The PDZ-GEF Dizzy regulates cell shape of migrating macrophages via Rap1 and integrins in the *Drosophila* embryo

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In *Drosophila* embryos, macrophages originate from the cephalic mesoderm and perform a complex migration throughout the entire embryo. The molecular mechanisms regulating this cell migration remain largely unknown. We identified the *Drosophila* PDZ G-nucleotide exchange factor (PDZ-GEF) Dizzy as a component essential for normal macrophage migration. In mutants lacking Dizzy, macrophages have smaller cellular protrusions, and their migration is slowed down significantly. This phenotype appears to be cell-autonomous, as it is also observed in embryos with a dsRNA-induced reduction of *dizzy* function in macrophages. In a complementary fashion, macrophages overexpressing Dizzy are vastly extended and form very long protrusions. These cell shape changes depend on the function of the small GTPase Rap1: in rap1 mutants, Dizzy is unable to induce the large protrusions. Furthermore, forced expression of a dominant-active form of Rap1, but not of the wild-type form, induces similar cell shape changes as Dizzy does overexpression. These findings suggest that Dizzy acts through Rap1. We propose that integrin-dependent adhesion is a Rap1-mediated target of Dizzy activity: in integrin mutants, neither Dizzy nor Rap1 can induce cell shape changes in macrophages. These data provide the first link between a PDZ-GEF, the corresponding small GTPase and integrin-dependent cell adhesion during cell migration in embryonic development.

**KEY WORDS:** PDZ-GEF, Dizzy (Gef26), Rap1, Integrin, Cell adhesion, Cell motility, Macrophage, *Drosophila*

**INTRODUCTION**

From worms to humans, cell migration is an essential cellular process: during embryonic development cells migrate to specific locations, where they subsequently differentiate to form tissues and organs. Then later in adult organisms, cell migration is implicated in tissue renewal and immune response. Besides its crucial role during normal development of organisms, cell migration also contributes to pathological processes such as cancer.

Migrating cells undergo a continuous cycle of integrated cellular events that are initiated by migration-promoting cues. Such cues lead to a polarization of the cell and to the formation of protrusions in the direction of migration. The protrusions adhere to the substrate via transmembrane receptors linked to the cytoskeleton. The sites of adhesion then provide traction during the contraction of the cell, leading to the forward movement of the cell body. At the rear of the cell, adhesion sites become disassembled, allowing the cell to detach and to efficiently migrate toward guidance cues (Friedl and Wolf, 2003; Lauffenburger and Horwitz, 1996; Ridley et al., 2003). Thus, cell adhesion and its regulation are of crucial importance for migrating cells.

Many different factors regulate cell adhesion during cell migration, including cell surface receptors that mediate adhesion. A major family of these receptors are the integrins, heterodimers composed of one α and one β subunit, which link the migratory substrate to the cytoskeleton (Bökel and Brown, 2002; Hynes, 1992). Integrin-mediated adhesion can be regulated by modulation of their affinity to ligands or by changing their local concentration at the membrane. This regulation occurs either at the outside of the cell by ligands or from the inside by cytoplasmic signals (Kinbara et al., 2003; Liddington and Ginsberg, 2002; van der Flier and Sonnenberg, 2001). Important mediators of the latter signals toward the integrins are small GTPases. For instance, Rap1 is involved in the regulation of integrin-mediated cell adhesion in several cases (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000). In *Drosophila* the molecule is required for many aspects of morphogenesis, including invagination of embryonic mesoderm, migration of mesoderm precursors and positioning of adherens junctions (Asha et al., 1999; Boettner et al., 2003; Knox and Brown, 2002). However, Rap1 has not yet been linked to the activity of integrins in *Drosophila*.

The functional state of small GTPases such as Rap1, the active GTP-bound versus the inactive GDP-bound state is determined by two classes of proteins: G-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Ridley, 2001). While GAPs are responsible for the inactivation of small GTPases, GEFs are the activating components and stimulate specific small GTPases.

