Ena drives invasive macrophage migration in *Drosophila* embryos

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**SUMMARY**

It is seldom the primary tumour that proves fatal in cancer, with metastasis the fundamental pathological process for disease progression. Upregulation of Mena, a member of the evolutionarily conserved Ena/VASP family of actin cytoskeletal regulators, promotes metastasis and invasive motility of breast cancer cells in vivo. To complement in vitro studies of Ena/VASP function in fibroblasts, we manipulated levels of Ena, the *Drosophila* homologue of Mena, in migrating embryonic macrophages (haemocytes). Consistent with data from fibroblasts in vitro, Ena localises to regions of actin dynamics within migrating haemocytes, stimulates lamellipodial dynamics and positively regulates the number and length of filopodia. However, whereas Ena overexpression in fibroblasts reduces migration speeds, overexpressing Ena in haemocytes leads to a dramatic increase in migration speeds, more closely resembling the increased motility of breast cancer cells that overexpress Mena. We provide evidence that this key difference is due to spatial constraints imposed on cells within the three-dimensional environment of the embryo; this might explain how Mena can be used to promote aggressive migratory behaviour during cancer progression.

**INTRODUCTION**

The metastasis of cancer cells from the primary tumour is the key step in cancer progression and requires invasive cell migration, a process also important during development (Hanahan and Weinberg, 2000; Franz et al., 2002). Actin polymerisation drives the formation of protrusions at the leading edge of a migrating cell, and adhesion of protrusions coupled with retraction and disassembly of adhesions at the rear of the cell enable continued migration. In many cell types, actin filaments are organised into broad, sheet-like lamellipodia, which consist of branched arrays of filaments at the leading edge, or are bundled into finger-like filopodial protrusions (Pollard and Borisy, 2003; Ridley et al., 2003). One actin-regulatory protein recently implicated in several human cancers is Mena (Di Modugno et al., 2004; Toyoda et al., 2009), a member of the Ena/VASP family (*Drosophila* Enabled, vasodilator-stimulated phosphoprotein) (Gertler et al., 1996), which antagonise capping of barbed ends of actin filaments, facilitating their continued elongation and stimulating cell protrusion (Bear et al., 2002).

Recent studies using rat breast carcinoma cells revealed that elevated levels of Mena increase cell motility in vivo and also metastasis to the lungs. Furthermore, overexpression of Mena increased invasive migration into collagen gels in vitro (Philippar et al., 2008). Additionally, Ena/VASP proteins are targeted downstream of numerous guidance receptors (Bashaw et al., 2000; Evans et al., 2007; Lebrand et al., 2004), with the misregulation of many of these implicated in cancer (Brantley-Sieders et al., 2008; Kaufmann et al., 2009; Legg et al., 2008). Members of the Ena/VASP family are also regulated by the kinases Abl and PKC (Gertler et al., 1990; Wentworth et al., 2006), which are themselves heavily implicated in cancer progression (Mauro et al., 2010; Srinivasan et al., 2008).

Much of our understanding of Mena function is derived from studies of fibroblasts in vitro, where Ena/VASP proteins negatively regulate cell migration, potentially by antagonising capping at the barbed ends of actin filaments (Barzik et al., 2005; Bear et al., 2002). This activity is thought to facilitate actin polymerisation, enhancing the generation of actin-rich protrusions; however, in these cells exuberant Ena/VASP activity actually decreases migration speed because the protrusions formed are unstable and tend to be lost as membrane ruffles (Bear et al., 2000; Bear et al., 2002).

To determine how Ena/VASP proteins regulate cell migration in vivo, we have manipulated the levels of Ena, the *Drosophila* homologue of Mena (Gertler et al., 1996), within the macrophages (haemocytes) of developing embryos. Loss of zygotic Ena in *Drosophila* was initially shown to disrupt axon guidance (Wills et al., 1999). Subsequently Ena has been demonstrated to play an important role in embryonic morphogenesis, with removal of maternal and zygotic Ena disrupting processes such as germ band retraction, head involution and dorsal closure; during the latter, Ena regulates filopodial number and length as well as lamellipodial area in leading edge epithelial cells (Gates et al., 2007; Homem and Peifer, 2009). Furthermore, Ena plays important roles at the cortex of nurse cells during dumping and within border cells during their collective migration (Gates et al., 2009).

During *Drosophila* development, embryonic haemocytes undergo invasive and highly stereotyped migrations to distribute fully throughout the embryo and are capable of mounting rapid chemotactic and phagocytic responses to epithelial wounds and pathogens (Stramer et al., 2005; Tepass et al., 1994; Vlisidou et al., 2009; Wood et al., 2006). Here we have used time-lapse microscopy to visualise haemocytes as they migrate between the ventral nerve cord (VNC) and epidermis both during and after their developmental dispersal.

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Received 17 March 2010; Accepted 11 August 2010
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Similar to findings in fibroblasts in vitro, we show that Ena localises to sites of dynamic actin reorganisation in haemocytes in vivo. However, despite stimulating protrusion and lamellipodial dynamics in a similar fashion to that seen in fibroblasts, we find that Ena overexpression increases haemocyte migration speed, resembling the effect of Mena overexpression in cancer cells in vivo. We show evidence that a three-dimensional (3D) constraint in vivo prevents loss of protrusions as membrane ruffles, allowing a more concerted migration, which might also underlie the enhancement of cancer cell motility and metastasis upon Mena overexpression in vivo.

