

# 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  $\alpha$  and one  $\beta$  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  $\beta$ PS 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

*dizzy<sup>EP</sup>*, synonyms EP(2)388, *pdz-gef<sup>2</sup>* (Lee et al., 2002); *dizzy<sup>P</sup>*, synonyms l(2)k13720, *pdz-gef<sup>1</sup>* (Lee et al., 2002); *Df(2L)BSC5*, 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-gfp<sup>S65T</sup>* (Bloomington #1521, #1522); *UAS-actin::gfp* (Verkhusha et al., 1999); *UAS-rap1<sup>WT</sup>*, *UAS-rap1<sup>V12</sup>*, *UAS-rap1<sup>N17</sup>* (Boettner et al., 2003); *rap1<sup>P5709</sup>* (Knox and Brown, 2002); *UAS-rho<sup>N19</sup>* (Strutt et al., 1997); *UAS-Rac<sup>N17</sup>*, *UAS-cdc42<sup>N17</sup>* (Luo et al., 1994); *UAS-p35* (Hay et al., 1994); *mys<sup>XG43</sup>* (Bunch et al., 1992);  $\Delta 2-3$  *Ki* (Robertson et al., 1988). *dizzy<sup>\Delta 1</sup>/CyO,actGFP;srph-gal4 UAS-gfp<sup>S65T</sup>, UAS-gfp<sup>S65T</sup>;srph-gal4 UAS-gfp<sup>S65T</sup>* and *dizzy<sup>EP</sup>/UAS-gfp<sup>S65T</sup>;srph-gal4 UAS-gfp<sup>S65T</sup>/+*, 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 *dizzy<sup>EP</sup>* was mobilized by crossing-in  $\Delta 2-3$ , and the F<sub>2</sub> generation was scored for semi-lethality in trans to *Df(2L)BSC5*. In total, 11 lines were established with strong eye and wing phenotypes of adult escapers; seven were used for this study: *dizzy<sup>\Delta 1</sup>* and *dizzy<sup>\Delta 5</sup>* remove 174 and 1187 bp upstream of the former insertion site, *dizzy<sup>\Delta 7</sup>* and *dizzy<sup>\Delta 8</sup>* 2253 bp and 2632 bp around the site, and *dizzy<sup>\Delta 3</sup>*, *dizzy<sup>\Delta 10</sup>* and *dizzy<sup>\Delta 12</sup>* 2328, 2380 and 2969 bp downstream only. One revertant (*dizzy<sup>P-RV4</sup>*), obtained upon mobilization of the *P*-element of *dizzy<sup>P</sup>*, 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 TGTTCAAGTTGCGGGTACCGCGT. 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 pHBS and subsequently cloned in normal and in reverse orientation, separated by the *Hairless* intron, in pUAST (Brand and Perrimon, 1993). This construct was introduced in the *Drosophila* germline by *P*-element-mediated transformation and gave several transgenic lines (*UAS-ds.dizzy*). Two of these were chosen, which led to lethality when ubiquitously expressed under control of *da-gal4*.

### Crosses to assess the role of integrin $\beta$ PS *mys*

To examine the role of integrins in cell shape changes induced by *dizzy<sup>EP</sup>* or by *Rap1<sup>V12</sup>*, we crossed *mys<sup>XG43</sup>/FM7<sup>fz::lacZ</sup>;+;srph>cd2* females with *w/Y;dizzy<sup>EP</sup>;+* or with *w/Y;UAS-rap1<sup>V12</sup>;UAS-rap1<sup>V12</sup>/TM6B* males. Immunostaining with anti-CD2 antibodies visualized the morphology of the macrophages. With progeny carrying *FM7<sup>fz::lacZ</sup>*, all embryos gave the cell shape phenotype, but that phenotype was only visible in about 50% of embryos lacking *FM7<sup>fz::lacZ</sup>*.

### Immunohistochemistry and in-situ hybridization

Embryos for histochemistry were fixed and immunostained according to standard procedures. Primary antibodies: mouse  $\alpha$ -CD2 (Serotec), rabbit  $\alpha$ - $\beta$ Gal (Cappel), mouse  $\alpha$ - $\beta$ Gal (Promega), rabbit  $\alpha$ -pFak<sup>Tyr397</sup> (Biosource), mouse  $\alpha$ - $\beta$ PS (Brower et al., 1984) (DSHB). Secondary antibodies: labeled with Alexa 488, Alexa 555 (Molecular Probes) or biotin (Jackson Labs). The signal of the biotinylated antibodies was enhanced using Vectastain 'Elite' and detected either with DAB or with the TSA technique (PerkinElmer). Embryos stained with fluorescent dyes were counterstained with DAPI, mounted in Vectashield and documented with a Leica SP2 confocal system

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 (Jenoptik).

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 RH54455, for *Pez* (CG9493) from EST RE59091 and for *Cpr* (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 *xyt*-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 *dizzy<sup>EP</sup>*, *dizzy<sup>P</sup>* or homozygous *dizzy<sup>\Delta n</sup>* 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 *dizzy<sup>\Delta n</sup>* alleles, locus-specific primers were designed, and PCR products were sequenced.

