

# Fascin is required for blood cell migration during *Drosophila* embryogenesis

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Fascin is well characterized in vitro as an actin-bundling protein and its increased expression is correlated with the invasiveness of various cancers. However, the actual roles and regulation of Fascin in vivo remain elusive. Here we show that Fascin is required for the invasive-like migration of blood cells in *Drosophila* embryos. Fascin expression is highly regulated during embryonic development and, within the blood lineage, is specific to the motile subpopulation of cells, which comprises macrophage-like plasmotocytes. We show that Fascin is required for plasmotocyte migration, both as these cells undergo developmental dispersal and during an inflammatory response to epithelial wounding. Live analyses further demonstrate that Fascin localizes to, and is essential for the assembly of, dynamic actin-rich microspikes within plasmotocyte lamellae that polarize towards the direction of migration. We show that a regulatory serine of Fascin identified from in vitro studies is not required for in vivo cell motility, but is crucial for the formation of actin bundles within epithelial bristles. Together, these results offer a first glimpse into the mechanisms regulating Fascin function during normal development, which might be relevant for understanding the impact of Fascin in cancers.

**KEY WORDS:** Actin, *Drosophila*, Fascin (Singed), Hemocytes, Migration, Wound healing

## INTRODUCTION

The dynamic regulation of cell shape underlies many cell behaviors, in particular that of cell migration, which in turn is pivotal for the development and maintenance of animal tissues. However, the ways in which a cell integrates genetic programs and extracellular signals to remodel its actin cytoskeleton and direct the assembly of surface protrusions in order to move are still poorly understood. We thus require a better comprehension of the key regulators of actin polymerization, severing and bundling in cells undergoing their normal activities in vivo.

Although it is clear that a meshwork of actin filaments is necessary for plasma membrane lamellar protrusion (Pollard and Borisy, 2003), a precise role for parallel actin bundles in regulating various cellular morphologies remains more elusive. Bundles of actin filaments are well characterized in dynamic membrane extensions, such as filopodia (Mattila and Lappalainen, 2008), as well as in static protrusions such as *Drosophila* bristles (Tilney et al., 2000). Bundles of actin have also been observed to be interspersed within the lamellar actin meshwork of numerous motile cell types, from melanoma cells (Nemethova et al., 2008; Rottner et al., 1999; Vignjevic et al., 2006) to growth cones (Cohan et al., 2001). However, the function of these filopodia-like bundles that barely protrude beyond the leading edge (also known as microspikes) during in vivo cell migration remains unclear.

Fascin was one of the first actin-bundling proteins to be biochemically characterized (Bryan et al., 1993; DeRosier and Edds, 1980) and defines a highly conserved family in vertebrates (Adams, 2004). Fascin contains two separate actin-binding domains in its N-

and C-terminal regions (Ono et al., 1997), which are likely to mediate its actin-bundling activity. In vitro studies indicate that Fascin can switch the activity of the actin nucleation complex, Arp2/3, from the production of branched microfilaments towards that of parallel bundles (Vignjevic et al., 2003). Most of our knowledge of Fascin function and regulation primarily stems from assays utilizing a variety of cell lines and suggests a major role for Fascin in generating filopodial extensions (Adams, 2004; Mattila and Lappalainen, 2008; Vignjevic and Montagnac, 2008). That overexpression of Fascin increases the two-dimensional motility of cells in culture (Hashimoto et al., 2005; Yamashiro et al., 1998) has led to the hypothesis that Fascin might play a role in enhancing cell migration and invasiveness in vivo. In contrast to most transformed cell lines, Fascin is generally absent from normal epithelial cells in adults (Adams, 2004) and is restricted to specific tissues during development (De Arcangelis et al., 2004), showing that actin bundling within a cell is transcriptionally controlled. In addition, Fascin activity also appears to be post-translationally regulated as phosphorylation of a serine residue (S39 in the human protein) (Ono et al., 1997) leads to a decrease in actin bundling in vitro (Vignjevic et al., 2003; Yamakita et al., 1996). Expression of a phosphomimetic (Ser→Asp) variant of Fascin reduces the motility of cells in xenografts (Hashimoto et al., 2007), whereas a non-phosphorylatable mutation (Ser→Ala) enhances the actin-bundling capacity of the protein (Vignjevic et al., 2006). How these different levels of Fascin regulation contribute to modifying the migratory capacity of cells is unclear.

There are three human Fascin genes, two of them having documented roles in pathologies. Mutations in *FSCN2* (fascin 2) lead to autosomal dominant retinitis pigmentosa (Wada et al., 2001), and several studies have shown that fascin 1 (or fascin) is highly overexpressed in a variety of carcinomas (Adams, 2004; Vignjevic and Montagnac, 2008). Furthermore, Fascin levels in cancer cells are often prognostic of their invasive and metastatic properties, making Fascin a potential marker of aggressive tumors (Hashimoto et al., 2005). Our understanding of how Fascin functions in vivo has mainly been gained from studies in *Drosophila*, which contains a single Fascin gene [*fascin*; *singed* (*sn*)], thus facilitating genetic analyses. Fascin knockdown in flies leads to abnormal morphology of epithelial cells owing to alterations to the supporting actin bundles

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that form the shaft of cellular protrusions (Cant et al., 1994; Tilney et al., 2000; Chanut-Delalande et al., 2006; Dickinson and Thatcher, 1997). Fascin is also required for actin bundle formation in nurse cells during oogenesis and loss of Fascin leads to sterility (Cant and Cooley, 1996; Cant et al., 1994). Although Fascin is highly expressed in various motile cells during development in *Drosophila* (Borghese et al., 2006) and mice (Hayashi et al., 2008), a direct assay of the role of Fascin in physiological cell migration is still lacking. In addition, the mechanisms regulating Fascin activity remain to be deciphered.

We show here that during *Drosophila* embryonic development, high levels of *fascin* characterize the motile subpopulation of blood cells, which comprises the macrophage-like plasmatocytes. Within live plasmatocytes in vivo, we show that the Fascin protein associates with polarized actin microspikes that extend beyond the leading edge lamellae. Time-lapse in vivo imaging reveals a requirement for Fascin during developmental and wound migratory responses of plasmatocytes. This reduced migratory capacity of plasmatocytes is the result of a loss of dynamic Fascin-decorated actin bundles leading to unpolarized cells with static lamellae. Furthermore, we show that the activity of Fascin is also regulated in a tissue-specific manner. Whereas the conserved phosphorylation site (S52 in flies) is crucial for bristle formation, nurse cells and macrophages do not require this site in order to bundle actin filaments. This in vivo dissection of Fascin reveals its physiological role in cell migration and shows that Fascin activity relies on distinct post-translational regulatory mechanisms between tissues.

