Germ cell-autonomous Wunen2 is required for germline development in *Drosophila* embryos

Kazuko Hanyu-Nakamura[1], Satoru Kobayashi[2,3,*] and Akira Nakamura[1,*]

[1]Laboratory for Germline Development, RIKEN Center for Developmental Biology, Kobe, Hyogo 650-0047, Japan
[2]Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, Okazaki, Aichi 444-8787, Japan
[3]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan
*Authors for correspondence (e-mail: skob@nibb.ac.jp and akiran@cdb.riken.jp)

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Summary

In many animals, primordial germ cells (PGCs) migrate through the embryo towards the future gonad, a process guided by attractive and repulsive cues provided from surrounding somatic cells. In *Drosophila*, the two related lipid phosphate phosphatases (LPPs), Wunen (Wun) and Wun2, are thought to degrade extracellular substrates and to act redundantly in somatic cells to provide a repulsive environment to steer the migration of PGCs, or pole cells. Wun and Wun2 also affect the viability of pole cells, because overexpression of either one in somatic cells causes pole cell death. However, the means by which they regulate pole cell migration and survival remains elusive. We report that Wun2 has a maternal function required for the survival of pole cells during their migration to the gonad. Maternal *wun2* RNA was found to be concentrated in pole cells and pole cell-specific expression of *wun2* rescued the pole cell death phenotype of the maternal *wun2* mutant, suggesting that *wun2* activity in pole cells is required for their survival. Furthermore, we obtained genetic evidence that pole cell survival requires a proper balance of LPP activity in pole cells and somatic cells. We propose that Wun2 in pole cells competes with somatic Wun and Wun2 for a common lipid phosphate substrate, which is required by pole cells to produce their survival signal. In somatic cells, Wun and Wun2 may provide a repulsive environment for pole cell migration by depleting this extracellular substrate.

Key words: Germ cells, Lipid phosphate phosphatase, Cell survival, Cell migration, *Drosophila*

Introduction

In many animals, PGCs are specified and segregated from somatic cells at an early stage in development. PGCs are often formed in regions that are distant from the site where the gonad will form, and subsequently migrate through the embryo towards the future gonad. It has become clear that interactions between PGCs and surrounding somatic cells are essential for the proper migration of PGCs to the gonads.

Drosophila PGCs, which are known as pole cells, are formed at the posterior end of the blastoderm embryo. During gastrulation, pole cells are carried inside of the embryo with the invaginating posterior midgut primordium. Pole cells then actively pass through the midgut epithelium to enter the interior of the embryo, and move dorsally along the midgut surface towards the mesoderm. Next, pole cells migrate from the midgut to the mesoderm, where they make contact with the somatic gonadal precursors (SGPs). Pole cells adhere tightly to the SGPs and finally coalesce into the embryonic gonad (Starz-Gaiano and Lehmann, 2001).

Nanos and its co-factor Pumilio (Pum) are well-characterized maternal factors that are essential in a cell-autonomous manner for the proper migration of pole cells. Pole cells lacking Nanos or Pum are able to pass through the midgut. After exiting the midgut, however, they remain clustered on its outer surface, fail to enter the mesoderm and are lost during subsequent development (Asaoka-Taguchi et al., 1999; Forbes and Lehmann, 1998; Kobayashi et al., 1996). In addition, Nanos or Pum are essential for transcriptional regulation in pole cells. Pole cells lacking either of them show premature activation of several germline-specific enhancers that are normally expressed at much later stages (Asaoka et al., 1998; Asaoka-Taguchi et al., 1999; Kobayashi et al., 1996), as well as the ectopic expression of genes normally active only in somatic cells (Deshpande et al., 1999). The product of the *polar granule component* (*pgc*) gene also regulates transcriptional repression in pole cells. In embryos with reduced *pgc* function, pole cells ectopically express soma-specific genes such as *zerknullt* and *tailless*. In these pole cells, there is a premature phosphorylation of the Ser2 residue in the C-terminal domain (CTD) repeats of RNA polymerase II (Deshpande et al., 2004; Martinho et al., 2004). As this phosphorylation reflects the active transcription state (Dahmus, 1996), it has been suggested that *pgc* regulates global transcription in pole cells. As with *nanos* and *pum*, in embryos with reduced *pgc* function, pole cells fail to migrate into the gonads and die after exiting the midgut (Nakamura et al., 1996).

In addition to these cell-autonomous mechanisms, intercellular communication between pole cells and somatic cells is crucial for pole cell development and migration. For example, *trapped in endoderm-1* (*tre1*) acts in the passage of pole cells through the midgut. *tre1* encodes a G-protein-coupled receptor that is expressed in pole cells, and is thought to respond to a signal from the midgut to direct trans-epithelial migration (Kunwar et al., 2003). Furthermore, pole cells also receive directional cues from somatic cells that guide their migration to the gonads. Enzymes involved in isoprenoid biosynthesis are essential for attracting pole cells toward the gonads (Santos and Lehmann, 2004; Van Doren et al., 1998a). Conversely, zygotic *wun* and *wun2*, which encode a putative ectoenzyme, lipid phosphate phosphatase (LPP), are expressed in the ventral region of the midgut, and provide a repulsive environment for pole cell migration (Burnett and Howard, 2003; Starz-Gaiano et al., 2001; Zhang et al., 1996; Zhang et al., 1997). In addition to repelling pole cells, Wun and Wun2 activities also affect pole cell survival. Overexpression of either of them in somatic tissues leads to the drastic loss of pole cells during their migration (Burnett and Howard, 2003; Starz-Gaiano et al., 2001). However, it remains elusive how zygotic Wun and Wun2 activities in somatic cells exert their functions on both pole cell migration and survival. We report that *wun2* has a maternal function that is required in pole cells for their survival. The *wun2* function in pole cells was different from those of *nanos*, *pum* or *pgc*, because transcriptional regulation in early pole cells was intact in the maternal *wun2* mutant embryo. Furthermore, we provide evidence that pole cell survival requires a balance of LPP activities in pole cells and somatic cells. These results indicate that Wun2 in pole cells competes with somatic Wun and Wun2 for pole cell survival.

Materials and methods

Mutagenesis and screening

Isogenized *w; FRT42B* males were treated with 25 mM ethyl methanesulfonate (EMS) (Sigma) for 18-24 hours using standard methods. Mutagenized males were crossed with *w; T(2;3)ap^{Xa}/CyO* virgin females. Single male progeny with the genotype *w; FRT42B, */T(2;3)ap^{Xa}* or *CyO* (where the asterisk indicates the mutagenized chromosome) were crossed with *w; T(2;3)ap^{Xa}/CyO* virgin females to establish balanced stocks. The average frequency of lethal hits per chromosome was 1.2.

To generate germline clones, *w; FRT42B, */T(2;3)ap^{Xa}* females were crossed with *y w hs-FLP; FRT42B ovo^{D1}/CyO* males. The progeny were heat-shocked twice daily at 37°C for 2 hours during the early pupal stage. Embryos from females with the genotype *y w hs-FLP; FRT42B, */FRT42B ovo^{D1}* crossed to wild-type males were collected, fixed and stained for Vasa. Using this strategy, 1156 independent mutagenized lines were screened.

