The PDGF/VEGF Receptor Controls Blood Cell Survival in *Drosophila*

Katja Brückner,[2,*] Lutz Kockel,[2]
Peter Duchek,[3,4] Carlos M. Luque,[3,5]
Pernille Rørth,[3] and Norbert Perrimon[1,2]
[1]Howard Hughes Medical Institute
[2]Department of Genetics
Harvard Medical School
77 Avenue Louis Pasteur
Boston, Massachusetts 02115
[3]Developmental Biology Programme
European Molecular Biology Laboratory
Meyerhofstrasse 1
69117 Heidelberg
Germany

Summary

The *Drosophila* PDGF/VEGF receptor (PVR) has known functions in the guidance of cell migration. We now demonstrate that during embryonic hematopoiesis, PVR has a role in the control of antiapoptotic cell survival. In *Pvr* mutants, a large fraction of the embryonic hemocyte population undergoes apoptosis, and the remaining blood cells cannibalistically phagocytose their dying peers. Consequently, total hemocyte numbers drop dramatically during embryogenesis, and large aggregates of engorged macrophages carrying multiple apoptotic corpses form. Hemocyte-specific expression of the pan-caspase inhibitor p35 in *Pvr* mutants eliminates hemocyte aggregates and restores blood cell counts and morphology. Additional rescue experiments suggest involvement of the Ras pathway in PVR-mediated blood cell survival. In cell culture, we demonstrate that PVR directly controls survival of a hemocyte cell line. This function of PVR shows striking conservation with mammalian hematopoiesis and establishes *Drosophila* as a model to study hematopoietic cell survival in development and disease.

Introduction

Studies in recent years have highlighted the similarity between the *Drosophila* blood cell system and the hematopoietic system of vertebrates (Evans et al., 2003). *Drosophila* blood cells, or hemocytes, participate in innate immunity (Hoffmann and Reichhart, 2002), have important functions in the removal of apoptotic cells (Abrams et al., 1993; Franc, 2002; Franc and White, 2000; Tepass et al., 1994), and produce extracellular matrix components (Nelson et al., 1994).

During *Drosophila* development, generation of blood cells occurs at different physical locations (Evans et al., 2003), similar to the shift of hematopoietic sites during

*Correspondence: kbrueckn@genetics.med.harvard.edu
[4]Present address: Ludwig Institute for Cancer Research, 91 Riding House Street, London, W1W 7BS, United Kingdom.
[5]Present address: Parc Científic de Barcelona – IRBB, C/Josep Samitier, 1-5, E-08028 Barcelona, Spain.

vertebrate development. Two independent waves of hematopoiesis can be distinguished: First, embryonic hemocytes arise from the procephalic mesoderm, a specific region of the head mesoderm (Tepass et al., 1994), and persist into larval and adult stages (Holz et al., 2003). Second, larval hemocytes originate in the lymph glands, mesodermal structures that develop in tight association with the dorsal vessel during late embryonic stages (Lanot et al., 2001; Rizki, 1978) and that originate from a distinct subset of blood cell precursors (Holz et al., 2003). Hemocytes present in the adult derive from both populations of embryonic and larval hemocytes (Holz et al., 2003; Lanot et al., 2001). In the embryo, blood cells are distributed solely by migration, while at larval and adult stages, action of the dorsal vessel perpetuates streaming of the endolymph and dispersal of hemocytes.

Similar to the vertebrate hematopoietic system, the *Drosophila* blood cell system comprises myeloid cells, but in contrast to vertebrates lacks a lymphoid branch. At least three different blood cell lineages can be distinguished (Lanot et al., 2001; Rizki, 1978): Macrophages, or plasmatocytes, phagocytose apoptotic corpses and bacteria, and represent the majority of hemocytes both in the embryo (Tepass et al., 1994) and during later stages of development (Lanot et al., 2001). Crystal cells, which mediate melanization during encapsulation of parasites or wounding, form a minor fraction of hemocytes from embryonic stages onward (Lebestky et al., 2000). Finally, lamellocytes differentiate during encapsulation and metamorphosis, but are not present in the embryo or adult (Lanot et al., 2001; Rizki and Rizki, 1992).

Genetic analysis of *Drosophila* hematopoiesis has revealed similarities with mammalian hematopoiesis at the molecular level (Evans et al., 2003; Fossett and Schulz, 2001). Still, major questions remain to be answered. For example, while in mammalian biology trophic survival of cells inside and outside the hematopoietic system is a recurring theme (Raff et al., 1993), such pathway(s) involved in *Drosophila* embryonic and larval blood cell survival have not been identified yet.

In vertebrates, Receptor Tyrosine Kinases (RTKs) are important players that transduce trophic cues in many systems (Jacobson et al., 1997). Members of the PDGF and VEGF RTK families have been studied in numerous in vitro and cell culture systems and have been implicated in cell survival and other cellular responses such as proliferation and differentiation (Hoch and Soriano, 2003). Mouse genetics has demonstrated a requirement for these RTKs in multiple hematopoietic lineages: c-Kit in multipotent stem/progenitor cells, mast cell, erythroid, and other lineages (Ashman, 1999); VEGFR2 (KDR, Flk1) in the earliest hematopoietic/angioblastic precursors (Ferrara and Davis-Smyth, 1997); and c-Fms (CSF-1R, M-CSFR) in the monocyte-macrophage lineage (Stanley et al., 1997). Dissecting functions of these RTKs in cell survival versus proliferation is often impossible or technically challenging, as early embryonic lethality frequently interferes with the analysis (Gerber et

al., 2002; Waskow et al., 2002). Improved characterization of these in vivo functions is desirable, since hyperactivation of PDGF/VEGF receptors (PVR) is frequently associated with tumorigenesis and leukemogenesis (Scheijen and Griffin, 2002) and the underlying mechanisms are being debated (Gerber and Ferrara, 2003; Kelly and Gilliland, 2002).

Drosophila embryonic hematopoiesis offers a number of experimental advantages: (1) Blood cell proliferation is separated in time from other developmental events such as blood cell migration, differentiation, and survival. With the exception of a small number of crystal cell precursors (Lebestky et al., 2000), blood cell proliferation is largely completed by stage 11, resulting in about 600–700 blood cells per embryo. Subsequently, blood cells migrate out of the anterior area where they originated, and a large number of hemocytes differentiate into macrophages (Tepass et al., 1994). (2) *Drosophila* has a low genomic complexity. Only a single PDGF/VEGF receptor is encoded by the genome (Cho et al., 2002; Duchek et al., 2001; Heino et al., 2001), thus allowing analysis without genetic redundancy.

Previously, PVR was shown to mediate guidance of border cell migration in the *Drosophila* egg chamber (Duchek et al., 2001; McDonald et al., 2003). Likewise, initial reports implicated PVR and its ligands PVF1-3 in embryonic hemocyte migration (Cho et al., 2002; Heino et al., 2001). Here, we dissect the functions of PVR during embryonic development and investigate parallels between the *Drosophila* and vertebrate hematopoietic system. Isolating and analyzing *Pvr* mutants, we demonstrate that the predominant role of PVR in the *Drosophila* embryo is to mediate trophic survival of blood cells. Cell culture experiments with a *Drosophila* hemocyte cell line confirm direct control of cell survival by PVR.

Results

Pvr Mutant Embryos Form Macrophage Aggregates

Previously, in situ hybridizations showed high expression of *Pvr* mRNA in embryonic hemocytes (not shown and Cho et al., 2002; Heino et al., 2001). To determine which subpopulation of the embryonic hemocytes expresses PVR protein, immunohistochemical stainings for PVR and known blood cell markers were done. Beginning at stage 10 of embryonic development, PVR protein was highly expressed in a hemocyte-specific pattern. The macrophage marker Croquemort (Crq) (Franc et al., 1996) colocalized with virtually all strongly PVR-positive cells (Figure 1A), indicating that PVR is expressed in the macrophage lineage.

