SCAR/WAVE-mediated processing of engulfed apoptotic corpses is essential for effective macrophage migration in *Drosophila*

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*In vitro* studies have shown that SCAR/WAVE activates the Arp2/3 complex to generate actin filaments, which in many cell types are organised into lamellipodia that are thought to have an important role in cell migration. Here we demonstrate that SCAR is utilised by *Drosophila* macrophages to drive their developmental and inflammatory migrations and that it is regulated via the Hem/Kette/Nap1-containing SCAR/WAVE complex. SCAR is also important in protecting against bacterial pathogens and in wound repair as SCAR mutant embryos succumb more readily to both sterile and infected wounds. However, in addition to driving the formation of lamellipodia in macrophages, SCAR is required cell autonomously for the correct processing of phagocytosed apoptotic corpses by these professional phagocytes. Removal of this phagocytic burden by preventing apoptosis rescues macrophage lamellipodia formation and partially restores motility. Our results show that efficient processing of phagosomes is critical for effective macrophage migration *in vivo*. These findings have important implications for the resolution of macrophages from chronic wounds and the behaviour of those associated with tumours, because phagocytosis of debris may serve to prolong the presence of these cells at these sites of pathology.

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Numerous *in vitro* studies have shown SCAR/WAVE to have an important role in the generation of actin-based structures thought to be required for cell motility;¹ fewer studies have addressed its role *in vivo*, particularly dynamically and in the context of individually migrating cells. *Drosophila* embryonic macrophages (hemocytes) are highly migratory and represent a relevant cell type to probe the regulation of the actin cytoskeleton,² not least because of their evolutionary relationship to vertebrate macrophages, in terms of their specification,³ conserved mechanisms utilised to detect and engulf apoptotic cells⁴ and their potent inflammatory responses to wounds.⁵

Hemocyte dispersal is crucial to embryogenesis as these cells are largely responsible for secretion of matrix⁶ and removal of apoptotic corpses,⁷ much like their vertebrate counterparts.⁸ With dispersal controlled by PDGF- and Vegf-related factors (Pvfs),⁹ matrix¹⁰ and contact inhibition¹¹ and their ability to respond to apoptotic corpses, wounds and pathogens, hemocytes represent a tractable genetic system to probe signal integration,¹² understanding which would be of enormous assistance in developing therapies against diseases involving immune cell dysfunction.

Despite recent studies showing roles for Fascin and Ena in regulating the hemocyte actin cytoskeleton,¹²,¹³ it remains an open question as to how the structures these actin regulators act on are generated in these cells. Previous studies using *Drosophila* S2 cells indicated a requirement for SCAR in the formation of actin-rich lamellae downstream of Nck and Rac,¹⁴,¹⁵ nonetheless a role within hemocytes has not yet been shown.

*Drosophila* SCAR/WAVE belongs to the WASp family of actin nucleation promoters. Diverse upstream signals activate WASp family members to activate Arp2/3-dependent actin polymerisation; SCAR/WAVE is regulated as part of a pentameric complex downstream of Rac, phosphatidyl inositol lipids and the adaptor Nck.¹⁶ Several studies suggest phosphorylation of SCAR is important, with phosphorylation of the C-terminal tail recently shown to modulate its activity in *Dictyostelium*.¹⁷ Analysis of SCAR mutants in *Drosophila* previously revealed it to have an essential role in processes including oogenesis, cell division, gastrulation, axonal guidance and muscle development.²⁰

Here we investigate the role of SCAR in hemocyte migration within the *Drosophila* embryo. SCAR is required by hemocytes for the formation of lamellipodia and efficient migration, including to epithelial wounds. Surprisingly, SCAR mutant hemocytes become highly vacuolated because of numerous phagocytic corpses within their cell bodies, uncovering a novel role in apoptotic cell clearance. Most importantly, removing this defect by blocking apoptotic cell death helped restore hemocyte motility, revealing that apoptotic cells can modulate the dynamic behaviour of macrophages *in vivo*.

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**Abbreviations:** H₂O₂, hydrogen peroxide; Pvf, PDGF- and Vegf-related factor; Pvr, PDGF- and Vegf-related receptor; VNC, ventral nerve cord

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**Results**

**SCAR is required autonomously within hemocytes for their developmental dispersal.** The dispersal of hemocytes during embryogenesis is co-dependent on development of the ventral nerve cord (VNC) and the dynamics of these cells is strongly influenced by their surrounding environment.13 Previously, Zallen et al.21 found that maternal and zygotic (M/Z) SCAR \textsuperscript{137} mutants exhibited early embryonic lethality because of defects during blastodermal stages, whereas even M/Z mutants of the weak SCAR\textsuperscript{1281} allele gave rise to a highly deformed VNC by stage 14/15. Therefore, to analyse the role of SCAR in hemocyte motility within a relatively unperturbed environment, hemocyte migration was probed in zygotic SCAR\textsuperscript{137} mutants. By stage 13 of embryonic development, hemocytes normally occupy the length of the VNC (Figure 1a),23 however, in SCAR\textsuperscript{137} mutants they were absent from the ventral side of several neuromeres (segments of the VNC), indicating a failure in their dispersal (Figure 1b). Hemocyte movement along the VNC from both anterior and posterior ends, the latter population having first penetrated the germ band before its retraction,24 dynamic imaging revealed that hemocytes from these two populations make contact at the boundary between segments 6 and 7 (4/10 movies) or 7 and 8 (6/10 movies) during stage 13 in wild types (Figures 1f and g; Supplementary Movie 1). In contrast, both posterior and anterior hemocytes were retarded in SCAR\textsuperscript{137} embryos, suggesting a general defect in hemocyte progression along the VNC (Figures 1f–h; Supplementary Movie 1).