Mapping and identification of the *N14* gene

The *N14* chromosome was outcrossed to an unmutagenized *FRT42B* chromosome to recombine away second mutation(s). A lethal mutation on the *N14* chromosome was placed between *FRT42B* and *curved* by meiotic recombination mapping. *N14* was crossed to the deficiency kit for 2R (Bloomington Stock Center) and tested for complementations based on lethality and on the maternal-effect mutant phenotype. During this process, the maternal-effect phenotype of the *N14* mutation was found to be separable from the lethal mutation, and was uncovered by five overlapping deficiencies in the chromosome region 45C-D: *Df(2R)Np5*, *Df(2R)w45-30n*, *Df(2R)w73-1*, *Df(2R)wun-GL* and *Df(2R)w45-19g*. The breakpoints of these deficiencies were determined by semi-quantitative Southern hybridization using PCR-amplified genomic fragments as the probes.

A series of genomic DNA fragments in the *N14* candidate locus was isolated from a *Drosophila* genomic DNA library in λFIXII (a gift from B. Suter, McGill University) and subcloned into pCaSpeR3. These constructs were introduced into flies by a standard method, and were checked for their ability to rescue the *N14* mutant phenotype.

Immunostaining, in situ hybridization and β-galactosidase staining of embryos

Immunostaining was carried out as described (Kobayashi et al., 1999), except that the embryos were devitellinized by hand in the tracer experiment using the caged fluorescein. The following primary antibodies were used: rabbit and rat anti-Vasa, rabbit anti-Nanos (K.H.-N. and A.N., unpublished), rabbit anti-cleaved caspase 3 (Asp175) (Cell Signaling Technology), mouse anti-RNA polymerase II H5 (Babco) and rabbit anti-β-galactosidase (Cappel). Antibody detection was performed using either a biotinylated secondary antibody followed by the ABC Kit (Vector Laboratory) and DAB staining, or Alexa Fluor 488-, 568-, 594- and 660-conjugated secondary antibodies (Molecular Probes). Fluorescence signals were observed under a laser-scanning confocal microscope (Leica TCS-SP2 AOBS). In situ hybridization with DIG-labeled RNA probes was carried out as described (Kobayashi et al., 1999). For the *nanos*, *gcl* or *pgc* probes, hybridized signals were detected with an alkaline phosphatase-conjugated anti-DIG antibody (Roche). For *wun2*, hybridized signals were detected with a horseradish peroxidase-conjugated anti-DIG antibody (Roche), amplified with the TSA Biotin System (Perkin Elmer) and visualized with alkaline phosphatase-conjugated streptavidin (Vector Laboratory). To detect β-galactosidase expression, embryos were stained with X-Gal as described (Sano et al., 2001).

Labeling of pole cells using a photoactivatable lineage tracer

A photoactivatable lineage tracer, DMNB-caged fluorescein (Molecular Probes), was injected into early cleavage stage embryos at a concentration of 1 mg/ml. The injected embryos were allowed to develop until the cellular blastoderm stage. To photoactivate the tracer, pole cells were exposed to a two-second pulse of UV irradiation from an epifluorescence microscope fitted with a DAPI optical filter set. Embryos with pole cells that were successfully marked with fluorescein were allowed to develop until the indicated stages. These embryos were then fixed and stained with antibodies.

Generation of a new *wun2* allele

EP2650 is a viable insertion that is located ~30 bp upstream of the *wun2* locus. Excision lines were generated by crossing virgin *EP2650* females with males possessing *Δ2-3* transposase. Genomic DNA from the excision lines was prepared and used as PCR templates to screen lines that carried a deletion within the *wun2* locus. The breakpoints of the deletion were determined by sequencing the PCR products.

Genetic interactions between maternal *wun2* and zygotic *wun* and *wun2*

To test the phenotype of *wun^{m+z-} wun2^{m-z-}* mutant embryos, *wun2^{N14}/Df(2R)w45-19g* females were crossed to *wun^{CE}/CyO, P{en-lacZ}* males. *Df(2R)w45-19g* uncovers both *wun* and *wun2* loci, and *wun^{CE}* allele lacks zygotic expression of both *wun* and *wun2* (Starz-Gaiano et al., 2001). Embryos that received a *wun^{CE}* chromosome were identified by the loss of β-galactosidase expression. In this experiment, half of the *lacZ^{-}* embryos showed a less severe pole cell death phenotype than that observed in the *wun2^{m-z-}* embryos. Thus, we scored embryos with weaker pole cell death as well as mis-migrated pole cell phenotypes as *wun^{m+z-} wun2^{m-z-}* embryos. For the overexpression of *wun2* both in pole cells and the mesoderm, *twist-*

Gal4/+; *nanos-Gal4-VP16/+* females were crossed to *EP2650* males. Half of the embryos obtained from this cross were expected to overexpress *wun2* in both pole cells and the mesoderm. These embryos were identified by a strong pole cell migration defect, owing to the overexpression of *wun2* in the mesoderm.

Results

Maternal *N14* mutation affects pole cell maintenance

To identify new maternally acting genes required for pole cell development, we performed a genetic screen for maternal mutations causing defects in this process. We carried out a screen for EMS-induced mutations on the chromosome arm 2R using the FLP/FRT/DFS system, which produces homozygous germline clones in heterozygous mothers (Chou and Perrimon, 1996). Embryos derived from homozygous germline clones were stained for Vasa, a marker protein for the germline. From the screening of 1156 independent lines, we obtained one line, which we named *N14*, that exhibited developmental defects in pole cells during their migration.

Deficiency mapping revealed that *N14* was uncovered by *Df(2R)w45-19g*. In embryos derived from *N14/Df(2R)w45-19g* mothers (hereafter referred to as $N14^{m-}$ embryos), normal numbers of pole cells were formed (stage 5, Fig. 1A,B; Table 1). These pole cells were carried into the embryo along with the invagination of the posterior midgut, passed through the midgut epithelium, and migrated dorsally along the surface of the midgut (Fig. 1C,D). However, at stage 11, when pole cells normally associate with the mesoderm, the number of Vasa-positive pole cells was dramatically reduced in $N14^{m-}$ embryos (Fig. 1E,F). In these embryos, the remaining pole cells, if any, associated with the surface of the midgut (Fig. 1F), and in subsequent development, few or no pole cells were incorporated into the gonads (Fig. 1G,H; Table 1). $N14^{m-}$ embryos showed no discernible morphological defects in somatic tissues and developed into adults. However, consistent with the loss of pole cells during embryogenesis, over 80% of the adult females developed from $N14^{m-}$ embryos had agametic ovaries (Table 1). This defect in pole cell development was not rescued by a paternally supplied wild-type copy of the *N14* gene, and zygotic *N14* mutation did not affect the maternal *N14* mutant phenotype. These results indicate that maternal *N14* function is required for the maintenance of pole cells during their migration to the gonads.

