Spatially restricted activity of a *Drosophila* lipid phosphatase guides migrating germ cells

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**SUMMARY**

Temporal and spatial controls of cell migration are crucial during normal development and in disease. Our understanding, though, of the mechanisms that guide cells along a specific migratory path remains largely unclear. We have identified *wunen 2* as a repellent for migrating primordial germ cells. We show that *wunen 2* maps next to and acts redundantly with the previously characterized gene *wunen*, and that known *wunen* mutants affect both transcripts. Both genes encode *Drosophila* homologs of mammalian phosphatidic acid phosphatase. Our work demonstrates that the catalytic residues of Wunen 2 are necessary for its repellent effect and that it can affect germ cell survival. We propose that spatially restricted phospholipid hydrolysis creates a gradient of signal necessary and specific for the migration and survival of germ cells.

Key words: *Drosophila*, Germ cell migration, *wunen*, *wunen 2*, Lipid phosphatase, Phosphatidic acid phosphatase.

**INTRODUCTION**

Cell migration is fundamental to the proper development and survival of animals. Migrating cells depend upon cell-cell and cell-matrix interactions for guidance, and upon cytoskeletal rearrangements for movement. Much remains unclear, though, about exactly how cells receive and interpret cues from their environment and translate them into directional movement. We are using the primordial germ cells (PGCs) of *Drosophila* to study directed cell migration. The PGCs provide an excellent model system for understanding migration, as they are easily identified morphologically and, as in most organisms, they arise far from their somatic gonadal partners and must travel to the developing gonad (Dixon, 1994).

Thirty to forty PGCs form in the early *Drosophila* embryo, and these are passively swept into the posterior midgut by the movements of gastrulation. The PGCs actively move through the midgut endoderm starting at stage 9 (Callaini et al., 1995; Jaglarz and Howard, 1995) and then reorient on the basal surface of the gut towards the dorsal side of the embryo. At stage 10, the germ cells begin to migrate into the mesoderm to seek out the somatic gonadal precursors (Boyle et al., 1997; Broihier et al., 1998; Moore et al., 1998). These two cell types remain associated during germ band retraction and, by stage 14, coalesce to form the embryonic gonad. Unlike germ cells in vertebrates, *Drosophila* PGCs do not divide while migrating, and only about 10-15 of them are incorporated into each gonad, while the remaining ‘lost’ germ cells fail to give rise to any other structures and are presumed to die (Hay et al., 1988; Technau and Campos-Ortega, 1986; Underwood et al., 1980).

Analysis of germ cell migration in *Drosophila* has uncovered several genes important to this process (Moore et al., 1998; Warrior, 1994; Zhang et al., 1997). In particular, previous work has identified both repulsive and attractive signals for the germ cells. In *wunen* (*wun*) mutants, the germ cells fail to move towards the mesoderm and instead spread over the entire surface of the midgut. In wild-type embryos, the PGCs orient away from the area of the gut that expresses *wun* mRNA. Moreover, when this gene is ectopically expressed in the mesoderm, the germ cells avoid this otherwise attractive tissue. *wun*, which encodes a *Drosophila* homolog of mammalian phosphatidic acid phosphatase type 2, has been proposed to produce a repellant for the germ cells (Zhang et al., 1996; Zhang et al., 1997). Further, an attractive signal for the germ cells is produced by HMGCoA reductase (Van Doren et al., 1998a). *Hmgcr* (the gene for HMGCoA reductase) is expressed in the gonadal mesoderm, and in flies that are mutant for the catalytic activity of HMGCoAR, the germ cells fail to move properly into the mesoderm and do not associate with their somatic partners. Moreover, ectopic expression of HMGCoAR can direct migrating germ cells towards its site of expression. It therefore seems that spatially restricted activation of the HMGCoAR pathway leads to the production of an attractant signal that guides germ cells towards the somatic gonadal mesoderm.