To isolate *Pvr* loss-of-function mutations, we conducted an EMS mutagenesis. Seven independent alleles were recovered from this screen (*Pvr*[1-7], see Supplemental Data at http://www.developmentalcell.com/cgi/content/full/7/1/73/DC1). Using in situ hybridizations of embryos and antibody staining in follicle cell clones, PVR mRNA and protein was detected with *Pvr*[4] but not with *Pvr*[1] (data not shown), suggesting that *Pvr*[1] is a complete null and that *Pvr*[4] might be a hypomorphic allele.

We analyzed the *Pvr* mutant phenotype by staining macrophages with anti-Crq antibody. By stage 12, *Pvr*[1]

homozygous embryos displayed large clusters of macrophages (Figure 1C). Embryos homozygous for *Pvr*[4] showed macrophage aggregates at later stages (Figure 1G), consistent with the hypomorphic character of this allele. In both *Pvr*[1] and *Pvr*[4], phenotypes increased in severity over time (Figures 1I and 1J). Interestingly, clustered macrophages showed intensified Crq staining, indicative of elevated phagocytic activity (Franc et al., 1999). Staining with anti-Lz antibody (Lebestky et al., 2000) revealed no obvious effect of *Pvr* loss-of-function on crystal cells (data not shown). Taken together, our results indicate that in the absence of PVR activity, embryos display defects in hemocyte distribution and bear anterior macrophage aggregates with elevated Crq expression.

Hemocyte Aggregation Is Blood Cell Autonomous

To test whether the *Pvr* mutant phenotype was blood cell autonomous, we wanted to use a GAL4 line that drives hemocyte-specific expression of UAS transgenes (Brand and Perrimon, 1993). As none of the available GAL4 lines was sufficiently specific and active at all stages of embryonic hemocyte development, we constructed *srp*HemoGAL4 based on selected regulatory regions of *serpent* (*srp*), a GATA factor essential for all embryonic blood cell development (Rehorn et al., 1996).

The expression pattern of *srp*HemoGAL4 driving UAS-EGFP was compared to stainings for Crq and the hemocyte/macrophage marker Peroxidasin (Pxn) (Abrams et al., 1993; Nelson et al., 1994). *srp*HemoGAL4 activity was highly specific for blood cells, as EGFP expression was detected in procephalic mesoderm of stage 9 embryos and hemocytes/macrophages of all embryonic stages thereafter (Figures 2A and 2B and data not shown). Double stainings with Lozenge (Lz) (Lebestky et al., 2000) showed that *srp*HemoGAL4 is also active in the crystal cell lineage (Figure 2C). Importantly, *srp*HemoGAL4 activity matches the hemocyte expression domain of PVR (Figures 2D–2E).

To determine whether the *Pvr* mutant phenotype is blood cell autonomous, we used *srp*HemoGAL4 to express PVRΔC, a dominant-negative form of PVR that lacks the entire cytoplasmic domain of the receptor. Ubiquitous expression of PVRΔC induced hemocyte aggregation as seen in *Pvr* mutants, consistent with PVRΔC dominantly interfering with endogenous PVR activity (data not shown). Hemocyte-specific disruption of PVR signaling was sufficient to mimic *Pvr* loss of function: Embryos showed the typical blood cell aggregation phenotype from stage 14 onward (Figure 2F). Expression of PVRΔC by *srp*HemoGAL4 was lethal, resembling the lethality associated with the *Pvr* mutant phenotype. These findings demonstrate that the hemocyte aggregation phenotype seen upon decrease of PVR signaling is cell autonomous and is based on an inherent blood cell defect.

Ectopically Induced Hemocyte Apoptosis Phenocopies *Pvr* Loss of Function

To determine the basis of the hemocyte defect associated with loss of *Pvr* activity, we expressed various UAS lines under control of *srp*HemoGAL4 (Supplemental Figure S1A). Blood cell aggregation was assessed by
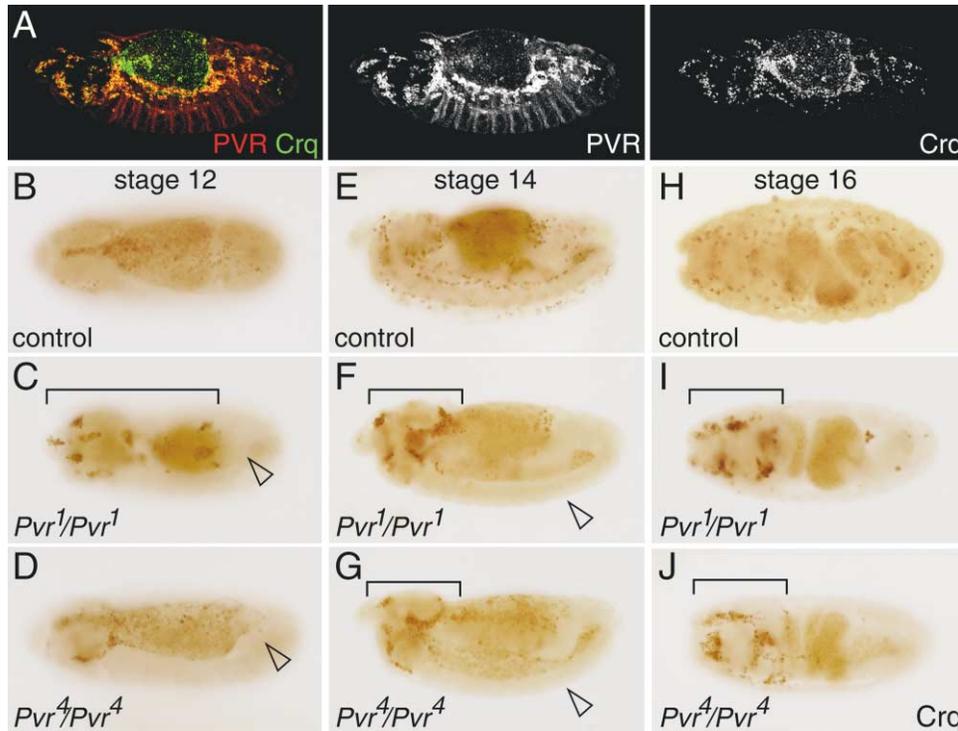
Figure 1. *Pvr* Mutants Show Macrophage Defects

(A) PVR is expressed in embryonic macrophages. Antibody staining for PVR (red) and Crq (green). PVR expression is highest in macrophages. Low-level expression is observed in the developing tracheal system (not shown) and the epidermis. PVR is not expressed in additional Crq expression domains such as the amnioserosa. Stage 14 embryo, dorsal-lateral view, anterior to the left. For technical reasons, genotype is *lz*GAL4, UAS-EGFP.

(B–J) Hemocyte distribution in wild-type and *Pvr* mutant embryos. (B–D) Stage 12 dorsal-lateral view, (E–G) stage 14 lateral view, (H–J) stage 16 dorsal view, anterior to the left. Hemocytes are visualized by anti-Crq (low-level staining of gut and amnioserosa). Central focal planes of embryos were selected. *Pvr* mutants (*Pvr1* in [C], [F], [I] and *Pvr4* in [D], [G], [J]) display large macrophage aggregates (in regions of the amnioserosa, head, and proventriculus; see brackets), while hemocytes of wild-type control (w1118) embryos (B, E, and H) are evenly dispersed. Blood cell aggregation becomes obvious around stage 12 in *Pvr1* and stage 14 in *Pvr4*. Hemocytes do not enter the posterior end (arrowhead in [C] and [D]) and appear sparse or absent in ventral-posterior areas at later stages (arrowhead in [F] and [G]).