Pole cells die during migration in $N14^{m-}$ embryos

The above observations allow two possible interpretations: that the pole cells are eliminated after exiting the midgut in $N14^{m-}$ embryos, or that they survive but lose pole cell-specific markers, such as Vasa. To discriminate these alternative possibilities, we examined the fate of pole cells in $N14^{m-}$ embryos by using a photoactivatable lineage tracer, caged fluorescein. We injected caged fluorescein into cleavage-stage embryos and photoactivated it in pole cells at the cellular blastoderm stage. We successfully marked the majority of the pole cells, and traced the marked cells until they formed the embryonic gonads in wild-type embryos (data not shown).

In $N14^{m-}$ embryos, pole cells, which were marked with both fluorescein and anti-Vasa antibody, were observed on the surface of the midgut at stage 10 (Fig. 2A-C). However, at stage 11, the fluorescein-marked cells rapidly disappeared (Fig. 2D-F). We occasionally observed fluorescein-marked pole cells with no or very faint Vasa signals on the surface of the

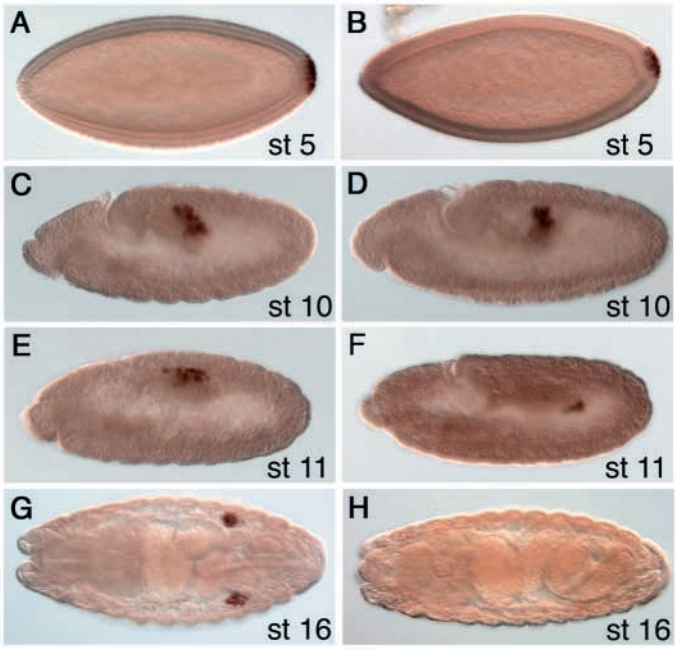

Fig. 1. Maternal *N14* mutant embryos have a defect in pole cell maintenance. Wild-type embryos (A,C,E,G) and $N14^{m-}$ embryos from *N14/Df(2R)w45-19g* females (B,D,F,H) were stained for Vasa to visualize pole cells. Anterior is towards the left in all panels. (A-F) Lateral views. (G,H) Top views. In wild-type embryos, pole cells were formed at the posterior pole of the blastoderm-stage embryo (A), and carried into the posterior midgut primordium. At stage 10, pole cells moved through the midgut epithelium and migrated dorsally along its basal surface (C). Next, pole cells moved into the mesoderm at stage 11 (E), and were finally incorporated into the embryonic gonad at stage 16 (G). In $N14^{m-}$ embryos, a normal number of pole cells was formed (B). These pole cells migrated normally through the midgut epithelium and moved dorsally along its surface (D). However, the number of Vasa-positive pole cells was rapidly reduced at stage 11 (F), and few or no pole cells were incorporated into the gonad (H).

Table 1. Phenotypes of maternal *wun2* mutants

	Number of pole cells				Female progeny with agametic ovaries (%)		
	Stage 5		Stage 14				
Maternal genotype	Per embryo	*n*	Per gonad	*n*	Unilateral	Bilateral	*n*
$wun2^{N14}/Df(2R)w45\text{-}19g$	29.0±3.5	20	1.1±1.6	36	9.4	83.4	181
$wun2^{\Delta}/Df(2R)w45\text{-}19g$	28.3±1.2	3	1.6±2.3	51	32.0	12.0	25
$wun2^{N14}/wun2^{\Delta}$	29.8±3.5	11	2.1±1.4	29	25.5	5.6	161
$+/Df(2R)w45\text{-}19g$	29.0±3.8	20	12.3±2.0	22	0	0	122

midgut (Fig. 2D-F; arrowheads). At stage 11, the pole cells appeared to have experienced a significant reduction in size accompanied by an increase in the intensity of fluorescein signal (Fig. 2D-F; arrows), and few fluorescein-marked pole cells were detectable by stage 12 (Fig. 2G-I). From these observations, we conclude that pole cells in *N14^{m-}* embryos die after they pass through the midgut epithelium.

Pole cell death in *N14^{m-}* embryos might occur in a caspase-3-independent manner

We next examined whether pole cell death in *N14^{m-}* embryos was caused by apoptosis. To detect apoptotic cells, we used an antibody against cleaved caspase 3. It has been shown that an antiserum against the same epitope reacts with the active form of the *Drosophila* effecter caspase, Drice, and labels dying cells in *Drosophila* (Brennecke et al., 2003; Yu et al., 2002). At stage 11, pole cells marked by the uncaged fluorescein with reduced Vasa signals were frequently observed in *N14^{m-}* embryos. However, active caspase 3 immunoreactivity was never detected in these dying cells (Fig. 3; arrowheads). We did observe a few caspase 3/Vasa double-positive cells (Fig. 3; arrows). Even in wild-type embryos, however, a small number of mis-migrated pole cells, which were usually seen in or on the surface of the midgut, were double-stained for the active caspase 3 and Vasa. The average number of active caspase 3/Vasa double-positive cells per stage 11 embryo was 1.7±2.2 in the *N14^{m-}* mutant (n=24) and 2.2±2.4 in wild-type (n=40). These observations indicate that caspase 3-mediated cell death occurs in mis-migrated pole cells even in wild-type embryos, and that it is not sufficient to explain the dramatic increase in pole cell death in *N14^{m-}* embryos. These results suggest that pole cell death in *N14^{m-}* embryos occurs via a caspase 3-independent pathway.

N14 mutation does not affect early events in pole cell development

A reduction in pole cell number during migration is also observed in embryos from females mutant for *nanos*, *pum* or *pgc* (Asaoka-Taguchi et al., 1999; Kobayashi et al., 1996; Nakamura et al., 1996). As these maternally acting genes have roles in earlier events in pole cell development, such as transcriptional repression (Asaoka-Taguchi et al., 1999; Deshpande et al., 1999; Martinho et al., 2004), we examined whether such events are also disrupted in *N14^{m-}* embryos.