To test whether SCAR was required cell autonomously, dominant-negative GFP-SCAR (dn SCAR)\textsuperscript{20} was specifically expressed within hemocytes. Dn SCAR expression phenocopied the defects in hemocyte dispersal observed in SCAR\textsuperscript{137} mutants (Figures 1c–f), including the delay in both anterior and posterior populations. The presence of two copies of the dn SCAR transgene further enhanced this phenotype (Figures 1e and f). In addition, hemocyte-specific expression of wild-type SCAR completely rescued development of the ventral nerve cord (VNC) and the dynamics of hemocyte numbers since by stage 15 there was a comparable number of hemocytes on the ventral midline in SCAR\textsuperscript{137} and wild-type embryos (data not shown; also see Figure 7). We also found no significant difference in the numbers of hemocytes in the field of view in wild-type and SCAR mutants immediately post-wounding when normalised according to the area of the field of view. Importantly, a statistically significant decrease in the numbers of hemocytes at wounds was maintained when these values were normalised against both wound size and density of hemocytes present immediately after wounding (data not shown), comparable to the decrease presented in Figure 2d.

Hemocytes remain motile following their dispersal, exhibiting contact-dependent repulsion at stage 15.\textsuperscript{11} Tracking SCAR\textsuperscript{137} mutant hemocyte movement at this stage revealed a dramatic reduction in motility compared with wild types (Figures 3a, b and f); once again hemocyte directionality was unaffected by loss of SCAR (data not shown). Expression of wild-type SCAR specifically in hemocytes in a SCAR\textsuperscript{137} mutant background (SCAR rescue) significantly rescued hemocyte speed, demonstrating a cell autonomous requirement of SCAR for effective migration. Thus, SCAR is important in driving hemocyte motility during three crucial behaviours: dispersal, patrolling and inflammation.

**SCAR regulates hemocyte morphology in vivo.** SCAR is an important activator of the Arp2/3 complex that has a key role in the formation of lamellipodia in vitro, structures considered to drive cell motility in many cell types.\textsuperscript{1} To examine the influence of SCAR on morphology we imaged GFP- and mCherry-moesin-labelled hemocytes live. Wild-type hemocytes typically extend large, dynamic, F-actin-rich lamellipodia at stage 15 of development (Figures 3a and a'). These lamellipodia contain Ena at their tips and Fascin-enriched F-actin bundles/microspikes extending radially from the cell body towards the periphery;\textsuperscript{13,14} some of these microspikes extend beyond the leading edge to form filopodia (Figure 3a'). SCAR\textsuperscript{137} hemocytes exhibited a dramatic loss of lamellipodia and were instead dominated by long, motile filopodia-like protrusions (Figures 3b and b'); Supplementary Movie 2), giving rise to a stellate morphology strongly reminiscent of SCAR-depleted S2 cells in culture.\textsuperscript{15–17}

The effects of SCAR\textsuperscript{137} on lamellipodia were cell autonomous as lamellipodial area could be restored to wild-type levels in SCAR rescue hemocytes (Figures 3c, c' and g). In addition, lamellipodial area was reduced in hemocytes expressing dn SCAR (Figure 3d and g), with a more severe phenotype observed in those carrying two copies of this transgene (Figure 3e).