## MATERIALS AND METHODS

### Fly stocks

We used the following lines obtained from the Bloomington Stock Center and the community: *sn*<sup>2</sup>; *sn*<sup>28</sup> (Cant and Cooley, 1996); *sn*<sup>X2</sup>; *Df**sn*<sup>C128</sup>; *Pxn*-Gal4, UAS-GFP (Wood et al., 2006); *srp*-Gal4, UAS-MoeGFP (Dutta et al., 2002). Mutations were kept over balancers carrying *K7*-Gal4, UAS-GFP transgenes and mutant embryos lacking the GFP balancer were hand-selected under a dissecting microscope equipped for epifluorescence (Nikon).

### Plasmids and transgenesis

Transgenic lines were generated by P-element-mediated transformation according to standard protocols. UAS-Fascin lines were generated by inserting a full-length *sn* cDNA (RH62992) into the pUASp vector. GFP-Fascin fusions were obtained by fusing eGFP sequences to the N-terminal part of full-length *sn* cDNA, and site-directed mutagenesis on serine 52 was performed by PCR. All constructs were verified by sequencing. For each individual construct, we established and tested a minimum of three independent lines. Details of primers and cloning procedures used are available upon request.

### Antibody staining and in situ hybridization

Embryos were fixed and stained as previously described (Wood et al., 2006). Antibodies used were: anti-Singed 7C (Development Studies Hybridoma Bank) at 1/100 dilution; anti-β-galactosidase (Cappel) at 1/4000; Alexa Fluor 488- or 555-conjugated secondary antibodies (Molecular Probes) at 1/500. Embryos were mounted in Vectashield (Vector Laboratories) and imaged with a Leica TCS SP5 confocal microscope. The antisense *fascin* probe was synthesized in vitro according to the manufacturer's protocol (Roche). In situ hybridization was performed by standard procedures on 0- to 24-hour embryos collected at 25°C and fixed with 37% formaldehyde. Embryos were mounted in glycerol-containing medium and photographed with a Nikon Eclipse 2000 microscope using a 20× Plan Apo na 0.5 objective.

### Live imaging and polarity quantification

UAS transgenic constructs encoding fluorescent proteins (GFP or mCherry) fused to the actin-binding domain of Moesin were expressed in hemocytes using the *srp-Gal4* driver line (Bruckner et al., 2004). Live embryos were mounted as previously described (Wood et al., 2006) and imaged using a confocal microscope (TCS SP5, Leica Microsystems) with a 63× Plan Apo na 1.32 objective using the scanner resonant mode. To quantify hemocyte

polarity, the cell contour was outlined and the protrusive area within each quadrant was measured using ImageJ. The index of polarity and the dynamics of protrusive areas were calculated as described in the figures.

### Wounding and imaging of wounded embryos

Live stage 15 embryos were wounded by laser ablation as described previously (Stramer et al., 2005). Images of hemocyte recruitment to the wound site were recorded at 30-second intervals for 1 hour, using an Axioplan 2a microscope equipped with a 63× Plan-Neo objective (Zeiss) and Openlab software (Improvision). Cell tracking was performed using ImageJ (manual tracking plug-in). For each time point, the center of the cell body was tracked manually and positional data were then used to calculate the mean velocity of individual hemocytes.

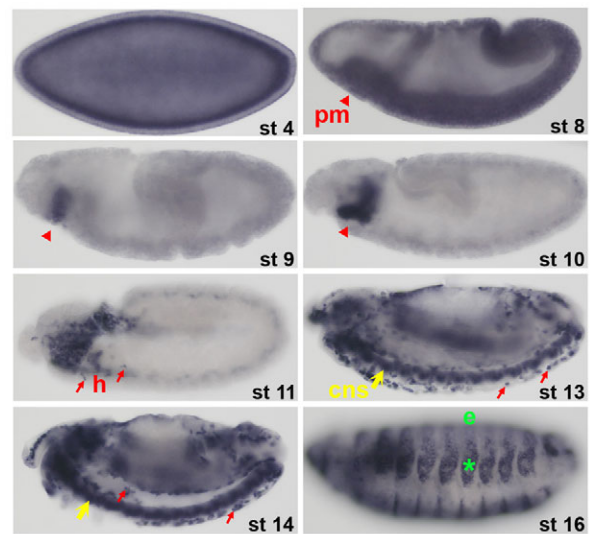
### Egg chamber and bristle staining procedures

Ovaries were dissected in ice-cold PBS, fixed in 4% formaldehyde for 30 minutes and permeabilized for 2 hours in 1% Triton X-100 in PBS followed by staining with phalloidin-SR101 (FluoProbes). Prepupae were dissected at 40-44 hours of pupal development (Cant and Cooley, 1996) and fixed in 4% paraformaldehyde for 30 minutes and then rinsed in 0.3% Triton X-100 and 0.5% BSA (bovine serum albumin) in PBS (PBT) for 2 hours. Actin was stained with phalloidin-SR101 and the tissue then incubated overnight at 4°C with anti-GFP. The tissue was rinsed extensively in PBT and incubated for 3 hours at room temperature in Alexa 488-conjugated secondary antibody. Ovaries and bristles were mounted in Vectashield and imaged using a Leica SP5 confocal microscope. Scanning electron microscopy was performed according to Delon et al. (Delon et al., 2003).

## RESULTS

### *fascin* is specifically expressed in migratory blood cells during *Drosophila* embryogenesis

In the course of our previous studies on epidermal cell morphogenesis (Chanut-Delalande et al., 2006), we noticed that *fascin* was expressed in several other embryonic tissues, suggestive of unexplored functions. As a first step, we defined the pattern of *fascin* expression throughout embryogenesis.



**Fig. 1. Tissue-specific expression of *fascin* during embryogenesis.**

In situ hybridization of *fascin* mRNA as shown on lateral views of developmentally staged wild-type *Drosophila* embryos. *fascin* expression in the procephalic mesoderm (pm) starts at stage 8 and becomes prominent by stage 9 (arrowhead). *fascin* is highly expressed in migrating hemocytes (h) from stage 11 to 16 (arrow), whereas expression in the epidermis (e) starts at stage 14 and persists until stage 16 (asterisk). *fascin* is also expressed at various levels in the central nervous system (cns) from stage 11 to 16 (arrow).

In early embryos, *fascin* mRNA was ubiquitous, reflecting a maternal contribution (Paterson and O'Hare, 1991). *fascin* mRNA became progressively restricted to the anteroventral part of the embryo and, at stage 10, it was specifically detected in the procephalic mesoderm (Fig. 1). This region is the origin of blood cell precursors (Wood and Jacinto, 2007), suggesting that *fascin* might also be expressed in mature blood cells (hemocytes). Indeed, we observed strong *fascin* expression in hemocytes as they commenced their dispersal throughout embryonic tissues, and *fascin* remained highly expressed in hemocytes until the end of embryogenesis (Fig. 1). *fascin* was also detected in the central nervous system from stage 11, and, at stage 14-16, *fascin* mRNA accumulated in the epidermal cells forming apical extensions (Fig. 1).