**RESULTS**

**Ena localises to regions of dynamic actin reorganisation in vivo**

Mena, a mammalian homologue of *Drosophila* Ena, typically localises to the tips of filopodia and leading edges of lamellipodia in cells such as fibroblasts and neurons in vitro (Gertler et al., 1996; Lanier et al., 1999). Ena itself is widely expressed within *Drosophila* embryos; levels are particularly high within the central nervous system (CNS) (Gertler et al., 1995) and the protein is also present within haemocytes (supplementary material Fig. S1A-C). To determine Ena localisation in vivo, Ena-GFP and mCherry-Moesin were co-expressed in *Drosophila* haemocytes using the GAL4-UAS system (Brand and Perrimon, 1993). At stage 15 of embryonic development Ena was enriched at the tips of filopodia and lamellipodia in haemocytes (Fig. 1A; supplementary material Movie 1). Time-lapse movies of filopodial extension and retraction revealed Ena at the tips of filopodia during extension, with retention at the tip in 25% of cases (n=77) during retraction (Fig. 1B); in the remainder Ena was lost from filopodial tips upon retraction (supplementary material Movie 1). Kymographic analysis of haemocytes in the embryo indicated that Ena was enriched at lamellipodial leading edges during extension but upon retraction this localisation was lost (Fig. 1C).

To investigate the distribution of Ena during directed migration, we analysed haemocytes undergoing lateral migration away from the ventral midline to the edges of the developing VNC, easily visualised in stage 14 embryos (Wood et al., 2006). Observation of laterally migrating haemocytes expressing Ena-GFP and mCherry-Moesin clearly showed that Ena localised to the leading edge of the lamellipodia during this directed migration (Fig. 1D,E; supplementary material Movie 2). Therefore, Ena is present at regions of dynamic actin reorganisation in migrating haemocytes in vivo and these locations are similar to those previously reported in both fibroblasts and neural growth cones in vitro (Gertler et al., 1996; Lanier et al., 1999).

**Fig. 1. Ena localises to the tips of filopodia and the leading edge of lamellipodia in vivo.** Haemocytes co-expressing Ena-GFP (green) and mCherry-Moesin (red) were imaged at stage 15 of embryonic development. (A) Ena-GFP localises to the tips of filopodia (arrowhead) and the leading edge of lamellipodia (bracket) in haemocytes at the midline. (B) Ena-GFP at a filopodial tip during extension and retraction (see supplementary material Movie 1 for an example of Ena-GFP being lost during retraction). (C) Kymograph generated along the axis of protrusion (solid line in C), demonstrating the presence of Ena-GFP at the leading edge during lamellipodial extension and its loss from this site upon retraction (arrowheads); the dotted line indicates the time point in the kymograph corresponding to the still image in C. (D) Ena-GFP is present at the lamellipodial leading edge during lateral migration (see supplementary material Movie 2 for corresponding time-lapse). Dots and lines reveal progress of the haemocyte when moving from the midline to the edge of the VNC. (E) Images showing the GFP channel of the haemocyte from D; in these images Ena-GFP is clearly localised at the lamellipodial leading edge (arrowheads). Scale bars in C represent 2 μm (vertical) and 60 seconds (horizontal); all other scale bars represent 10 μm.
Filopodia and lamellipodial dynamics are regulated by Ena in vivo

Ena regulates the formation and dynamics of both filopodia and lamellipodia in vitro (Applewhite et al., 2007; Bear et al., 2002), and we sought to determine what effect Ena has on these structures within haemocytes. To this end, Ena or FPPPMito-GFP (FP4Mito) (Gates et al., 2007) were specifically overexpressed in haemocytes to supplement or inactivate Ena function, respectively. FP4Mito has previously been shown to phenocopy genetic ablation of Ena/VASP function via sequestration of endogenous Ena at mitochondria away from sites of actin dynamics (Bear et al., 2000; Gates et al., 2007) and did so efficiently in haemocytes (supplementary material Fig. S1A). FP4Mito and Ena were co-expressed with cytoplasmic GFP to detail haemocyte morphology. Typically, wild-type haemocytes produced large lamellipodia, from which numerous filopodia protrude (Fig. 2B), whereas very few filopodia were produced when Ena was inactivated by expression of FP4Mito, with residual filopodia drastically shortened (Fig. 2A,D,E; Table 1). By contrast, Ena overexpression increased both the number and length of filopodia (Fig. 2C,E; Table 1).

Whereas Ena exerted striking effects on filopodia, its regulation of lamellipodia was more subtle. Ena was not required to form lamellipodia because these structures remained after its inactivation (Fig. 2A) and intriguingly both overexpression and inactivation of Ena increased average lamellipodial area (Fig. 2F; Table 1). Instead Ena regulated lamellipodial dynamics: Ena inactivation led to stable lamellipodia that fluctuated very little in area over time when compared with the more dynamic lamellipodia of wild-type haemocytes. Consistently, overexpression of Ena increased lamellipodial dynamics compared with wild-type cells (Fig. 2A,C,G; Table 1). To determine how Ena regulates dynamics at the level of individual lamellipodia, we carried out kymography on specific lamellipodial regions in single cells. Kymographs showed that wild-type lamellipodia protruded and retracted at regular intervals (Fig. 2I); Ena inactivation decreased the speed of lamellipodial protrusion and prolonged the persistence of these protrusions (Fig. 2H,K,L; Table 1), whereas Ena overexpression increased lamellipodial protrusion speeds but these faster-growing protrusions were less persistent (Fig. 2J,L; Table 1).