**SCAR is required for the inflammatory response and motility of hemocytes.** Drosophila hemocytes mount rapid inflammatory responses to laser-induced epithelial wounds, responding to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}).\textsuperscript{5,27} To test whether SCAR is necessary for this process, we laser wounded the ventral epithelium of stage 15 embryos just laterally to the midline, and assessed hemocyte recruitment 1 h later, finding a substantial reduction in hemocytes recruited to wounds in SCAR\textsuperscript{137} mutants compared with wild types (Figures 2a–d), demonstrating that SCAR is an important regulator of this in vivo inflammatory migration. Dynamic imaging indicated that the few hemocytes that did migrate to wounds in SCAR\textsuperscript{137} mutants did so at a reduced rate, but displayed an identical directionality to wild types (Figure 2e). This suggests SCAR does not have an important role in guiding hemocytes or sensing wound-induced damage cues, but instead powers their migration in response to those signals. Decreased hemocyte recruitment was not a consequence of an overall reduction in hemocyte numbers since by stage 15 there was a comparable number of hemocytes on the ventral midline in SCAR\textsuperscript{137} and wild-type embryos (data not shown; also see Figure 7). We also found no significant difference in the numbers of hemocytes in the field of view in wild-type and SCAR mutants immediately post-wounding when normalised according to the area of the field of view. Importantly, a statistically significant decrease in the numbers of hemocytes at wounds was maintained when these values were normalised against both wound size and density of hemocytes present immediately after wounding (data not shown), comparable to the decrease presented in Figure 2d.
Other members of the WASp family of nucleation promoting factors could be responsible for the formation of residual lamellipodia observed in SCAR<sup>−/−</sup> mutants, particularly as WASp itself is recruited to Dictyostelium pseudopods in SCAR’s absence. However, neither WASp nor WASH appeared necessary for these residual lamellipodia because both SCAR<sup>−/−</sup>, WASp<sup>EY06238</sup> and SCAR<sup>−/−</sup>, WASH<sup>−/−</sup> double mutants were indistinguishable from SCAR<sup>−/−</sup> mutants in

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**Figure 1** SCAR is required by migrating macrophages for their developmental dispersal and interacts genetically with Pvr. (a–d) Lateral views of stage 13/14 embryos immunostained to show distribution of GFP-labelled hemocytes. Hemocytes migrate from the head to form a single line atop the VNC in wild-type (WT) embryos (a); shown between arrows. Hemocytes failed to migrate as far in SCAR<sup>−/−</sup> zygotic mutants (b) or when dominant-negative SCAR (dn SCAR) was specifically expressed in hemocytes (c). Regions between arrowheads indicate absence of hemocytes (b and c). Expression of wild-type SCAR specifically in hemocytes in a SCAR<sup>−/−</sup> background (SCAR rescue) rescued this defect (d). (e) Quantification showing mean number of empty segments in fixed embryos at st13/14; each SCAR mutant, with the exception of SCAR rescue embryos, was significantly different from wild types (P<0.001, Kruskal–Wallis test, Dunn’s multiple comparison post test, n≥29 embryos per genotype), whereas hemocyte-specific expression of the anti-apoptotic gene p35 in a SCAR<sup>−/−</sup> mutant background (SCAR + p35) failed to rescue. N.B. 2x dn SCAR represents a 50:50 mix of embryos with hemocytes expressing from 1 or 2 copies of UAS-dn SCAR (see Materials and methods section). (f) Quantification of mean position of lead hemocytes for anterior and posterior populations from fixed embryos; where hemocytes were present all along the VNC they were assumed to have met at segment 7.6, a value that corresponds to the mean meeting point taken from ten movies of WT embryos. (g and h) Projections of all time points of 3 h-long movies of WT and SCAR<sup>−/−</sup> mutant embryos with red stinger-labelled hemocytes (see also Supplementary Movie 1) revealed that hemocytes from both posterior and anterior populations were retarded in SCAR<sup>−/−</sup> mutants; hemocytes of these two populations met at the arrowhead in (g); representative of at least three movies per genotype. (i) Mean number of empty segments per embryo in SCAR<sup>−/−</sup>+/+ and Pvr<sup>+/+</sup> heterozygotes and SCAR<sup>−/−</sup>+/+ Pvr<sup>−/−</sup> transheterozygotes showing genetic interaction between Pvr<sup>−/−</sup> and SCAR<sup>−/−</sup>. Each genotype is significantly different from the others (P<0.05, Kruskal–Wallis test, Dunn’s multiple comparison post test, n≥35 embryos per genotype). Anterior is to the right and ventral is up in all images; scale bars represent 50 μm; error bars represent the S.D.
Any remaining lamellipodia may therefore be driven by maternal SCAR or a partially redundant mechanism. Loss of SCAR function leads to defective phagosome processing. As hemocytes disperse through the embryo they engulf dying cells, consequently containing numerous vacuoles by stage 15 (Figures 3a, h and 4a). Both SCAR<sup>D37</sup> mutant and dn SCAR hemocytes exhibited striking increases in the number of vacuoles per cell, especially when two copies of the dn SCAR transgene were present (compare Figure 3d and e), whereas SCAR rescue hemocytes were no more vacuolated than wild types (Figures 3c' and h). Acridine orange staining of wild-type and SCAR<sup>D37</sup> mutants showed the majority of these vacuoles contained apoptotic corpses (Figure 4b and f); this was validated by the absence of both vacuoles and acridine orange staining from apoptosis-null H99 mutant embryos (Figure 4c and d), which lack the pro-apoptotic genes hid, reaper and grim.<sup>29–31</sup> This increase in engulfed apoptotic corpses was not a consequence of hemocyte death or gross increases in apoptosis, thus uncovering a novel function for SCAR in the processing of phagocytosed apoptotic corpses.