Following their determination, hemocytes differentiate into two subpopulations (Wood and Jacinto, 2007). The crystal cells remain in the head region as a group of tightly packed cells that are involved in immune responses at later stages, whereas the main population of hemocytes differentiates into plasmatocytes, which migrate throughout embryonic tissues and participate in the elimination of apoptotic corpses. Fascin was detected in all migrating hemocytes (Fig. 2A), but we observed no Fascin expression in the related, non-motile, crystal cells (Fig. 2B). It thus appears that Fascin is specifically expressed in the migrating subpopulation of *Drosophila* blood cells, i.e. only in plasmatocytes.

### Fascin is required for the guided migration of plasmatocytes

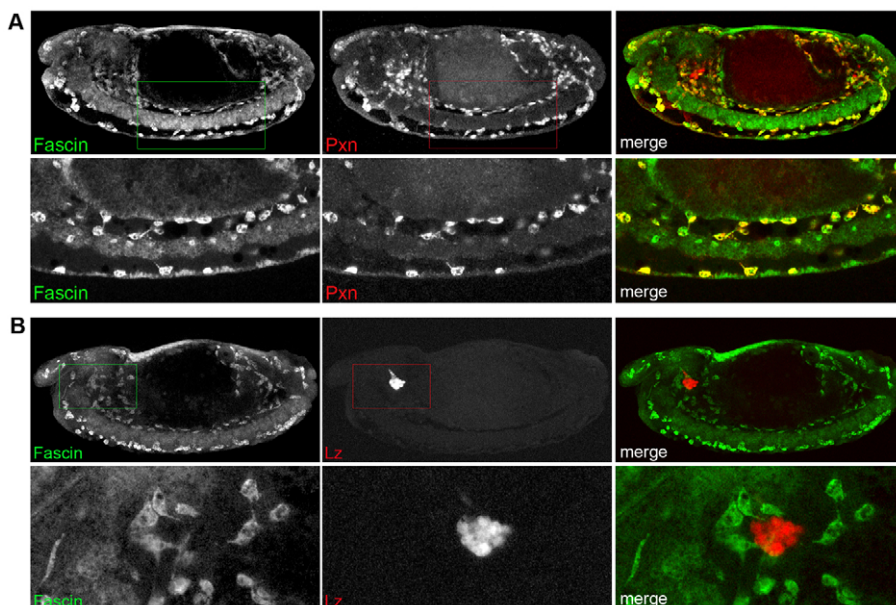
The specific expression of *fascin* in plasmatocytes strongly suggests that, in addition to epidermal cell morphogenesis, Fascin contributes to blood cell differentiation and/or function during embryogenesis. To explore this hypothesis, we analyzed the consequences of *fascin* inactivation and investigated a putative function of Fascin in plasmatocyte migration.

Hemocytes normally start to migrate from the procephalic mesoderm and eventually populate the whole embryo. This developmental dispersal of plasmatocytes was severely reduced in the absence of Fascin activity, as observed in fixed embryos (Fig. 3A). This phenotype is unlikely to be due to a massive decrease in

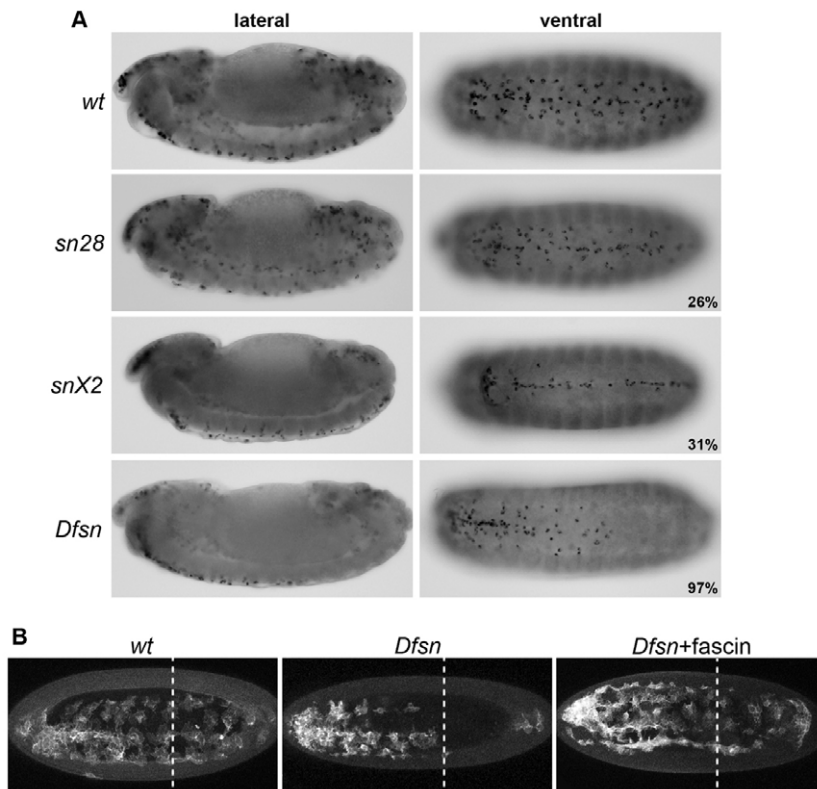
the population of hemocytes, although our estimation of the number of blood cells (data not shown) did not allow a slight effect to be ruled out. In stage 14 embryos, we indeed observed a substantial delay in the migration of plasmatocytes along the ventral midline (Fig. 3A). We analyzed several *fascin* mutations, including *sn<sup>28</sup>* and *sn<sup>X2</sup>* (two strong alleles), as well as a small deficiency, *Df(1)sn<sup>C128</sup>*, that removes the entire *fascin* locus. The penetrance of the hemocyte dispersal phenotype correlated with the severity of the allele; 31% and 28% of embryos displayed strong migration defects in *sn<sup>X2</sup>* and *sn<sup>28</sup>*, respectively, and 97% for *Df(1)sn<sup>C128</sup>* (Fig. 3A). These migration defects persisted in later stages, as plasmatocytes remained aggregated in the ventral part of the embryo and were unable to reach the posterior region.

To further evaluate the migratory behavior of plasmatocytes, we performed confocal microscopy on live embryos. Consistent with our observations on fixed embryos, in vivo imaging confirmed that the absence of Fascin impinges on plasmatocyte migration (Fig. 3B). Whereas hemocytes dispersed throughout the whole embryo in controls, embryos lacking Fascin displayed a prominent migration defect, with most hemocytes remaining in the anteroventral region of the embryo (Fig. 3B). Furthermore, reintroducing wild-type Fascin specifically in hemocytes efficiently rescued their migration towards the posterior (Fig. 3B). These Fascin-rescued hemocytes did not however migrate along the stereotypical routes observed in controls, suggesting that the *Df(1)sn<sup>C128</sup>* deficiency removes additional genes involved directly, or indirectly, in hemocyte migration and/or guidance.