First, we analyzed the distributions of several maternal RNAs and proteins that localize in pole plasm. At the blastoderm stage, distributions of *nanos* (Fig. 4A,E), *germ cell-less* (*gcl*) (Fig. 4B,F) and *pgc* RNAs (Fig. 4C,G), and the Nanos protein (Fig. 4D,H) in *N14^{m-}* embryos were indistinguishable from those in wild-type embryos, indicating that these pole plasm components are normally incorporated into pole cells in *N14^{m-}* embryos.

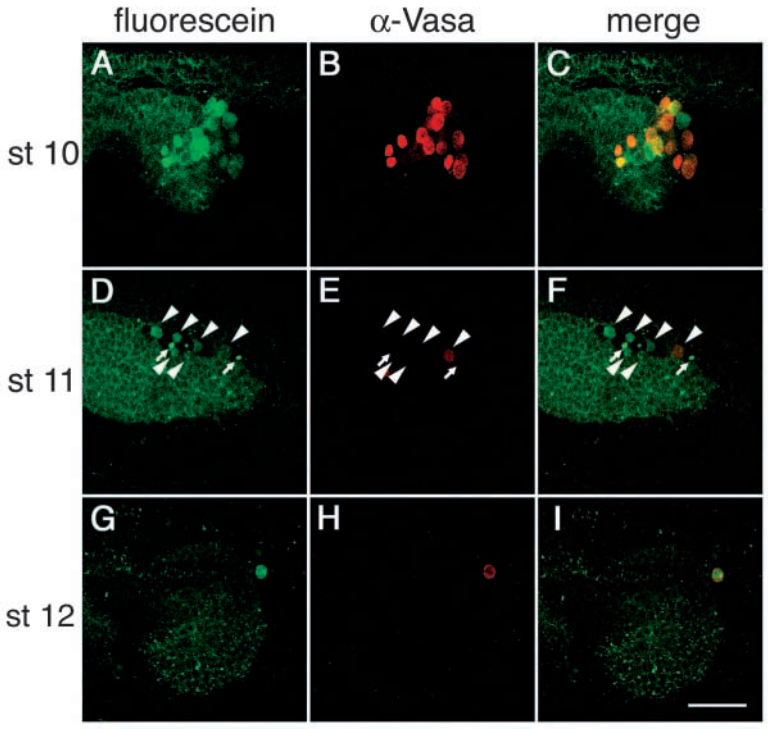

Fig. 2. Pole cells in *N14^{m-}* embryos die after exiting the midgut. Pole cells in *N14^{m-}* embryos were labeled with a photoactivatable lineage tracer, caged fluorescein (green), and an antibody against Vasa (red). Anterior is towards the left and dorsal is upwards. (A-C) At stage10, uncaged-fluorescein/Vasa double-positive pole cells were observed on the surface of the midgut. (D-F) From stage 11 onwards, the fluorescein-marked cells disappeared. The remaining fluorescein-positive cells were left on the dorsal side of the midgut surface. (G-I) At stage 12, few fluorescein-marked cells were observed. Scale bar: 40 μm.

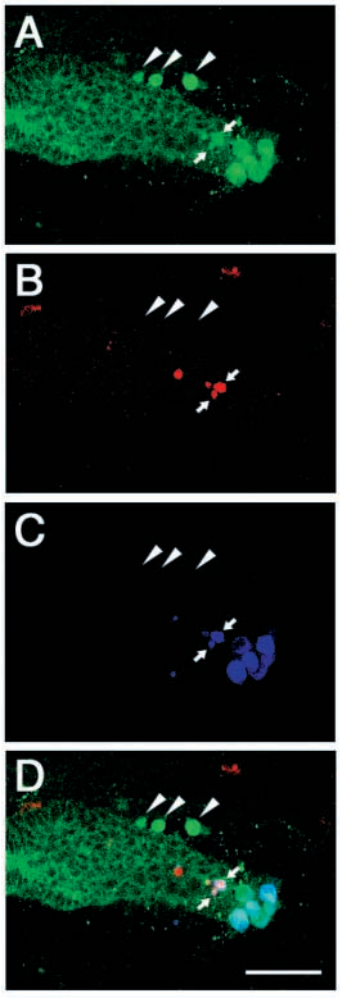

Fig. 3. Pole cell death in *N14^{m-}* embryos might occur via a caspase 3-independent pathway. Pole cells in *N14^{m-}* embryos were directly labeled using a caged fluorescein (A, green), and were stained for cleaved caspase 3 (B, red) and Vasa (C, blue). (D) Merged image of A-C. Cleaved caspase 3 was never detected in the dying pole cells on the surface of the midgut, in which the fluorescein signal remained but the Vasa signal was reduced (arrowheads). Arrows indicate the active caspase 3-positive mis-migrated pole cells, which are usually seen even in wild-type embryos. Scale bar: 40 μm.

We next examined whether transcriptional control in early pole cells is disrupted in *N14*$^{m-}$ embryos. It has been shown that transcriptional repression in pole cells correlates with the absence of phosphorylation of the Ser2 residue in the CTD repeats of RNA polymerase II (Seydoux and Dunn, 1997). To test global transcriptional activity in pole cells, we stained embryos with the antibody H5, which specifically reacts with the phospho-Ser2 version in the CTD of RNA polymerase II. In wild-type blastoderm embryos, H5 staining was not detected in pole cells, in contrast to the marked staining observed in somatic cells (Fig. 5A). This pattern of H5 staining was not altered in *N14*$^{m-}$ embryos (Fig. 5B), indicating that global transcriptional repression in pole cells remains intact in *N14*$^{m-}$ embryos.

Finally, we asked whether the *N14* mutation affects zygotic gene expression in pole cells. We introduced a *nanos-Gal4-VP16* transgene into an *N14* mutant background to supply a potent transcriptional activator in pole cells, and analyzed the Gal4-VP16-dependent *UAS-lacZ* target gene expression in pole cells (Van Doren et al., 1998b). In *N14*$^{m-}$ embryos, we did not detect precocious expression of β-galactosidase in early pole cells (Fig. 5C-E). The expression of β-galactosidase was normally repressed until stage 8, and was initiated in pole cells from stage 9 onwards (Fig. 5C-E). After stage 12, owing to the pole cell loss in *N14*$^{m-}$ embryos, the number of embryos expressing β-galactosidase was diminished (Fig. 5E). These results indicate that the regulation of zygotic gene expression in pole cells is normal in *N14*$^{m-}$ embryos. Thus, the *N14* mutation is unlikely to affect early events in pole cells, i.e. the sequestration and maintenance of pole plasm components, global transcriptional repression and the initiation of zygotic gene expression, leading us to conclude that *N14* represents a novel class of maternal mutations affecting pole cell survival.