We have performed an EP misexpression screen (Rørth, 1996) in order to identify genes involved in the regulation and in the execution of cell migration of embryonic macrophages in *Drosophila*. Here we report the gene *dizzy* (Gef26 – FlyBase) encoding the *Drosophila* PDZ-GEF to be required for proper cell shape and cell migration of macrophages in the *Drosophila* embryo. Furthermore, we show that Dizzy has the capacity to induce cell shape changes in migrating macrophages depending on the function of Rap1 and BPS integrins. Our data suggest that the *Drosophila* PDZ-GEF Dizzy is a GEF for Rap1, regulates integrin-dependent adhesion via Rap1, and stabilizes cellular protrusions during the migration of embryonic macrophages in *Drosophila*.
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MATERIALS AND METHODS
Fly stocks
dizzyEP, synonyms EP(2)388, psc-gef2 (Lee et al., 2002); dizzyP, synonyms l(2)k13720, psc-gef1 (Lee et al., 2002); Df(2L)BS5C.5, breakpoints 26B1-2-26D1-2 (Deal and Cook, FlyBase); UAS-cd2 (Dunin-Borkowski et al., 1995); da-gal4 (Hinz et al., 1994); srph>cd2, recombinant of srph-gal4 and UAS-cd2; UAS-gfpSBST (Bloomington #1521, #1522); UAS-actin: gfp (Verkuhsha et al., 1999); UAS-rap1W7, UAS-rap1V12, UAS-rap1N17 (Boettner et al., 2003); rap1P579 (Knox and Brown, 2002); UAS-rhoN17 (Strutt et al., 1997), UAS-RacN17, UAS-cdc42N17 (Luo et al., 1994); UAS-p35 (Hay et al., 1994); myoXG43 (Bunch et al., 1992); da-3 Ki (Robertson et al., 1988), dizzyd1/2Cy0, actGFP, srph-gal4 UAS-gfpSBST, UAS-gfpB57, srph-gal4 UAS-gfpSBST and dizzyP(UAS-gfpSBST; srph-gal4 UAS-gfpSBST)+, for time-lapse recordings.

EP screen
Fifteen to 30 virgins of the stock srph>cd2 were crossed with three to seven males of one genotype of the EP collection (Szeged). Embryos were collected and immunostained for CD2 following the methods described in Hummel et al. (Hummel et al., 1997), were mounted in methyl salicylate and analyzed for macrophage migration, cell form and cell number.

Generation of dizzy alleles
The P-element insertion of dizzyEP was mobilized by crossing-in Δ2-3, and the F1 generation was scored for semi-lethality in trans to Df(2L)BS5C.5. In total, 11 lines were established with strong eye and wing phenotypes of adult escapers; seven were used for this study: dizzyd1 and dizzyP remove 174 and 1187 bp upstream of the former insertion site, dizzyA7 and dizzyA8 2253 bp and 2632 bp around the site, and dizzyd1, dizzyd10 and dizzyd12 2328, 2380 and 2969 bp downstream only. One revertant (dizzyP-Rv), obtained upon mobilization of the P-element of dizzyP, has lost the insertion completely and is fully viable.

Qi He and colleagues introduced the gene name dizzy. dizzy is identical to Gef26 and dpDZ-GEF (Lee et al., 2002; Wang et al., 2006).

Generation of dizzy RNAi transgenics
For the tissue-specific downregulation of dizzy function, we generated a transgene allowing the expression of dizzy double-stranded RNA under Gal4 control. A fragment of the dizzy EST AT08279 was amplified by PCR, using the 5’-primer GCAGTTAAAAAGATGCTATCGCTG and the 3’- primer TGGTCAAGTGGGCTGGATC GCGT. This fragment contains a portion of exon 3, the exon 4 and a part of exon 5. Following the strategy of Nagel et al. (Nagel et al., 2002), a 485 bp long piece of the fragment (positions 4328 to 4812 of cDNA DQ423241) was first cloned in pHIBS and subsequently by Ratn V12, we crossed myoXG43/FM7ftz::lacZ or srph>cd2 females with w/Y; srph-gal4 UAS-gal4 (CG11567) from EST LD4659. In-situ hybridization was performed essentially as described in Tautz and Pfeifle (Tautz and Pfeifle, 1989). DIG-labeled dizzy RNA probes were generated from EST RHS4455, for Psc (CG9493) from EST RE59091 and for Psp (CG11567) from EST LD46590.

