.6 and 14.4, respectively, $P<0.05$). (F) Quantification reveals no difference in the lamellipodial area of WT and *mys* mutant haemocytes ($n=47$ haemocytes for both genotypes).

components. In order to carry out these important functions, embryonic macrophages must disseminate throughout the embryo. As of yet very little is known about the molecular mechanisms governing these early macrophage migrations within the developing embryo. Utilising *Drosophila* haemocytes as a model system to study the role of integrins in mediating embryonic macrophage migration, we show a role for this family of transmembrane receptors in regulating several responses in embryonic macrophages: developmental dispersal, contact repulsion and chemotactic inflammatory migration towards wounds.

Integrins have always been proposed to play an essential role in the recruitment of macrophages to sights of infection or injury. However, recent work has shown that, although there is an absolute requirement for integrins in leukocyte migration *in vitro*, *in vivo* they are only required for crossing tissue barriers and are dispensable for interstitial migration within the lymph node (Lämmermann et al., 2008). Authors show that leukocyte migration is driven solely by expansion of the actin network, which drives protrusion of the leading edge independently of adhesion, allowing leukocytes to migrate autonomously in the wide variety of tissue environments encountered *in vivo*. These results challenge the classical view about the role of integrins, and cell adhesion in general, during cell migration within the context of a living organism. Our results show that, although integrins are not essential for the initial movements of haemocytes away from their point of origin and along the anterior ventral nerve cord, they are required for all subsequent developmental migrations within the embryo. It is likely therefore that the requirement for integrins in cell migration is cell type as well as environment specific. Whereas leukocytes show stochastic, swarming migration across a wide variety of extra cellular environments, *Drosophila* haemocytes undertake highly orchestrated migrations over tissues with a basement membrane. Therefore although performing common functions *in vivo*, environmental interactions may have forced these

cell types to adapt different modes of migration. For example although leukocytes are able to switch to contraction-driven ‘squeezing’ through a confined 3D environment, fibroblasts rely solely on integrin-dependant anchorage to track along components of the extracellular matrix (Renkawitz and Sixt, 2010). This linkage-based guidance of migration could aid in the control of haemocyte developmental migration along pre-determined pathways. Interestingly, the ability of integrin mutant haemocytes to undergo very early dispersal from the head mesoderm to occupy the anterior region of the ventral midline indicates that during embryonic development haemocytes are also able to switch from an integrin-independent to an integrin-dependant mode of migration.

By analysing cell migration within the developing embryo, we have shown a dual requirement for integrins in both the migrating haemocytes and the surrounding tissues. Haemocyte migration along the ventral midline is dependent upon correct development of the VNC. In *slit* and *robo1*, 2 mutant embryos, haemocytes fail to progress along the midline due to a failure in the separation of the VNC and epidermis (Evans et al., 2010). Authors propose that this failed separation event may result from disruptions in axonal path finding and glial positioning within the VNC, leading to a failure of these structures to separate from the epidermis. We show here that *mys* mutant embryos also display a failure in the separation of the VNC and given that *mys* mutant embryos display axonal pathfinding defects (Stevens and Jacobs, 2002), the separation defect observed in *mys* mutants may be a consequence of this disrupted axonal wiring. Alternatively, integrin-dependant assembly of ECM components, as in several cellular contexts (Narasimha and Brown, 2004; Tanentzapf et al., 2007), may be required for correct separation of the VNC. Consistent with this, haemocytes fail to migrate along the VNC in laminin mutant embryos (Urbano et al., 2009). It would be interesting to see whether this migration defect may also be due to a failure in VNC separation.