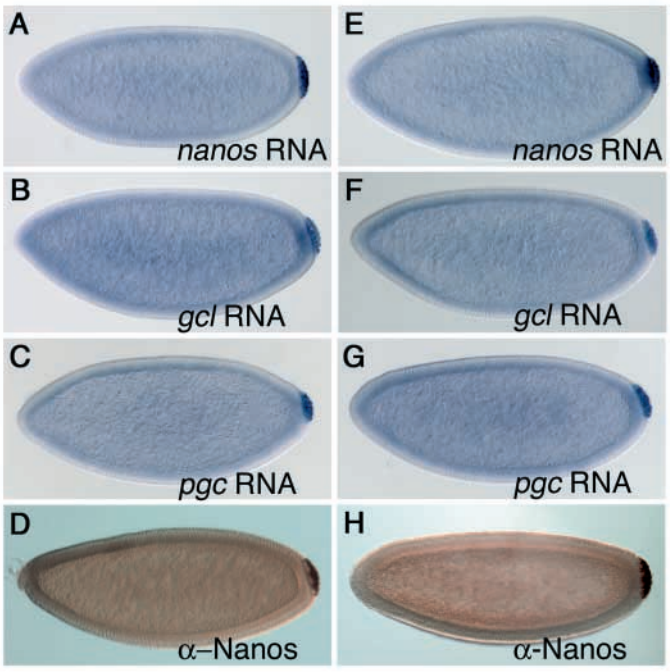

Fig. 4. *N14* mutation does not affect the distribution of pole plasm components. Wild-type (A-D) and *N14*$^{m-}$ (E-H) embryos at the cellular blastoderm stage. Embryos were stained for *nanos* RNA (A,E), *gcl* RNA (B,F), *pgc* RNA (C,G) or Nanos protein (D,H). These RNAs and protein were incorporated normally into pole cells in *N14*$^{m-}$ embryos (E-H) as in wild-type embryos (A-D).

wun2 is the gene responsible for the *N14* mutant phenotype

Through complementation tests, we identified five overlapping deficiencies that uncovered the *N14* mutant (see Materials and methods). The breakpoints of these deficiencies defined the *N14* locus within a ~100 kb genomic region containing nine identified or predicted genes (Fig. 6A). To identify the gene responsible for the *N14* mutant phenotype, we generated a series of transgenes containing genomic DNA fragments in this region (Fig. 6A). Of these, a ~17 kb genomic DNA fragment, which contained two genes, *wun2* and *CG13955*, completely rescued the *N14* mutant. To determine which gene was responsible for the *N14* mutant phenotype, we next made constructs, in which only one of the genes was intact. Only the 8 kb *Hin*cII fragment (*Pwun2-8k*), containing the entire *wun2*

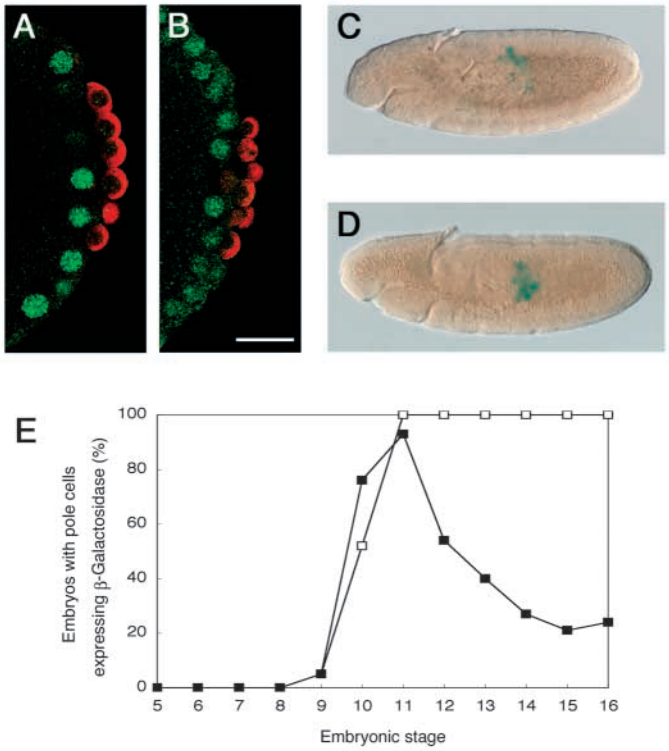

Fig. 5. *N14* mutation does not affect zygotic gene expression in pole cells. (A,B) Embryos at the syncytial blastoderm stage were stained with the antibody H5 (green) and an antibody against Vasa (red). The H5 antibody labeled somatic nuclei, but not pole cell nuclei in *N14*$^{m-}$ embryos (B) as well as in wild-type embryos (A). Scale bar: 20 μm. (C-E) Expression of β-galactosidase in pole cells was examined in embryos from *nanos-Gal4-VP16* females crossed with *UAS-lacZ* males. (C,D) Lateral views of stage 10 embryos. In both *N14*$^{m-}$ (D) and wild-type (C) embryos, β-galactosidase was expressed in pole cells. (E) The stage-dependent expression of β-galactosidase was examined in *N14*$^{m-}$ background (black squares) and wild-type background (white squares). The percentage of embryos with β-galactosidase-positive pole cells was plotted against the embryonic stage. In both *N14*$^{m-}$ and wild-type background, the expression of zygotic β-galactosidase was detected from stage 9. After stage 12, the number of embryos expressing β-galactosidase was diminished in *N14*$^{m-}$ embryos, owing to pole cell loss.

locus, rescued the *N14* mutant. Furthermore, a genomic fragment that had a partial deletion in the *wun2* RNA-coding region (*Pwun2-8kΔ*) failed to rescue the *N14* mutant. Finally, we found that the *N14* mutant chromosome had a nonsense mutation in the *wun2* gene at the 111th Trp codon (Fig. 6B). Thus, we refer to the *N14* mutant as the *wun2*^{N14} allele.