Having shown that Fascin is required for the developmental dispersal of blood cells, we then tested its putative function in cell motility outside of developmental dispersal. Laser wounding of the embryonic epidermis triggers an active recruitment of hemocytes to the wound site, providing a useful in vivo chemotaxis assay (Stramer et al., 2005). When compared with wild-type embryos, the number of recruited plasmatocytes was significantly reduced in *fascin* mutants (Fig. 4A,B). In addition, tracking the paths of individual plasmatocytes allowed their migratory behavior to be evaluated (Fig. 4A,C) and revealed their migratory speed to be significantly reduced: from ~4.2  $\mu\text{m}/\text{minute}$  in the wild type to ~2  $\mu\text{m}/\text{minute}$  in *sn<sup>28</sup>* or *sn<sup>X2</sup>* *fascin* mutants.



**Fig. 2. Fascin expression in blood cells is restricted to plasmatocytes.** Co-localization of Fascin with markers of hemocyte differentiation. Fascin protein was detected by immunostaining (green). (A) *Peroxidase* (*Pxn*) is expressed in both plasmatocytes and crystal cells, as shown by GFP (red) driven by the *Pxn* promoter. The lower row shows the ventral abdominal region at higher magnification. Cells positive for both *Pxn* and Fascin appear in yellow. (B) Crystal cells marked by *lozenge* (*Lz*) expression (red) co-localized with *fascin*-expressing cells (green). The lower row shows high-magnification views of the head region revealing that expression of *fascin* and *Lz* is mutually exclusive.



**Fig. 3. Plasmotocyte migration is impaired in the absence of Fascin.** (A) Hemocyte dispersal in wild-type (*wt*) *Drosophila* embryos versus those carrying *fascin* mutations (*Dfsn*, *snX2* and *sn28*), as visualized by in situ hybridization for *Peroxidase* mRNA. Lateral (left) and ventral (right) views of stage 14 embryos reveal abnormal migratory dispersal of hemocytes in *fascin* mutants. Percentages indicate the proportion of embryos displaying the mutant phenotype. (B) Ventral view of stage 14 embryos (*srp-Gal4*, *UAS-GFP-Moe*). Compared with the wild type, *Dfsn* mutants displayed a delayed migration of hemocytes, which remained in the anterior region of the embryo (to the left). Re-expression of wild-type Fascin (driven by *srp-Gal4*) in hemocytes of *Dfsn* embryos rescued their migration towards the posterior of the embryo (to the right of the dashed line).

These results show that Fascin activity is required for proper plasmotocyte migration and thus provide the first evidence for a role of Fascin in cell motility in vivo, both during normal embryonic development and for an inflammatory cell response.

### Fascin controls polarized cytoskeletal dynamics within living cells

The migration of hemocytes is accompanied by the assembly and remodeling of broad lamellae with long actin-rich ribs or microspikes (Paladi and Tepass, 2004). The role of Fascin in actin bundling in vitro prompted us to examine whether Fascin influenced the cytoskeletal organization of migrating hemocytes in live embryos.

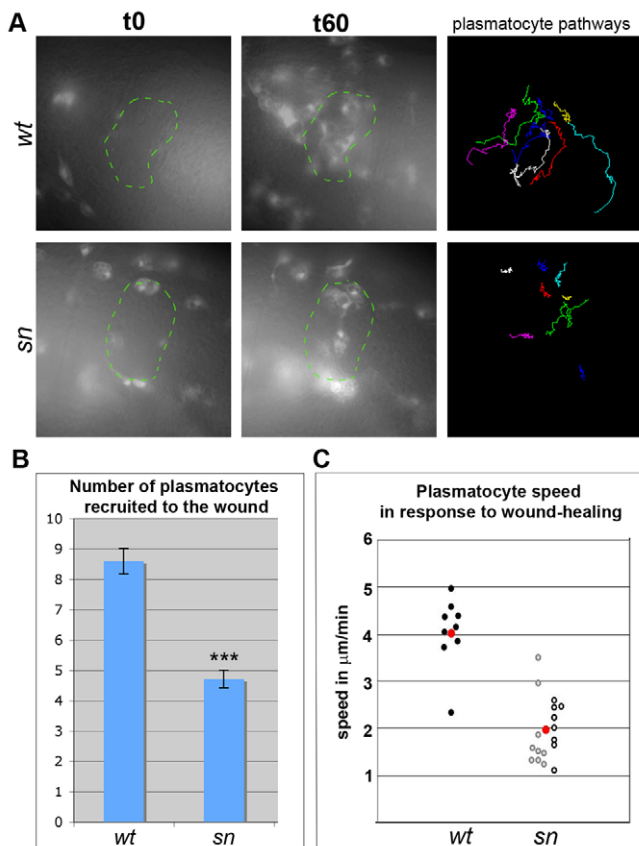
To image cytoskeletal dynamics in vivo, we used transgenic constructs that allow the directed expression in hemocytes of the F-actin-binding domain of Moesin fused to fluorescent moieties (Dutta et al., 2002). We focused on stage 15 embryos, when individual plasmotocytes leave the ventral midline and migrate towards stereotyped lateral positions. In wild-type embryos, live hemocytes showed a highly polarized morphology during lateral migration (Wood et al., 2006). These cells are characterized by a broad lamellipodium that projects long actin-rich microspikes beyond the leading edge, with a condensed cell body containing the nucleus at the trailing edge (Fig. 5A). Fascin associated specifically with the ribs of actin (20–30 per cell) that sustain the lamellipodia and extend into filopodia-like extensions (Fig. 5A), as revealed by Fascin-GFP expressed from a functional transgenic construct (see below). Time-lapse confocal microscopy further showed that Fascin accumulation was highly dynamic during migration and exhibited an apparent treadmilling throughout the lamellipodia (see Movie 1 in the supplementary material). Fascin-rich filaments displayed polarized growth from the cell body towards the migratory front, then retracted and eventually disappeared (estimated half-life of  $84 \pm 35$  seconds).

Loss of Fascin dramatically altered the polarized organization of the actin cortex in live plasmotocytes. It prevented the formation of cell extensions and, instead, a flaccid lamella formed all around the cell, with no sign of leading versus trailing edge polarization (Fig. 5B and see Movie 2 in the supplementary material). To quantify the polarization of plasmotocytes along the direction of their migration, we measured the respective areas of the trailing and leading edges. The ratio between the leading and trailing surfaces of the cell allowed an index of cell polarity to be evaluated. This morphometric analysis demonstrated a drastic reduction in the polarity of plasmotocytes lacking Fascin (Fig. 5C), whereas their total cell area was similar to that of the wild type (data not shown). Time-lapse confocal microscopy showed that wild-type plasmotocytes displayed highly dynamic lamellipodia, with rapid variations in the area of the local cytoplasm. By contrast, plasmotocytes lacking Fascin were characterized by a static cell cortex, which exhibited only a reduced variation of its surface over time (Fig. 6 and see Movie 2 in the supplementary material).