Overexpression of Ena increases haemocyte speed in vivo

Our findings regarding lamellipodial dynamics are consistent with results from fibroblasts in vitro, where Ena overexpression has been shown to increase rates of lamellipodial protrusion but decrease persistence, translating to an overall reduction in the speed of migration (Bear et al., 2000). This negative effect on migration speed is difficult to reconcile with recent data suggesting that cancer cells with elevated levels of Mena display a higher propensity to metastasise (Philippar et al., 2008), which would be consistent with a positive effect on migratory speed. In light of this apparent paradox we wondered how Ena upregulation in vivo might affect the overall speed of haemocyte migration in a variety of contexts, given that lamellipodial dynamics in haemocytes in vivo seem to be regulated in a similar Ena-dependent manner to fibroblasts in vitro.

We began by looking at lateral migration of haemocytes, an example of a highly stereotyped, directed migratory process (Fig. 3A). Live analysis of individual haemocytes revealed that Ena overexpression increased speed compared with wild-type haemocytes during lateral migration, whereas Ena inactivation reduced migration speed (Fig. 3E; Table 1). A similar decrease in lateral migration speed was observed in ena mutant embryos.
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Table 1. Ena-dependent regulation of hemocyte morphology and behaviour in vivo

| Expression | Filopodia | Lamellipodia | Migration speed |
|------------|-----------|--------------|-----------------|
|            | Number    | Length (µm)  | Area (µm²)      | Change in area (µm²/second) | Rate of protrusion (µm/minute) | Persistence of protrusion (minutes) | Lateral migration (µm/minute) | Migration to wounds (µm/minute) | Random migration (µm/minute) |
| FP4Mito    | 0.13      | 1.6          | 380            | 0.46              | 2.4                        | 0.46                         | 1.4                          | 1.3                           | 0.87                           |
|            |           |              | (1.7×10⁻²²)    | (2.5×10⁻¹³)       | (1.2×10⁻¹¹)                | (0.000144)                   | (0.042)                      | (1.1×10⁻⁷)                    | (0.012)                        | (4.3×10⁻¹⁰)                    |
| WT         | 4.9       | 2.9          | 330            | 0.76              | 5.3                        | 0.34                         | 1.8                          | 1.6                           | 1.4                           |
|            |           |              | (2.1×10⁻¹⁰)    | (0.00010)         | (1.2×10⁻¹⁵)                | (0.0034)                     | (0.026)                      | (0.0021)                      | (0.017)                        | (0.044)                        |
| Ena        | 7.8       | 4.0          | 380            | 0.94              | 8.0                        | 0.25                         | 2.4                          | 1.9                           | 1.6                           |
|            |           |              | (2.1×10⁻¹⁰)    | (0.00010)         | (1.2×10⁻¹⁵)                | (0.0034)                     | (0.026)                      | (0.0021)                      | (0.017)                        | (0.044)                        |

All values are averages, with P-values shown in parentheses if applicable.

(supplementary material Fig. S1D). Surprisingly, neither Ena inactivation nor overexpression affected directionality (compare Fig. 3B-D), suggesting Ena controls cell speed independently of signalling events required to direct this migration. Furthermore, it implies that filopodia might not be crucial in sensing lateral cues or mechanically in lateral migration itself, given that Ena inactivation significantly reduced filopodial numbers.

Haemocytes also migrate in a directed fashion towards laser-induced, epithelial wounds in response to H2O2 production (Moreira et al., 2010; Stramer et al., 2005). Tracking haemocyte migration for an hour post-wounding, we found that Ena overexpression increased haemocyte speed towards wounds, whereas inactivation reduced speed compared with wild-type controls (Fig. 3F-J; Table 1). Interestingly, neither overexpression nor inactivation of Ena affected haemocyte directionality towards wounds (compare Fig. 3G-I), nor wound healing itself, although inactivating Ena did slightly reduce the number of haemocytes recruited to wounds [eight haemocytes for wild type (n=35) compared with seven for FP4Mito (n=45); P=6.04×10⁻⁶].

The fact that Ena overexpression increased the rate of two distinct examples of directed migration in vivo whereas inactivation decreased migration speed is in stark contrast to similar experiments conducted with fibroblasts in vitro. However, fibroblast speed was measured during random migration. We

Fig. 3. Ena positively regulates haemocyte migration speed in vivo. Haemocytes expressing GFP and FP4Mito (FP4; green boxes in graphs), GFP alone (WT; blue boxes), or GFP and Ena (Ena; purple boxes) were imaged live as they migrated laterally, to laser-induced wounds, and randomly. (A) WT haemocytes migrate from the midline (arrow) to the edges of the VNC (arrowheads) from stage 14 of development. (B-D) Tracks of FP4, WT and Ena haemocytes undergoing lateral migration reveal the directionality of migration. (E) Box and whisker plot of lateral migration speed for each genotype. (F) WT haemocytes migrate to laser-induced, epithelial wounds. (G-I) Tracks of FP4, WT, and Ena haemocytes as they migrate towards a wound (asterisk). (J) Box and whisker plots of haemocyte migration speed to wounds for each genotype. (K) WT haemocytes migrate randomly over the VNC after lateral migration from stage 15 onwards; red shading illustrates position of the cell body of an individual haemocyte at 0 and 30 minutes in the time-lapse movie. (L-N) Tracks of FP4, WT and Ena haemocytes showing random motility over the course of a 30 minute movie. (O) Box and whisker plot of the speed of random migration for each haemocyte genotype. Scale bars represent 20 µm; asterisks on graphs denote that the probability of a significant difference being <0.05 (t-test).
therefore monitored migration of haemocytes at stage 15 of development, when they too migrate randomly on the ventral side of the embryo (Fig. 3K,M). Manipulation of Ena caused no obvious differences in haemocyte trajectories but, consistent with wounding studies, Ena overexpression increased haemocyte speed whereas inactivation reduced it (Fig. 3L-O; Table 1).