Since both *wun* and *Hmgcr* can cause aberrant migration phenotypes when ectopically expressed, we conducted a
mismatched restriction map to identify other genes involved in germ cell migration. This screen took advantage of the Gal 4-UAS system, as adapted by Rørth et al. (Rørth et al., 1998), to identify genes that lead to germ cell migration defects when activated either in the germ cells or in the mesoderm. In this manner, we identified a homolog of the Drosophila wun gene, which we named wunen-2 (wun2). wun and wun2 map within 5 kb of each other, have the same mRNA expression pattern, and are both affected in the original wun mutants. In addition, we showed that disruption of either gene alone does not result in a severe germ cell migration defect, suggesting that the two genes act redundantly to guide the germ cells. We demonstrated that mutations in the predicted catalytic domain of Wun2 eliminate repellent activity and that the protein is localized to the cell membrane. Biochemical studies of vertebrate lipid phosphatases have shed light on how these enzymes act, but the specificity and physiological roles for these proteins are not clear. Our data strongly suggest that Wun and Wun2 act enzymatically in a manner similar to those identified in vertebrates and that they have a specific role in Drosophila germ line development.

MATERIALS AND METHODS

Misexpression screens
For misexpression in the germ cells, females carrying the fai facets-lacZ (fai-lacZ; Fischer-Vize et al., 1992; Moore et al., 1998) and the nanos-Gal 4 VP16 (nos-Gal 4 VP16; Van Doren et al., 1998b) transgenes were generated and tested for a germ cell migration phenotype. These were kept in a heat shock-hid Y (psh-hidY) background (M. VanDoren and R. L., unpublished). The flies were heat shocked in bottles twice over 2 days for 2 hours to kill males and to produce large numbers of fai-lacZ; Nos-Gal 4 VP16 virgins. The few males that ‘escaped’ were fertile. 2300 EP lines (generated by P. Rørth (Rørth et al., 1998)) were generously provided by the Berkeley Drosophila Genome Project. For viable lines, EP homozygous male flies were used, otherwise we used EP/balancer males. ~10 males were crossed to ~20 females in vials, mated for 2 days and then placed into 18-well blocks. Embryo collection and staining was carried out as described previously (Moore et al., 1998). Stained embryos were examined after dissecting blocks under a dissecting microscope. EP lines that caused a phenotype were kept.

In the case of misexpression in the mesoderm, we tested approximately 200 EP lines, including 56 that had a phenotype in the larger screen. In this case, we used females bearing the twi-24B Gal 4 driver. Embryo collection was as described above, but anti-Vasa antibody (generously provided by Anne Williamson and Hélène Zinszner) was used to detect the germ cells. Antibody staining was performed in 18-well staining blocks; embryos were then screened for germ cell migration defects directly using a dissecting microscope. Images were obtained using a Zeiss Axiophot microscope and Sony Digital Camera with Adobe Photoshop software.

Molecular identification of wun2
Plasmid rescue and sequencing of inverse-PCR products were carried out as described at the BDGP web site (http://www.fruitfly.org/about/methods/inverse.pcr.html). In brief, for plasmid rescue, genomic DNA was prepared from homzygous EP flies, digested with EcoRI and ligated in a large volume. This ligation was concentrated then transformed into super-competent bacteria (Stratagene) and grown on ampicillin plates. A ~12 kb plasmid was rescued independently for both EP 2217 and EP 2650. Sequence analysis and restriction mapping confirmed that these were almost identical genomic pieces. This construct was digested with HindIII/EcoRI, and the pieces were subcloned into pBluescript. These subclones were used to make probes for in situ hybridization and filter screening. High density Drosophila P1 filters (Genome Systems) were screened to determine that the cytological location of both insertions is 45D. Two embryonic cDNA libraries were screened to find full-length clones: 9-12 hour embryonic library made by Kai Zinn in 1988 (a kind gift from Helen Sink) and the 0-22 hour embryonic library made by Ling Hong (LD library, BDGP website, a generous gift from Greg Beitel). Clones were excised from the lambda phage using Stratagene Lambda Excision Kit. Similar cDNAs were also identified in an expression pattern screen, as clone CK02248 (Kopeczynski et al., 1998) and by the Howard lab, who entered it in the NCBI database as ‘Tunen’ (AF236058). The full-length clone used for in situ and in the transgenics, LD 7, is from the LD library. For in situ, this clone was linearized with EcoRI and transcribed with T7 using the DIG mRNA labeling kit (Roche). The wun in situ hybridizations were carried out using the wunC19 cDNA, kindly provided by Ken Howard. Predicted transmembrane domains were determined by comparison with other PAP 2 family members and the following programs: HMMPHMM (www.enzim.hu/hmmpath/hmmpath.cgi); DAS (www.biokemi.su.se/~server/DAS/dasserver/); TopPred2 (www.biokemi.su.se/~server/toppred2/toppredServer.cgi), TMPred (dot.imagen bcm.tmc.edu:9331/cgi-bin/seq-search/); and TMHHMM(130.225.67.199/services/TMHHMM-1.0/). Some programs predicted only five transmembrane domains.