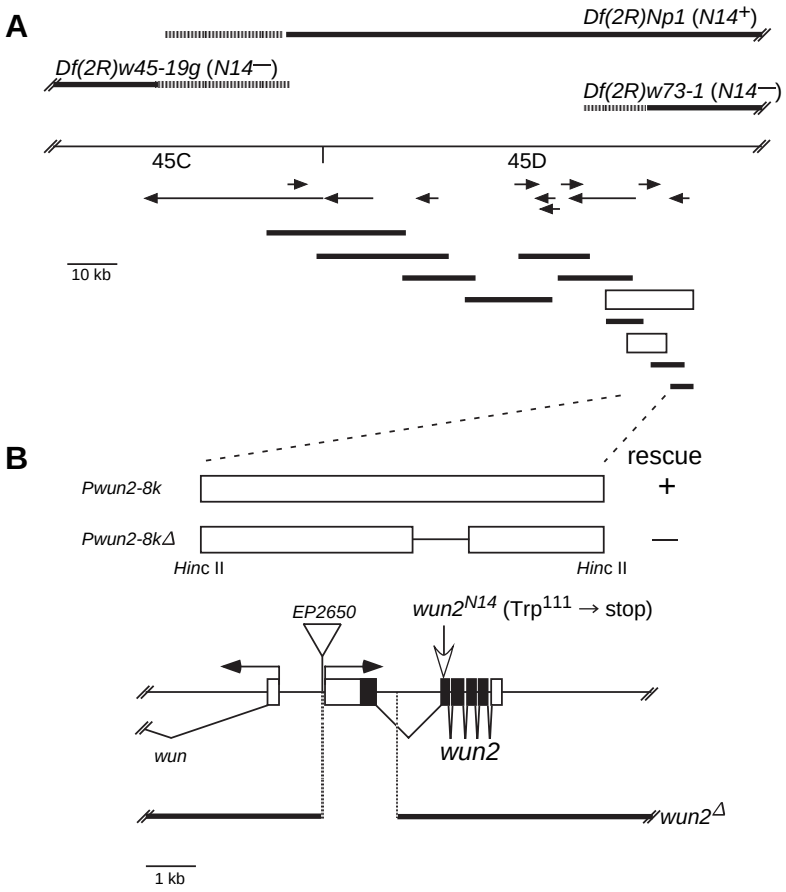

Fig. 6. *N14* is an allele of *wun2*. (A) Gene organization of the *N14* locus. The *N14* mutant phenotype was not complemented by *Df(2R)w45-19g* or *Df(2R)w73-1*, but was by *Df(2R)Np1*. These deficiencies defined the *N14* locus within ~100 kb of a genomic region that contains nine identified and predicted genes. A series of transgenic constructs that was used to identify the responsible gene for the *N14* mutant phenotype is shown below. Only the constructs in which *wun2* was intact (white boxes), rescued the *N14* mutant phenotype. (B) An 8 kb *Hin*cII fragment (*Pwun2-8k*) rescued the *N14* mutant phenotype, but the *Pwun2-8kΔ* fragment, which had a partial deletion in the *wun2* RNA-coding region, did not. The *N14* chromosome had a nonsense mutation at the 111th Trp codon of the *wun2*-coding region. Mobilization of a P-element insertion, *EP2650*, generated a *wun2* deletion (*wun2*$^{\Delta}$). Embryos from *N14*/*wun2*$^{\Delta}$ females showed the *N14* mutant phenotype, confirming that *wun2* is the responsible gene for the *N14* mutation (Table 1).

To generate new *wun2* alleles, we mobilized a P element insertion, *EP2650*, which locates ~30 bp distal to the 5′ side of the *wun2* locus. As a result, we isolated a deletion of *wun2* (*wun2*$^{\Delta}$), in which one-third of the *wun2* locus was deleted (Fig. 6B). The *wun2*$^{\Delta}$ mutant was homozygous viable, and lacked *wun2* RNA expression (see below). In embryos derived from *wun2*$^{\Delta}$/*Df(2R)w45-19g* and *wun2*$^{\Delta}$/*wun2*^{N14} mothers, pole cells formed normally, but most of them died after exiting the midgut; phenotypes essentially the same as those of the *N14*$^{m-}$ embryos (Table 1). The maternal *wun2*$^{\Delta}$ mutant phenotype was rescued by introducing the *Pwun2-8k* transgene (data not shown). These data demonstrate that maternal *wun2* is essential for pole cell survival.

wun2 encodes an LPP that dephosphorylates a number of phospholipids in vitro (Burnett and Howard, 2003; Starz-Gaiano et al., 2001). We next asked whether the phosphatase activity of Wun2 is responsible for maternal Wun2 function. It has been shown that the putative catalytic residues of Wun2, His274 or His326, are essential for its activity (Starz-Gaiano et al., 2001). We mutated His274 or His326 into Lys (H274K or H326K) and examined the ability of such mutant transgenes to rescue the *wun2*^{N14} phenotype. The two mutant *Pwun2-8k* transgenes with H247K or H326K failed to rescue the *wun2*^{N14} phenotype (data not shown), indicating that the effect of Wun2 on pole cell survival is dependent on its phosphatase activity.

Maternal *wun2* activity is required in pole cells

We next examined the distribution of *wun2* RNA during early embryogenesis. *wun2* RNA was detected ubiquitously in cleavage-stage embryos (stage 2, Fig. 7A). Although the signal in the somatic cell region became undetectable by the cellular blastoderm stage, it remained at high levels in pole cells (stage 4, Fig. 7B,B′). At stage 5, *wun2* signal was also detected in the somatic region in a posterior stripe pattern (Fig. 7C). These observations were consistent with a previous report (Renault et al.,

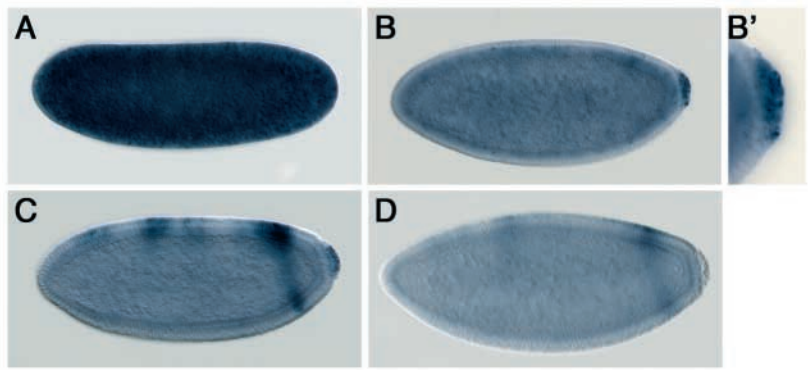

Fig. 7. Distribution of *wun2* RNA. Wild-type embryos (A-C) and embryos from *wun2*$^{\Delta}$/*Df(2R)w45-19g* females (*wun2*$^{\Delta}$ embryos; D) were stained for *wun2* RNA. (A) *wun2* RNA was detected throughout the stage-2 embryo. (B,B′) At stage 4, *wun2* RNA remained in pole cells, while the signal in somatic cells disappeared. (B′) Highlighting of the posterior pole of the embryo shown in B. (C) At stage 5, *wun2* RNA was detected in pole cells and in a posterior stripe. (D) Stage 5 *wun2*$^{\Delta}$ embryo showing the absence of *wun2* RNA in pole cells but its presence in a posterior stripe.