### Cloning of *dizzy* full-length cDNA

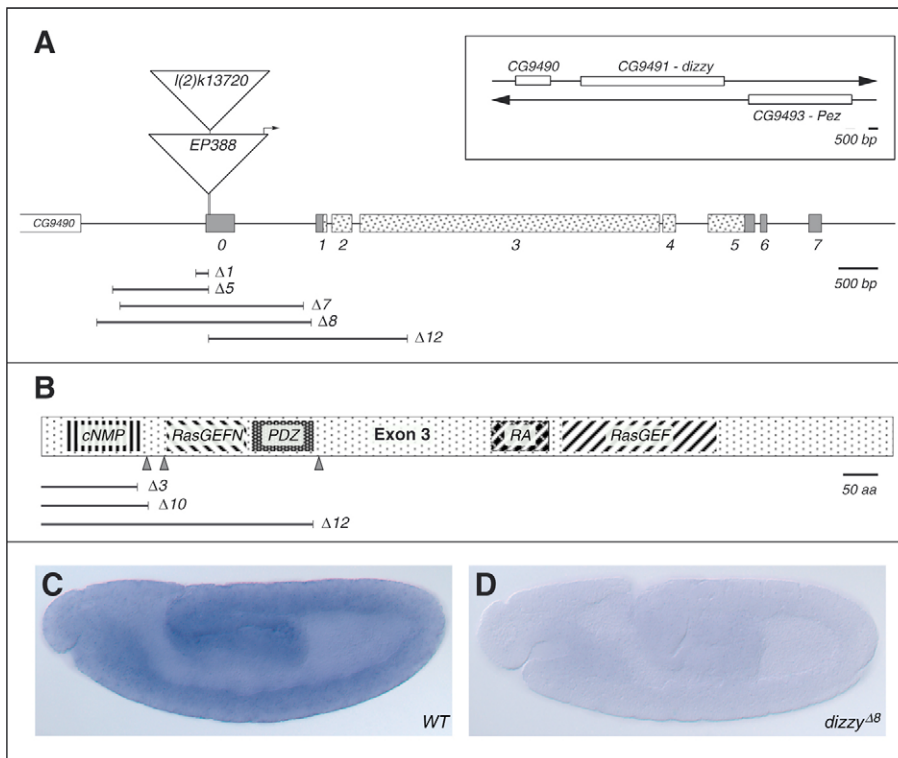
Total RNA was isolated from *w* embryos of *Drosophila*, selected for poly-A<sup>+</sup> RNA and reverse-transcribed to cDNA. Then, the 5' and 3' ends of *dizzy* cDNA were amplified using the RACE systems of Invitrogen and were subcloned in TOPO TA. Two independent clones were obtained, which had an identical 5' end. The central parts 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, *dizzy<sup>EP388</sup>* and *dizzy<sup>l(2)k13720</sup>* (abbreviated as *dizzy<sup>EP</sup>* and *dizzy<sup>P</sup>*). Strikingly, even though the insertion sites are very close (Fig. 1A), the phenotypic effects of the *P*-elements on viability are quite different: *dizzy<sup>EP</sup>* is fully viable, whereas *dizzy<sup>P</sup>* 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 *dizzy<sup>EP</sup>* and *dizzy<sup>P</sup>* phenotypes is probably due to the distinct properties of the *P*-elements. In contrast to *dizzy<sup>P</sup>*, *dizzy<sup>EP</sup>* 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 *dizzy<sup>EP</sup>* and obtained a series of small deletions in the *dizzy* locus. Seven representative deletions were used for further analysis. *dizzy<sup>\Delta 1</sup>* and *dizzy<sup>\Delta 5</sup>* removed genomic DNA upstream of the former insertion site, including the transcription start site (Fig. 1A). *dizzy<sup>\Delta 7</sup>* and *dizzy<sup>\Delta 8</sup>* deleted the entire exon 0, the transcription start site, most of the putative *dizzy* enhancer regions and almost the complete first intron (Fig. 1A). *dizzy<sup>\Delta 12</sup>* left the transcription start site intact, but removed the original translation start site and about



**Fig. 1. Genomic structure of the *dizzy* locus and embryonic *dizzy* expression.**

(A) The *dizzy* locus comprises eight exons (UTRs dark gray). The *P*-elements of *dizzy*<sup>EP</sup> and of *dizzy*<sup>P</sup> are inserted into exon 0 at positions +39 and +46, respectively. The various alleles *dizzy*<sup>Δn</sup> have been obtained by imprecise excision of *dizzy*<sup>EP</sup> and delete the transcription start site or the translation start site. The inset shows neighboring genes of *dizzy*. (B) All conserved domains of the PDZ-GEF Dizzy are encoded by exon 3: cNMP, cyclic nucleotide binding domain; RasGEFN, N-terminal Ras-GEF domain; PDZ, PDZ domain; RA, Ras association domain; RasGEF, Ras-GEF domain. The deletions of the alleles *dizzy*<sup>Δ3</sup> and *dizzy*<sup>Δ10</sup> predict the expression of truncated Dizzy proteins lacking the cNMP domain (gray triangles: predicted translation starts); *dizzy*<sup>Δ12</sup> might lead to the expression of a protein without cNMP, RasGEFN and PDZ domains. (C) *dizzy* RNA is ubiquitously expressed during embryogenesis at relatively low levels. (D) Embryos homozygous or hemizygous for one of the alleles associated with a deletion of the transcription start site (here *dizzy*<sup>Δ8</sup> homozygous) show no expression: the signal is indistinguishable from a signal obtained by the sense probe (not shown).

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*<sup>Δ3</sup> and *dizzy*<sup>Δ10</sup> 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).