Live-imaging and measurements
Dechorionated embryos were mounted in a drop of water-saturated 3S Voltalef oil on a slide, properly oriented and covered with a coverslip based on two lateral coverslips. For time-lapse recordings, we performed x-y sections using the Leica confocal microscope. Images were processed using IPlab (Scanalytics) and then evaluated using a software tool developed with Macromedia Director. The positions of individual cells were recorded in 4D image stacks by mouse click. Then travel distance and cell speed were calculated and the recorded tracks were visualized. Time-lapse movies were exported from IPlab, and processed in QuickTime Player. The lengths of the cellular protrusions were measured on digital photomicrographs in IPlab and exported to Excel for numerical analysis.

Preparation and analysis of genomic DNA
Genomic DNA was isolated from dizzyEP, dizzyP or homozygous dizzyan adults, amplified by PCR and sequenced essentially following the protocol of J. Rehm (http://www.fruitfly.org/about/methods/inverse.pcr.html). For the molecular characterization of the dizzyan alleles, locus-specific primers were designed, and PCR products were sequenced.

Cloning of dizzy full-length cDNA
Total RNA was isolated from w embryos of Drosophila, selected for poly-A RNA and reverse-transcribed to cDNA. Then, the 5’ and 3’ ends of dizzy cDNA were amplified using the RACE systems of Invitrogen and were subcloned in TOPO TA. Two independent clones were obtained, which had an identical 5’ end. The central part of the dizzy cDNA were amplified with gene-specific primers and then subcloned (accession number of full-length cDNA: DQ423241).

RESULTS
Molecular genetics of the Drosophila PDZ-GEF dizzy
We started the functional characterization of dizzy by confirming the insertion sites of the two dizzy P-element alleles, dizzyEP388 and dizzyA7/813726 (abbreviated as dizzyEP and dizzyA7). Strikingly, even though the insertion sites are very close (Fig. 1A), the phenotypic effects of the P-elements on viability are quite different: dizzyEP is fully viable, whereas dizzyA7 is recessive lethal (Lee et al., 2002). To understand the molecular basis for this discrepancy, we isolated full-length cDNAs of dizzy and defined the transcription start site. Thereby we mapped an additional exon, termed exon 0, which is located 5’ to the first exon annotated in FlyBase (Fig. 1A). The P-elements are inserted into exon 0, a few base pairs downstream of the transcription start site. The difference between dizzyEP and dizzyA7 phenotypes is probably due to the distinct properties of the P-elements. In contrast to dizzyEP, dizzyA7 has an hsp70 promoter at its 3’ end that could allow a sufficient level of dizzy transcription controlled by endogenous enhancers.

In order to obtain loss-of-function alleles of dizzy, we mobilized the P-element of dizzyEP and obtained a series of small deletions in the dizzy locus. Seven representative deletions were used for further analysis. dizzyd4 and dizzyd5 removed genomic DNA upstream of the former insertion site, including the transcription start site (Fig. 1A). dizzyA7 and dizzyA8 deleted the entire exon 0, the transcription start site, most of the putative dizzy enhancer regions and almost the complete first intron (Fig. 1A). dizzyd12 left the transcription start site intact, but removed the original translation start site and about