Integrins allow traction in migrating cells by acting as a molecular clutch that links the actin cytoskeleton to the ECM, allowing the transmission of acto-myosin contraction to the substratum (Alexandrova et al., 2008). Consistent with this role for integrins, we find that removal of functional integrins from haemocytes results in a severe reduction in migratory velocity during both random and directional migration. In migrating leukocytes and fibroblasts, loss of integrins results in increased actin retrograde flow as a consequence of the integrin 'clutch' (Renkawitz and Sixt, 2010). Within 3D environments, integrin mutant leukocytes are able to overcome this loss of traction by increasing the polymerisation of actin, reaching migration velocities indistinguishable to WT. Although haemocyte spreading is not affected by integrin loss *in vivo*, perhaps attributable to the close confinement of haemocytes within the 3D context of the embryo (Tucker et al., 2011), we do however see subtle changes in the structure of the actin cytoskeleton. Surprisingly, the increase in the number of microspikes, actin bundles within the lamellipodia, seen in *mys* mutant haemocytes usually correlates with increased migration speed (P. K. Tucker, I. E. and W. W., unpublished data). If the compensatory mechanism observed in integrin mutant leukocytes is conserved in haemocytes, the increase in microspikes within lamellipodia could reflect an attempt to overcome slippage due to loss of the integrin 'clutch'. It would therefore be interesting to compare retrograde flow in *mys* mutant haemocytes to determine whether the function of integrins between mammalian and *Drosophila* blood cells is conserved.

Applying this clutch theory could also aid in explaining the microtubule phenotype observed in *mys* mutant haemocytes. It is appreciated that migration requires close co-ordination between the actin and microtubule network, however the nature of these interactions during cell migration remains poorly understood. We have previously shown that microtubules are closely associated with actin microspikes as they extend and probe the lamellipodia of an advancing haemocyte (Stramer et al., 2010). An increase in actin retrograde flow within the lamellipodia could lead to the rapid and repeated collapse of the microtubule arm as seen in integrin mutants. Alternatively, these microtubule defects could reflect a direct requirement for integrins in regulating microtubule dynamics, via previously identified downstream targets such as ILK, FAK and diaphanous (Palazzo et al., 2004; Wickström et al., 2010).

In summary, our results highlight an essential role for integrins in mediating many embryonic macrophage functions, including developmental migration, inflammatory migration towards wounds and contact repulsion. The possibility of examining these processes *in vivo* in a genetically tractable organism such as *Drosophila* will significantly assist in the dissection of the molecular mechanisms by which integrins exert these different functions in this prominent cell type so important in many biological processes. Furthermore, in addition to their role in the clearance of apoptotic cells, macrophages are being increasingly recognised for their role in providing trophic support in many tissues, their contribution to tissue regeneration and their pivotal role in tumour angiogenesis and metastasis. Pro-metastatic roles have even been demonstrated by haemocytes in *Drosophila* cancer models (Cordero et al., 2010). Recent expression profiling of embryonic macrophages revealed similarities with tumour associated macrophages (TAM), independent of their tissue of origin (Rae et al., 2007). Hence, a better characterization of the biology of embryonic macrophages may also provide clues to

TAM function in cancer and may lead to the identification of new molecular targets to inhibit their proangiogenic and protumoral activities in neoplasia and other diseases.

Materials and Methods

Drosophila strains and techniques

Flies were raised at room temperature. Embryos were collected from laying cages kept overnight at 25 °C. For RNAi experiments laying cages were kept overnight at 29 °C. The following stocks were used for fixing and staining to analyse defects in haemocyte developmental dispersal: *mys*^{XG43} FRT101 (Bunch and Brower, 1992), *rhea*⁷⁹ FRT 2A (Prout et al., 1997), *if*^{B4} (Brown, 1994), *scab*^{HG} (Stark et al., 1997), *mew*^{m6} (Brower et al., 1995), β v (Yee and Hynes, 1993), *UAS-mys* (Martin-Bermudo and Brown, 1996), *UAS-Torso*^{P/β_{cyt}} (Martin-Bermudo and Brown, 1999), *UAS-p35* (Hay et al., 1994), *srph-GAL4* (Huelsmann et al., 2006), *srp-HemoGAL4* (Bruckner et al., 2004), *sim-gal4* and *slit-gal4* (Scholz et al., 1997), *UAS-gfp*^{S65T}, *ovo*^{D1} FRT101 (Bloomington). For the RNAi knockdown experiments, we used the following UAS-RNAi lines; UAS-RNAi *mys* and UAS-RNAi *italin* (VDRC).