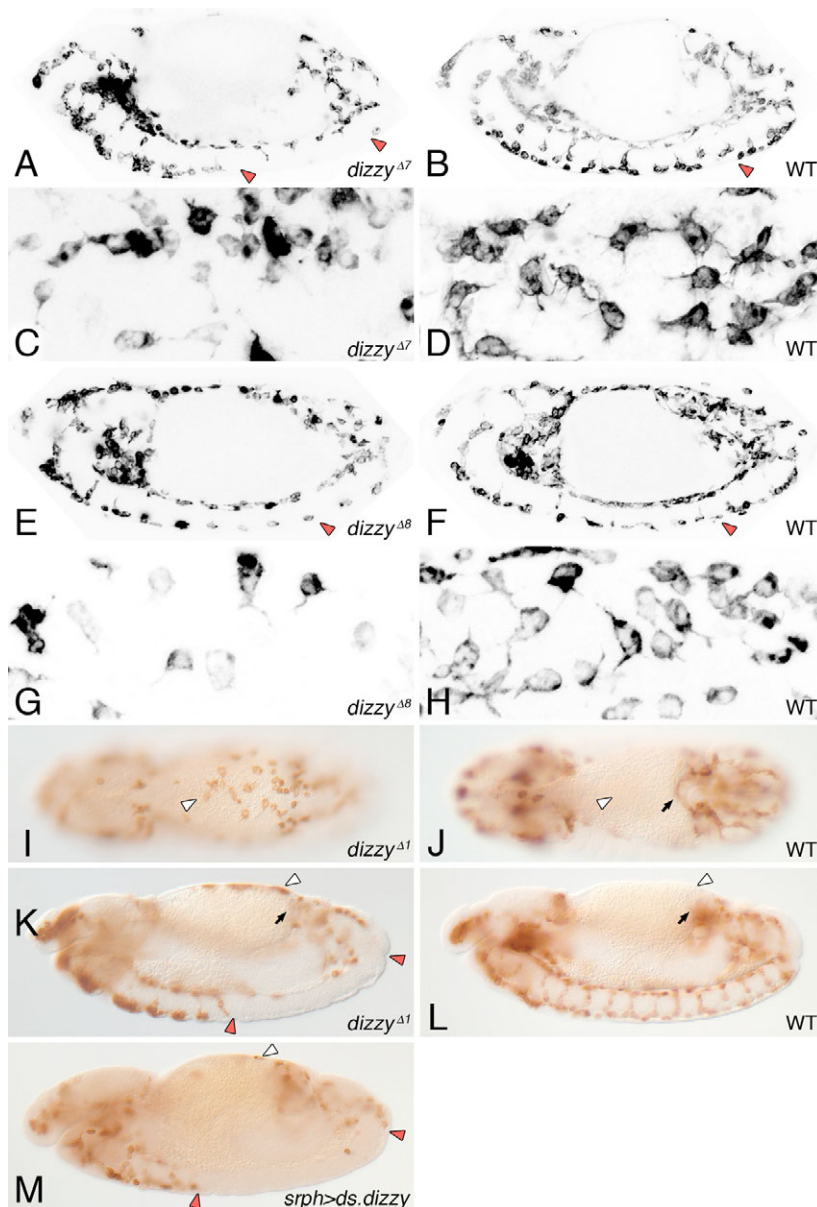
### ***dizzy* is required for cell shape and cell migration of embryonic macrophages**

Next we tested the function of *dizzy* for macrophage migration in embryos either homozygous for any of the *dizzy*<sup>Δn</sup> alleles, hemizygous for *dizzy*<sup>Δn</sup>, or trans-heterozygous for *dizzy*<sup>Δn</sup> and *dizzy*<sup>P</sup>. 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. 2I,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*<sup>Δn</sup> 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.



**Fig. 2. *dizzzy* is required for proper cell migration and cell shape of embryonic macrophages.** (A,C,E,G) Hemizygous *dizzzy*<sup>Δ7</sup> mutant embryos; (I,K) homozygous *dizzzy*<sup>Δ1</sup> mutant embryos; (B,D,F,H,J,L) embryos, wild-type for *dizzzy*; (M) embryo carrying *srph-Gal4 UAS-ds.dizzzy*. (A,E,I,K) Mutations in *dizzzy* affect the migration of macrophages. (A) At stage 14, the posterior part of the ventral nerve cord (VNC) lacks macrophages in *dizzzy* mutants (between red arrowheads), whereas in wild-type embryos (B), the VNC is completely surrounded by macrophages. (E) In the *dizzzy* mutants the gap eventually disappears toward stage 15. (F) Wild-type embryos have an even distribution of macrophages around the VNC at this stage. (C,D,G,H) In addition, *dizzzy* mutant macrophages form smaller protrusions than wild-type cells, here shown for macrophages migrating along the dorsal edge of the epidermis at stage 14 (C,D) and at lateral position at stage 15 (G,H). At this stage there are fewer macrophages in lateral positions in mutant embryos (G) than in wild-type (H). (I,K) In *dizzzy* mutants, macrophages become trapped beneath the amnioserosa (white arrowhead, dorsal view). The hindgut primordium is devoid of macrophages (arrow, I, dorsal view; K, sagittal view). (J,L) In wild-type embryos, macrophages effectively enter the posterior end and leave the space between amnioserosa and yolk. A fraction of them surrounds the developing hindgut (arrow, J, dorsal view; L, sagittal view). (M) *dizzzy* function is required in macrophages for proper cell migration. In embryos, expressing a fragment of *dizzzy* dsRNA in macrophages, these cells do not populate the posterior nervous system at stage 14 and leave a gap (between red arrowheads), which is not entirely closed later. Like in the mutants, macrophages become trapped between yolk and amnioserosa (white arrowhead). Embryos with *srph>cd2* and stained for CD2 to visualize macrophages; anterior to the left and dorsal up. For A-H, each picture consists of several merged confocal images representing a slice of about 12 μm thickness.

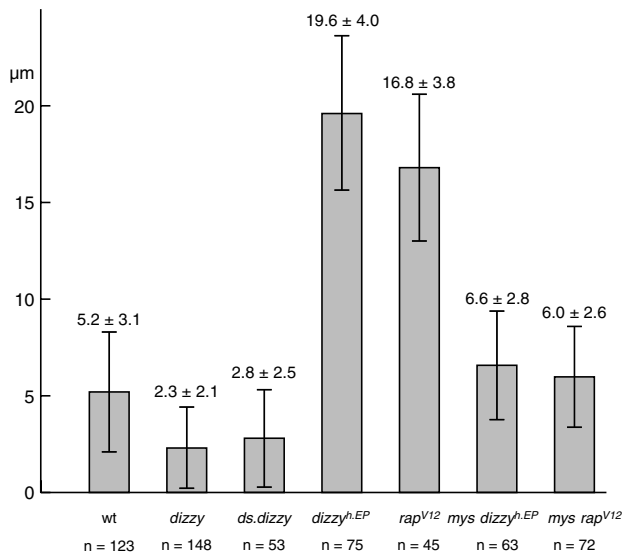
### Dizzzy is sufficient to change the cell form of macrophages

Next we wondered how crucial *dizzzy* activity is for cell shape and cell migration, and we specifically overexpressed *dizzzy* in macrophages (Fig. 4). For this purpose we used the EP-allele *dizzzy*<sup>EP</sup> and directed the expression by *srph-gal4* (termed *dizzzy*<sup>h,EP</sup> hereafter). The overexpression seen in *dizzzy*<sup>h,EP</sup> was confined to the *dizzzy* transcription unit: genes neighboring *dizzzy*, such as *Pez* or *Cpr*, were not influenced by the *dizzzy*<sup>EP</sup> allele. Macrophages in *dizzzy*<sup>h,EP</sup> embryos commenced their migration normally (Fig. 4A,B) but then formed very long protrusions, in the range of 20 μm 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 *dizzzy* was overexpressed from one copy of *dizzzy*<sup>EP</sup>, 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 μm per minute (Fig. 5; see Movies 1-4 in the supplementary material). However, when the level of *dizzzy* expression was increased further, by expression from two copies of *dizzzy*<sup>EP</sup>, 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 *dizzzy* level is of crucial importance for regulation of cell shape and migration of macrophages.