**Spatial constraints might underlie behavioural differences in vivo and in vitro**

Using three distinct in vivo migration assays we have shown that, in contrast to fibroblast migration in vitro, Ena positively regulates migration speed. This finding is relevant to cancer studies given the fourfold upregulation of Mena in rat breast carcinoma cells (Wang et al., 2004), which increases their motility and invasiveness (Philippar et al., 2008). This discrepancy might occur because protrusions created by Ena-overexpressing fibroblasts are frequently lost as ruffles in vitro (Bear et al., 2002); in contrast, in vivo cells are constrained by their 3D environment (Fig. 4D) (Evans et al., 2010), which might physically prevent ruffling, allowing protrusions to be used productively during cell migration and lead to increased cell speeds.

In order to investigate this further, we expressed mCD8-GFP to label the plasma membrane of haemocytes and observed their dynamic behaviour in vivo and in vitro. In vivo haemocytes never formed membrane ruffles as they migrated through the embryo (Fig. 4A; supplementary material Movie 3). However, distinctive wave-like ruffles were seen forming at the leading edge of lamellipodia and moving centripetally towards the cell body when mCD8-GFP-expressing haemocytes were cultured in vitro (Fig. 4B; supplementary material Movie 3). Strikingly, on addition of methylcellulose to thicken cell culture media and reconstitute a physical constraint, haemocytes no longer formed membrane ruffles in vitro (Fig. 4C; supplementary material Movie 3), more closely resembling haemocytes in vivo. Indeed, kymographic analysis of lamellipodial dynamics revealed no significant difference in speed or persistence of lamellipodial protrusions between wild-type haemocytes in vivo and those in vitro with methylcellulose (supplementary material Table S1). However, protrusions were less persistent in vitro without methylcellulose compared with in vivo, and speed of lamellipodial protrusion was increased (supplementary material Table S1). This suggests that protrusions are less stable in vitro than in vivo and that the addition of a physical constraint that prevents ruffling stabilises protrusions in a manner similar to the in vivo environment.

We then compared the dynamics of protrusions that are lost as membrane ruffles to those lost without ruffling in vitro and found that protrusions lost as ruffles had a faster speed of retraction and retracted for longer. We also found that the overall frequency of retraction in vitro was much higher than in vivo (supplementary material Table S1), with retraction coincident with membrane ruffles accounting for 58% of retraction events. Taken together these events demonstrated that in the absence of a 3D constraint protrusions are less persistent and retraction events more severe. The stabilisation of protrusions and inhibition of membrane ruffling thus enables efficient migration in vivo, because membrane ruffling impacts negatively on migration by increasing both the speed and persistence of lamellipodial retraction. Consistently, the in vivo environment was sufficient to inhibit membrane ruffling on overexpression of active Rac (Fig. 4E; supplementary material Movie 4), which is known to induce ruffling in vitro (Ridley et al., 1992); Rac overexpression led to the formation of exuberant
haemocyte protrusions, which formed ruffles only in vitro (supplementary material Fig. S2). Furthermore, we observed that when haemocytes were in an environment where the constraint from the overlying epithelium had been removed, such as in a wound, they actively ruffled (Fig. 4F; supplementary material Movie 5), supporting the hypothesis that the surrounding tissue provides the physical constraints that prevent ruffling in vivo (Fig. 4G).

In summary, we propose that 3D spatial constraints stabilise lamellipodial protrusions, such that the increased rate of protrusion can accelerate migration upon Ena overexpression in vivo. This offers a potential explanation as to how increased Mena levels might exacerbate metastasis and cancer progression. Furthermore, it illustrates the power of haemocytes as a model cell type to examine the roles of other modulators of the actin cytoskeleton that are implicated in cancer progression and their influence on cell migration in vivo.

**DISCUSSION**

Here we demonstrate that Ena increases the speed of lamellipodial protrusion but reduces the persistence of these protrusions in haemocytes in vivo, paralleling the role of Mena in fibroblasts cultured in vitro (Bear et al., 2002). However, despite regulating lamellipodial dynamics similarly, Ena overexpression increased haemocyte migration speeds in vivo, whereas it reduces fibroblast speeds in vitro (Bear et al., 2000). Consistent with our findings, Ena inactivation in border cells also decreased their migration speeds (Gates et al., 2009). Interestingly, our in vivo haemocyte data closely correlate with the finding that Mena upregulation in rat breast carcinoma cells increases metastases and invasion (Philippar et al., 2008). Furthermore, we show that the external environment plays a key role in determining haemocyte migration speeds, potentially by placing spatial constraints on haemocytes that inhibit membrane ruffling. Correspondingly, when haemocytes were cultured in vitro they began to produce membrane ruffles, but application of an artificial constraint blocked membrane ruffling. Although we observed retraction events in vivo, these events were less severe and occurred less frequently during migration; therefore, the inhibition of ruffling enables lamellipodia to advance further and in doing so increase cell migration speed. Our results might be particularly pertinent to invasive migration of cancer cells through stromal tissue in which extracellular matrix is highly constrictive (Wolf et al., 2007). Overexpression of Mena might therefore be advantageous for invasion in this spatially restricted microenvironment because protrusions that might otherwise be lost as membrane ruffles could be rendered competent to contribute to cell migration.