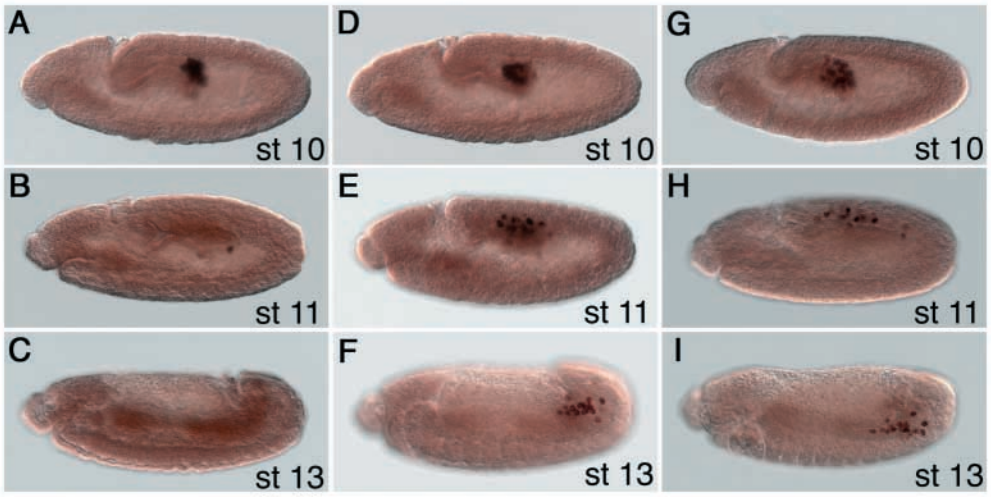

Fig. 8. Pole cell-specific expression of *wun2* rescues the maternal *wun2* mutant phenotype. Embryos were stained for Vasa to visualize pole cells. (A-C) Embryos from a cross of $wun2^{N14}$*/Df(2R)w45-19g; nanos-Gal4-VP16/+* females with *Oregon-R* males. (D-F) Embryos from a cross of $wun2^{N14}$*/Df(2R)w45-19g; nanos-Gal4-VP16/+* females with *EP2650* males. (G-I) Embryos from a cross of *nanos-Gal4-VP16* females with *EP2650* males. (D-F) Expression of *wun2* in pole cells fully rescued the maternal *wun2* mutant defect. These pole cells were moderately dispersed at the late stages of embryogenesis (F), as in embryos overexpressing *wun2* in pole cells (I) (Starz-Gaiano et al., 2001). The maternal *wun2* mutant phenotype was not rescued with *nanos-Gal4-VP16* driver alone (A-C) or with no driver (data not shown).

2002). In embryos from $wun2^{\Delta}$*/Df(2R)w45-19g* females crossed to wild-type males, *wun2* RNA was undetectable in pole cells, but was expressed in the posterior stripe (Fig. 7D). These results indicate that *wun2* RNA in pole cells is supplied maternally, while it is expressed zygotically in the posterior stripe.

To examine whether *wun2* activity is required in pole cells for their survival, we tested whether the pole cell-specific zygotic expression of *wun2* rescues the maternal *wun2* mutant phenotype. For this purpose, $wun2^{N14}$ mutant females carrying the *nanos-Gal4-VP16* transgene were crossed with males possessing *EP2650*, which expresses *wun2* under UAS control (Starz-Gaiano et al., 2001). We found that, in embryos resulting from this cross, pole cells escaped from cell death (Fig. 8D-F). Although these pole cells were moderately dispersed, as in $wun2^{+}$ embryos that overexpressed *wun2* in pole cells (Fig. 8G-I), they were incorporated into the gonads.

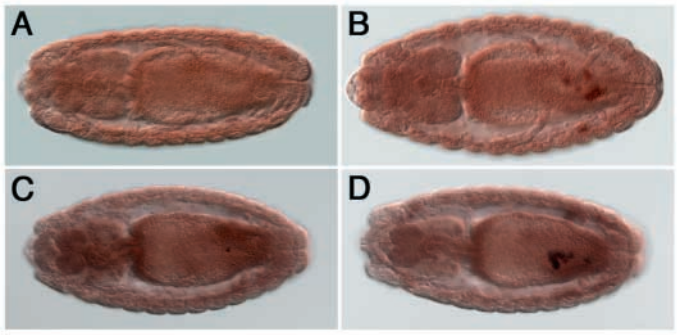

Fig. 9. Wun2 activity in pole cells competes with somatic Wun and Wun2 activities for pole cell survival. Embryos were stained for Vasa to visualize pole cells. (A) A $wun2^{m-}$ embryo showing the drastic pole cell loss phenotype. (B) An embryo lacking both maternal Wun2 and zygotic Wun and Wun2 showing partial suppression of the pole cell death phenotype of $wun2^{m-}$ embryos. (C) Overexpression of *wun2* in the mesoderm leads to pole cell death. (D) An embryo overexpressing *wun2* both in pole cells and the mesoderm shows a partially suppressed pole cell death phenotype caused by the overexpression of *wun2* in the mesoderm alone (C).

These results indicate that the expression of *wun2* in pole cells is sufficient to rescue pole cell death in $wun2^{m-}$ embryos, and that *wun2* is required in pole cells for their survival.

Wun2 activity in pole cells competes with zygotic Wun and Wun2 activities in somatic tissues to promote pole cell survival

It has been shown that zygotic *wun2* acts redundantly with *wun* in somatic tissues to guide pole cell migration. In embryos that are double mutants for *wun* and *wun2*, pole cells are dispersed on the midgut at stage 10, and fail to migrate to the mesoderm (Starz-Gaiano et al., 2001; Zhang et al., 1996; Zhang et al., 1997). Interestingly, somatic Wun and Wun2 are also capable of affecting pole cell survival. Overexpression of either of them in somatic tissues causes dramatic pole cell loss after stage 11 (Burnett and Howard, 2003; Starz-Gaiano et al., 2001). As this latter phenotype is very similar to that observed in $wun2^{m-}$ embryos, we asked whether there are any functional links between maternal *wun2* and zygotic *wun* and *wun2* in regard to pole cell survival.

First, we examined the effect of the loss of both zygotic *wun* and *wun2* on pole cell death caused by a maternal *wun2* mutation. Embryos lacking both maternal *wun2* and zygotic *wun* and *wun2* (referred to as wun^{m+z-} $wun2^{m-z-}$ embryos) showed a partial rescue of the pole cell death phenotype of the maternal *wun2* mutation (Fig. 9A,B). In wun^{m+z-} $wun2^{m-z-}$ embryos, an average of 10.5±3.5 pole cells were survived at stage 14-15 (n=28), although they failed to migrate toward the gonads as in the wun^{m+z-} $wun2^{m+z-}$ embryos. This result revealed that maternal *wun2* interacts genetically with zygotic *wun* and *wun2* in regulating pole cell survival.

We next examined whether the overexpression of *wun2* in pole cells affects the pole cell loss phenotype caused by the overexpression of *wun2* in somatic cells. We crossed females carrying both the *nanos-Gal4-VP16* transgene and a mesoderm driver *twist-Gal4* with males possessing *EP2650* to overexpress *wun2* both in pole cells and the mesoderm. The embryos that overexpressed *wun2* both in pole cells and the mesoderm had an average of 10.4±4.0 pole cells (n=64), while embryos overexpressing *wun2* in the mesoderm alone had an

average of 4.4±3.6 pole cells (n=99) per embryo at stage 14-15 (Fig. 9C,D). This result revealed that the overexpression of *wun2* in pole cells suppressed the pole cell loss caused by the overexpression of *wun2* in somatic cells. These observations show that pole cell survival requires a balance between LPP activities in pole cells and somatic cells. This further suggests that Wun2 activity in pole cells competes with the Wun and Wun2 activities in somatic cells to promote pole cell survival.