Analysis and generation of wun and wun2 mutants
Southern blots and sequence information from BDGP and Celera showed that wun and wun2 are about 5 kb apart. The wunC19 stock and the LDN-wun rescue line and DNA were gifts from Ken Howard. LSNa DNA was used in Southern blots and partially sequenced to confirm that it does not contain wun2. Df(2R) wun2B, Df(2R) 73-1, and 73-2 were obtained from the Bloomington and Umea stock collections. An X-ray mutagenesis screen was carried out to generate excisions of the EP P-elements. 82,000 males (half EP 2217, half EP 2650) were screened for loss of the white+ EP element by eye color. 80 white flies were generated and 41 were recovered as stocks. Of these, all but one had wild-type wun2 mRNA expression by in situ hybridization. This one, Df(2R) NYX-D15, lacked both wun2 and wun mRNA expression. Nucleic Acid Protection Assays were performed using the Ambion Multi-Nuclease Protection Kit and probes were made against the first 200-300 bases of the wun and wun2 transcripts, and to the 5' end of RP 49 as a loading control. For lethal mutations, homozygous mutant embryos were obtained by balancing the mutation over CyO Kr-GFP (Bloomington) and sorting under a fluorescence dissection microscope against the GFP expression. mRNA was then made from these embryos using Trizol (Gibco BRL).

Transgenic flies
The UAS-wun2-myc construct was made by overlapping PCR to add restriction sites to the ends of the cDNA. The β-globin 5'UTR was also added to the 5' end to enhance message stability and translation. This PCR product was subcloned into pBluescript and sequenced to confirm there were no errors. This was then subcloned along with the Myc tag (Myc tag DNA was kindly provided by Brad Jones) into the pUAST vector (a kind gift from Andrea Brand and Norbert Perrimon). The DNA was injected into embryos along with pTurbo (Mullins et al., 1989) to generate transformants. Embryos carrying this transgene were stained with anti-Myc antibody (Santa Cruz) to confirm expression of the transgene. The β-globin-wun2-Myc was used as a template to make site-directed point mutants using the Stratagene QuickChange Mutagenesis Kit. These point mutations were confirmed by sequencing.

Overexpression and germ cell counts
The line EP2650 was crossed to various drivers to examine the effects
DNA revealed that the two P-elements are inserted adjacent to the EP P-elements (see Materials and Methods). We used plasmid rescue to clone the genomic region of 2300 Drosophila germ cell migration. A misexpression screen for genes affecting germ cell migration

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We conducted a screen in Drosophila of 2300 independent ‘EP’ lines (Rørth et al., 1998) to find gain-of-function effects on germ cell migration (Fig. 1). These lines have a P-element that contains the Gal 4 upstream activation sequences (UAS) inserted randomly into their genome. To activate genes through the UAS, we used a germline-specific activator consisting of Gal 4-VP16 under the control of the promoter and localization elements of the nanos gene (nos-Gal 4-VP16; Van Doren et al., 1998b). This transcriptional activator is maternally deposited into the germ cells. Offspring from each EP line crossed to the nos-Gal 4-VP16 strain express a random gene specifically in the germ cells at stage 9, when transcription in these cells is initiated. Of the lines tested, 56 affect germ cell migration when crossed to the germ cell specific driver. Two lines, EP 2217 and EP 2650, have a dramatic effect, causing many germ cells to migrate incorrectly when crossed to nos-Gal 4-VP16 (Fig. 1C) compared with wild-type or to the EP lines without the driver (Fig. 1E). This phenotype is seen in about 30% of the embryos.