on an inverted Leica DM IRBE microscope. DAB-labeled embryos were mounted in araldite and analyzed on a Zeiss Axioplan 2 equipped with a Progress 3012 camera (Zeissoptik).
1.3 kb of the ORF. Therefore, it could code for a truncated Dizzy protein that lacks the cNMP, N-terminal RasGEF and PDZ domain (Fig. 1B). dizzy\textsuperscript{AB} and dizzy\textsuperscript{A10} are associated with smaller deletions, affecting translation start and cNMP domain only. Concerning the adult phenotype, all alleles behave like amorphic alleles, as homozygous escapers show the same phenotypes as animals carrying one of these dizzy alleles in trans to the deficiency Df(2L)BSC5: eyes are rough and reduced in size, wing blades are bent downward, and the male genitalia are distorted, probably contributing to the male sterility (Lee et al., 2002; Wang et al., 2006). Consistent with this genetic argument for the amorphic nature of the alleles is our finding that alleles with deleted transcription start sites lacked endogenous dizzy RNA expression in the embryo and therefore appeared to be null alleles (Fig. 1D). The normal expression of dizzy in the embryo was ubiquitous and of a comparably low level (Fig. 1C).

**dizzy is required for cell shape and cell migration of embryonic macrophages**

Next we tested the function of dizzy for macrophage migration in embryos either homozygous for any of the dizzy\textsuperscript{AB} alleles, hemizygous for dizzy\textsuperscript{AB}, or trans-heterozygous for dizzy\textsuperscript{AB} and dizzy\textsuperscript{A10}. In all cases the migration of macrophages in the embryo was similarly disturbed, specifically the migration along the ventral nerve cord (VNC). Macrophages from the anterior and from the posterior part of the embryo migrated along the midline of the VNC toward each other. Then at stages 13 and 14, wild-type macrophages surrounded the entire midline of the VNC (Fig. 2B). By contrast, macrophages of dizzy mutant embryos failed to migrate properly and did not succeed in completely surrounding the midline of the VNC at this time (arrowheads in Fig. 2A.K). In some embryos, the resulting ventral gap persisted until even later stages, whereas in others the defect disappeared (Fig. 2E). In addition, macrophages were found in an aberrant, dorsal position beneath the amnioserosa in mutant embryos after germ band retraction (Fig. 2L.K). This indicates that these macrophages did not properly adhere to the posterior end of the germ band and failed to enter the posterior germ band before and during its retraction. Later, during the phase of ‘central spreading’ at stage 14, macrophages in wild-type embryos migrated laterally and ended up rather evenly distributed throughout the interstitial space at stage 15 (Fig. 2F.H). By contrast, dizzy mutant macrophages did not achieve that even distribution, and the posterior-ventral part of the embryo contained less macrophages than the equivalent area of a wild-type embryo (Fig. 2E-H). Thus, dizzy mutant macrophages appeared to be slower and did not reach their destinations in time. Other aspects of the migration, such as the migration along the dorsal epidermis, were not noticeably affected in dizzy\textsuperscript{AB} mutants.

**dizzy is required not only for proper macrophage migration, but also for the cells to adopt their normal size and shape.** In dizzy mutants, macrophages formed smaller protrusions than those in wild-type embryos (Fig. 2C,D,G,H). The average lengths of the protrusions per cell were about 5 μm at stage 14 at lateral positions in fixed preparations of wild-type compared with less than half this size in dizzy mutants (Fig. 3). Hence, the analysis of the dizzy mutant phenotype demonstrates that dizzy is required for proper cell migration and proper cell form of macrophages during Drosophila embryogenesis and indicates a function of dizzy in cell adhesion.