To probe this role in phagocytic processing, a dominant-negative form of Rab5 was specifically expressed within hemocytes;<sup>33</sup> the Rab family of GTPases have important roles in trafficking, with Rab5 involved in phagosome maturation.<sup>34</sup> Dn Rab5-expressing hemocytes became highly vacuolated, phenocopying SCAR<sup>D37</sup> mutants (Figure 4g). Furthermore, the fraction of vacuoles within both dn Rab5 and SCAR<sup>D37</sup> mutant hemocytes that stained positively using LysoTracker, an indicator of phagosome acidification, was reduced compared with wild types (Figure 4h). In contrast, we found no striking accumulation of mCherry-Moesin around the persistent vacuoles observed in SCAR mutants suggesting that a failure to remove F-actin from the surfaces of vesicles, a process that precedes both Rab5 activation and subsequent acidification steps,<sup>35</sup> does not contribute to phagosomal maturation defects (Figure 4e; data not shown), whereas the dynamics of this process also appeared unaffected (data not shown). In summary, these results show SCAR as critical for the normal maturation and acidification of phagocytosed apoptotic corpses in macrophages in vivo.

Loss of the SCAR/WAVE complex component Hem phenocopies SCAR defects. The activity, stability and localisation of SCAR is regulated via incorporation into the evolutionarily conserved SCAR/WAVE complex, one component of which is Hem (also referred to as Kette or Nap1 in Drosophila).<sup>16,17</sup> Drosophila Hem mutants exhibit defects in

Figure 2  SCAR drives inflammatory migration of hemocytes. (a and b) Stage 15 wild-type (a) and SCAR<sup>D37</sup> mutant embryos (b) with GFP-labelled hemocytes (green) were wounded and imaged 1 h later (purple borders show wound position). (c and d) Scatter (c) and box and whisker plots (d) reveal that significantly fewer hemocytes were present per µm<sup>2</sup> of wound area in SCAR<sup>D37</sup> mutants (P<0.001, t-test, n=20 embryos per genotype). (e,e') Box and whisker plots of hemocyte speed (e) and directionality (e') show that SCAR<sup>D37</sup> mutant hemocytes migrated to wounds significantly more slowly (P<0.001, Heat, 50 WT and 20 SCAR<sup>D37</sup> tracks taken from seven movies of each genotype), but as directionally as wild types (NS, not significant). Scale bars represent 20 µm. Lines represent the median, whereas boxes and whiskers show the interquartile and 2.5–97.5 percentile ranges, respectively, in these and all subsequent box and whisker plots.
axonal wiring and muscle cell fusion. The same complex operates in vertebrates; in particular an almost exclusively haematopoietic-specific mouse homologue of Hem has a key role in neutrophil migration in vitro. To verify the vacuolation phenotype and further understand the regulation of SCAR in hemocytes, we tested the role of Hem in hemocyte function. Analysis of Hem mutants and Hem rescues revealed Hem is necessary for normal dispersal (Figures 5a and b), migration at stage 15 (Figures 5c–e) and lamellipodia formation by hemocytes (Figures 5f–k). Similarly to SCAR mutants, Hem mutant hemocytes became highly vacuolated (Figures 5f–k). These data strongly suggest that SCAR is regulated via the SCAR/WAVE complex in hemocytes.

**Figure 3** SCAR drives hemocyte migration and is necessary for normal hemocyte morphology. (a–c) Hemocyte tracks representing migration for 30 min at stage 15 in WT (a), SCAR (b) and SCAR rescue embryos (c); arrows show position of ventral midline. (a–c) Morphology of GFP-labelled hemocytes at stage 15 on the ventral midline; arrowheads illustrate large lamellipodia, asterisks indicate spindly protrusions. (d–e) F-actin morphology of hemocytes at the ventral midline shown via mCherry-moesin expression. (d and e) Hemocytes expressing GFP and dn SCAR from one UAS construct showed a mild reduction in lamellipodia (d), but a more severe phenotype was frequently seen in embryos that expressed dn SCAR from either 1 or 2 copies of the UAS insertion (e). (f–h) Box and whiskers plots showing quantification of hemocyte speeds (f; n ≥ 75 hemocytes from 7 embryos per genotype) and lamellipodial area (g; n ≥ 25 hemocytes from 15 embryos per genotype) and numbers of vacuoles per hemocyte (h; n ≥ 34 hemocytes from 13 embryos per genotype); each category is significantly different from the rest (P ≤ 0.05, one-way ANOVA, Bonferroni’s post test). All scale bars represent 20 μm.

The same complex operates in vertebrates; in particular an almost exclusively haematopoietic-specific mouse homologue of Hem has a key role in neutrophil migration in vitro. To verify the vacuolation phenotype and further understand the regulation of SCAR in hemocytes, we tested the role of Hem in hemocyte function. Analysis of Hem and Hem rescue mutants revealed Hem is necessary for normal dispersal (Figures 5a and b), migration at stage 15 (Figures 5c–e) and lamellipodia formation by hemocytes (Figures 5f–k). Similarly to SCAR mutants, Hem mutant hemocytes became highly vacuolated (Figures 5f–k). These data strongly suggest that SCAR is regulated via the SCAR/WAVE complex in hemocytes.