### Dizzzy assists in stabilization of cellular protrusions during the migrational cycle of macrophages

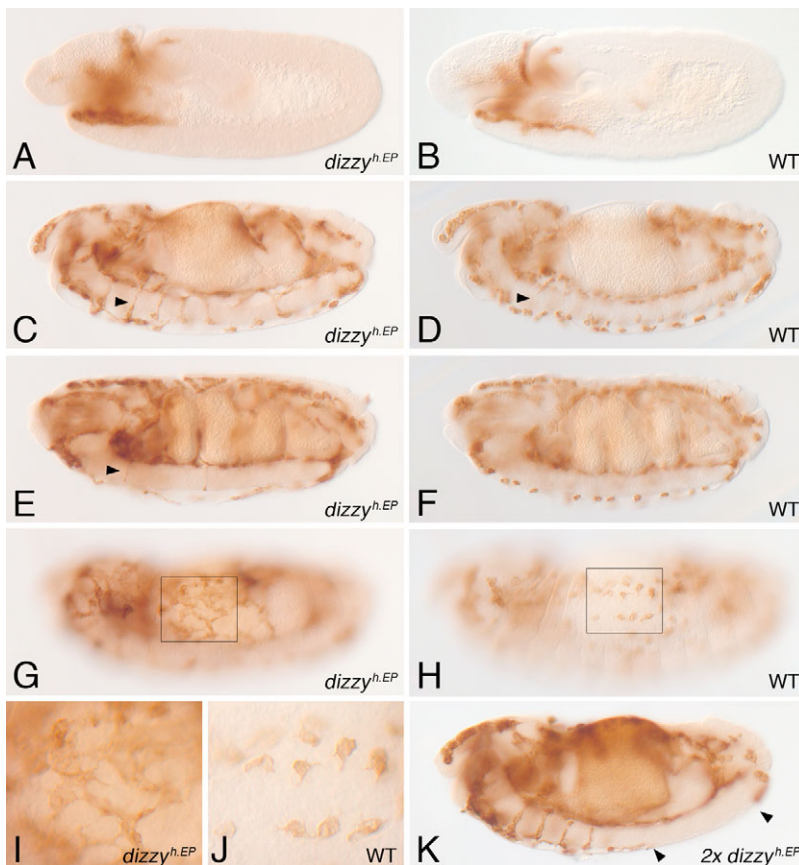
Using time-lapse video microscopy, we intended to assess the origin of the large protrusions of *dizzzy*<sup>h,EP</sup> embryos. Live macrophages are much bigger than expected from their appearance



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

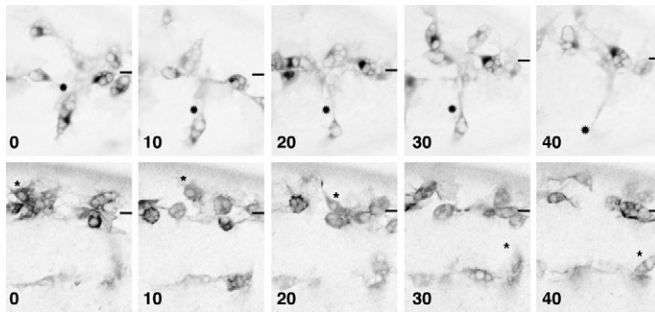
**Fig. 3. The length of the cellular protrusions of macrophages depends on *dizzy*.** Cellular protrusions per macrophage have been measured for the given genotypes in fixed and immunostained embryos beneath the dorsolateral epidermis at stage 14. In *dizzy* mutants and in embryos expressing *ds.dizzy* 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 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  $\beta$ PS 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.

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. 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 dorsolateral 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.



**Fig. 5. Macrophages overexpressing *dizzo* leave long stable protrusions behind during their migration.** 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) *dizzo*<sup>h,EP</sup> 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  $\mu$ m in width.

### Dizzo acts via Rap1

The phenotypes seen in macrophages of *dizzo* mutants, of *ds.dizzo* embryos and of embryos overexpressing *dizzo* suggest that Dizzo modulates adhesive properties of macrophages during their migration, and that the cell shape changes of macrophages reflect this modulation. As *dizzo* is predicted to be a GEF, based on its protein structure, we investigated by genetic means whether *dizzo* 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 Dizzo in macrophages. When we overexpressed *dizzo* in macrophages of zygotic *rap1* mutants, these macrophages showed a substantial rescue of the cell shape changes induced by Dizzo (Fig. 6F,G). This rescue effect became even more evident in *rap1* mutants that expressed *dizzo* from two copies of *dizzo*<sup>EP</sup> in macrophages. While macrophages in these embryos looked similar to wild-type macrophages (Fig. 6I), the macrophages in *rap1*<sup>+</sup> embryos expressing *dizzo* to the same extent formed a dense net of cells beneath the dorsal epidermis at the time of dorsal closure and in other regions of the embryo (Fig. 6H). This supports the argument that Dizzo acts upstream of Rap1. If Dizzo and Rap1 act in the same pathway, Rap1 overactivity should lead to a similar phenotype to the overactivity of Dizzo. To test this we expressed dominant-active *rap1*<sup>V12</sup> in macrophages. This expression led to significantly enlarged cellular protrusions (Fig. 3) and to a dense net of cells along the dorsal edge of the epidermis (Fig. 6J), very much like overexpression of *dizzo* from two copies of *dizzo*<sup>EP</sup> (Fig. 6H). Thus, Dizzo and Rap1 appear to act in the same pathway. The amount of Rap1 was not the limiting factor in this respect, as overexpression of wild-type *rap1* did not affect migrating macrophages (Fig. 6K).