We also conducted a smaller screen where the EP lines were crossed to a Gal 4 line that drives expression in the mesoderm (twist-24B Gal 4, Fig. 1G; Greig and Akam, 1995). 200 EP lines were tested, including all lines that gave a phenotype in the first screen. EP 2217 and EP 2650 both have a strong and completely penetrant germ cell migration phenotype when crossed to the mesoderm Gal 4. In the progeny from these crosses, the germ cells are repelled from their normal target tissue and instead remain on the midgut (Fig. 1F).

The EP lines control expression of wun2

We used plasmid rescue to clone the genomic region adjacent to the EP P-elements (see Materials and Methods). Surprisingly, the sequence of the flanking DNA revealed that the two P-elements are inserted approximately 200 bases apart. This DNA was also used to isolate several cDNAs. Comparison of the cDNA sequence with the database revealed that although the predicted protein has 80 additional residues at the N terminus, the remainder of the protein is 50% identical and 67% similar to Wun (Fig. 2B).

RESULTS

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Fig. 1. A misexpression screen identified EP lines which cause aberrant germ cell migration. (A) Females carrying the fat facets-lacZ and nanos-Gal 4-VP16 transgenes were mated to males from 2300 lines, each having a randomly-inserted EP element containing the Gal 4 binding site (Upstream Activation Sequence, UAS). Thus, the progeny from this cross have the endogenous downstream gene specifically activated in the germ cells (indicated in blue-green). β-Galactosidase activity in the germ cells is maternally supplied by fat-facets-lacZ, so the embryos can be directly stained and assayed for germ cell migration defects. (B-F) Anterior is towards the left in all panels, and embryos are stained with anti-Vasa antibody. (B,D,F) lateral views; (C,E) dorsal view. (B,C) Embryos from the cross fat-facets-lacZ; nos-Gal4-VP16 x EP 2650. (B) At stage 11, migration appears normal (compare with D). (C) By stage 15, many germ cells are found outside of the gonad (brackets), indicating that they did not migrate properly. An identical phenotype was observed using EP 2217 (not shown). (D,E) Embryos from EP 2650 homozygous parents. Germ cell migration is wild type in this strain, both at stage 11 (D) and stage 15 (E). (F) Germ cells are repelled when EP 2650 is expressed in the mesoderm using twist-24B Gal 4 and stay on the midgut. Normally, germ cells would enter the mesoderm at this stage (see B,D). We see an identical result using EP 2217 (not shown). (G) Misexpression system for the mesoderm. A smaller misexpression screen was carried out by crossing females carrying the twist-24B Gal 4 transgenes to males from about 200 EP lines. The progeny from this cross have the endogenous gene downstream from the UAS turned on in the mesoderm (blue-green).
In addition, genomic Southern blot analysis and partial sequencing revealed that the transcriptional start sites of the two genes are approximately 5 kb apart and that they are transcribed in opposite orientations. Thus, we named the new gene wunen-2 (wun2). Both genes are Drosophila homologs of the mammalian protein phosphatidic acid phosphatase type 2 α2 (PAP2α2, also known as lipid phosphate phosphohydrolase 1 – LPP1). wun2 is 38% identical and 52% similar to human PAP2α2 over the core catalytic and transmembrane domains.

To confirm that the germ cell migration defect we observe with the two EP lines is caused by ectopic expression of wun2, we generated transgenic lines with the cDNA under UAS control. When crossed to nos-Gal 4-VP16 (not shown) or mesoderm drivers, the pUAST-wun2 transgenic lines gave germ cell migration phenotypes identical to those of the original EP lines (see Fig. 6A).