**SCAR drives phagocytosis of bacteria and is necessary for efficient clearance of pathogens and damage repair to promote survival.** One of the first functions ascribed to SCAR was in phagocytosis; indeed SCAR is important in this regard in larval hemocytes. To test whether SCAR...
carries out a similar function within embryos, RFP-expressing *E. coli* were microinjected into SCAR37 and wild-type stage 15 embryos containing GFP-labelled hemocytes. Live imaging 1 h post-injection revealed that SCAR37 mutant hemocytes phagocytosed *E. coli* at substantially reduced levels compared with wild types (Figures 6a–c). Non-phagocytosed bacteria were frequently observed adhered to the surface of SCAR37 mutant hemocytes, suggesting defects in phagocytosis rather than detection of bacteria.

The biological cost of defective clearance was probed by monitoring viability 24 h post-injection with the pathogenic bacteria *Erwinia carotovora carotovora* 15 (ecc15). Infection with ecc15 caused an increase in mortality in both wild types and SCAR37 mutants on top of that observed in PBS-injected embryos. A typical experiment is shown in Figure 5.

**Figure 4** SCAR mutant hemocytes contain numerous apoptotic corpses and have defective phagosome processing. (a and b) Maximum projections (a) and zooms of single confocal slices from indicated box region (a') reveal that vacuoles within mCherry-moesin-labelled (ch moe) wild-type (WT) hemocytes appear as regions excluding this F-actin marker (asterisks indicate examples); co-staining with acridine orange (ao = red and/or green spheres, arrowheads indicate examples; red = ch moe) shows the majority of these vacuoles contain apoptotic corpses (b). (c and d) H99 mutants neither contained vacuoles (c–c'), nor stained with acridine orange (d). (e and f) SCAR137 mutants were highly vacuolated (e–e') and the majority of vacuoles stained positively for acridine orange (f). (g) Stage 15 hemocytes co-expressing dominant-negative Rab5 and GFP also became highly vacuolated, although they retained lamellipodia (indicated by arrows in zoomed region). (h) Quantification revealed that the mean fraction of vacuoles positively labelled with LysoTracker red within hemocytes per embryo was significantly reduced in both SCAR137 homozygotes and embryos that contained dn Rab5-expressing hemocytes at stage 15 compared with wild types (*P* ≤ 0.01, one-way ANOVA, Bonferroni's post test, *n* ≥ 12 embryos per genotype); error bars represent the S.D.; scale bars show 10 μm. NS, not significant.
controls, but crucially this mortality was significantly enhanced in SCAR^{D37} mutants (Figure 6d), indicating that failures in SCAR-dependent phagocytosis substantially compromised innate immune responses. Interestingly, there was a significant reduction in the viability of SCAR^{D37} mutants when non-injected and PBS-injected embryos were compared (Figure 6d), paralleling the defects in larval healing observed on RNAi of SCAR,^{41} and suggesting that SCAR has an important role in repair processes. To further confirm that ecc15 and not simply the injection process itself accounted for...
the enhanced drop in survival, we normalised according to the appropriate PBS-injected control, which conclusively showed that, despite a decreased tolerance to the injection process, there was a further drop in survival because of the presence of ecc15 (Figure 6d’) and presumably its defective clearance.

Separating vacuolation and lamellipodial phenotypes in SCAR mutants. Loss of SCAR results in two clear defects: failure in the processing of apoptotic corpses and dramatic reductions in lamellipodial area. To investigate the contribution of the failure in phagosome processing to lamellipodial and motility defects in SCAR mutants we analysed SCAR37,H99 double mutants, which lack all apoptosis. Strikingly, removal of the apoptotic clearance burden rescued both migration along the VNC and lamellipodial area to the levels observed in wild-type and H99 mutant hemocytes (Figures 7a–f).

SCAR,H99 double mutant hemocytes moved significantly faster than SCAR37 mutants at stage 15, but did not move as quickly as either SCAR rescue hemocytes (compare speeds with Figure 3g) or H99 mutants (Figure 7g). Importantly, this partial rescue of hemocyte migration at stage 15 in SCAR37,H99 double mutants revealed that, whereas vacuolation is a major contributing factor to the hemocyte motility defects observed in SCAR37 mutants, it cannot be the sole explanation.

In order to examine the nature of the rescued lamellipodia in more detail, the rate of lamellipodial area change was calculated from timelapse movies of wild-type hemocytes, the occasional SCAR37 mutant hemocytes that possessed large lamellipodia and SCAR37,H99 double mutant hemocytes at stage 15. Of those hemocytes analysed, there was no difference in average lamellipodial areas (data not shown), however, SCAR37 mutant lamellipodia were more dynamic with greater changes in lamellipodial area over time, indicating a reduction in their stability. Surprisingly, the presence of the H99 deficiency reverted lamellipodial behaviour in these SCAR mutant cells to that observed in wild types (Figure 7h). Therefore, even the occasional lamellipodia that appear morphologically normal in SCAR37 mutant hemocytes behave very differently to wild-type lamellipodia and this altered behaviour can be dramatically rescued simply by removing apoptosis, further highlighting the delicate balance between the machineries controlling phagosome processing and migration.