Crq antibody staining (Figure 3, Supplemental Figure S1, and data not shown).

Previously, expression of λPVR, a constitutively active form of PVR, was shown to arrest border cell migration in female egg chambers, reflecting requirement for locally asymmetric receptor activation in border cell guidance (Duchek et al., 2001). In contrast, we found that hemocyte-specific expression of λPVR did not result in blood cell aggregation (Figure 3C) and did not affect adult viability. A low percentage of embryos showed a mild deficiency of hemocytes in the ventral-posterior region (Figure 3C) and in the posterior end of the elongated germ band (data not shown). Despite these minor irregularities in blood cell distribution, we conclude that uniform activation of PVR does not cause hemocytes to form aggregates.

Among all lines tested, we found that only the proapoptotic Head Involution Defect (HID) in its nonrepressable ala5 version (Bergmann et al., 1998) induced a macrophage aggregation phenotype similar to dominant-negative PVRΔC (Figures 3B and 3D). When HID^ala5 was expressed, blood cell clusters appeared at earlier stages, but resembled the *Pvr* loss-of-function phenotype: Hemocytes had fewer extensions, were dramatically enlarged, and carried multiple inclusions typical for

apoptotic corpses (Figures 3E–3G). These observations suggest that loss of PVR signaling may lead to an increase in blood cell death, similar to the hemocyte death induced by ectopic expression of the proapoptotic gene HID^ala5. As a consequence, dead blood cells may be phagocytosed by still viable peers, resulting in the observed increase in incorporated apoptotic corpses. This hypothesis of "cannibalistic phagocytosis" is supported by elevated levels of Crq protein (Figures 1 and 3), indicating enhanced phagocytic activity of hemocytes (Franc et al., 1999).

Pvr Mutant Macrophage Aggregates Result from Apoptotic Blood Cell Death and Cannibalistic Phagocytosis

To test our hypothesis, we sought a method to demonstrate apoptosis of hemocytes. A major function of macrophages is to phagocytose dead cells. Therefore, apoptotic stains like TUNEL or acridine orange do not distinguish between phagocytosed apoptotic corpses naturally occurring within macrophages and phagocytosed dead hemocytes themselves. We therefore chose an alternative approach to test our hypothesis in vivo. By coexpressing nuclear β-gal and cytoplasmic EGFP
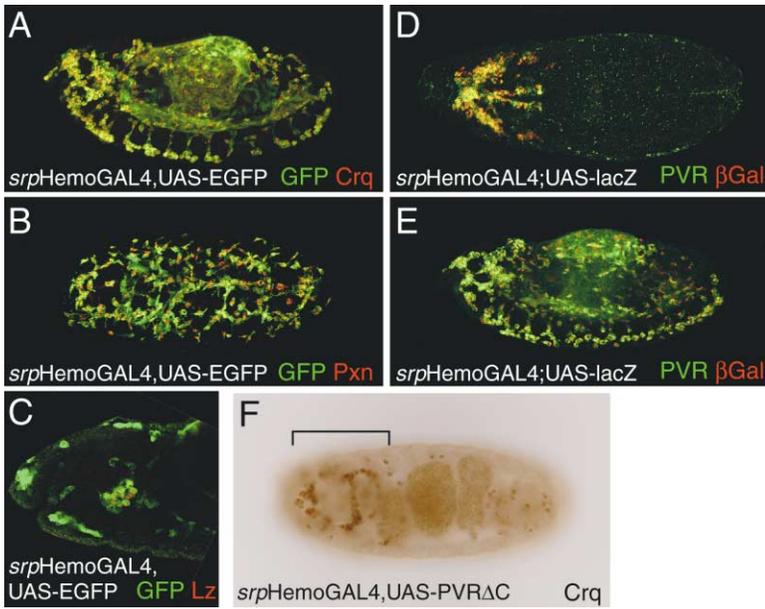
Figure 2. The *Pvr* Macrophage Aggregation Phenotype Is Blood Cell Autonomous

(A–C) Colocalization of the hemocyte-specific driver *srp*HemoGAL4 with known blood cell markers. Genotype *srp*HemoGAL4, UAS-srcEGFP. (A) EGFP (green) colocalizes with Crq (red). Stage 14 embryo, lateral view. Other Crq expression domains such as amnioserosa and gut do not show colocalization. (B) EGFP (green) colocalizes with Pxn (red). Stage 16 embryo, dorsal view. (C) Lz-positive cells (red) are EGFP positive. Stage 14 embryo, lateral-dorsal view, single optical section.

(D and E) *srp*HemoGAL4 driven UAS-*lacZ*-nls (nuclear β-gal, red) marks the same set of blood cells that expresses PVR (green) from stage 10/11 onward. (D) Stage 11, dorsal view. (E) Stage 14, lateral view. In contrast to the *srp* gene (Rehorn et al., 1996), *srp*HemoGAL4 does not drive expression in other tissues, such as midgut primordia and the amnioserosa, and shows only marginal expression in some areas of the fat body.

(F) Hemocyte-specific expression of dominant-negative PVRΔC phenocopies *Pvr* loss of function. Embryos *srp*HemoGAL4/+; UAS-PVRΔC/+, stained with anti-Crq. Dorsal view of a stage 16 embryo (central focal plane) is shown. Bracket marks anterior accumulation of macrophage aggregates. Hemocyte aggregation prior to stage 14 is less obvious, presumably due to a lag in the production of sufficient levels of PVRΔC protein. When the early mesodermal driver *twi*GAL4 is used, hemocyte aggregates appear at earlier stages (not shown).

using the hemocyte-specific *srp*HemoGAL4, we differentially marked the nuclei and cytoplasmic compartment of hemocytes in wild-type and *Pvr* mutant embryos (Figure 4). This staining allowed us to determine whether hemocytes would maintain single β-gal-positive nuclei or whether they would accumulate multiple β-gal-positive inclusions indicative of incorporating hemocytes that were dying. Further, it allowed us to quantify labeled hemocytes and visualize nuclear morphology as an indicator of apoptotic fragmentation and cell death (Abrams et al., 1993; Jacobson et al., 1997).

Wild-type embryos showed evenly distributed blood cells, each with a single β-gal-positive nucleus (Figure 4H). In *Pvr¹* mutant embryos, hemocytes appeared sparse and localized to clusters (Figure 4A). As predicted for a macrophage that has engulfed other dying hemocytes, we found that many EGFP-marked hemocytes contained multiple β-gal-positive inclusions. Typical hallmarks of programmed cell death, such as nuclear fragmentation, were seen (Figures 4I and 4J). In many cases, β-gal staining expanded to structures beyond normal nuclear size and colocalized with EGFP, presumably due to a loss of nuclear integrity and diffusion of the β-gal and EGFP proteins (Peterson et al., 2003). An antibody against the cleaved form of Caspase 3 was used as a marker for apoptotic cells. It costained with β-gal and EGFP in some hemocytes of *Pvr¹*, but not wild-type embryos (Figures 4M and 4N). Taking the data together, we conclude that hemocytes in *Pvr* mutant embryos phagocytose dying hemocytes, consistent with cannibalistic phagocytosis.