Ectopic expression of Wun or Wun2 results in a reduction in the number of germ cells

Ectopic expression of wun2 in the mesoderm causes germ cells to avoid this normally attractive tissue. The same phenotype was previously observed for wun using a similar misexpression construct (pUAST-wun218; Zhang et al., 1997). While this repellant activity is apparent as early as stage 10 of embryogenesis, we observed a second phenotype at later stages. Beginning at stage 11, the number of germ cells declines dramatically as assayed by a variety of markers, including anti-Vasa antibody (Fig. 3A,E) and in situ hybridization for nanos mRNA (not shown). In wild type approximately 25 germ cells are present at stage 13 of embryogenesis, whereas in embryos where wun2 or wun are ectopically expressed, only two to four germ cells are detected at this stage. To test whether the reduction in germ cell number is correlated with a defect in the development of the mesoderm, specifically the gonadal mesoderm, we analyzed the expression of the general mesoderm marker Mef2 (Bour et al., 1995; Lilly et al., 1995) and the gonadal mesoderm marker Clift (Boyle et al., 1997). In embryos with ectopic wun2 expression, muscles and the somatic gonadal mesoderm form apparently normally (Fig. 3C and data not shown). Furthermore, these embryos develop to adulthood, hatch, and are often sterile. The fact that these germ cells do not associate with the gonad is not sufficient to explain their loss, because in other mutants with significant germ cell migration defects we observe a normal number of germ cells throughout embryogenesis. Thus, ectopic expression of wun and wun2 specifically affects germ cell migration and survival.

To analyze the effect of Wun and Wun2 on germ cell survival in more detail, we expressed the protein in a variety of cell types and during different stages of development (Fig. 3E). Expression in tissues that the germ cells do not normally contact, including the nervous system (with an elav Gal 4) or the trachea (breathless Gal 4), results in a decrease in germ cell number. Using embryos carrying a heat-shock promoter driving Gal 4 expression, we determined that expression of wun2 is necessary prior to stage 11 for germ cell loss (data not shown). These results suggest that germ cell loss occurs independently of cell-cell contact between the Wun2-expressing tissue and the germ cells and that wun2 must be expressed early to cause a reduction in germ cell numbers. Expression in the germ cells themselves does not result in a sharp decline in their numbers. We believe that this is caused by a combination of the late onset of this driver, and the fact that the levels of enzymatic activity (see below) are probably lower because fewer cells are expressing the transgene than is the case for other drivers. This may also explain the weakness of the repellant phenotype when wun2 is expressed in the germ cells.
Wunen 2 activity guides germ cells

wun2 mRNA has the same expression pattern as wun mRNA in the embryo (Fig. 4). At stage 10 of embryogenesis, both gene products are detected in the hindgut and in those regions of the posterior midgut that normally exclude germ cells. In addition, the mRNA has a dynamic expression pattern in the epidermis, some lateral neuroblast cells of the nervous system and in the anterior midgut.

Given the conservation between wun and wun2, their close juxtaposition in the genome, and identical expression patterns and misexpression phenotypes, we wanted to determine whether mutations in wun2 would result in germ cell migration defects similar to those in wun alleles. To this end, we characterized the existing deficiencies in this genomic region as well as the previously described wun allele, wunCE, and also generated new mutations. All mutations and deficiencies that cause a wun germ cell migration phenotype lack wun as well as wun2 mRNA expression, as assayed by in situ hybridization and RNase protection analysis (Fig. 5C). These data strongly suggest that the wun mutant phenotype is caused by lack of both wun and wun2 transcripts. To determine whether the two genes act redundantly with respect to germ cell migration, we characterized two additional EP P-element insertion lines, EP 2607 and EP 2527. EP 2607 is inserted just upstream of the wun transcription unit; EP 2527 is inserted into the 5’ UTR in the first exon of wun2 (Fig. 2). RNase protection assays indicate that the P-element insertion EP 2607 specifically affects the wun transcript, while EP 2527 affects only wun2.