The phenotype observed for macrophages in dizzy mutants could be due to a function of dizzy in the macrophages themselves or might be indirect, due to a requirement of dizzy in other tissues. We therefore specifically reduced dizzy function in the macrophages by expressing a dsRNA-fragment of dizzy. This expression led to the same phenotype as seen in the dizzy mutants: macrophage migration was severely delayed, macrophages failed to reach the posterior end of the germ band in time (Fig. 2M), and the cellular protrusions were smaller than in wild type (Fig. 3). We therefore conclude that the activity of dizzy is required within the macrophages for proper cell shape and motility.
Dizzy is sufficient to change the cell form of macrophages

Next we wondered how crucial dizzy activity is for cell shape and cell migration, and we specifically overexpressed dizzy in macrophages (Fig. 4). For this purpose we used the EP-allele dizzy\textsuperscript{EP} and directed the expression by srph-gal4 (termed dizzy\textsuperscript{h,EP} hereafter). The overexpression seen in dizzy\textsuperscript{h,EP} was confined to the dizzy transcription unit: genes neighboring dizzy, such as Pez or Cpr, were not influenced by the dizzy\textsuperscript{EP} allele. Macrophages in dizzy\textsuperscript{h,EP} embryos commenced their migration normally (Fig. 4A,B) but then formed very long protrusions, in the range of 20 \mu m per cell (Fig. 3, Fig. 4C,D). In addition, the protrusions of different macrophages contacted each other, resulting in the formation of a network that spanned the nervous system in a dorsoventral direction (Fig. 4C,E) or that was seen below the epidermis at lateral positions (Fig. 4G,I). In wild-type embryos, the macrophages migrating along the midline or beneath the dorsal edge of the epidermis had smaller protrusions and formed fewer or no contacts with each other (Fig. 4H,J).

Astonishingly, the motility of the macrophages was not significantly affected when dizzy was overexpressed from one copy of dizzy\textsuperscript{EP}, although the cell shape was changed dramatically. The cells migrated along their normal paths and made contacts with their normal substrates. Also, when followed by time-lapse microscopy, the cells showed the normal average speed of about 2 \mu m per minute (Fig. 5; see Movies 1–4 in the supplementary material). However, when the level of dizzy expression was increased further, by expression from two copies of dizzy\textsuperscript{EP}, migration of macrophages was slowed down (Fig. 4K). In embryos after stage 13, a significant gap remained at the VNC that was not closed later in embryogenesis. We conclude that the dizzy level is of crucial importance for regulation of cell shape and migration of macrophages.

Dizzy assists in stabilization of cellular protrusions during the migrational cycle of macrophages

Using time-lapse video microscopy, we intended to assess the origin of the large protrusions of dizzy\textsuperscript{h,EP} embryos. Live macrophages are much bigger than expected from their appearance...
in fixed material (Fig. 5) (Paladi and Tepass, 2004; Stramer et al., 2005). They have large, very dynamic lamellopodia, which extend in the direction of migration, multiple small filopodia and a short tail, depending on their state in the migrational cycle. These structures are not well preserved during the fixation procedure used for immunohistochemistry. Live macrophages overexpressing dizzy showed protrusions with principally the same size and the same dynamics as wild-type cells, but beyond that they also had the long cellular extensions seen in fixed preparations (Fig. 5; see Movies 1 and 3 in the supplementary material). Time series indicated that these extensions were not formed as independent entities, but originated either from retracted lamellopodia or more frequently from the tail of migrating cells (Fig. 5). This latter aspect contributes to the formation of the net-like appearance of the macrophages: in a group of cells, often only one cell at a time moved along and, upon dizzy overexpression, stayed in contact with the other, remaining cells of the group by its extended tail. Also at later stages, the long extensions were maintained in parallel to lamellopodia and did not disturb the normal local mobility of the cells (see Movie 3 in the supplementary material). We therefore suggest that dizzy contributes to the stabilization of cellular protrusions.

Fig. 3. The length of the cellular protrusions of macrophages depends on dizzy. Cellular protrusions per macrophage have been measured for the given genotypes in fixed and immunostained embryos beneath the dorsolateral epidermis at stage 14. In dizzy mutants and in embryos expressing ds.dizzy in macrophages the average length of cellular protrusions per cell is about half of the length seen in wild-type macrophages. Overexpression of dizzy (dizzy<sup>h.EP</sup>) or of dominant-active Rap1<sup>V12</sup> in macrophages leads to an increase of total length per cell by a factor of about four and three, respectively. As the protrusions of macrophages in these embryos span from cell body to cell body, standard deviations are here based on the lengths of individual protrusions rather than on the overall length per cell. The increase in protrusion length depends in both cases, dizzy<sup>h.EP</sup> and Rap1<sup>V12</sup>, on the zygotic expression of βPS integrin mys. In zygotic mys mutants the length per cell is similar to wild-type even when dizzy or dominant-active Rap1<sup>V12</sup> is expressed in the macrophages.