Discussion

SCAR has long been thought to drive the activation of the Arp2/3 complex to generate actin structures to drive migration. Here, we show that SCAR is crucial for the generation of lamellipodia in macrophages in vivo, structures whose presence is critical for effective motility of these cells to a variety of cues including H2O2 and Pvs. SCAR is necessary for inflammatory responses and hemocyte dispersal, a process necessary for embryogenesis and immune surveillance. However, lamellipodial defects aside, the absence of SCAR causes an extreme vacuolation phenotype, which appears to be a consequence of a failure to adequately process ingested material, resulting in a build-up of undigested apoptotic corpses. Suppression of apoptosis and the consequent relief of this clearance burden prevents vacuolation and in turn completely rescues lamellipodia and partially restores normal migration speeds. How might increased vacuolation suppress cell motility in SCAR mutants? One potential explanation might involve sequestration/titration of...
components that would otherwise be able to rescue these defects. Proteomic characterisation of phagosomes has shown recruitment of SCAR and Ena, and the latter regulates lamellipodial dynamics and migration in hemocytes. However, unlike the H99 deficiency, overexpression of Ena in SCAR mutants failed to rescue migration along the VNC, whereas enaGC1 failed to interact genetically with SCAR in transheterozygous embryos (data not shown). It remains possible that rescue is via the presence of low levels of maternal SCAR that would otherwise be held at vacuoles.

An alternative hypothesis is that competition for the Arp2/3 complex by another activator on the surface of vacuoles might prevent lamellipodial formation via residual SCAR. WASH is another member of the WASp family and can activate Arp2/3 downstream of Rho1. In Dicyostelium WASH has a role in the removal of V-ATPase to facilitate neutralisation of vacuoles before expulsion of indigestible waste and therefore blocking phagosome maturation could drive WASH’s accumulation on vacuoles. However, SCAR;WASH double mutants showed no rescue of lamellipodia appearing to rule this hypothesis out. We were also unable to find any obvious hemocyte dispersal or morphological defects in WASH mutants (Supplementary Figure 2). Similarly, SCAR;WASp double mutants neither rescued nor further perturbed lamellipodia in hemocytes.

Other potential explanations as to how vacuolation might suppress migration include a reduced availability of membrane to add to protrusions, cell signalling feedback mechanisms or even steric hindrance of migration through constricted spaces. Alternatively, increased ingested material to process (for instance cholesterol) or other changes in cellular lipid composition/distribution (e.g., phosphoinositides) could underlie migratory defects associated with vacuolation in SCAR mutants. Further investigation is required to determine the precise molecular mechanisms underpinning this intriguing balance of power. The fact that the removal of the apoptotic clearance burden only partially restores migration speeds in SCAR mutant hemocytes demonstrates that none of these explanations can fully explain the motility defects in SCAR mutants and that SCAR is indeed necessary in cells that utilise lamellipodia to move as quickly as possible.

**Figure 7** Removing apoptosis rescues lamellipodia and partially rescues speed in SCAR mutant hemocytes. (a–d) GFP expression in hemocytes at stage 15 in wild-type (a), SCARD37 (b), SCARD37;H99 double mutant (c) and H99 mutant (d) embryos revealed that the H99 deficiency rescued lamellipodia (arrows). Regions with reduced levels of GFP in cell bodies in (c) and (d) are nuclei (asterisks); scale bars represent 20 μm. (e) Bar graph showing mean number of segments lacking hemocytes at stage 13; error bars represent the S.D. SCARD37 mutants exhibited a significant defect compared with the other genotypes (P < 0.01; Kruskal–Wallis test, Dunn’s multiple comparison post test, n = 15 embryos per genotype), whereas there were no significant differences amongst the remaining comparisons. (f–h) Box and whisker plots of hemocyte lamellipodial area (f; n > 50 hemocytes from ≥ 12 embryos per genotype), speed (g; n > 90 hemocytes from ≥ 5 embryos per genotype) and rate of lamellipodial change per hemocyte per min (h; > 12 hemocytes were analysed from three different movies) at stage 15. Asterisks indicate a significant difference between SCARD37 mutants and the other genotypes in (f) (P < 0.001) and with WT and SCAR;H99 in (h) (P < 0.01). P-values determined by one-way ANOVA and Bonferroni’s post test (f–h).
The severely defective lamellipodia observed on loss of SCAR function suggests that Arp2/3’s primary activator in hemocytes is SCAR. That some F-actin persists and migration remains possible in the absence of SCAR seemingly indicates that other actin nucleators operate within hemocytes, but that the network that other actin regulators influence is overwhelmingly Arp2/3 dependent. A loss of lamellipodia on depletion of Arp2/3 has recently been demonstrated in vitro in fibroblasts, correlating with a reduction in their migratory capabilities.\(^{45,46}\) Interestingly, cells lacking Arp2/3 moved more slowly but still chemotaxed efficiently towards soluble PDGF,\(^{45}\) paralleling the slower but highly directional routes SCAR mutant hemocytes take towards H\(_2\)O\(_2\) at wounds. In addition, Arp2/3-depleted cells exhibited increased protrusion and retraction rates,\(^{46}\) consistent with the increased lamellipodial dynamics of SCAR mutant hemocytes (Figure 7h).