These data show that Fascin sustains the formation of highly dynamic cell extensions at the leading edge of migrating plasmotocytes. In addition, the absence of Fascin precludes polarization of the cell shape along the migration route. Therefore, Fascin appears to be involved at multiple levels in the dynamic reorganization of the F-actin network, all of which are necessary for the migration of plasmotocytes in vivo.

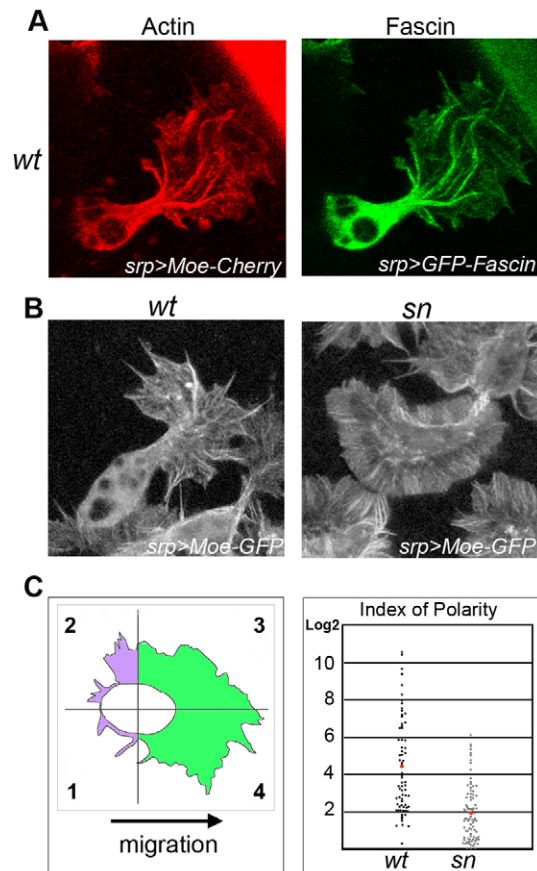
### Differential influence of serine 52 on Fascin functions throughout development

Phosphorylation of human fascin on serine 39 (Ono et al., 1997) decreases its bundling activity, as measured in vitro (Vignjevic et al., 2003). To explore whether Fascin is regulated in a similar way in vivo, we generated transgenes expressing phosphovariants and assayed their activity during *Drosophila* development.



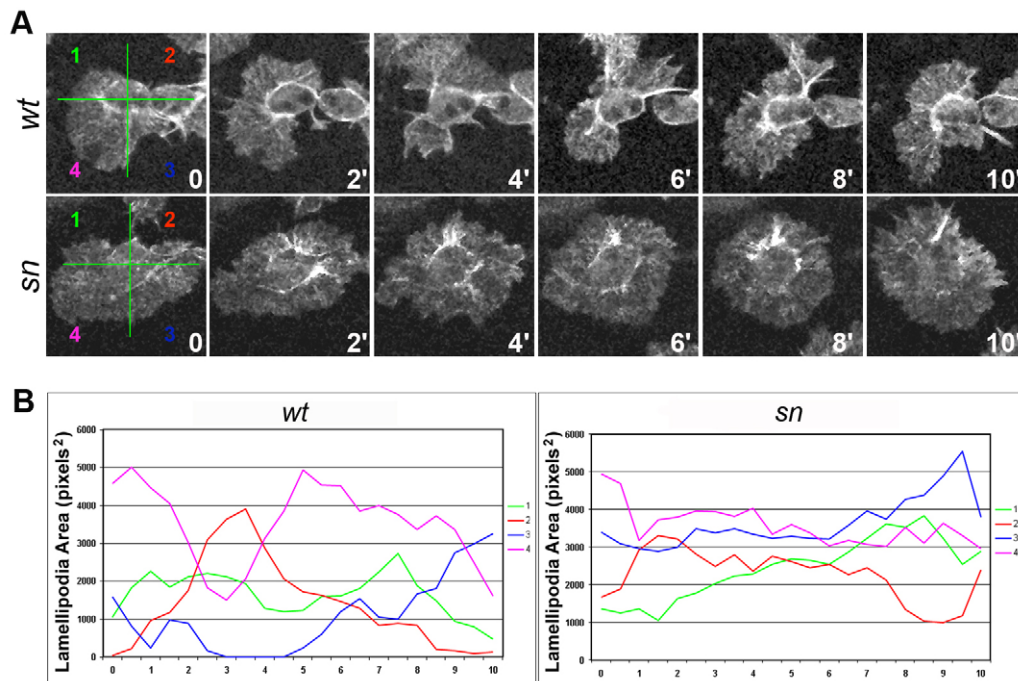
**Fig. 4. Fascin is involved in plasmatocyte migration in response to wound inflammation.** Time-lapse analysis of plasmatocyte recruitment to a laser-induced epithelial wound in live *Drosophila* embryos. **(A)** Images were taken immediately after wounding (t0) and after a 1-hour recovery period (t60) in wild-type and *sn*<sup>28</sup> mutant embryos; the wound site is outlined (green dashed line). Individual plasmatocytes labeled with GFP-Moe were tracked over this period and imaged every 30 seconds. Paths of individual plasmatocytes (color coded) recruited to the wound were manually tracked and are shown in the right-hand panels. **(B)** The number of plasmatocytes recruited to wounds in wild-type ( $n=45$ ) and *sn*<sup>X2</sup> ( $n=64$ ) embryos. \*\*\* $P<10^{-7}$  by Student's *t*-test. **(C)** The speed of plasmatocyte migration to the wound in the wild type (black dots) and in two *sn* mutant embryos [*sn*<sup>28</sup> (gray circles) and *sn*<sup>X2</sup> (black circles)]. Red dots indicate average values of plasmatocyte speed.