To revert apoptotic hemocyte death and cannibalistic phagocytosis, we coexpressed antiapoptotic rescue transgenes (Figures 4 and 5). Apoptotic cell death is executed by the action of a family of caspases, that activate one another in a proteolytic cascade and mediate the breakdown of other cellular proteins (Danial and Korsmeyer, 2004). We asked whether hemocyte-specific expression of the baculovirus p35 pan-caspase inhibitor (Hay et al., 1994) would be sufficient to rescue the *Pvr¹* mutant phenotype. Indeed, expression of p35 eliminated macrophage clusters, and blood cells were distributed evenly throughout anterior and dorsal areas (Figure 4B). Hemocytes carried single β-gal-marked nuclei that showed no signs of nuclear fragmentation or loss of nuclear integrity (Figures 4K and 4L). Despite these dramatic improvements, some minor abnormalities in hemocyte distribution remained (Figure 4B), which point to residual survival-independent functions of PVR in cell migration and/or invasion (see Figure 7). Nevertheless, we conclude that the predominant defect in *Pvr* loss-of-function hemocytes is a lack of trophic survival.

Activated RTK Signaling Mediators Rescue Hemocyte Death and Aggregation

PVR may trigger a number of signaling pathways to promote trophic blood cell survival. One candidate is the Ras/MAPK pathway, as PVR signaling induces MAPK activation in Schneider cells (Duchek et al., 2001) and correlates with MAPK activation in embryonic blood cells (Cho et al., 2002). MAPK signaling was shown to suppress apoptosis of both HID-induced photoreceptor death and naturally occurring apoptosis of midline glia (Bergmann et al., 1998, 2002) and smooth cuticle cells (Urban et al., 2004). By analogy with mammalian PDGF/VEGF receptors, PVR may also activate the PI3K/Akt pathway, which mediates trophic survival of mammalian cells (Franke et al., 1997) and promotes cell survival during *Drosophila* embryogenesis (Scanga et al., 2000).
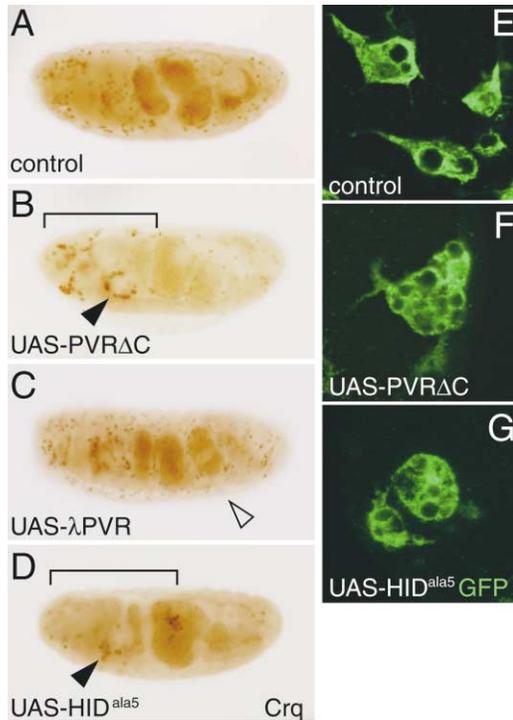
Figure 3. Hemocyte Death, but Not Uniform PVR Activation, Leads to Blood Cell Aggregation

(A–D) Wild-type control embryo (A) and embryos expressing trans-genes under control of *srp*HemoGAL4. Staining with anti-Crq, stage 16 lateral view. (B) Dominant-negative PVRΔC (*srp*HemoGAL4/+; UAS-PVRΔC/+) induces hemocyte aggregation in the anterior region (bracket) and around the proventriculus (arrowhead). (C) Uniform hemocyte-specific expression of activated λPVR (*srp*Hemo-GAL4/+; UAS-λPVR /+, or *srp*HemoGAL4/UAS-λPVR, not shown) does not result in blood cell aggregation. In 20% (UAS-λPVR on III) and 45% (UAS-λPVR on II) of stage 16 embryos, cell distribution was mildly aberrant with fewer cells in the ventral-posterior area (arrowhead). Earlier expression of λPVR by the mesodermal driver *twi*GAL4 results in a comparably mild hemocyte distribution defect (not shown). (D) UAS-HID^ala5/+; *srp*HemoGAL4/+). Hemocyte aggregates (bracket) are found in the head region, around the proventriculus (arrowhead), and in areas of the amnioserosa or gut.

(E–G) Hemocyte morphology. Hemocytes were marked by *srp*HemoGAL4, UAS-srcEGFP, and anti-EGFP staining. Single optical sections of comparable regions in the anterior region of stage 16 embryos are shown. (E) Wild-type background, (F) expression of PVRΔC, (G) expression of HID^ala5.

We therefore tested activated Ras^V12 and an activated form of the PI3K catalytic subunit, p110^CAAX, for their ability to rescue hemocyte aggregation in *Pvr* mutant embryos.

Hemocyte-specific expression of the constitutively active λPVR served as a control for *Pvr¹* rescues that promote uniform activation of signaling pathways: Cell morphology was reverted to dispersed EGFP-positive hemocytes with single β-gal-positive nuclei, and blood cells were distributed evenly in all anterior and dorsal areas of the embryo (Figure 4C). Expression of activated Ras1 rescued *Pvr¹* with similar efficiency (Figure 4D). On the contrary, expression of p110^CAAX had only mild effects on *Pvr¹* blood cell aggregates of stage 16 embryos (Figure 4E), and combination of p35 and activated p110^CAAX

was comparable to p35 alone (Figure 4F). In all rescues by p35 or activated signaling components, we noted persistence of local abnormalities in hemocyte distribution in the ventral-posterior area (Figures 4B–4F) and at the posterior end during germ band elongated state (data not shown). No defects in blood cell distribution remained when *Pvr* mutants were rescued by hemocyte-specific expression of wild-type PVR (Figure 4G). Thus, we conclude that uniformly activated PVR is sufficient to rescue blood cell death and aggregation associated with *Pvr* mutants, although it may fail to substitute for an additional function(s) of PVR that would require differential activation of the receptor (see Figure 7). Activated Ras rescues *Pvr* dependent hemocyte death and aggregation, suggesting that signaling through the Ras pathway may be a route of PVR-mediated trophic cell survival.

To confirm hemocyte loss and quantify the effect of coexpressed rescue transgenes, we analyzed embryonic blood cell numbers. Hemocytes were counted in embryos at stage 11/12, and subsequently at stage 15/16, to analyze stability of cell numbers over time. Initial blood cell counts of wild-type embryos and embryos with hemocyte expression of PVRΔC were comparable. At stage 15/16, however, counts of PVRΔC hemocytes had dropped to about 50% of wild-type (Figure 5), consistent with cell loss due to apoptotic death. In *Pvr¹* homozygous mutants, hemocyte numbers were already reduced at stage 11/12, and dropped further over time. Asking whether the early reduction in blood cell counts was due to an impairment of hemocyte proliferation or early apoptotic cell death, we coexpressed the baculovirus p35 pan-caspase inhibitor. Consistent with hemocyte morphology and distribution (Figures 4B, 4K, and 4L), p35 expression was sufficient to restore initial blood cell numbers to almost wild-type, indicating that zygotic PVR is not required for embryonic hemocyte proliferation. Blood cell counts diminished slightly over time, suggesting that under the experimental conditions used, p35 did not block cell death completely. Uniform activation of PVR signaling by λPVR resulted in an efficient rescue of blood cell counts, and coexpression of activated Ras^V12 had comparable effects (Figure 5). The activated form of the PI3K catalytic subunit, p110^CAAX, was not potent in *Pvr* rescues when stage 15/16 embryos were inspected (Figure 4E), while at early stages partial rescue of blood cell counts was seen (Figure 5). Combining expression of the two partially rescuing transgenes p35 and p110^CAAX had only marginally additive effects (Figure 5). Taking the data together, we conclude that *Pvr* mutants show apoptotic loss of hemocytes over the course of embryonic development. The underlying deficiency in trophic cell survival can be rescued by p35-mediated caspase inhibition, activated Ras, and, in the early stages of embryonic development, activated PI3K signaling.