The phagocytic processing defect in SCAR mutants has not been reported previously, although a potentially related role for both SCAR and WASp downstream of Cip4/Toca in actin comet-driven movement of vesicles from the plasma membrane has been shown in S2 cells.\(^{47}\) Cells in other assays of SCAR/WAVE function are unlikely to have been exposed to as many apoptotic corpses as hemocytes are during development; alternatively upstream roles in phagocytosis may have hidden downstream functions of SCAR in processing.

Our results highlight a fine balance between processing of phagocytosed debris and formation of actin-rich protrusions necessary for driving migration within macrophages. It has been well documented that exposure to apoptotic corpses can switch phagocytes from pro-inflammatory to anti-inflammatory phenotypes\(^{48,49}\) and we have previously shown that apoptotic cells can dictate the direction in which macrophages will migrate and represent their most highly prioritised cue in the Drosophila embryo.\(^{27}\) Here, we demonstrate that even after engulfment apoptotic corpses can influence the migration machinery in an inflammatory cell, hijacking key components of that machinery if the clearance burden becomes too great and thereby disabling the macrophages’ migration programme. In light of these findings, it is now critical to understand further the mechanisms underlying this crosstalk between debris clearance and migration if we are to design therapeutics to stimulate macrophage resolution away from areas such as chronic wounds or tumours where levels of debris and cell death may be very high.

### Materials and Methods

**Fly genetics.** Twi-Gal4 and Gal4-independent fluorescent balancers\(^{30,51}\) were used to maintain mutant lines and discriminate homozygous mutant embryos. Fly lines were previously generated in the lab or obtained from the Bloomington Stock Centre (University of Indiana, IN, USA); lines unavailable from Bloomington were kindly sent by the labs that originally made them (see references below). Stp-Gal4 (ref. 25), cpr-Gal4 (ref. 26) and pnx-Gal4 (ref. 5) were used to drive expression of UAS constructs specifically in hemocytes. UAS-GFP-SCAR\(^{20}\) and UAS-Red d\(^{33}\) were used as dominant-negative constructs; UAS-p35 (ref. 52) and UAS-SCAR\(^{13}\) were used as rescue constructs; insertions of UAS-GFP, UAS-mCherry-Moesin\(^{53}\) and UAS-red stinger\(^{21}\) were used to visualise hemocyte morphology and dynamics. Mutant alleles used were SCAR\(^{13}\) (ref. 21), Hem\(^{26,28}\) (ref. 36), Hem\(^{2030}\) (ref. 54) and Df(3L)H99 (ref. 31), WASp\(^{34,50}\) (ref. 55) and WASh\(^{17,65}\) (ref. 56); see Supplementary Table 1 for a full list of genotypes used. SCAR\(^{53,57}\) was recombined with srp-Gal4 (Figures 1–6) or srp-Gal4,UAS-GFP and srp-Gal4,UAS-red stinger (Figure 7).

**Quantification of developmental migration.** Fixation and subsequent immunostaining of embryos was carried out as per Evans et al.,\(^{22}\) using rabbit anti-GFP (Abcam, Cambridge, UK) as a primary antibody. The presence or absence of GFP- or red stinger-labelled hemocytes on the ventral side of the VNC was scored by eye in either live or fixed and immunostained embryos using a Leica MZ16F fluorescent dissection microscope with a 5X/0.5 PL APO objective (Leica, Wetzlar, Germany). Low magnification images of representative embryos were taken on the same microscope.

**Live imaging.** Embryos were mounted on slides via Scotch double-sided sticky tape (3M, Maplewood, MN, USA) in voltaoil as previously described,\(^{22}\) halocarbon oil 700 (Sigma, St Louis, MO, USA) may be used as an alternative as voltaoil is no longer commercially available. All live imaging was carried out at 22 ± 1 °C. Time-lapse movies of red stinger-labelled hemocytes at stage 15 were taken of ventrally orientated embryos on a Leica DMI8 inverted microscope fitted with an HCX PL APO 40X objective, with a z-stack of three slices that captured red stinger-labelled hemocytes moving between the epidermis and VNC collected every 30 s for 30 min using ImagePro software (Media Cybernetics, Bethesda, MD, USA). Movies to determine when anterior and posterior hemocytes were met were taken on the same set-up using a Leica HC PL APO 20X/0.70 multi-immersion objective lens (with oil); embryos were mounted laterally at mid-stage 12 and a z-stack of five slices was taken every 2 min to capture hemocyte progression along the VNC.