The regulatory serine of Fascin is highly conserved across species (Fig. 7A). In the *Drosophila* protein, it corresponds to serine 52, which was mutated to generate unphosphorylatable (S52A) and phosphomimetic (S52D or S52E) forms. We assayed their rescuing activities, first in the hemocytes of *Df(1)sn*<sup>C128</sup> embryos that lack wild-type Fascin and display very penetrant migration defects. As with wild-type Fascin, re-expression of Fascin S52A restored polarity and the dynamic actin cortex at the leading edge of plasmatocytes, and rescued their developmental migration (Fig. 7A,B). Unexpectedly, the phosphomimetic variants (S52D or S52E) displayed a rescuing activity indistinguishable from that of the wild-type or unphosphorylatable forms (Fig. 7A,B). We then tested the consequences of S52 modifications on cell motility using the in vivo chemotaxis assay triggered by laser wounding. Again, we found that the two reciprocal S52A and S52E forms of Fascin both restored plasmatocyte migration to the same extent as the wild-type protein



**Fig. 5. Fascin is required for polarity of the plasmatocyte cytoskeleton.** **(A)** Live confocal imaging of stage 15 hemocytes co-expressing GFP-Fascin (green) and a Moe-Cherry (red) construct that allows for visualization of F-actin organization. **(B)** Architecture of the actin cytoskeleton in live hemocytes from wild-type and *fascin* (*sn*<sup>28</sup>) mutant embryos expressing GFP-Moe driven by *srp*-Gal4. The absence of *fascin* prevents the formation of polarized lamellar extensions, which are replaced by an abnormal lamella that surrounds the whole cell. **(C)** Morphometric analysis of hemocyte polarity. To quantify cell polarity, the surface of each cell was divided into four quadrants (the center is the middle of the cell body) along their direction of migration. The respective areas of quadrants 1 and 2 (trailing edge, purple) and 3 and 4 (leading edge, green) were measured. The ratio between the leading and trailing edge areas was used to calculate an index of polarity and  $\log_2$  values are plotted on the graph. The polarity index was calculated for individual living hemocytes that were selected for being well isolated from other hemocytes in the same ventral region of embryos. Pictures were taken from 30 different wild-type or *sn*<sup>28</sup> embryos (the total number of analyzed hemocytes was 72 and 90, respectively). The difference in polarity index between wild-type and *sn* hemocytes is highly significant ( $P<0.006$ , Student's *t*-test). Black dots, individual wild-type plasmatocyte; gray dots, individual *sn* plasmatocytes; red dots, index of polarity average value of wild-type and *sn* plasmatocytes.

(Fig. 7C). Using GFP-tagged versions, we observed that the three Fascin variants displayed indistinguishable localization, in both the cell body and actin-rich extensions at the leading edge. We did not detect any differences in the length, shape or number of filopodia-like extensions that were formed upon re-expression of the three Fascin variants (Fig. 7B).



**Fig. 6. Fascin inactivation slows down lamellipodia dynamics.** (A) Analysis of lamellipodia dynamics by time-lapse confocal imaging of wild-type and *sn*<sup>28</sup> plasmotocytes (images were acquired at 2-minute intervals). In wild-type plasmotocytes, the lamellae display a rapid variation in size and location, whereas *sn*<sup>28</sup> mutants show a static lamella that spreads around the entire cell body. (B) Dynamics of the cytoplasmic area of the two cells shown in A. The cytoplasmic region was arbitrarily divided into four quadrants (color coded in A and B) and their respective areas were measured at each time point. Live cells were imaged over a period of 10 minutes at 30-second intervals.

These data indicate that S52 does not influence Fascin function during plasmotocyte migration, thus challenging a putative regulatory role *in vivo*. We then examined the activity of Fascin S52 phosphovariants in other developmental processes in which Fascin is involved. First, we focused on late oogenesis, when Fascin organizes transient actin cables that maintain the nucleus of nurse cells. Lack of Fascin prevents the formation of these bundled actin filaments and leads to female sterility (Cant et al., 1994). Re-expression of wild-type Fascin (or Fascin-GFP) in germ cells was sufficient to restore the building of actin cables (Fig. 8A), as well as fertility (data not shown). Using this assay, we found that the S52A and S52E phosphovariants could equally substitute for wild-type Fascin (Fig. 8A). Furthermore, each of the three Fascin S52 variants displayed a similar association with actin bundles in germ cells (Fig. 8B).

Finally, we analyzed the activity of Fascin S52 phosphovariants during bristle formation. The absence of Fascin disrupts the large actin cables that support each sensory cell extension, leading to gnarled and wavy sensory bristles (Cant et al., 1994; Tilney et al., 2000; Tilney et al., 1995). Re-expression of wild-type Fascin in pupae rescued this bristle phenotype (Fig. 8C). Whereas Fascin S52A rescued bristles to the same extent as the wild-type protein, Fascin S52D failed to compensate, even partially, for the lack of the wild-type protein (Fig. 8C). These results show that mutation of S52 impinges on Fascin activity in bristles, strongly suggesting that phosphorylation of this serine mediates a conserved downregulation of its bundling activity. Supporting this interpretation, Fascin S52E was no longer associated with actin cables as observed in growing bristles during metamorphosis (Fig. 8D).

Together, these data show that S52 indeed regulates Fascin activity *in vivo*, albeit in a specific way that is strictly dependent on the cellular context. Whereas the phosphomimetic substitution

prevents both actin association and the bundling activity of Fascin in bristle cells, it does not affect Fascin function in nurse cells or in plasmotocyte migration.

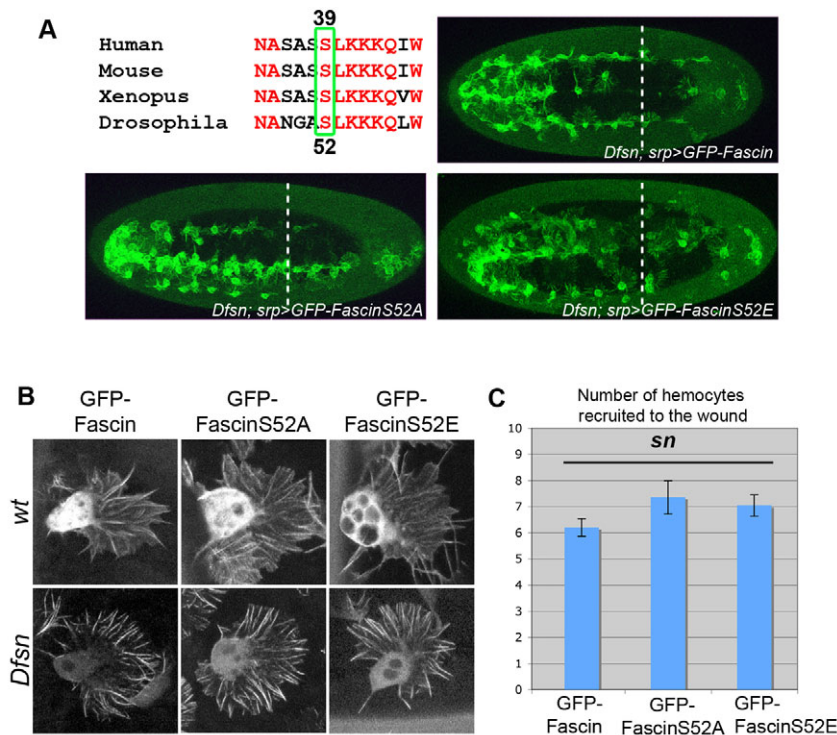
## DISCUSSION

Guided cell migration is essential for normal embryonic development, as well as for tissue repair and the immune response in adult organisms. Furthermore, corruption of the mechanisms of cell migration plays an important role in a number of pathologies, notably in cancer cell invasion and metastasis. A general feature of the migration of individual cells is the reorganization of the cytoskeleton/membrane compartment at the leading edge, which acts as the main driver of forward motility (Pollard and Borisy, 2003). Using a combination of functional approaches *in vivo*, we show here that Fascin is required for the polarized morphology and migration of blood cells during *Drosophila* embryogenesis.