PVR Signaling Is Essential for Trophic Survival of a Hemocyte Cell Line

To distinguish whether PVR signaling is required for hemocytes to reach a trophic target within the embryo, or whether PVR itself can trigger antiapoptotic cell survival, we studied survival of a hemocyte cell line in culture. The embryonic cell line Kc 167 (Echalier and
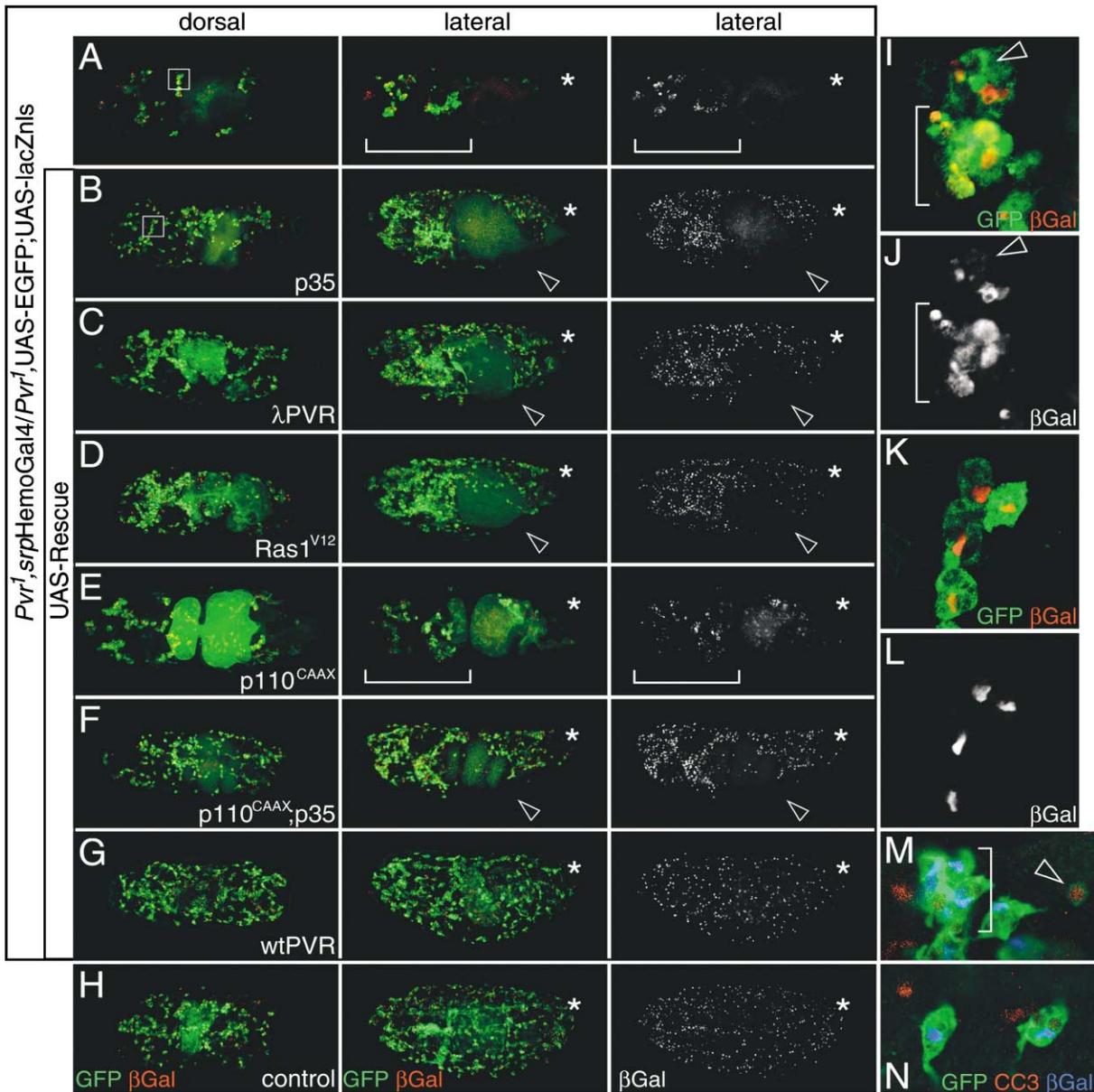
Figure 4. Rescue of Hemocyte Apoptosis Reverts Blood Cell Aggregation

(A–G) In a *Pvr¹/Pvr¹* mutant background, the indicated rescue constructs were expressed using the hemocyte-specific *srp*HemoGAL4. Blood cells were marked by expression of membraneous/cytoplasmic srcEGFP and nuclear β-gal (UAS-*lacZ*nls). Stage 16 embryos are shown. Dorsal views in left panels, and lateral views of projections through the whole embryo in middle panels. Right panels show red channel (β-gal) of middle panels. Asterisks indicate posterior end of embryos. Brackets mark hemocyte aggregates. Some images show autofluorescense of the gut. (A) *Pvr¹/Pvr¹*. (B) Rescue by p35. (C) Rescue by λPVR. (D) Rescue by Ras^V12. (E) Rescue by p110^CAAX. (F) Rescue by p35 and p110^CAAX. In (B)–(D) and (F), no blood cell aggregates are seen and cells migrate dorsally up to the posterior end (asterisk). Cell distribution into the ventral-posterior area remains incomplete (arrowheads). (G) Rescue by wtPVR. (H) Wild-type control (*srp*HemoGAL4,UAS-srcEGFP; UAS-*lacZ*nls).
(I–L) Close-up images of the marked areas in (A) and (B), single optical sections. (I and J) Hemocyte morphology of *Pvr¹/Pvr¹* shown in (A). (K and L) Blood cell morphology of *Pvr¹*, rescued by p35 expression as shown in (B).
(M and N) Costaining with anti-cleaved Caspase 3 (CC3) to mark apoptotic cells. Hemocytes in the anterior region of stage 14 embryos, single optical sections. (M) *Pvr¹*,UAS-srcEGFP/ *Pvr¹*,UAS-srcEGFP;UAS-*lacZ*nls. CC3signal colocalizes with both EGFP and β-gal in some (bracket, arrowhead) but not all hemocytes. (N) wild-type control (*srp*HemoGAL4,UAS-srcEGFP; UAS-*lacZ*nls) shows CC3 staining in apoptotic corpses of non-hemocyte origin only.