Fig. 4. The overexpression of dizzy in macrophages changes their cell shape. Macrophages overexpressing dizzy from a single copy of the allele dizzy<sup>EP</sup> under the control of sph-gal4 (A,C,E,G,I; hereafter named dizzy<sup>h.EP</sup>) have a significantly different cell shape from macrophages of wild-type embryos (B,D,F,H,J). (A,B) At stage 11, dizzy<sup>h.EP</sup> macrophages migrate in a similar way to wild-type cells. (C) During and after migration through the embryo, dizzy<sup>h.EP</sup> macrophages form long protrusions that contact each other and the substrate. These protrusions span dorsoventrally through the entire VNC (arrowhead). (D) In wild-type embryos, the protrusions are much smaller. (E) The change in cell form is maintained also in late embryos affecting the clearance of the VNC from macrophages (arrowhead). (F) In a wild-type embryo, macrophages have left the inner region of the VNC at stage 16. (G) In dorsalateral positions, the dizzy<sup>h.EP</sup> macrophages form a network with their large cellular extensions. (H) In wild-type embryos, the cells are smaller and do not touch each other. (I,J) Magnifications of areas indicated in G and H. (K) Expression of dizzy from two copies of dizzy<sup>EP</sup> also affects migration of macrophages, most strongly at stage 14.
Dizzy acts via Rap1

The phenotypes seen in macrophages of dizzy mutants, of ds.dizzy embryos and of embryos overexpressing dizzy suggest that Dizzy modulates adhesive properties of macrophages during their migration, and that the cell shape changes of macrophages reflect this modulation. As dizzy is predicted to be a GEF, based on its protein structure, we investigated by genetic means whether dizzy acts upstream of one of the small GTPases Cdc42, Rho, Rac or Rap1. Among these, we found Rap1 to be essential for the activity of Dizzy in macrophages. When we overexpressed dizzy in macrophages of zygotic rap1 mutants, these macrophages showed a substantial rescue of the cell shape changes induced by Dizzy (Fig. 6F,G). This rescue effect became even more evident in rap1 mutants that expressed dizzy from two copies of dizzyEP in macrophages. While macrophages in these embryos looked similar to wild-type macrophages, at stage 13; numbers are minutes lapsed after the start of the series. The cells are located on the ventral side of the midline of the VNC (indicated by black line; anterior is to the left).

The panels show time series of pictures taken from embryos expressing actin::gfp in the macrophages, at stage 13; numbers are minutes lapsed after the start of the series. The cells are located on the ventral side of the midline of the VNC (indicated by black line; anterior is to the left).

(Top row) dizzyEP embryo; one of the cells (below asterisk) moves to the left edge of the VNC and maintains contact with the cells at the midline by a long cellular extension. (Bottom row) Wild-type embryo; one of the cells (asterisk) is followed along the midline and from there to the left edge of the VNC. Although the cell occasionally forms a short tail (20’ panel), it does not maintain contact to cells it has passed on its path (pictures from Movies 1 and 2 in the supplementary material). Each panel represents 70 μm in width.

Dizzy activity requires integrin function

Dizzy activity leads to cell shape changes in migrating macrophages, and we assume that these changes reflect modulations of adhesive properties of macrophages. Therefore, we tested whether βPS integrins are required for the cell shapes induced by Dizzy activity. mys mutants solely lacking the zygotic βPS gene function did not have a phenotype in macrophage shape or migration during embryogenesis (Fig. 8D). The mys requirement became noticeable only in germline clones also lacking the maternal contribution (data not shown). Nevertheless, dizzy overexpression did not lead to large protrusions and the net-like appearance of macrophages in zygotic mys mutant embryos (Fig. 8A,B). The cells had the appearance of wild-type macrophages regarding size and protrusions (Fig. 3, Fig. 7B). This shows that Dizzy requires the full function of βPS and indicates that integrins act downstream of Dizzy.