Fig. 3. Overexpression of wun2 leads to a reduction of germ cells. Orientation is anterior to the left and dorsal up in this and all following figures. (A,C) Embryos from the cross twi-24B Gal4 × EP 2217. (A,B) Germ cells are stained with anti-Vasa antibody. At stage 13, only four germ cells can be seen in a wun2 misexpression embryo, compared to 17 on one side of a wild-type embryo (B). Similar results were seen using EP 2650 (not shown). (C,D) Gonadal mesoderm (white brackets) develops normally in a misexpression embryo (C) compared with wild type (D) as revealed by anti-Clift antibody (α-Eya). (E) Graph indicates the decline in Vasa-positive germ cell numbers over time in response to wun2 expression in various tissues. For each Gal4 driver, the number of germ cells at stage 10, 11, 12 and 13 is shown. A small decline in number is normal, as is seen with no driver (EP 2217 alone, blue), with twi-Gal4 alone crossed to EP 2217 (turquoise) or with the nos-Gal4-VP16 driver (nos, green). The number of germ cells rapidly declines when wun2 is misexpressed in the mesoderm (twi, dark blue), the trachea (btl, orange), or the gut (48Y, yellow). There is also a reduction of germ cells when wun is expressed in the mesoderm (red). Error bars reflect the standard error for each data set. All drivers alone are similar to no driver (see twi alone).

Fig. 4. wun2 and wun mRNAs have identical expression patterns. (A) At stage 10, wun2 mRNA can be seen in the epidermis, some neuroblasts, the hindgut and in the bottom of the midgut by in situ hybridization. Note the gap in expression in the midgut juxtaposed to the mesoderm (between arrows), where the germ cells exit the gut. (B) A dorsal view of the CNS expression at stage 11. (C) Stage 13. wun2 is still expressed in the epidermis and in the gut. (D) wun mRNA expression is also seen in the epidermis and gut at stage 10. At later stages, the wun expression pattern is the same as that for wun2 (not shown).
Fig. 5. wun and wun2 provide redundant cues to guide migrating germ cells away from the midgut. (A) In wun<sup>CE</sup> mutant embryos at stage 10, the germ cells exit the midgut and remain spread out on its basal surface instead of reorienting towards the mesoderm as in wild type (B). The wun<sup>CE</sup> lesion has not been molecularly characterized. (C) wun mutants, which have a strong germ cell migration defect, also lack the wun2 transcript by RNase protection analysis. The protected fragment for wun2 and the loading control RP 49 are marked. Unprotected probe, seen strongly in the yeast/no nuclease control, is asterisked. The wun2 transcript is detected in wild type, but not in homozygous wun mutants. This includes two alleles originally identified based on the wun phenotype, wun<sup>Gl</sup> and wun<sup>CE</sup>, and a deficiency, Df(2R) 73-1. In an effort to make a wun2 mutant, we conducted an X-ray screen and generated the allele NYX-D15; however, this also removes expression of both genes. The deficiency Df(2R) 73-2 breaks near the two genes but both mRNAs are expressed and this allele is normal for germ cell migration. All lanes contained 5 μg target mRNA unless otherwise indicated. RNase protection using a probe for wun transcript is detected (arrow) in wild type (OR), EP 2527 homozygous embryos, and NYX-D15 heterozygotes, but not in EP 2607 or NYX-D15 homozygotes. (E) The wun2 transcript is detected in wild type (OR), EP 2607 homozygous and heterozygous embryos, and wun<sup>CE</sup> heterozygotes, but not in EP 2527 or wun<sup>CE</sup> homozygotes. Neither EP 2607 nor EP 2527 homozygous embryos show the germ cell migration phenotype seen in wun<sup>CE</sup> (A).

Germ cell migration requires Wun2 phosphatase activity

The closest mammalian homologs of wun and wun2 are the PAP 2α class of lipid phosphate phosphatases, which have been shown to hydrolyze several phospholipids in vitro (Brindley and Waggoner, 1998; Roberts et al., 1998). Sequence alignments with other phosphatases and structure-function analysis suggest that PAP 2α traverses the plasma membrane six times and that regions required for catalytic activity are located in two extracellular domains (Zhang et al., 2000). The finding that exogenously provided phospholipids can be rapidly hydrolyzed by the enzyme further supports that PAP 2α acts as an ecto-enzyme (Ishikawa et al., 2000; Jasinska et al., 1999). Sequence conservation between wun<sup>2</sup> and PAP 2α in the second and third extracellular loops includes the catalytic domains, as predicted from the analysis of the structurally-related enzyme glucose-6-phosphatase (G-6-Pase, Figs 2B, 6F; Lei et al., 1995; Pan et al., 1998). The catalytic activity of Wun, however, had not been tested for its function in germ cell guidance.