**Laser ablation of epithelial cells was performed as previously\(^{21}\) on a Zeiss LSM510 confocal microscope with a Zeiss FLUAR 40X/1.3 oil DIC objective (Zeiss, Oberkochen, Germany). The number of GFP-labelled hemocytes at or in contact with the wound margin was quantified 1 h post-ablation, with wound area measured from widefield images of transmitted laser light (channel D) in NIH ImageJ (http://rsweb.nih.gov/ij/index.html). To determine speed and directionality of migration movies were taken immediately after wounding with a z-stack of five images collected every 2 min for 1 h. All tracking was performed on maximal projections of time-lapse movies using ImageJ (manual tracking and chemotaxis tool plugins).

Stills of fluorescently labelled hemocytes on the ventral midline were taken of ventrally orientated, stage 15 embryos on either a Zeiss LSM510 confocal microscope with a Zeiss FLUAR 40X/1.3 oil immersion DIC objective or a Leica DMI8 6000B UltraVIEW VoX spinning disk confocal imaging system with an HCX PL APO 40X/1.3 oil immersion objective; images were captured using Volocity (Perkin Elmer, Waltham, MA, USA). The latter set-up was also used to determine lamellipodial dynamics with GFP-labelled hemocytes between the epidermis and VNC imaged every 30 s for 10 min.

**Acridine orange and LysoTracker staining.** Dechorionated embryos were transferred to a glass vial containing 500 ml heptane (Sigma) plus 500 ml 5 μg/ml acridine orange (Sigma) or 50 μg LysoTracker red DND-99 (Molecular Probes, Life Technologies, Carlsbad, CA, USA) in PBS (Sigma) and agitated for 5 or 30 min, respectively. Embryos were then removed from the interface in a minimal volume of fluid to halocarbon oil 700 and subsequently transferred in a small oil droplet to slides for immediate imaging. We found that acridine orange and LysoTracker both produced highly variable intensity of staining between embryos, even within the same treatment. It was therefore necessary to use different exposure times during image capture to avoid under/overexposure. Images were collected on the spinning disk system outlined above. To quantify acidification of phagosomes, the fraction of vacuoles >1.5 μm in diameter within hemocytes with clear LysoTracker labelling was determined from single confocal slices (LysoTracker positive vacuoles/total vacuoles in field of view).

**Image quantification and statistical analysis.** Lamellipodial area (total area – cell body area) was measured manually in ImageJ from contrast-enhanced maximum projections of confocal stacks of hemocytes expressing cytoplasmic GFP; vitelline membrane autofluorescence was manually removed from more superficial (ventral) confocal slices where it obscured hemocyte morphology before assembly of projections. Only hemocytes lying in the pseudo-2D plane between epidermis and VNC, bounded by the edges of the VNC, which could be distinguished from their neighbours were analysed. The values herein are likely an under representation of SCAR phenotypes as the most severely affected hemocytes tended to clump together and thus eluded analysis. Vacuole numbers per hemocyte were determined by scanning though z-stacks of confocal images. Lamellipodial dynamics were calculated as follows: the lamellipodial area of individual GFP-labelled hemocytes was determined every 30 s over the course of a
10 min movie (as described above). The absolute change in area (i.e., the modulus) between subsequent timepoints was then averaged per hemocyte and this was normalised according to the average lamellipodial area for that hemocyte over the entire movie to take into account differences in hemocyte size. For this analysis only SCAR\(^{13,17}\) mutant hemocytes with large lamellipodia were quantified.

Statistical analysis was performed using Graph Pad Prism for Mac (Graph Pad Software, La Jolla, CA, USA). In the box and whiskers plots, the lines represent the medians, whereas the boxes and whiskers show the interquartile and 2.5–97.5 percentile ranges, respectively.

**Bacterial injections.** DH5\(^{\text{a}}\) E. coli and ecc15 bacteria were grown for 18 h to stationary phase at 37 and 30 °C, respectively. After washing in sterile endotoxin-free PBS (Sigma), cultures were adjusted to the appropriate density (OD\(_{600}\) = 10 for E. coli and OD\(_{600}\) = 0.1 for ecc15) and microinjected into stage 15 embryos as per Vlisidou et al.\(^{21}\). Injection of sterile, endotoxin-free PBS was used as a negative control. For analysis of phagocytosis, a coverslip was mounted over injected embryos and z-stacks with a 1 μm spacing of GFP-labelled hemocytes and RFP-labelled E. coli taken at 1 h post-injection using a Zeiss LSM510 confocal microscope and a PL APO 63X/1.4 oil immersion objective. These z-stacks were then used to calculate the percentage of phagocytosed E. coli per embryo. For viability studies, injected embryos were transferred to apple juice agar plates and stored at 25 °C in humid conditions. Viability was scored at 24 h post-injection based on larval hatching or movement.

**Conflict of Interest**

The authors declare no conflict of interest.

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