### Fascin controls *in vivo* cell migration

Besides their role in innate immune responses, *Drosophila* blood cells have attracted a growing interest as a genetically tractable model of directed cell migration. Coupled with powerful genetic tools, recent advances in live imaging of hemocytes (Stramer et al., 2005) have provided novel insights into the regulatory mechanisms of the migration of individual cells in a live embryo (Wood and Jacinto, 2007). However, the nature and function of hemocyte cytoskeletal effectors remain unexplored.

Our results show that the actin-bundling protein Fascin mediates several aspects of the cytoskeletal reorganization required for blood cell migration. Fascin is expressed specifically in the motile subpopulation of embryonic hemocytes, the plasmotocytes. Furthermore, Fascin expression is eventually lost in plasmotocytes



**Fig. 7. Phosphorylation mutations of Fascin S52 rescue plasmatocyte morphology and migration.**

(A) The regulatory serine (S39 in human fascin) has been strongly conserved throughout evolution and corresponds to S52 in *Drosophila*. Expression of the wild-type protein, unphosphorylatable (S52A) Fascin, or the phosphomimetic (S52E) form (using the *srp-Gal4* driver), rescues plasmatocyte migration towards the posterior (to the right of the dashed line) in *Dfsn* embryos. (B) Subcellular distribution of S52 variants of GFP-Fascin expressed in wild-type or *Dfsn* mutant plasmatocytes. Each phosphovariant displayed a similar accumulation in filopodia-like actin bundles at the leading edge, which was enhanced in the absence of endogenous Fascin. The three GFP-Fascin variants (S52, S52A, S52E) led to a similar rescue of plasmatocyte polarity when expressed in *Dfsn* mutant embryos. (C) Effect of S52 Fascin variants on plasmatocyte migration induced by laser wounding of the embryonic epithelia. The bar chart shows the number of plasmatocytes recruited to the wound in *sn<sup>X2</sup>* mutants re-expressing GFP-Fascin ( $n=54$  embryos), or GFP-FascinS52A ( $n=49$ ) and GFP-FascinS52E ( $n=43$ ). Each of the three forms provides significant rescue of the mutant phenotype ( $P<0.001$ ), and no statistical difference was observed between the rescuing efficiency of the wild-type, S52A and S52D Fascin proteins.

at larval stages (J.Z., unpublished), when these cells become immobile (Babcock et al., 2008; Brock et al., 2008), showing that Fascin is characteristic of motile populations of blood cells. Consistent with observations in cultured cells (Adams, 2004; Mattila and Lappalainen, 2008), we show that Fascin is enriched in filopodia-like cellular extensions, or microspikes, within the lamellipodia of migrating plasmatocytes in vivo. The absence of Fascin impaired their migration, leading to delayed and incomplete developmental dispersal of plasmatocytes. In addition to their developmental dispersal, *Drosophila* plasmatocytes are rapidly responsive to epithelial wounding and are drawn to the damaged tissue where they may contribute to defense against septic infection (Stramer et al., 2008). Interestingly, the chemotaxis of inflammatory-induced migration relies on different signaling mechanisms to those that guide developmental dispersal (Wood et al., 2006). Nevertheless, Fascin deprivation also disrupts the migration of plasmatocytes to a wound site, showing that Fascin exerts a general function in the motility of blood cells, beyond the differential nature of guidance cues.

A prominent characteristic of living plasmatocytes in vivo is their polarization along their direction of migration, a feature that is generally lost in fixed specimens because of the fragility of actin-rich protrusions. Confocal movies show that the trailing edge of wild-type plasmatocytes displays a condensed organization of the cytoskeleton and cytoplasm that surround the nucleus. By contrast, the leading region organizes dynamic cell processes that are remodeled continuously during migration. These highly motile extensions are likely to contribute to exploration of the microenvironment for guidance cues as well as to forward propulsion of the cell (Wood and Jacinto, 2007). In the absence of Fascin, this polarized organization is disrupted and mutant plasmatocytes display an extended lamella that surrounds the whole cell. In addition, the abnormal lamella of Fascin-depleted plasmatocytes undergoes only limited reorganization over time, as

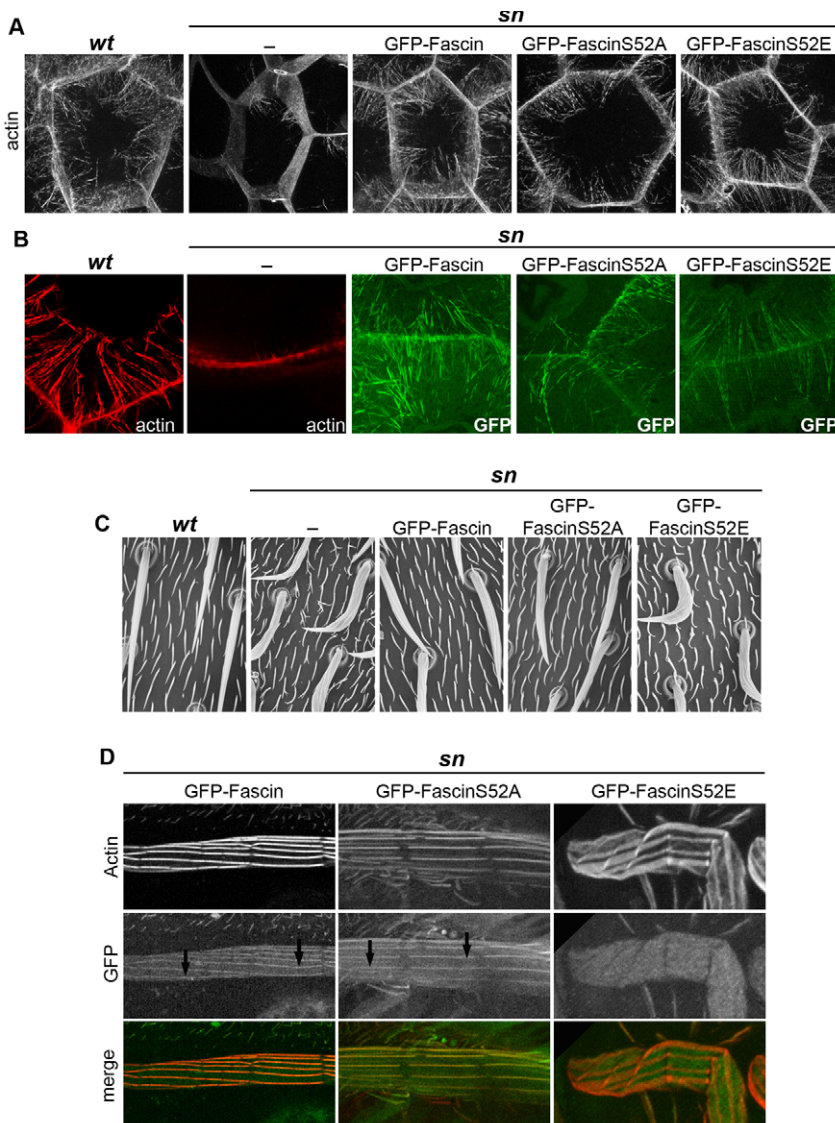
compared with wild-type cells. Fascin displays a reversible and highly dynamic interaction with actin, with a half-life of 6-9 seconds as estimated in vitro (Aratyn et al., 2007). Our in vivo results suggest that this dynamic Fascin-actin interaction underlies the formation of highly motile lamellipodia/filopodia at the leading edge of migrating cells. Thus, a major role of Fascin in blood cells is to mediate the polarized organization of actin filaments at the migration front, supporting the proposed role of Fascin in invasive tumor cells (Mattila and Lappalainen, 2008; Vignjevic et al., 2006).