Ohanessian, 1970) displays hemocyte features, as cells are able to phagocytose bacteria (Amy Kiger, Buzz Baum, and N.P., unpublished data) and express the macrophage marker Crq (data not shown). RT PCR analysis revealed endogenous expression of *Pvr* and its ligand *Pvf2* (not shown). Consistent with this finding, we
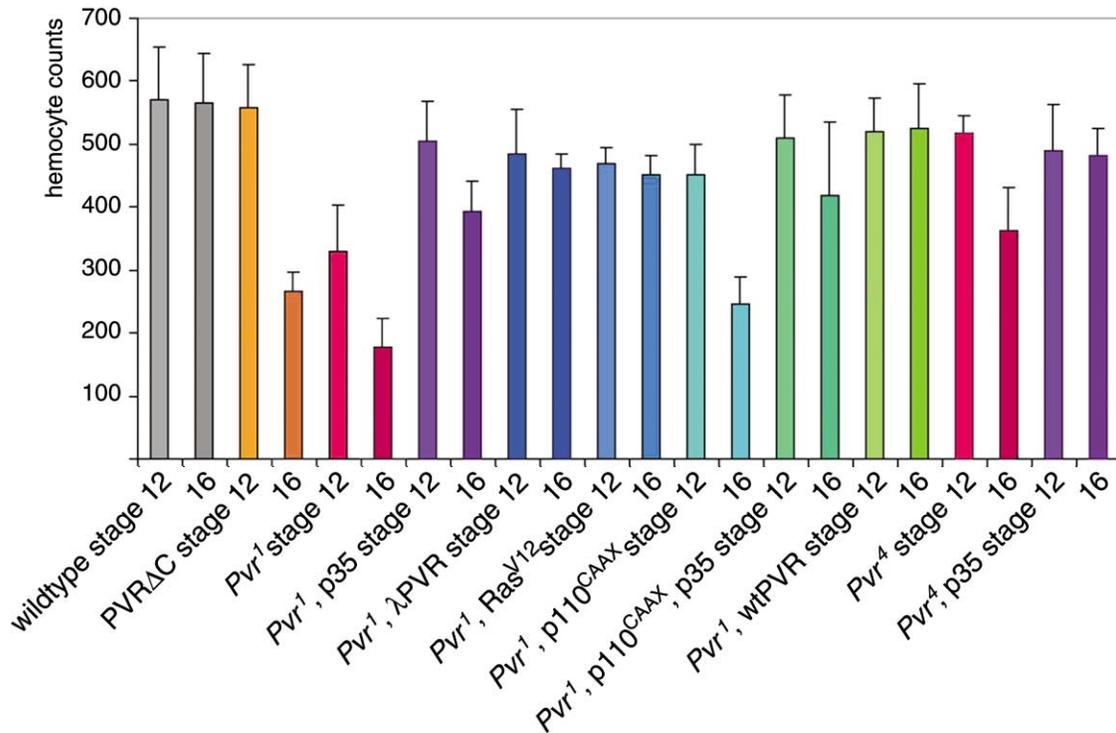
Figure 5. Rescue of Hemocyte Apoptosis Restores Blood Cell Counts

Statistical comparison of blood cell counts in wild-type, *Pvr* loss-of-function, and rescued embryos. Cells were marked by nuclear β-gal driven by *srp*HemoGAL4. Error bars represent standard deviation from average. Indistinguishable misshapen β-gal-positive structures were counted as single cells. See Supplemental Data for average hemocyte numbers. See Experimental Procedures for genotypes.

detected PVR protein in its tyrosine phosphorylated form in extracts of normally growing Kc cells (not shown), indicating constitutive PVR activation, as it was previously described for *Drosophila* S2 cells (Duchek et al., 2001). To disrupt PVR signaling in Kc cells, we utilized RNA interference (RNAi), treating cells with double-stranded RNAs (dsRNAs) corresponding to the *Pvr* gene. Loss of PVR protein was confirmed by Western blot (Supplemental Figure S2A). Upon *Pvr* silencing, we observed reduced total cell numbers and an increasing rate of dead cells, as characterized by their permeability to trypan blue. Cell death occurred over the course of several days (Figure 6A). Cell cycle analysis quantifying DNA contents by propidium iodide staining and FACS analysis confirmed that *Pvr* RNAi did not lead to a general cell cycle arrest (Figure 6B). We found an elevation in the sub-G0 peak of the profile (Figure 6B and Supplemental Figure S2B), indicative of increased cell death. To specifically characterize apoptotic cell death, we performed TUNEL staining for fragmented nuclear DNA (Figure 6C). As indicated by a shift in fluorescent emission, an increasingly large proportion of cells tested TUNEL-positive in *Pvr*-silenced cultures after 2 and 4 days of treatment (Supplemental Figure S2C), consistent with DNA fragmentation preceding the breakdown of cellular integrity. Taken together, these results demonstrate a requirement for PVR signaling in the trophic survival of Kc cells in culture and support an antiapoptotic function for PVR in embryonic hemocytes in vivo.

Discussion

PVR Controls Antiapoptotic Survival of Blood Cells

Our results demonstrate that PVR controls the trophic survival of embryonic blood cells in vivo and an embryonic hemocyte cell line in vitro. Inhibition of PVR signaling or loss of *Pvr* function results in caspase-dependent apoptotic hemocyte death and formation of large blood cell aggregates. Blood cell clustering in *Pvr* mutant embryos has previously been interpreted as a migration defect (Cho et al., 2002). Based on our findings we propose a different scenario: Dying hemocytes lose their ability to migrate, which may happen in many cases before cells leave the anterior locations of their origin. The resulting apoptotic corpses pose a strong attractant for their still-viable peer macrophages, which migrate toward them to fulfil their phagocytic function. Subsequently, cannibalistic phagocytosis leads to engorgement of macrophages with multiple blood cell corpses and results in the overall appearance of large hemocyte aggregates (Figure 7).

Blood cell death in *Pvr* mutants occurs progressively throughout embryonic development. While hemocyte clustering in *Pvr*[1] and *Pvr*[4] becomes evident at different stages of embryonic development, blood cell aggregates persist until the end of embryogenesis and intensify over time. This is consistent with our observations in the hemocyte cell line Kc 167, where silencing of *Pvr*
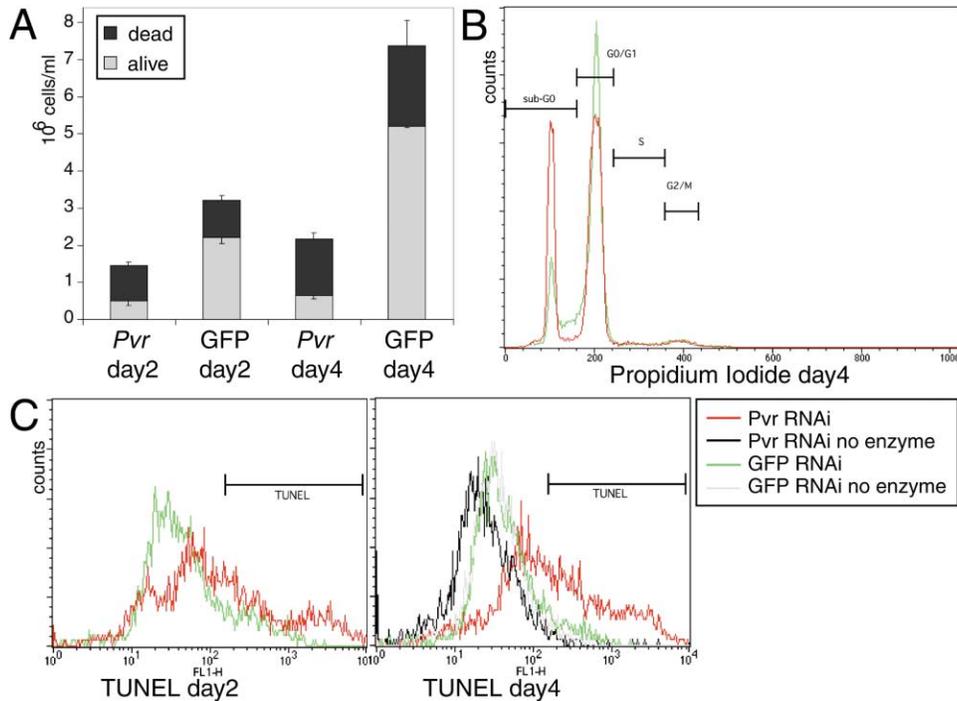
Figure 6. PVR Signaling Mediates Survival of the Hemocyte Cell Line Kc 167

Endogenous PVR signaling of Kc cells was disrupted by *Pvr* RNAi. For controls, GFP dsRNA was used.

(A) Loss of PVR results in reduced total cell numbers and elevated fractions of dead cells over time. In *Pvr* silenced cultures, live cell numbers were reduced to 23% of control cells at day 2, and 13% at day 4 after treatment. A fraction of live cells continued to be present beyond the fourth day of culture (not shown).