If Rap1 acts downstream of Dizzo and is the relevant target for Dizzo activity, the lack of Rap1 function should give the same phenotype as the lack of Dizzo. This hypothesis is not easily tested, because Rap1 has a strong maternal contribution (Asha et al., 1999). Zygotic *rap1* mutants had only a very subtle and transient macrophage migration phenotype (Fig. 6B), far weaker than the

phenotype caused by the amorphic *dizzo* alleles. An influence on the size of the cellular protrusions was not detectable. After germ band retraction, the migration along the midline of the VNC was slightly disturbed, resulting in a small area that was free of macrophages at stage 14 (Fig. 6B). This defect was compensated during later development. Other aspects of migration, such as the migration along the dorsal edge of the epidermis, were not noticeably affected in zygotic *rap1* mutants. By contrast, embryos lacking both maternal and zygotic *rap1* contribution are severely affected in all aspects of morphogenesis, including gastrulation (Asha et al., 1999), making it impossible to judge the specific function of *rap1* for macrophage migration. We therefore used the dominant-negative Rap1<sup>N17</sup> to test macrophage-specific function of *rap1*. In embryos expressing Rap1<sup>N17</sup> in macrophages, the migration was severely disrupted. Few macrophages reached the posterior region of the embryo, and a major gap remained in their distribution (Fig. 7A). Additionally, the cells were smaller and had smaller extensions than wild-type cells, similar to *dizzo* mutant macrophages (Fig. 7C). Therefore, *rap1* is unambiguously essential for proper migration of macrophages and has a similar influence on cell shape to *dizzo*.

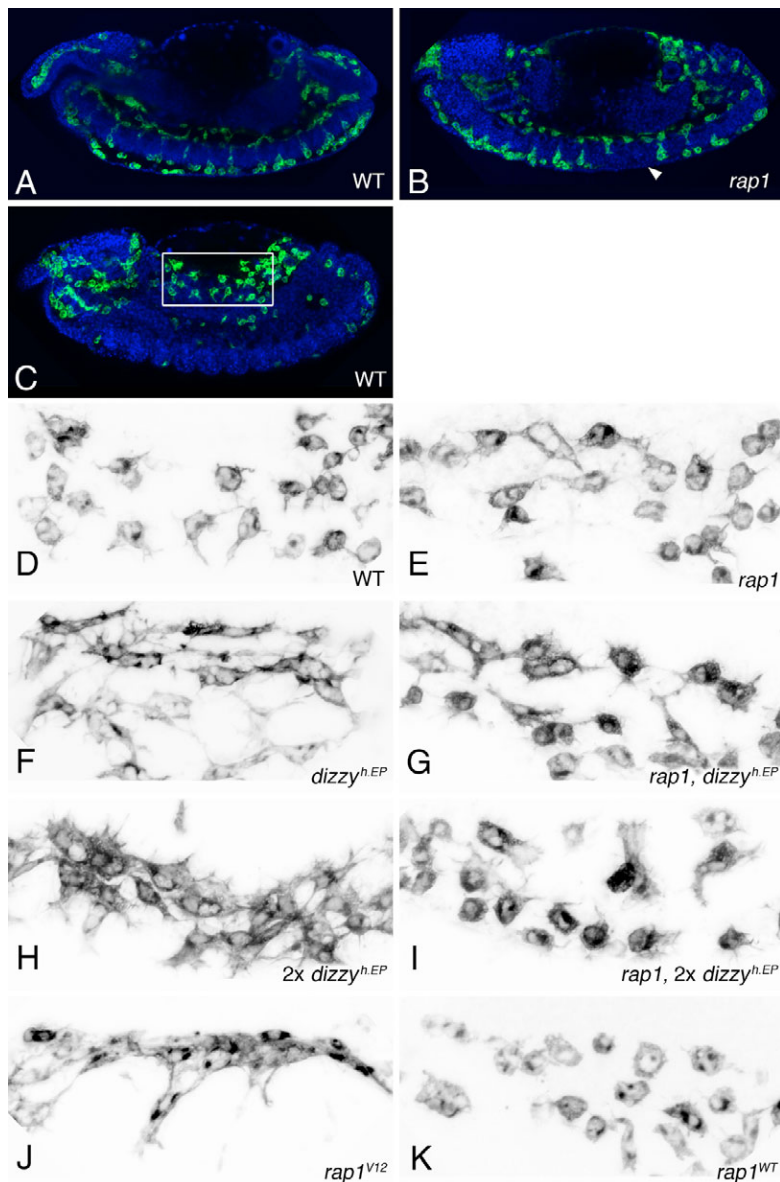
Next we wanted to know whether Dizzo acts *in vivo* like a GEF for Rap1. To test this we simultaneously expressed Dizzo and Rap1<sup>N17</sup> in migrating macrophages. Rap1<sup>N17</sup> is thought to act as a dominant-negative form of Rap1 by sequestering the limited amount of activating GEF. If Dizzo acts as a GEF for Rap1, the simultaneous expression should rescue the *rap1*<sup>N17</sup> phenotype. Indeed, while Rap1<sup>N17</sup> on its own strongly disrupted the migration of macrophages, the simultaneous expression of Dizzo and Rap1<sup>N17</sup> substantially rescued the phenotype of Rap1<sup>N17</sup>. Only a minimal gap was seen in the distribution of the macrophages around the VNC at stage 14, and the cells formed protrusions larger than normal (Fig. 7B,D).

Taken together, these data reveal a novel function of Rap1 in macrophages during migration. They show that active Rap1 is able to provoke cell shape changes in macrophages in a similar way to Dizzo. We have also demonstrated that Dizzo activity requires Rap1 function and have provided evidence that Dizzo is either itself a GEF for Rap1 or activates one.

### Dizzo activity requires integrin function

Dizzo 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  $\beta$ PS integrins are required for the cell shapes induced by Dizzo activity. *mys* mutants solely lacking the zygotic  $\beta$ PS 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, *dizzo* 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 Dizzo requires the full function of  $\beta$ PS and indicates that integrins act downstream of Dizzo.