Our results above suggest that Dizzy acts via Rap1 to induce the changes of cell shape in macrophages. One therefore would expect that Rap1 acts between Dizzy and the integrins in the genetic hierarchy and that the changes in cell morphology caused by dominant-active Rap1V12 also depend on integrin function. Indeed, in zygotic mys mutants Rap1V12 overexpression does not suffice in enlarging the protrusions (Fig. 3) or changing the cell shape of macrophages (Fig. 8F) as it does in mys+ embryos (Fig. 3, Fig. 8E).
Thus, we conclude that in migrating macrophages Dizzy acts upstream of Rap1, and Rap1 in turn regulates cell adhesion and cell shape via a pathway that requires the function of βPS integrins.

**DISCUSSION**

We identified the *Drosophila* PDZ-GEF *dizzy* as an essential gene for cell shape regulation and cell migration of macrophages in *Drosophila* embryos. On the cellular level, Dizzy activity appears to contribute to the stabilization of cellular protrusions such as the cell tail. We showed that it requires the function of the small GTPase Rap1 and provided evidence that Dizzy behaves like a GEF for Rap1 during macrophage migration in vivo. Furthermore, the activities of Dizzy and Rap1 require the function of βPS integrins to induce cell shape changes. These data provide the first evidence of a pathway via a PDZ-GEF and Rap1 that regulates integrin-dependent activity during embryogenesis and expands the current picture of the migration of macrophages in the *Drosophila* embryo (Brückner et al., 2004; Cho et al., 2002; Stramer et al., 2005; Tepass et al., 1994).

The PDZ-GEF Dizzy is essential for proper migration and cell shape of macrophages

We characterized the *dizzy* locus and found that lack of *dizzy* function results in a relatively subtle phenotype in the embryo: the macrophages had cellular protrusions about half the size of normal cells in fixed preparations and did not reach their destination in the central region of the embryo in time (Figs 2, 3). Surprisingly, the difference in the size of the protrusions was not obvious in live *dizzy* macrophages. We assume that the difference in fixed preparations is caused by a reduced stability of protrusions in *dizzy* mutants that leads to a diminished preservation during fixation. Also the measurement of the speed of individual *dizzy* macrophages showed no significant reduction in their average speed during stages 12 and 13. However, due to the stochastic behavior of individual cells during migration it is difficult to assess in live embryos whether the cells commence their migration too late or whether they stop prematurely. The observed phenotypes in macrophage shape and migration are due to the lack of *dizzy* function, as they are seen in embryos homozygous or hemizygous for *dizzy* or *dizzy* alleles, i.e. they are independent of the genetic background. Moreover, the revertant
Dizzy acts via Rap1

Experiments in mammalian cell culture demonstrated that PDZ-GEFs specifically activate the small GTPases Rap1 and Rap2 (de Rootj et al., 1999; Gao et al., 2001; Kuiperij et al., 2003; Liao et al., 2001; Rebhun et al., 2000). Also recent reports from Caenorhabditis and Drosophila place PDZ-GEFs upstream of Rap1 (Lee et al., 2002; Pellis-van Berkel et al., 2005; Wang et al., 2006). Our data suggest that the PDZ-GEF Dizzy is a GEF for the small GTPase Rap1 in Drosophila macrophages: the level of Dizzy expression is crucial (Figs 2-4), the cell shape changes induced by high levels of Dizzy in macrophages are Rap1-dependent (Fig. 6), and high levels of Dizzy rescue the phenotype of dominant-negative Rap1N17 (Fig. 7). We assume that in migrating macrophages most, if not all, of the activity of Dizzy goes through Rap1 for two reasons: (1) the appearance of macrophages of Dizzy overexpressing embryos and of embryos overexpressing dominant-active Rap1V12 were very similar (Figs 3, 4, 6); (2) overexpression of Dizzy was effective only in rap1+ embryos (Fig. 6). The lack-of-function phenotypes of dizzy and rap1 were quantitatively different: rap1 mutants had a far weaker macrophage migration defect than dizzy mutant embryos because of the compensation by maternal contribution. However, dominant-negative Rap1N17 caused a strong migrational phenotype (Fig. 7), supporting our notion that Dizzy and Rap1 act in the same pathway.