Discussion

The two related LPPs, Wun and Wun2, act redundantly in somatic cells to provide a repulsive environment to steer pole cell migration (Starz-Gaiano et al., 2001; Zhang et al., 1996; Zhang et al., 1997). The directional migration of pole cells is disrupted only when the expression of both *wun* and *wun2* is lost in somatic cells. Wun and Wun2 also influence pole cell viability, because the overexpression of either of them in somatic cells causes a severe loss of pole cells (Burnett and Howard, 2003; Starz-Gaiano et al., 2001). These observations had led to the proposal that Wun and Wun2 were indistinguishable in their somatic function, which is required for pole cell migration and survival. However, our results show that Wun and Wun2 have separable functions, and that Wun2 plays a novel cell-autonomous role in pole cell survival. Furthermore, we have shown that a balance between the LPP activities in pole cells and the surrounding somatic cells is crucial for pole cell survival. We propose that this balance is also involved in the directional migration of pole cells.

Maternal Wun2 has a novel function in pole cell survival

Several maternal mutations that affect pole cell survival have been isolated. These include *nanos*, *pum* and *pgc* (Asaoka-Taguchi et al., 1999; Forbes and Lehmann, 1998; Kobayashi et al., 1996; Nakamura et al., 1996). However, our results indicate that the phenotypes of maternal *wun2* mutant embryos are different from those of *nanos*, *pum* or *pgc* mutant embryos. For example, although *nanos*, *pum* and *pgc* are required for transcriptional repression in early pole cells (Asaoka et al., 1998; Asaoka-Taguchi et al., 1999; Deshpande et al., 2004; Deshpande et al., 1999; Martinho et al., 2004), maternal *wun2* plays no role in either transcriptional repression or in the onset of zygotic gene expression in pole cells (Fig. 5). Thus, it is likely that maternal *wun2* is required for pole cell survival at a different developmental step from *nanos*, *pum* and *pgc*.

Surprisingly, dying pole cells in $wun2^{m-}$ embryos were negative for cleaved caspase 3 (Fig. 3). We also found that the overexpression of the caspase inhibitor p35 (Hay et al., 1994) in pole cells did not rescue the pole cell death phenotype in $wun2^{m-}$ embryos (data not shown). Furthermore, we have never detected TUNEL-positive pole cells in $wun2^{m-}$ embryos (data not shown). Thus, the pole cell death in $wun2^{m-}$ embryos seems to occur via a mechanism different from typical caspase-dependent apoptosis. It has become evident that caspase-independent cell death pathways do exist (Golstein et al., 2003; Leist and Jäättela, 2001; Lockshin and Zakeri, 2002), and we suppose that such caspase-independent cell death might be occurring in these pole cells. Further morphological study may reveal how pole cell death occurs in $wun2^{m-}$ embryos.

A balance between LPP activities in pole cells and somatic cells is crucial for pole cell survival and migration

Wun2 belongs to a conserved family of LPPs (Burnett and Howard, 2003; Starz-Gaiano et al., 2001). LPPs are integral membrane proteins that dephosphorylate a number of bioactive lipid phosphates in vitro, such as lysophosphatidic acid, phosphatidic acid, sphingosine-1-phosphate and ceramide-1-phosphate. These lipid phosphates act as extracellular signaling molecules and/or intracellular second messengers and affect a variety of cellular processes, including cell survival and motility (Brindley et al., 2002; Sciorra and Morris, 2002). LPPs can attenuate cell activation by dephosphorylating bioactive lipid phosphates and/or they can generate alternative signals from dephosphorylated lipids, such as diacylglycerol, sphingosine and ceramide. The active sites of LPPs are exposed either on the outer surface of the plasma membrane or on the luminal surface of intracellular organelles, depending on their subcellular localization (Brindley et al., 2002; Sciorra and Morris, 2002). It has been proposed that LPPs promote the incorporation of lipid phosphate substrates into the outer leaflet of the membrane before their dephosphorylation (Roberts and Morris, 2000). Although the exact subcellular distribution of the endogenous Wun2 protein remains elusive, it localizes to the plasma membrane in *Drosophila* embryos when overexpressed (Starz-Gaiano et al., 2001). Therefore, Wun2 is likely to be an ecto-enzyme that promotes the uptake and the dephosphorylation of extracellular lipid phosphate substrates.

Our data indicate that maternally supplied Wun2 acts in a cell-autonomous manner to promote the survival of pole cells (Figs 7, 8). By contrast, zygotically expressed Wun and Wun2 act in somatic cells to direct pole cell migration (Starz-Gaiano et al., 2001; Zhang et al., 1996; Zhang et al., 1997). Furthermore, we have shown that pole cell survival requires a balance between LPP activities in pole cells and somatic cells (Fig. 9). Considering that Wun and Wun2 are likely to function as ecto-enzymes (Burnett and Howard, 2003; Starz-Gaiano et al., 2001), the same extracellular lipid phosphate could be degraded by both Wun2 in pole cells, and Wun and Wun2 in somatic cells. Thus, we propose that Wun2 activity in pole cells competes with somatic Wun and Wun2 activities for the uptake and the dephosphorylation of a common substrate. When Wun or Wun2 is overexpressed in somatic cells, the extracellular substrate would be depleted in the hemocoel surrounding the pole cells, so that Wun2 in pole cells is unable to produce the survival signal any longer. In embryos overexpressing Wun2 in both somatic cells and pole cells, increased Wun2 activity in pole cells leads to the increased incorporation of the substrate, promoting pole cell survival. In wun^{m+z-} $wun2^{m-z-}$ embryos, pole cells escaped from cell death (Fig. 9B), suggesting that pole cells are capable of producing the survival signal even in the absence of pole cell-autonomous Wun2 activity. There are eight LPP genes, including *wun* and *wun2*, in the *Drosophila* genome; *wun* mRNA is also maternally supplied in cleavage-stage embryos (Renault et al., 2002). Although *wun* mRNA does not become concentrated in pole cells, it is conceivable that trace amounts of Wun and/or other LPP become partitioned into pole cells, promoting their survival in the wun^{m+z-} $wun2^{m-z-}$ embryo. However, in normal embryos, such activity would have only a subtle effect on pole cell survival,

because it would promote the survival of only a small number of pole cells.

Somatic LPP activity also functions to repel pole cells. An extracellular substrate might direct somatic cells to produce a repellant molecule, while directing pole cells to produce a distinct survival signal. However, based on our finding that LPP activity in somatic cells competes with that in pole cells for an extracellular substrate, we favor the idea that LPP activity in somatic cells provides a repulsive environment that directs pole cell migration by depleting a substrate that is required by pole cells for their survival. We expect that similar LPP-mediated mechanisms of cell migration and survival may be widely used, as LPPs play important roles in various developmental processes such as axon growth, axis patterning and extra-embryonic vasculogenesis in mammals (Bräuer et al., 2003; Escalante-Alcalde et al., 2003). Future work will focus on identifying the endogenous substrate for Wun2 and resolving the mechanism by which Wun2 exerts its effects on pole cell survival.

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