Our results above suggest that Dizzo acts via Rap1 to induce the changes of cell shape in macrophages. One therefore would expect that Rap1 stands between Dizzo and the integrins in the genetic hierarchy and that the changes in cell morphology caused by dominant-active Rap1<sup>V12</sup> also depend on integrin function. Indeed, in zygotic *mys* mutants Rap1<sup>V12</sup> overexpression does not suffice in enlarging the protrusions (Fig. 3) or changing the cell shape of macrophages (Fig. 8F) as it does in *mys*<sup>+</sup> embryos (Fig. 3, Fig. 8E).



**Fig. 6. Dizzy acts via Rap1 during the migration of macrophages.** (A,B) The migration of macrophages is only slightly disturbed in zygotic *rap1* mutant embryos. (A) In wild-type embryos, the macrophages are already found in the entire midline after stage 13. (B) The macrophages of zygotic, homozygous *rap1* mutants fail to completely surround the midline of the VNC at stage 14 (arrowhead). (C) Confocal section of an embryo, highlighting the dorsal edge as shown in D-K. (D-K) Macrophages migrating along the dorsal edge. (D,E) Macrophages are not significantly affected in zygotic *rap1* mutants. (F,G) Dizzy fails to induce cell shape changes in a *rap1* mutant background. (H,I) Similarly, the even stronger cell shape phenotype induced by overexpression from two copies of *dizzy*<sup>hEP</sup> is rescued in *rap1* mutants. (J) The expression of dominant-active Rap1<sup>V12</sup> induces a phenotype similar to *2xdizzy*<sup>hEP</sup>. (K) Overexpression of wild-type Rap1 fails to induce cell shape changes in migrating macrophages. Embryos at stage 14.

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  $\beta$ PS integrins.

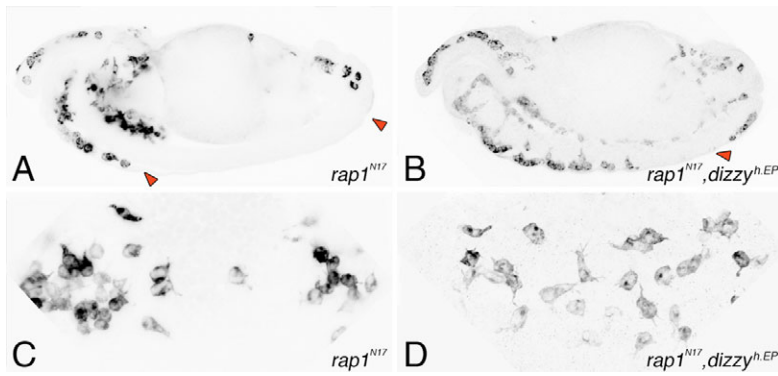
## DISCUSSION

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  $\beta$ PS 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*<sup>P</sup> or *dizzy*<sup>Δn</sup> alleles, i.e. they are independent of the genetic background. Moreover, the revertant



**Fig. 7. Dizzy behaves like a Rap1-GEF in vivo.**

Embryos solely expressing dominant-negative  $rap1^{N17}$  (A,C) compared with embryos simultaneously expressing  $rap1^{N17}$  and  $dizzy^{EP}$  (B,D) in macrophages. (A) The expression of  $rap1^{N17}$  severely disrupts the migration of macrophages along the VNC. (B) This effect of  $rap1^{N17}$  is rescued by the simultaneous expression of  $dizzy$ , presumably by substituting for GEFs sequestered by Rap1<sup>N17</sup>. (C,D) Similarly, the effect of Rap1<sup>N17</sup> on macrophages migrating along the dorsal edge (C) is rescued by the coexpression of  $dizzy$  (D). A,B: sagittal optical sections; C,D: magnifications of similar areas as indicated by the rectangle in Fig. 6C.

$dizzy^{P-RV4}$ , which had lost the *P*-element insertion, no longer showed the phenotypes. Furthermore, the dsRNA-induced reduction of *dizzy* function specifically in the macrophages led to the same phenotypes. Here the extent is more variable than in mutants, presumably due to the intrinsic inhomogeneity of the UAS-Gal4 system. In any case, we have no indication that inappropriate apoptosis contributes to the phenotypes. The number of macrophages is normal in the mutants throughout embryogenesis. A rescue by the expression of the pancaspase inhibitor p35 is not observed (data not shown).

Overexpression of *dizzy* in the macrophages led to effects on cell shape complementary to the *dizzy* loss of function. The protrusion length per cell was increased by a factor of four, and more protrusions per cell were visible. In live cells we saw extraordinarily long, stable extensions, which apparently connected the macrophages with each other. These protrusions were formed in addition to normal tails and leading edges, resulting in the increase of protrusion number per cell seen in fixed preparations. They seemed to originate from lamellopodia, which were incompletely retracted or often from cell tails that failed to retract properly. We propose that Dizzy normally stabilizes the protrusions, explaining the smaller protrusions in fixed preparations of *dizzy* mutants. When overexpressed, *dizzy* might inhibit the proper disassembly of cellular protrusions, explaining their vast enlargement and persistence. Whether Dizzy acts in a particular subcellular location, e.g. tail versus leading edge, remains to be seen.

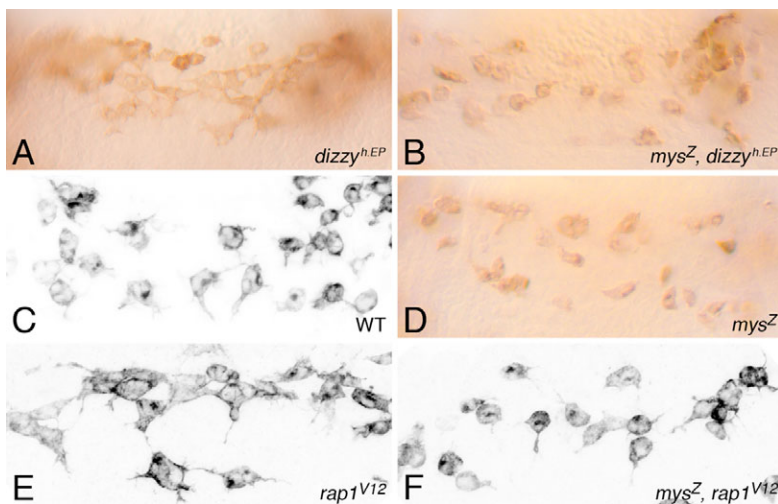
### Dizzy acts via Rap1

Experiments in mammalian cell culture demonstrated that PDZ-GEFs specifically activate the small GTPases Rap1 and Rap2 (de Rooij 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 Rap1<sup>N17</sup> (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 Rap1<sup>V12</sup> 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 Rap1<sup>N17</sup> 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  $\beta$ PS integrins (Fig. 8). Already in *mys* mutants that solely lack the zygotic contribution of  $\beta$ PS integrin, neither Dizzy nor dominant-active Rap1<sup>V12</sup> 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,