It has been suggested that levels of membrane ruffling in combination with adhesion strength might underlie differing migration speeds between cell types (Small et al., 2002). The spatial constraints imposed on migrating haemocytes could restrict the detachment of Ena-regulated lamellipodia from their adhesions to the substrate, enabling faster migration. Alternatively, Ena could assume a more direct role in modulating adhesion as Mena localises to focal contacts and adhesions in fibroblasts (Gertler et al., 1996). However, selective removal of Mena from adhesions did not affect cell migration speed in fibroblasts (Bear et al., 2000). Integrins are clearly important for haemocyte migration in vivo (Siekhaus et al., 2010; Huelsmann et al., 2006; Urbano et al., 2009), but the nature of adhesions made by these cells has yet to be addressed and Ena-positive, focal-adhesion-like structures do not seem to be present. Furthermore, a GFP-tagged version of integrin-linked kinase, a kinase that associates with integrins at adhesion sites (Zervas et al., 2001), did not localise to adhesion-like puncta within haemocytes (data not shown). It will be intriguing to see exactly what types of adhesive structures haemocytes make and whether there is a relationship between Ena levels and integrins as observed in epithelial cells (Delon and Brown, 2009). We cannot rule out disruption of adhesions on overexpression of Ena or FP4Mito, although any such effect would be relevant to misregulation of Ena/VASP levels given the reduction in migratory speeds in both ena mutants and embryos containing FP4Mito-expressing haemocytes.

In this study we show that Ena overexpression and inactivation increase total lamellipodial area. A similar increase in lamellipodial area is seen in Drosophila leading edge epithelial cells upon Ena overexpression; however, inactivation of Ena reduces lamellipodial area in these cells (Homem and Peifer, 2009), implying Ena has some cell-type-specific effects, most probably dependent upon the levels and activities of other actin-regulatory proteins within the cell. In addition to lamellipodial area, we demonstrate that Ena regulates filopodial number and length in haemocytes in vivo; a similar role for Ena has been observed in epithelial cells and neurons (Gates et al., 2007; Lebrand et al., 2004), reiterating the importance of Ena for efficient initiation and elongation of filopodia. However, Ena inactivation did not totally preclude the formation of filopodia in haemocytes, suggesting that Ena-independent filopodia can be produced by other actin-regulatory proteins in these cells. One candidate for this role is the formin Diaphanous (Dia), because this protein is thought sufficient to initiate filopodial formation (Block et al., 2008; Steffen et al., 2006) and a similar Dia-regulated compensatory mechanism was recently demonstrated in leading edge epithelial cells during dorsal closure upon Ena inactivation (Homem and Peifer, 2009). Dia is expressed in haemocytes and much, but not all, endogenous Dia was recruited in an Ena-dependent fashion to mitochondria upon expression of FP4Mito and colocalised with Ena-GFP at filopodia, raising the possibility that Ena might be required to target Dia to sites of dynamic actin rearrangement (supplementary material Fig. S3A–E). Nonetheless, the comparable reduction in migration speeds observed in FP4Mito-expressing haemocytes and in ena mutants indicates that the effects of FP4Mito are specific to Ena. We cannot, however, rule out a role for Dia in concert or downstream of Ena, although zygotic dia mutants failed to show a significant reduction in lateral migration speeds (supplementary material Fig. S3F). Further analysis of the role of Dia and its crosstalk with Ena will illuminate how haemocytes form appropriate protrusions for migration in vivo.

This work demonstrates the importance of investigating cell migration in vivo, and highlights the role that the surrounding environment can exert on invasive migration. Indeed, we reveal that disrupting one actin regulatory protein can have drastically different effects on in vitro and in vivo migration. Recent work has shown dramatic differences in the migration machinery operating within mammalian leukocytes as they migrate through different environments, with an absolute requirement for integrins.
in vitro but no requirement for these proteins during their interstitial migration in vivo (Lammermann et al., 2008). It is therefore becoming increasingly important to develop systems in which researchers can study cell migration in the complex setting of a living organism. Haemocytes represent such a system, as they undergo stereotyped migrations in response to a variety of stimuli, enabling the contributions of actin regulators to be probed in several different circumstances and within a genetically tractable animal. Here we have used these cells to understand how Ena functions to promote cell motility in vivo, mirroring its pro-migratory role during metastasis. Similarly, Fascin, another actin regulatory protein implicated in cancer invasiveness, was also recently demonstrated to be important in mediating haemocyte migration (Zanet et al., 2009). Further studies using these cells will lead to a better understanding of cell migration in vivo and in doing so could provide insight into how misregulation of the actin cytoskeleton might contribute to metastasis and tumour progression.