For time-lapse imaging *SerpentHemoGAL4* (*srp-GAL4*) (Bruckner et al., 2004), *croquemort-GAL4* (*crq-GAL4*) (Stramer et al., 2005) and *singed-GAL4* (*sn-GAL4*) (Zanet et al., 2012) were used to drive haemocyte-specific expression of the following UAS constructs (obtained from Bloomington Stock Center unless otherwise stated): *UAS-GFP*, *UAS-GFP-Moesin* (Dutta et al., 2002), *UAS-mCherry-Moesin* (a gift from Paul Martin, University of Bristol), *UAS-mCherry-CLIP170* and *UAS-GFP-CLIP170* (Stramer et al., 2010) and *UAS-redstinger* (Barolo et al., 2004). The constructs and drivers were used to produce the following genotypes; *srp-GAL4,UAS-GFP-Moesin;UASCherryClip,srp-GAL4,UAS-GFP;crq-GAL4,UAS-GFP,srp-GAL4,UAS-mCherry-Moesin;crq-GAL4,UAS-GFP-CLIP170,srp-GAL4,UAS-mCherry-Moesin;crq-GAL4,UAS-mCherry-Moesin,sn-GAL4,UAS-LifeActGFP* and *srp-GAL4,UAS-redstinger;crq-GAL4,UAS-redstinger*.

Histochemistry

Antibody staining of embryos was performed using standard procedures. We used the following primary antibodies: Rb-GFP 1/100 (Molecular Probes), rabbit anti-β-Gal (1:6000; Cappel), rabbit anti-Srp (1:1000; Riechmann et al., 1998), rabbit anti-Crq (1:1000; Franc et al., 1996), mouse anti-βPS (1:300; DSHB, Iowa). Alexa-conjugated secondary antibodies used were Alexa Fluor 488 (green), Alexa Fluor 568 (red) (Molecular Probes™). For non-fluorescent staining, embryos were incubated in biotinylated secondary antibodies followed by incubation with Elite ABC complex (Vector Laboratories) and revealed with DAB (Gibco-BRL). Images were collected with a Zeiss Axioplan 2 microscope or a Leica TCS-SP2 confocal microscope.

Time-lapse recording

Embryos were prepared and mounted as previously described (Wood and Jacinto, 2005). To analyse random migration stage 15 red stinger-labelled haemocytes were imaged on Zeiss Axioplan 2 wide-field imaging system. Contact repulsion of stage 15 haemocytes expressing GFP was captured using a Zeiss 510 confocal laser-scanning microscope with a plan apochromat 63×/1.4 oil objective. All other imaging was conducted on a spinning disc confocal microscope (UltraView; PerkinElmer).

Wounding

Due to anterior localization of *mys* mutant haemocytes, stage 15 WT and *mys*^{XG43} mutant embryos were wounded in the anterior half of the embryo between the ventral midline and lateral lines of haemocytes by laser ablation (Wood et al., 2002).

Dextran injections

Stage 15 embryos were prepared and mounted ventral side up as per live imaging before 2.5 mg/ml 70 kDa Rhodamine-dextran (Molecular Probes/Invitrogen, Carlsbad, CA, USA) was injected between the epidermis and VNC as previously described (Evans et al., 2010). Embryos were then imaged live as described above.

Image processing and analysis

Cell tracking and cell area measurements were performed using image J (NIH). Graphs and statistical analysis was carried out using Prism for Mac (Graph Pad). Unless otherwise stated, haemocyte migratory behaviour was analysed using images acquired from five embryos of each genotype.

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Author contributions

K.C., S.H. and B.J.S.-S. performed experiments and contributed to the writing of the paper; I.E. performed experiments; A.C. and W.W. jointly supervised K.C.; R.R. designed the experiments, analyzed results and contributed to the writing of the paper; and W.W. and M.D. M-B designed the experiments, analyzed results and wrote the paper.

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