To determine whether the predicted catalytic residues of Wun2 are indeed necessary for the repulsion of the germ cells in vivo, we generated point mutations in the conserved domains and tested the effects. We mutated amino acids previously shown to be important for the catalytic activities of similar enzymes: His274 (corresponding to His171 of mLPP1 and His119 of G-6-Pase) and His326 (His223 of mLPP1 and His176 of G-6-Pase), which have been predicted to act as phosphate acceptors and covalent linkers in G-6-Pase (Hemrika and Wever, 1997; Lei et al., 1995; Messerschmidt and Wever, 1996; Pan et al., 1998; Zhang et al., 2000). In addition, the non-conserved amino acid Y225 adjacent to the catalytic domain was mutated, which should not affect phosphohydrolase activity. We generated transgenic flies carrying the wild-type or mutated wun2-coding region as Myc-tagged, UAS constructs (pUAST-wun2-myc). Wild-type and mutant proteins were expressed in the mesoderm and the embryos were assayed for the germ cell repulsion and germ cell loss phenotypes. Changes in the putative catalytic residues H274K or H326K resulted in a loss of repellant activity and a normal number of germ cells (Fig. 6C-D and data not shown). The ‘control’ substitution (Y225W) has wild-type activity with regard to repulsion and germ cell loss (Fig. 6B). The mutant and wild-type proteins were expressed at similar levels, as assayed by antibody staining for the Myc tag (Fig. 6). We conclude that the effect of Wun2 on germ cell migration and maintenance is dependent on its phosphohydrolase activity.

If Wun2 has the same membrane topology and distribution that has been shown for PAP 2α, we would expect Wun2 to be at the cell membrane. We used the C-terminally tagged wun2 expression constructs to analyze the distribution of Wun2 in embryos. We expressed the tagged protein in the midgut and the epidermis and co-labeled with markers for different cell compartments. We found that wild-type as well as Wun2...
We have identified a homolog of the *Drosophila wun* gene, *wun2*. These two genes map 5 kb apart, are transcribed in opposite orientations and have identical expression patterns. We show that the previously identified ‘wun’ mutations affect expression of both *wun* and *wun2*. Furthermore, mutations in either *wun* or *wun2* do not cause a ‘wun’ germ cell migration phenotype, suggesting that the two genes act redundantly. *wun* and *wun2* mRNAs are expressed in those regions of the gut that the germ cells avoid during their migration. Furthermore, ectopic expression of either gene repels the germ cells from the sites of expression and ultimately causes germ cell loss. These and previous data have led to the hypothesis that Wun and Wun2 produce a repellant signal for migrating germ cells, and that spatially restricted expression of *wun* and *wun2* in the midgut is required to orient germ cells towards the mesoderm.

Wun and Wun2 belong to a conserved family of phosphatidic acid phosphohydrolases. Human PAP 2α is thought to have its catalytic domains exposed on the cell surface (Jasinska et al., 1999) and has been shown in vitro to hydrolyze a variety of phospholipid substrates, including lysophosphatidic acid (LPA), phosphatidic acid (PA), sphingosine-1-phosphate (S-1-P) and ceramide-1-phosphate (C-1-P; Roberts et al., 1998). In support of the idea that Wun and Wun2 are functional homologs of mammalian PAP 2α, we have shown that Wun2 protein localizes to the cell membrane and that its catalytic activity is required for its non-cell-autonomous effects on the germ cells. Little is known about the in vivo function of PAP 2α in mammals, although in *Drosophila*, Wun and Wun2 seem to have a rather specific effect on germ cell migration and survival.