METHODS

Fly stocks

SerpentHemoGAL4 (srpGAL4) (Bruckner et al., 2004) and croquemortGAL4 (crqGAL4) (Stramer et al., 2005) were used to drive expression of UAS constructs specifically in haemocytes. The following UAS constructs were used in this study (obtained from Bloomington Stock Center unless otherwise stated): UAS-Ena-GFP, UAS-FPPPPmito-GFP (both obtained from M. Peifer, Chapel Hill) (Gates et al., 2007), UAS-mCherry-Moesin (obtained from P. Martin, University of Bristol) (Millard and Martin, 2008), UAS-mCD8-GFP (Lee and Luo, 1999), UAS-Ena (Ahern-Djamali et al., 1998) and UAS-RacV12 (Luo et al., 1994). The constructs and drivers were used to produce the following genotypes: w;srpGAL4, UAS-mCherry-Moesin; UAS-Ena-GFP and w;srpGAL4, UAS-mCD8-GFP flies were generated to probe Ena localisation and membrane ruffling, respectively; w;srpGAL4, UAS-GFP; crqGAL4, UAS-GFP (wild type, WT), w; srpGAL4, uas-GFP/+; crqGAL4, UAS-GFP/UAS-FPPPPmito-GFP (Ena inactivation, FP4Mito) and w;srpGAL4, UAS-GFP/+; crqGAL4, UAS-GFP/UAS-RacV12 embryos were used to investigate ruffling in vivo; finally, enaGC1 (Gertler et al., 1995), ena25, ena10 (Ahern-Djamali et al., 1998), dia2 or dia5 (Castrillon and Wasserman, 1994) homozygous embryos with GFP-labelled haemocytes were generated by selecting CTG-negative embryos from a stable stock of mutant/CTG;crqGAL4, UAS-GFP.

Live imaging

Embryos were prepared for imaging as previously described (Wood and Jacinto, 2005) and imaged live on a Zeiss LSM510 confocal microscope using Zeiss fluor 40 x 1.3 oil DIC, plan-apochromat 63 x/1.4 oil and c-apochromat 63 x/1.2 water immersion objectives at the Bath BioImaging Facility. The resulting stills and time-lapse movies were assembled and analysed using ImageJ (NIH).

Cell migration assays

For the wound assay the epithelium of stage 15 embryos was ablated dorsolaterally with respect to the VNC with a nitrogen laser-pumped dye laser (model no. VSL-337ND-S; Laser Science Inc.) as previously described (Wood et al., 2002). Haemocyte migration was then imaged live for 1 hour post-wounding. For live imaging of lateral migration and random motility of haemocytes, embryos were mounted ventral side up and imaged live from stage 14 or stage 15, respectively.

In vitro haemocyte culture

Ten stage 15 embryos were dechorionated and bled into a drop of 10% fetal bovine serum (FBS; Sigma) in S2 cell media (Sigma) ±0.8% methylcellulose (Sigma) inside a Nunclon A-coated 60 mm tissue culture dish (Nunc). A coverslip was then placed on top and sealed using nail varnish. Haemocytes were allowed to adhere for 60 minutes at room temperature before live imaging through the coverslip.

In vitro staining of haemocytes

For supplementary material data, stage 15 embryos of each genotype were dechorionated and dissociated by repeated pipetting in 125 μl of 10% FBS (Sigma) in S2 cell media (Sigma). (WT and FP4 haemocytes in the supplementary material are the same as wild type). The resulting stills and time-lapse movies were assembled and analysed using ImageJ (NIH).

TRANSLATIONAL IMPACT

Clinical issue

One of the key steps in cancer progression is the metastasis of carcinoma cells from the primary tumour. This process involves invasive cell migration, which requires the formation of actin-rich protrusions at the leading edge of migrating cells. An actin-regulatory protein that is upregulated in several human cancers is Mena, a member of the Ena/VASP family. Overexpression of Mena in rat breast carcinoma cells increases metastasis to the lungs. However, current understanding of Ena/VASP protein function comes mainly from in vitro studies of fibroblasts, in which overexpression of Mena decreases rather than increases cell migration speeds. It has therefore been unclear what effect the overexpression of Ena/VASP proteins might have in an in vivo setting.

Results

In this study, the authors investigate how altering the levels of Ena, the sole Drosophila Ena/VASP family member, affects the migration of macrophages (haemocytes) through the embryo during development, a process that is similar in many respects to tumour-cell migration. Using detailed analysis of the actin protrusions formed by the macrophages, the authors show that inactivation of Ena results in the formation of stable protrusions, whereas Ena overexpression results in very dynamic protrusions, revealing that Ena is a key regulator of lamellipodial dynamics in Drosophila macrophages, as has been observed for fibroblasts in vitro. However, although overexpression of Mena reduces fibroblast migration speeds in vitro, the authors show here that Ena overexpression increases macrophage migration speeds in the embryo, consistent with previous findings that Mena overexpression increases cell motility and metastasis in breast cancer. The authors also provide evidence that the difference in results obtained between in vivo and in vitro systems might be attributed to the difference in spatial constraints experienced by cells in vivo.

Implications and future directions

This paper provides insight into how increased Mena levels might contribute to cell motility and metastasis, and illustrates the importance of investigating cell migration in a living organism. In addition, this system should prove useful for determining the contribution of other actin regulators to cell migration in vivo, which might help to better understand how dysregulation of the actin cytoskeleton promotes metastasis and cancer progression.

doi:10.1242/dmm.006528
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genotype as per Figs 2,3; Ena haemocytes express Ena-GFP as per Fig. 1.) The cell suspension was then transferred to a Nuclon Δ-
coated 60 mm tissue culture dish (Nunc) and cells were allowed to
adhere for 2 hours in a humidified chamber at room temperature.
After 2 hours, the medium was removed and cells fixed with 4% paraformaldehyde (Sigma) made up in PBS. Adherent cells were
permeabilised by washing twice in 0.1% Triton-X 100 (Sigma) in
PBS. Rabbit anti-Dia (Afshar et al., 2000; a gift from M. Peifer, UNC, Chapel Hill) and mouse 5G2 anti-Ena (Bashaw et al., 2000)
supernatant obtained from Developmental Studies Hybridoma
Bank, University of Iowa (DSHB)) were used at 1:5000 and 1:50,
respectively. Goat anti-rabbit Alexa-Fluor-568 and goat anti-mouse
Alexa-Fluor-598 (Molecular Probes; both diluted 1:200) were used
to detect primary antibodies. PBS washes were performed between
each incubation, and stained cells mounted in mowiol (Calbiochem)
with a coverslip placed on top. Stained cells were imaged on the
Zeiss LSM510 confocal microscope, with residual GAL4-driven
GFP fluorescence used to identify haemocytes.