(B) Cell cycle analysis. Four days after addition of dsRNAs, adherent cells were stained with propidium iodide and were subjected to FACS analysis. Data are corrected for doublets.

(C) TUNEL staining for apoptotic cells. At day 2, 35% of *Pvr* dsRNA-treated cells stained TUNEL-positive (note left shoulder of live cells), while at day 4, 45% stained positive. Compare TUNEL-positive fluorescence to "no enzyme" control as reference for unspecific labeling background.

(B and C) For FACS histogram statistics, see Supplemental Figure S2.

results in apoptotic cell death over the course of days. Slow hemocyte death suggests activity of other partially redundant signaling pathways supporting cell survival. The Jak/Stat and Toll/Cactus pathways have been proposed to play roles in larval hemocyte survival (Govind, 1999; Mathey-Prevot and Perrimon, 1998), and it remains to be shown whether these pathways have a trophic function in the embryonic hematopoietic system. Further, it needs to be determined whether *Pvr* mutant blood cells die in a stochastic manner, or whether macrophages comprise different sublineages with variable dependence on trophic PVR signaling. While our studies indicate that both dying and actively phagocytosing blood cells express the same macrophage markers, identification of additional markers may reveal a distinction between sublineages.

Defects in hemocyte function may indirectly account for the associated lethality of *Pvr* mutants. Studies by Sears et al. (2003) have demonstrated that *Pvr* mutants suffer from secondary defects in the morphogenesis of the embryonic central nervous system. These defects are caused by a failure of hemocytes to remove dead cells, as similar effects were seen in embryos which lack *srp* function, or in which phagocytosis was impaired (Sears et al., 2003). It is likely that additional secondary defects of *Pvr* mutants will be found in other organ systems that rely on reshaping by the phagocytic activity of macrophages.

Relay of the PVR Trophic Survival Signal

Trophic survival is well known in mammalian systems, where it serves as an important mechanism to control cell number (Raff et al., 1993): Extracellular factors produced by target tissues trigger survival pathways that suppress apoptotic cell death and thereby control cell number. In *Drosophila*, few rare described cases of anti-apoptotic survival signaling are mediated through activation of the *Drosophila* EGFR (DER) by its ligands Vein or Spitz in the survival of glia cells (Bergmann et al., 2002; Hidalgo et al., 2001), and by both ligands in the survival of smooth cuticle cells (Urban et al., 2004). Other signaling pathways may have similar roles during development (Abrams, 2002; Hidalgo, 2002), but detailed analysis is often limited. In accordance with the classical model of trophic cues, the PVR ligands PVF1-3 are expressed dynamically in multiple embryonic tissues throughout embryogenesis (Cho et al., 2002). Only simultaneous RNAi silencing of all three ligands leads to hemocyte aggregation (Cho et al., 2002), consistent with PVF1-3 providing the crucial stimuli for hemocyte survival by activation of PVR.

Our *Pvr* mutant rescue experiments demonstrate that

A Wild type

B Pvr mutant

**C Pvr mutant
Rescue of cell viability**

Signals received

Survival
Invasion/Migration

Survival
Invasion/Migration

Survival
Invasion/Migration

stage 12

stage 17

Phenotype

Blood cells distributed throughout the embryo

✝ Blood cell aggregates form due to hemocyte death and cannibalistic phagocytosis.

Blood cells distributed throughout most parts of the embryo. No formation of blood cell aggregates.

normal hemocyte: nucleus red, cytoplasm green
engorged hemocyte after ingestion of apoptotic peer hemocytes: nucleus red, cytoplasm green, apoptotic corpses of hemocyte origin yellow

Figure 7. Model for PVR Function in Embryonic Blood Cell Development and Survival

(A) Wild-type embryo. During germ band retraction, hemocytes separate into two groups that populate the embryo from the anterior and posterior ends (arrows). PVR has at least two roles in embryonic blood cells: (1) Throughout embryogenesis, PVR function is essential for trophic survival of hemocytes. (2) PVR activity allows hemocytes to enter the posterior end, and to subsequently distribute within the ventral posterior area at later stages.

(B) Cannibalistic phagocytosis in Pvr mutants: Dying hemocytes stop migrating and attract viable peers, which phagocytose them according to their natural function as macrophages. As a result, large aggregates of mislocalized blood cell clusters form. Note enlarged macrophages with multiple apoptotic corpses.

(C) When viability is rescued, blood cells of Pvr mutants do not phagocytose each other any more and disperse from the anterior side (arrow). As PVR function is necessary for some aspect of cell entry or migration into the posterior end (stage 12), we propose that this subpopulation of hemocytes is not in reach to fill a gap in the ventral-posterior area, which is still evident at later stages (stage 17). Alternatively, PVR may support aspects of hemocyte migration necessary to reach targets in the ventral-posterior area.

Crystal cells shown in gray. Gut and nervous system (light gray) shown for orientation purposes.

activated Ras is sufficient to restore hemocyte survival. This result resembles findings from survival signaling by DER, which was shown to inhibit action of the proapoptotic protein HID by phosphorylation through Ras-activated MAPK (Bergmann et al., 1998). As PVR signaling triggers MAPK activation in Schneider cells (Duchek et al., 2001) and may have the same effect in embryonic hemocytes (Cho et al., 2002), it is likely that the Ras/MAPK pathway is a route of antiapoptotic PVR signaling. Expression of dominant-negative Ras^{N17} did not lead to large hemocyte aggregates but induced mild enlargement of hemocytes at a low penetrance. This mild phenotype points to weak defects in blood cell survival. The incomplete effect of Ras^{N17} may be due to a number of reasons. Ras^{N17} may be too weak to fully block endogenous Ras signaling, or Ras signaling may be redundant with other signaling pathways that are active in PVR-dependent cell survival. In Pvr^1 rescue experiments, activated Ras^{V12} was used, which was shown to ectopically activate other signaling pathways such as the PI3K pathway (Prober and Edgar, 2002). Therefore, rescue by Ras^{V12} may involve a number of downstream pathways, but the Ras/MAPK pathway itself may still be central to

the observed effect, consistent with a current model for apoptosis in *Drosophila* (Danial and Korsmeyer, 2004). Regardless of the upstream pathways involved, inhibition of caspases by the baculovirus inhibitor of apoptosis p35 was sufficient to rescue the *Pvr* mutant hemocyte death and aggregation phenotype. In these rescue experiments, p35 appeared slightly less potent than Ras^{V12}, which may be due to the inability of p35 to inhibit the upstream caspase Dronc (Hawkins et al., 2000), or partially insufficient expression levels, as p35 inhibits caspases by stoichiometric binding (Danial and Korsmeyer, 2004).

Other Functions of PVR in Hemocytes

While activated λPVR and other activated rescue transgenes reverted hemocyte clustering and cell death efficiently, embryos still retained residual defects in hemocyte distribution. In stage 11/12 embryos, blood cell entry into the posterior end of the elongated germ band was affected (Figure 1 and data not shown), and in stage 15/16 embryos, the posterior area of the ventral nervous system was lacking hemocytes. This is consistent with Cho et al. (2002) who described a role for PVR in the

entry/migration of hemocytes into the posterior end of stage 11/12 embryos. Since the natural route of hemocytes is to distribute from the posterior end into ventral posterior areas, we propose that the observed ventral-posterior lack in hemocytes may reflect the initial inability of blood cells to invade, or migrate within, the posterior end (Figure 7). Alternatively, PVR activity may be required at multiple steps of embryonic hemocyte migration. In this case, defects at early steps of hemocyte migration (i.e., entry into the posterior end) may be more readily visualized than defects at later steps, which may be masked by additional independent mechanisms of hemocyte dispersal. To distinguish between these possibilities, more refined techniques to study blood cell migration will be required. Consistent with both models, uniform expression of activated λPVR in hemocytes leads to a similar, yet milder, phenotype at a low penetrance, suggesting that correct hemocyte entry or migration requires tightly controlled or locally asymmetric activation of PVR. By analogy, migration of border cells in the egg chamber was dramatically affected by uniform activation of PVR (Duchek et al., 2001), revealing a role for locally asymmetric PVR activation in the guidance of border cells (Duchek et al., 2001; McDonald et al., 2003).