Nevertheless, removal of Fascin in plasmatocytes not only prevents the formation of cell extensions but also causes a general loss of trailing versus leading edge polarity. We propose that Fascin is required to respond to the guidance molecules that provoke the polarization of plasmatocytes and direct their migration. Since filopodia contain receptors for diffusible signals or extra cellular matrix (ECM) molecules (Mattila and Lappalainen, 2008), it is possible that the absence of Fascin impairs efficient receptor localization or downstream signaling. Lack of Fascin might also prevent the mechanical transmission of the forces that reorganize the cytoskeleton during migration, as there is evidence that Fascin provides stiffness to actin bundles (Vignjevic et al., 2006).

Taken together, these data, collected through functional analyses in live embryos, demonstrate the importance of Fascin in dynamic filopodia assembly during the migration of *Drosophila* embryonic macrophages.

### Differential regulation of Fascin activity during development

Fascin is likely to be controlled at the post-transcriptional level in *Drosophila* (Cant and Cooley, 1996). Studies in mammalian cells have shown that following ECM-mediated signaling, Fascin can interact with protein kinase C $\alpha$  (Anilkumar et al., 2003), which phosphorylates Fascin on a serine residue in the N-terminal actin-



**Fig. 8. Function of Fascin S52 during oogenesis and bristle morphogenesis.** (A) In stage 10 egg chambers, nurse cell nuclei are sustained by numerous actin bundles that are unable to form in *fascin* mutants (*sn*<sup>28</sup>). Expression (using NanosVP16-Gal4) of GFP-Fascin, GFP-FascinS52A or GFP-FascinS52E in *sn*<sup>28</sup> nurse cells fully restores the formation of actin bundles. Similar results were obtained with untagged forms of Fascin phosphovariants. (B) High-magnification view of nurse cells showing actin (red) organization in wild-type versus *sn*<sup>28</sup> nurse cells, and the specific association of GFP-Fascin, GFP-FascinS52A and GFP-FascinS52E (green) with actin cables. –, *sn* mutant phenotype in egg chambers in A and B. (C) Rescuing ability of Fascin S52 variants in adult sensory bristles, as visualized by scanning electron microscopy. Re-expression (using *hs*-Gal4) of wild-type (S52) or unphosphorylatable (S52A) forms of Fascin in *sn*<sup>3</sup> mutants allows full rescue of the gnarled bristle phenotype. By contrast, the phosphomimetic form (S52E) displays no rescuing activity under the same conditions. –, *sn* mutant phenotype in adult sensory bristles. (D) GFP-Fascin and GFP-FascinS52A are enriched within actin bundles during bristle morphogenesis (arrows), whereas GFP-FascinS52E displays diffuse staining throughout the cell extension. Actin is in red and GFP-Fascin in green in the merged image.

binding site (Ono et al., 1997). A phosphomimetic mutation weakens the actin-bundling activity of Fascin *in vitro* (Vignjevic et al., 2003) and reduces the number and length of filopodia when it is overexpressed in cultured cells (Vignjevic et al., 2006). Since this protein kinase C target site has been conserved throughout evolution, we evaluated the importance of this serine *in vivo* through the substitution of endogenous Fascin with mutants preventing (S52A) or mimicking (S52D/E) its phosphorylation. Consistent with *in vitro* assays, the phosphomimetic mutation blocks Fascin activity in bristles. By contrast, Fascin S52A is fully active, showing that the actin-bundling activity of Fascin in bristles relies on a non-phosphorylated form. Therefore, these data demonstrate the importance of this regulatory serine *in vivo*.

Unexpectedly, all Fascin forms (wild-type, S52A and S52E) display indistinguishable activities with respect to plasmotocyte migration. In both developmental and inflammatory-induced migration, the two reciprocal Fascin phosphovariants rescue plasmotocyte motility to the same extent as the wild-type protein. All Fascin variants further display a similar enrichment in filopodia and sustain the formation of a polarized lamella. Therefore, modifications of S52 do not influence Fascin activity for plasmotocyte migration.

An intriguing question is why S52E nullifies the function of Fascin in bristles and yet has no effect on blood cell migration. One possibility is that phosphomimetic mutations specifically inactivate the bundling activity of Fascin, which might be dispensable for cell migration. This is, however, not the case because both phosphovariants appear to fulfill wild-type bundling activity, at least for the formation of actin cables in nurse cells. We propose that the main difference between phosphorylation-sensitive and -insensitive developmental processes is linked to architectural differences in tissue-specific actin structures that might require different kinetic properties of Fascin. The formation of bristle cell extensions is a relatively slow process that would require a stable interaction of Fascin with actin filaments, which is prevented by S52E mutations. By contrast, actin cables of nurse cells display a dynamic reorganization that is required for dumping the nurse cell cytoplasm into the oocyte (Guild et al., 1997). The reorganization of the actin cytoskeleton that occurs even faster during plasmotocyte migration might also be insensitive to a decreased half-life of Fascin-actin interaction. It is noteworthy that phosphomimetic forms of Fascin have also been reported to associate with dynamic filopodia in other systems (Lin-Jones and Burnside, 2007; Vignjevic et al., 2006).



Thus, we show unexpected complexity in Fascin regulation in vivo, whereby the regulatory activity of the conserved serine appears crucial for the formation of stable cell extensions but dispensable for the dynamic actin reorganization that occurs during invasive-like cell migration.

Further studies in vivo will be essential to decipher the full repertoire of *fascin* regulation, a task that can directly benefit from genetic approaches in flies. *Drosophila* thus represents a valuable system in which to study how Fascin is regulated and how it functions in cells as they behave in situ, and this information will contribute to our understanding of how *fascin* misregulation contributes to cancer progression.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/15/2557/DC1>

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