Previously, several models were proposed to explain how germ cells are repelled by Wun (in the following, we will refer to the activities encoded by both the *wun* and *wun2* genes as ‘Wun activity’). Given our work and recent studies from other groups, we favor a model in which either the extracellular products or substrates of Wun activity are directly received on the surface of the germ cells and initiate a signaling cascade to direct these cells. For example, a dephosphorylated lipid may act as a repellant. Alternatively, a phospholipid may normally act as a diffusible attractant, and this signal may be destroyed by Wun activity. In this manner, germ cells would show no preference for sites of Wun activity and would instead be guided towards higher concentration of the attractive phospholipid. The HMGCoxAR pathway produces an attractant signal in the gonadal mesoderm which is necessary for germ cell migration (Van Doren et al., 1998a). The putative attractant on which Wun acts, however, is most likely provided generally, or by the midgut itself, and not by the gonadal mesoderm, because in embryos with no mesoderm, such as *twist, snail*,...
double mutants, the germ cells still move correctly to the top of the gut (Rongo et al., 1997; Warrior, 1994). Thus, it is unlikely that the Wun substrate is the product of the HMGCoAR cascade. Interestingly, phospholipid substrates for PAP 2α have been shown to promote a variety of cell responses in mammalian in vitro culture systems including cell migration, differentiation and apoptosis. Some of these responses are mediated by activation of G-protein coupled receptors of the epithelial differentiation gene (Edg) family (reviewed by Brindley and Waggner, 1998; Goetzl and An, 1998). In addition, a S-1-P receptor has recently been shown to be required for cell migration in zebrafish (Kupperman et al., 2000). Thus, the activation of a G-protein-coupled receptor in germ cells may mediate the migratory response to Wun activity.

In addition to affecting germ cell migration, overexpression of wun and wun2 during development leads to striking germ cell loss. This reduction in germ cell number seems to require that high levels of Wun activity are present early during embryogenesis. This effect is specific to germ cells, as we do not see any general effects on pattern formation, other cell migrations, or cell survival in these embryos. At this point, we have no direct evidence that links this reduction in germ cell number to the apoptotic pathway in germ cells, although we consider this the most likely explanation. TUNEL labeling indicates that wild-type germ cells can undergo apoptosis (M. S-G. and R. L., unpublished observations). Furthermore, expression of the cell death genes hid and reaper specifically in germ cells leads to their death (A. F. and R. L., unpublished observations). Thus, the downstream effectors of the apoptotic program are present in Drosophila germ cells. Our markers for germ cells seem to be lost quickly in dying cells, though, and the small number of germ cells makes it difficult to detect an increase in the number of apoptotic cells in misexpression experiments.

During the normal development of many organisms, some germ cells fail to find their way to the gonad, and it is probably important to prevent the survival of these lost germ cells, as they could differentiate into other tissues and potentially produce tumors. In the mouse, Steel factor provides both guidance and survival signals to germ cells (Dolci et al., 1991; Godin et al., 1991; Pesce et al., 1993). In Drosophila, the expression domains of wun and wun2 in the endoderm and ectoderm border the germ cell migratory path. A lipid substrate for Wun activity could act as a survival factor for germ cells, while this signal would be removed in areas with high levels of Wun activity and the germ cells would die. Interestingly, overexpression of a mammalian S-1-P phosphatase has been recently shown to promote cell death (Mandala et al., 2000). Mutants that lack wun and wun2 transcripts, though, still have the wild-type decline in germ cell numbers (data not shown). Thus, these genes may not normally act to control germ cell survival, or germ cell death may require very high levels of Wun activity. Alternatively, some residual lipid phosphatase activity may be present in the mutant embryos. Indeed, there are six other highly conserved wun-like genes in the Drosophila genome (Celera, BDGP) some of which could act to control germ cell number. No candidate phospholipid receptor has been identified yet in Drosophila that could mediate the guidance and survival cues to the germ cells. Ongoing maternal effect screens (C. B. Yohn and R. L., unpublished observations), as well as germ cell specific misexpression screens like the one reported here, should lead to the identification of such molecules.

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