Does Dizzy modulate integrin activity via Rap1?

The cell shape changes induced by Dizzy and Rap1 in macrophages are strictly dependent on the function of βPS integrins (Fig. 8). Already in mys mutants that solely lack the zygotic contribution of βPS integrin, neither Dizzy nor dominant-active Rap1V12 were able to trigger the formation of long cellular protrusions. Thus, the maternal contribution of mys in these embryos is insufficient to allow the Dizzy or Rap1 gain-of-function phenotype in the macrophages.
although this contribution suffices for normal shape and migrational behavior of the cells. Apparently, the dosage of integrins is decisive in what Dizzy/Rap1 execute in the cell. Therefore, it is reasonable to assume that the stabilization of the protrusions is achieved by a positive modulation of cell adhesion.

There are two possible roles for integrins in the Dizzy/Rap1-dependent stabilization of cellular protrusions: (1) integrins are the relevant targets of the pathway, and a modulation of their activity is responsible for the cell shape changes; (2) the prime target is another cellular component, and integrins are merely required to allow the stabilization. We favor the former hypothesis, as several lines of evidence from cell culture experiments suggest that Rap1 acts in such a fashion and regulates integrin activity via an ‘inside-out’ signaling pathway (Bos et al., 2003; Sebzda et al., 2002; Toyohama et al., 2003). Components of that pathway might be Rap1 effectors that provide the link between Rap1 and integrins. Two such effectors, RAPL and RIAM, have been described in vertebrates (Katagiri et al., 2003; Lafuente et al., 2004), but the function of the corresponding Drosophila orthologs are not yet known.

How do Dizzy/Rap1 act on integrins in migrating macrophages?

Above we proposed that Dizzy exerts a positive influence via Rap1 on the activity of integrins in Drosophila macrophages. This influence could be an immediate activation of integrins, but the nature of that activation is unclear. The level of expression or the influence could be an immediate activation of integrins, but the level of phosphorylated focal adhesion kinase was unchanged (pFak Tyr397; data not shown). The phosphorylation of Fak is dependent on integrin-mediated adhesion and therefore often used as a marker for integrin activity (for a review, see Mitra et al., 2005). Alternatively, the positive influence of Dizzy/Rap1 on integrin activity might be due to a repression of de-adhesion of integrins. This could be achieved by interaction with the small GTPase RhoA, which in monocytes is required for the retraction of the cell tail and appears to act by delimiting the adhesion to integrin ligands and by keeping the local levels of integrins low (Worthylake et al., 2001). However, initial experiments do not indicate a clear genetic interaction between RhoA and Dizzy in Drosophila during the migration of macrophages.

Whether as immediate activation or as repression of de-adhesion, the signaling via Dizzy/Rap1 to integrins appears to be permissive rather than instructive in nature for the guided motility of Drosophila macrophages. We have no indication that the directionality of the migration is changed upon overexpression of Dizzy. The additional protrusions are not an indication of perturbed polarity, nor do they represent multiple leading edges. Also, macrophages in dicky loss-of-function mutants appear to have normal polarity and migrate along the normal paths. They do not reach the central part of the embryo in time, but behave otherwise normally. Therefore, we conclude that the Dizzy/Rap1 pathway does not contribute to its directional action, but makes the migratory machinery of the cell efficient.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/15/2915/DC1

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