In mammals, PDGF/VEGF receptors are known to mediate additional cellular responses, such as cell proliferation and differentiation (Hoch and Soriano, 2003; Neufeld et al., 1999). *Drosophila* PVF2 has been described to promote proliferation of larval hemocytes (Munier et al., 2002). Our experiments do not point to a role for PVR in the proliferation of embryonic blood cells, as *Pvr* RNAi-treated Kc cells did not show an obvious arrest in the cell cycle, and p35 was sufficient to rescue blood cell counts in mutant embryos. As blood cell proliferation occurs very early during embryonic development, we cannot exclude, however, that an unnoticed maternal contribution of *Pvr* may obscure a role for the receptor in embryonic hemocyte proliferation in vivo. Likewise, with respect to blood cell differentiation, we did not detect effects of *Pvr* loss-of-function on macrophage differentiation as judged by the marker Crq. Nevertheless, a role for PVR in hemocyte differentiation may become apparent once more specific lineage markers will be available.

Taken together, PVR has at least two functions in the embryonic hematopoietic system (Figure 7): (1) PVR mediates antiapoptotic survival of blood cells throughout embryonic development, and (2) PVR is required for invasion into/migration within the posterior end of the embryo. Rescue of cell viability reverts the dramatic blood cell aggregation phenotype and leads to dispersal of cells in anterior and dorsal areas of the embryo.

A Model System for Blood Cell Survival in Development and Disease

The role of *Drosophila* PVR in trophic cell survival emphasizes the high degree of conservation between *Drosophila* and vertebrate PDGF/VEGF family receptor function. Our in vivo and cell culture work now provides the basis to study cell survival in a simple but highly conserved hematopoietic system. In vertebrates, control of cell survival is an important aspect of hematopoiesis and stem cell maintenance (Lotem and Sachs, 2002).

Antiapoptotic cell survival and its aberrant prolongation are a major mechanism in the formation of human neoplasias (Evan and Vousden, 2001). In many cases, connections to deregulated upstream signaling pathways remain unclear. Interestingly, in Acute Myeloid Leukemias (AML), more than one-third of cases are associated with specific activating mutations in the PDGF/VEGF receptors Flt3 and c-Kit, and activating fusions of PDGFβR replace the more common oncogenic BCR-ABL fusions in some cases of Chronic Myeloid Leukemia (CML) (Kelly and Gilliland, 2002). The contribution of these and other disease-associated genes to aspects of cell survival versus proliferation is still difficult to assess in vivo (Huettner et al., 2000), yet their mechanism of action is important for the selection of molecularly targeted therapies. *Drosophila* embryonic hematopoiesis allows the in vivo study of blood cell survival independent of cell proliferation, and our work has demonstrated the antiapoptotic potential of the *Drosophila* PDGF/VEGF receptor and activated signaling components such as Ras^{V12}. It will be interesting to exploit the system further by testing disease-related genes of the same and particularly other families for their in vivo potential to rescue blood cell survival in *Drosophila*. Complementary to our in vivo findings, we have established a *Drosophila* cell culture system for the study of PVR-dependent blood cell survival. Genome-wide RNAi screens (Boutros et al., 2004) will allow identification of modifiers of PVR-dependent blood cell survival.

Experimental Procedures

Constructs and Fly Stocks
For construction of transgenes pUAST-PVRΔC, pCaSpeR4-*srp* HemoGAL4, pCaSpeR-*tubulin*-PVR, and for EMS mutagenesis see Supplemental Data (http://www.developmentalcell.com/cgi/content/full/7/1/73/DC1). Genotypes in *Pvr* rescue experiments were: UAS-p110^{CAAX}/ +; *Pvr^1*,UAS-srcEGFP/ *Pvr^1*, *srp*HemoGAL4; UAS-*lacZ*nls/ + or *Pvr^1*,UAS-srcEGFP/ *Pvr^1*, *srp*HemoGAL4; UAS-rescue/ UAS-*lacZ*nls (for rescue constructs UAS-λPVR, UAS-p35, UAS-RasV12/ TM3,*ftzlacZ*). For the combined rescue by p110^{CAAX} and p35, the examined genotype was: UAS-p110^{CAAX}/ +; *Pvr^1*,UAS-srcEGFP/ *Pvr^1*, *srp*HemoGAL4; UAS-p35/ UAS-*lacZ*nls. The corresponding genotypes of heteroallelic combinations with *Pvr^4* contained instead: *Pvr^4*,UAS-srcEGFP/ *Pvr^1*, *srp*HemoGAL4.

Stainings and Microscopy
Embryo stainings were visualized by DAB (Vector) or fluorescently labeled secondary antibodies (Molecular Probes, Jackson) and confocal microscopy (TCSNT, Leica). Primary antibodies used were: anti-Crq (Franc et al., 1996), anti-Pxn (Abrams et al., 1993; Nelson et al., 1994), anti-Lz (Lebestky et al., 2000), anti-PVR (Duchek et al., 2001 and McDonald et al., 2003), anti-GFP (Molecular Probes, Cappel), anti-β-Gal (Promega), and anti-cleaved Caspase 3 (CC3) (Cell Signaling). For stainings with anti-CC3, embryos were devitelinized by hand. Confocal images are projections of eight optical sections representing approximately one-half of the embryo unless stated otherwise. Hemocyte counts were taken under fluorescent microscopy, assessing at least four independent embryos per genotype and stage. Embryo graphics of Figure 7 according to Hartenstein (1993).

Cell Culture and Flow Cytometry Analysis
Kc 167 cells (Echalier and Ohanessian, 1970) were grown under standard conditions (Brückner et al., 2000). dsRNAs were prepared from the following regions: *Pvr* internal, 501 nt (starting at GCA CAACCCT), *Pvr* 3′UTR, 429 nt (starting at GACGTCCCGG), GFP nt 3–549 of ORF, using the Ambion MegaScript kit. 2 × 10^{6}cells/6-well

were seeded. dsRNA treatment of cells was performed according to Clemens et al. (2000), using 12 µg dsRNA/well, with corresponding total amounts of dsRNA for *Pvr* and GFP controls. After incubations as indicated, all cells were collected, pelleted, and resuspended in Schneider's medium (GIBCO). For trypan blue stainings, cell suspensions were mixed at equal volume with trypan blue (0.4%) and counted in a hemacytometer. 2 ml of this suspension correspond to the culture of a 6-well. For each experiment, several fields were counted. Direct TUNEL labeling was done according to the manufacturer's instructions (Roche). For cell cycle analysis, adherent cells were washed and harvested, and 10^6 cells were incubated in 250 µl of staining solution (PBS, 1% Tween 20, 100 µg RNaseA [Sigma], 50 µg Propidium Iodide) for 2 hr. FACS analysis was performed on a FACSCalibur (Becton-Dickinson) and CellQuest software, analyzing 5000 (TUNEL)/50,000 (PI) events per measurement. SDS-PAGE and Western blot analysis were done as described (Brückner et al., 2000). Guinea pig anti-PVR antiserum was raised against the PVR C-terminal peptide NYVNMKPPRKNIPGKTTT.

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