**Fig. 8. Dizzy- and Rap1-induced cell adhesion require integrin function.**

Dizzy (A) and Rap1<sup>V12</sup> (E) can induce similarly strong cell shape changes in macrophages. (B,F) These changes depend on the function of  $\beta$ PS integrins, as both Dizzy (B) and Rap1<sup>V12</sup> (F) fail to show the effect in a *mys* mutant background. (C,D) Macrophages at the dorsal edge of a wild-type (C) and a *mys* mutant (D) embryo. The latter (lacking only the zygotic *mys* contribution) is indistinguishable from wild type concerning macrophage shape and motility. Embryos at stage 14; field of view as in Fig. 6D-K.



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; Tohyama 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 spatial distribution of  $\beta$ PS integrin is not obviously changed in macrophages overexpressing Dizzy or Rap1<sup>V12</sup> compared with wild-type cells. We observed neither clustering nor elevated protein expression of integrins. Likewise, the level of phosphorylated focal adhesion kinase was unchanged (pFak<sup>Tyr397</sup>, 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 *dizzy* 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>

### References

- Asha, H., de Ruiter, N. D., Wang, M. G. and Hariharan, I. K. (1999). The Rap1 GTPase functions as a regulator of morphogenesis in vivo. *EMBO J.* **18**, 605-615.
- Boettner, B., Harjes, P., Ishimaru, S., Heke, M., Fan, H. Q., Qin, Y., Van Aelst, L. and Gaul, U. (2003). The AF-6 homolog Canoe acts as a Rap1 effector during dorsal closure of the *Drosophila* embryo. *Genetics* **165**, 159-169.
- Bökel, C. and Brown, N. H. (2002). Integrins in development: moving on, responding to, and sticking to the extracellular matrix. *Dev. Cell* **3**, 311-321.
- Bos, J. L., de Bruyn, K., Enserink, J., Kuiperij, B., Rangarajan, S., Rehmann, H., Riedl, J., de Rooij, J., van Mansfeld, F. and Zwartkruis, F. (2003). The role of Rap1 in integrin-mediated cell adhesion. *Biochem. Soc. Trans.* **31**, 83-86.
- Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
- Brower, D. L., Wilcox, M., Piovant, M., Smith, R. J. and Reger, L. A. (1984). Related cell-surface antigens expressed with positional specificity in *Drosophila* imaginal discs. *Proc. Natl. Acad. Sci. USA* **81**, 7485-7489.
- Brückner, K., Kockel, L., Duchek, P., Luque, C. M., Rorth, P. and Perrimon, N. (2004). The PDGFR/EGF receptor controls blood cell survival in *Drosophila*. *Dev. Cell* **7**, 73-84.
- Bunch, T. A., Salatino, R., Engelsjerd, M. C., Mukai, L., West, R. F. and Brower, D. L. (1992). Characterization of mutant alleles of myospheroid, the gene encoding the beta subunit of the *Drosophila* PS integrins. *Genetics* **132**, 519-528.
- Caron, E., Self, A. J. and Hall, A. (2000). The GTPase Rap1 controls functional activation of macrophage integrin  $\alpha$ M $\beta$ 2 by LPS and other inflammatory mediators. *Curr. Biol.* **10**, 974-978.
- Cho, N. K., Keyes, L., Johnson, E., Heller, J., Ryner, L., Karim, F. and Krasnow, M. A. (2002). Developmental control of blood cell migration by the *Drosophila* VEGF pathway. *Cell* **108**, 865-876.
- de Rooij, J., Boenink, N. M., van Triest, M., Cool, R. H., Wittinghofer, A. and Bos, J. L. (1999). PDZ-GEF1, a guanine nucleotide exchange factor specific for Rap1 and Rap2. *J. Biol. Chem.* **274**, 38125-38130.
- Dunin-Borkowski, O. M., Brown, N. H. and Bate, M. (1995). Anterior-posterior subdivision and the diversification of the mesoderm in *Drosophila*. *Development* **121**, 4183-4193.
- Friedl, P. and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat. Rev. Cancer* **3**, 362-374.
- Gao, X., Satoh, T., Liao, Y., Song, C., Hu, C. D., Kariya Ki, K. and Kataoka, T. (2001). Identification and characterization of RA-GEF-2, a Rap guanine nucleotide exchange factor that serves as a downstream target of M-Ras. *J. Biol. Chem.* **276**, 42219-42225.
- Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* **120**, 2121-2129.
- Hinz, U., Giebel, B. and Campos-Ortega, J. A. (1994). The basic helix-loop-helix domain of *Drosophila* lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell* **76**, 77-87.
- Hummel, T., Schimmelpfeng, K. and Klämbt, C. (1997). Fast and efficient egg collection and antibody staining from large numbers of *Drosophila* strains. *Dev. Genes Evol.* **207**, 131-135.
- Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.
- Katagiri, K., Hattori, M., Minato, N., Irie, S., Takatsu, K. and Kinashi, T. (2000). Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase. *Mol. Cell. Biol.* **20**, 1956-1969.
- Katagiri, K., Maeda, A., Shimonaka, M. and Kinashi, T. (2003). RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nat. Immunol.* **4**, 741-748.
- Kinbara, K., Goldfinger, L. E., Hansen, M., Chou, F. L. and Ginsberg, M. H. (2003). Ras GTPases: integrins' friends or foes? *Nat. Rev. Mol. Cell Biol.* **4**, 767-776.
- Knox, A. L. and Brown, N. H. (2002). Rap1 GTPase regulation of adherens junction positioning and cell adhesion. *Science* **295**, 1285-1288.
- Kuiperij, H. B., de Rooij, J., Rehmann, H., van Triest, M., Wittinghofer, A., Bos, J. L. and Zwartkruis, F. J. (2003). Characterisation of PDZ-GEFs, a family of guanine nucleotide exchange factors specific for Rap1 and Rap2. *Biochim. Biophys. Acta* **1593**, 141-149.
- Lafuente, E. M., van Puijenbroek, A. A., Krause, M., Carman, C. V., Freeman, G. J., Berezovskaya, A., Constantine, E., Springer, T. A., Gertler, F. B. and Boussiotis, V. A. (2004). RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. *Dev. Cell* **7**, 585-595.
- Lauffenburger, D. A. and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.
- Lee, J. H., Cho, K. S., Lee, J., Kim, D., Lee, S. B., Yoo, J., Cha, G. H. and Chung, J. (2002). *Drosophila* PDZ-GEF, a guanine nucleotide exchange factor for Rap1 GTPase, reveals a novel upstream regulatory mechanism in the mitogen-activated protein kinase signaling pathway. *Mol. Cell. Biol.* **22**, 7658-7666.
- Liao, Y., Satoh, T., Gao, X., Jin, T. G., Hu, C. D. and Kataoka, T. (2001). RA-GEF-1, a guanine nucleotide exchange factor for Rap1, is activated by