To determine whether Ena was required for localisation of Dia
to FP4Mito, embryos with FP4Mito-labelled haemocytes from an ena
/CyO;CyO;GAL4;uas-FP4Mito/+ laying cage were dissected in 250
ml of media and then split between two tissue culture dishes.
One dish was stained for Dia to probe its recruitment to FP4Mito-
GFP-positive mitochondria, and the other for Ena to detect the
presence of ena
homozygotes in the culture on the basis of
absence of Ena at mitochondria.

Dye injections
Stage 15 embryos were prepared for live imaging ventral side up
as described above, but were dried in a box containing silica gel
for 4 minutes prior to covering with volatole oil. Embryos were then
injected anteriorly with 2.5 mg/ml rhodamine-conjugated 70 kDa
dextran (Molecular Probes), using Femtotips II and a
FemtoJet/InjectMan injection system (Eppendorf). Following
injection, coverslips were attached as per normal live imaging and
the position of haemocytes relative to extracellular space, as
marked by the injected rhodamine-dextran, was followed live.

Image analysis and quantification
All analysis was undertaken on six or more haemocytes from over
different embryos.

Data was plotted in ‘box and whisker’ plots; whiskers
conform to the 10th and 90th percentiles, the box corresponds to
the lower and upper quartiles, and the line shows the position of
the median.

ACKNOWLEDGEMENTS
We thank Brian Stramer (KCL), Paul Martin (University of Bristol), Mark Peifer (UNC,
Chapel Hill) and the Bloomington Stock Center for generously providing fly stocks.
W.W. is funded by a Wellcome Trust Senior Research Fellowship; P.K.T. is supported
by a BBSRC studentship.

COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
P.K.T. and I.R.E. carried out the experiments; P.K.T., I.R.E. and W.W. designed and
interpreted the experiments and prepared the manuscript.

SUPPLEMENTAL MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.005694/-/DC1

REFERENCES
Afshar, K., Stuart, B. and Wasserman, S. A. (2000). Functional analysis of the
Drosophila diaphanous FH protein in early embryonic development. Development
127, 1887-1897.
Ahern-Djamali, S. M., Comer, A. R., Bachmann, C., Kastenmeier, A. S., Reddy, S. K.,
Beckerle, M. C., Walter, U. and Hoffmann, F. M. (1998). Mutations in Drosophila
enabled and rescue by human vasodilator-stimulated phosphoprotein (VASP)
indicate important functional roles for Ena/VASP homology domain 1 (EVH1) and
EVH2 domains. Mol. Biol. Cell 9, 2157-2171.
Applewithe, D. A., Barzil, M., Kojima, S., Svittika, T. M., Gertler, F. B. and Borisy, G. G.
(2007). Ena/VASP proteins have an anti-capping independent function in
filopodia formation. Mol. Biol. Cell 18, 2579-2591.
Barzik, M., Kotova, T. I., Higgs, H. N., Hazelwood, L., Hanein, D., Gertler, F. B. and
Schafer, D. A. (2005). Ena/VASP proteins enhance actin polymerization in the
presence of barbed end capping proteins. J. Biol. Chem. 280, 28653-28662.
Bashaw, G. J., Kidd, T., Murray, D., Pavson, T. and Goodman, C. S. (2000). Repulsive
axon guidance: Abelson and Enabled play opposing roles downstream of the
roundabout receptor. Cell 101, 703-715.
Bear, J. E., Loueir, J. J., Libova, I., Fassler, R., Weihland, J. and Gertler, F. B. (2000).
Negative regulation of fibroblast motility by Ena/VASP proteins. Cell 101, 717-728.
Bear, J. E., Svittika, T. M., Krause, M., Schafer, D. A., Loueir, J. J., Strasser, G. A.,
Malv, I. V., Chaga, O. Y., Cooper, J. A., Borisy, G. G. et al. (2002). Antagonism
between Ena/VASP proteins and actin filament capping regulates fibroblast motility.
Cell 109, 509-521.
Block, J., Stradal, T. E., Hanisch, J., Geffers, R., Kostler, S. A., Urban, E., Small, J. V.,
Rottner, K. and Faix, J. (2008). Filopodia formation induced by active mDia2/Drf3.
J. Microsci. 231, 506-517.
Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of
altering cell fates and generating dominant phenotypes. Development 118, 401-415.
Brantley-Sieders, D. M., Zhuang, G., Hicks, D., Fang, W. B., Hwang, Y., Cates, J. M.,
Coffman, K., Jackson, D., Bruckheimer, E., Muraoka-Cook, R. S. et al. (2008).
The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis
and metastatic progression in mice by amplifying ErbB2 signaling. J. Clin. Invest. 118,
64-78.
Bruckner, K., Kockel, L., Duhcek, P., Luque, C. M., Rorth, P. and Perrimon, N.
(2004). The PDGF/VEGF receptor controls blood cell survival in Drosophila. Dev.
Cell 7, 73-84.
Castrillon, D. H. and Wasserman, S. A. (1994). Diaphanous is required for cytokinesis
in Drosophila and shares domains of similarity with the products of the limb
deformity gene. Development 120, 3367-3377.
Delon, I. and Brown, N. H. (2009). The integrin adhesion complex changes its
composition and function during morphogenesis of an epithelium. J. Cell Sci. 122,
4363-4374.
Di Maio, F., Bronzi, G., Scanlan, M. J., Del Bello, D., Cacioli, S., Venturo, I.,
Botti, C., Nicostra, M. R., Motolese, M., Natali, P. G. et al. (2004). Human Mena
protein, a serex-defined antigen overexpressed in breast cancer eliciting both
humoral and CD8+ T-cell immune response. Int. J. Cancer 109, 909-918.
Evans, I. R., Renne, T., Gertler, F. B. and Nobe, C. D. (2007). Ena/VASP proteins
mediate repulsion from ephrin ligands. J. Cell Sci. 120, 289-298.
Ena regulates macrophage speed in vivo

Pollard, T. D. and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell 112, 453-465.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70, 401-410.

Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. Science 302, 1704-1709.

Siekhause, D., Haesemeyer, M., Moffitt, O. and Lehmann, R. (2010). Rhok controls invasion and Rap1 localization during immune cell transmigration in Drosophila. Nat. Cell Biol. 12, 605-610.

Small, J. V., Stradal, T., Vignal, E. and Rottner, K. (2002). The lamellipodium: where motility begins. Trends Cell Biol. 12, 112-120.

Sriniwasan, D., Sims, J. T. and Plattner, R. (2008). Aggressive breast cancer cells are dependent on activated Abi kinases for proliferation, anchorage-independent growth and survival. Oncogene 27, 1100-1105.

Steffen, A., Faix, J., Resch, G. P., Linkner, J., Wehland, J., Small, J. V., Rottner, K. and Stradal, T. E. (2006). Filopodia formation in the absence of functional WAVE- and Arp2/3-complexes. Mol. Biol. Cell 17, 2581-2591.

Stramer, B., Wood, W., Gallo, M. J., Redd, M. J., Jacinto, A., Parkhurst, S. M. and Martin, P. (2005). Live imaging of wound inflammation in Drosophila embryos reveals key roles for small GTPases during in vivo cell migration. J. Cell Biol. 168, 567-573.

Tepass, U., Fessler, L. I., Aziz, A. and Hartenstein, V. (1994). Embryonic origin of hemocytes and their relationship to cell death in Drosophila. Development 120, 1829-1837.

Toyoda, A., Kawana, H., Azhukata, K., Yu, J., Omata, A., Kishi, H., Higashi, M. and Harigaya, K. (2009). Aberrant expression of human ortholog of mammalian enabled (hMena) in human colorectal carcinomas: implications for its role in tumor progression. Int. J. Oncol. 34, 53-60.

Urbano, J. M., Tortgol, C. M., Molnar, C., Tepass, U., Lopez-Varea, A., Brown, N. H., de Celis, J. F. and Martin-Bermudo, M. D. (2009). Drosophila lamins act as key regulators of basement membrane assembly and morphogenesis. Development 136, 4165-4176.

Visloidi, L., Dowling, A. J., Evans, I. R., Waterfield, N., fench-Constant, R. H. and Wood, W. (2009). Drosophila embryos as model systems for monitoring bacterial infection in real time. PLoS Pathog. 5, e1000518.

Wang, W., Goswami, S., Lapidus, K., Wells, A. L., Wyckoff, J. B., Sahai, E., Singer, R. H., Segall, J. E. and Cohen, J. S. (2004). Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. Cancer Res. 64, 8585-8594.

Wentworth, J. K., Pula, G. and Poole, A. W. (2006). Vasoconstrictor-stimulated phosphoprotein (VASP) is phosphorylated on Ser157 by protein kinase C-dependent and -independent mechanisms in thymus-stimulated human platelets. Biochem. J. 393, 555-564.

Wills, Z., Bateman, J., Korey, C. A., Comer, A. and Vactor, D. (1999). The tyrosine kinase Abi and its substrate enabled collaborate with the receptor phosphatase Dlar to control motor axon guidance. Neuron 22, 301-312.

Wolf, K., Wu, Y. I., Liu, V., Geiger, J., Tam, E., Overall, C., Stack, M. S. and Friedl, P. (2007). Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. Nat. Cell Biol. 9, 893-904.

Wood, W. and Jacinto, A. (2005). Imaging cell movement during dorsal closure in Drosophila embryos. Methods Mol. Biol. 294, 203-210.

Wood, W., Jacinto, A., Groze, R., Woolner, S., Gale, J., Wilson, C. and Martin, P. (2002). Wound healing recapitulates morphogenesis in Drosophila embryos. Nat. Cell Biol. 4, 907-912.

Wood, W., Faria, C. and Jacinto, A. (2006). Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in Drosophila melanogaster. J. Cell Biol. 173, 405-416.

Zanet, J., Stramer, B., Millard, T., Martin, P., Payre, F. and Plaza, S. (2009). Fascin is required for blood cell migration during Drosophila embryogenesis. Development 136, 2557-2565.

Zervas, C. G., Gregory, S. L. and Brown, N. H. (2001). Drosophila integrin-linked kinase is required at sites of integrin adhesion to link the cytoskeleton to the plasma membrane. J. Cell Biol. 152, 1007-1018.