- translocation induced by association with Rap1\*GTP and enhances Rap1-dependent B-Raf activation. *J. Biol. Chem.* **276**, 28478-28483.
- Liddington, R. C. and Ginsberg, M. H.** (2002). Integrin activation takes shape. *J. Cell Biol.* **158**, 833-839.
- Luo, L. Q., Liao, Y. J., Jan, L. Y. and Jan, Y. N.** (1994). Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.* **8**, 1787-1802.
- Mitra, S. K., Hanson, D. A. and Schlaepfer, D. D.** (2005). Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* **5**, 56-68.
- Nagel, A. C., Maier, D. and Preiss, A.** (2002). Green fluorescent protein as a convenient and versatile marker for studies on functional genomics in *Drosophila*. *Dev. Genes Evol.* **212**, 93-98.
- Paladi, M. and Tepass, U.** (2004). Function of Rho GTPases in embryonic blood cell migration in *Drosophila*. *J. Cell Sci.* **117**, 6313-6326.
- Pellis-van Berkel, W., Verheijen, M. H. G., Cuppen, E., Asahina, M., de Rooij, J., Jansen, G., Plasterk, R. H. A., Bos, J. L. and Zwartkruis, F. J. T.** (2005). Requirement of the *Caenorhabditis elegans* RapGEF pxf-1 and rap-1 for epithelial integrity. *Mol. Biol. Cell* **16**, 106-116.
- Rebhun, J. F., Castro, A. F. and Quilliam, L. A.** (2000). Identification of guanine nucleotide exchange factors (GEFs) for the Rap1 GTPase. Regulation of MR-GEF by M-Ras-GTP interaction. *J. Biol. Chem.* **275**, 34901-34908.
- Reedquist, K. A., Ross, E., Koop, E. A., Wolthuis, R. M., Zwartkruis, F. J., van Kooyk, Y., Salmon, M., Buckley, C. D. and Bos, J. L.** (2000). The small GTPase, Rap1, mediates CD31-induced integrin adhesion. *J. Cell Biol.* **148**, 1151-1158.
- Ridley, A. J.** (2001). Rho family proteins: coordinating cell responses. *Trends Cell Biol.* **11**, 471-477.
- Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. and Horwitz, A. R.** (2003). Cell migration: integrating signals from front to back. *Science* **302**, 1704-1709.
- Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Benz, W. K. and Engels, W. R.** (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics* **118**, 461-470.
- Rørth, P.** (1996). A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes. *Proc. Natl. Acad. Sci. USA* **93**, 12418-12422.
- Sebzda, E., Bracke, M., Tugal, T., Hogg, N. and Cantrell, D. A.** (2002). Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. *Nat. Immunol.* **3**, 251-258.
- Stramer, B., Wood, W., Galko, M. J., Redd, M. J., Jacinto, A., Parkhurst, S. M. and Martin, P.** (2005). Live imaging of wound inflammation in *Drosophila* embryos reveals key roles for small GTPases during in vivo cell migration. *J. Cell Biol.* **168**, 567-573.
- Strutt, D. I., Weber, U. and Mlodzik, M.** (1997). The role of RhoA in tissue polarity and Frizzled signaling. *Nature* **387**, 292-295.
- Tautz, D. and Pfeifle, C.** (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.
- Tepass, U., Fessler, L. I., Aziz, A. and Hartenstein, V.** (1994). Embryonic origin of hemocytes and their relationship to cell death in *Drosophila*. *Development* **120**, 1829-1837.
- Tohyama, Y., Katagiri, K., Pardi, R., Lu, C., Springer, T. A. and Kinashi, T.** (2003). The critical cytoplasmic regions of the alphaL/beta2 integrin in Rap1-induced adhesion and migration. *Mol. Biol. Cell* **14**, 2570-2582.
- van der Flier, A. and Sonnenberg, A.** (2001). Function and interactions of integrins. *Cell Tissue Res.* **305**, 285-298.
- Verkhusha, V. V., Tsukita, S. and Oda, H.** (1999). Actin dynamics in lamellipodia of migrating border cells in the *Drosophila* ovary revealed by a GFP-actin fusion protein. *FEBS Lett.* **445**, 395-401.
- Wang, H., Singh, S. R., Zheng, Z., Oh, S. W., Chen, X., Edwards, K. and Hou, S. X.** (2006). Rap-GEF signaling controls stem cell anchoring to their niche through regulating DE-cadherin-mediated cell adhesion in the *Drosophila* testis. *Dev. Cell* **10**, 117-126.
- Worthylake, R. A., Lemoine, S., Watson, J. M. and Burridge, K.** (2001). RhoA is required for monocyte tail retraction during transendothelial migration. *J. Cell Biol.* **154**, 147-160.