

# Quantifying the range of a lipid phosphate signal *in vivo*

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## Summary

Quantitative information about the range of influence of extracellular signaling molecules is critical for understanding their effects, but is difficult to determine in the complex and dynamic three-dimensional environment of a living embryo. *Drosophila* germ cells migrate during embryogenesis and use spatial information provided by expression of lipid phosphate phosphatases called Wunens to reach the somatic gonad. However, whether guidance requires cell contact or involves a diffusible signal is not known. We ectopically expressed Wunens in various segmentally repeated ectodermal and parasegmental patterns in embryos otherwise null for Wunens and used germ cell behavior to show that the signal is diffusible and to define its range. We correlated this back to the wild-type scenario and found that the germ cell migratory path can be primarily accounted for by Wunen expression. This approach provides the first quantitative information of the effective range of a lipid phosphate *in vivo* and has implications for the migration of other cell types that respond to lipid phosphates.

**Key words:** Germ cell, Somatic gonadal precursor, Cell migration, Lipid phosphate phosphatase, Wunen, *Drosophila*

## Introduction

Lipids can act as extracellular signaling molecules to influence many aspects of cell behavior, including cell migration. For example, the amoeboid cells of *Dictyostelium* migrate towards sources of lysophosphatidic acid (LPA) (Jalink et al., 1993), and the circulation of T lymphocytes is dependent on extracellular levels of sphingosine 1-phosphate (S1P) (Matloubian et al., 2004), whereas phosphatidic acid (PA) acts as a chemoattractant for leucocytes (Frondorf et al., 2010). When chemotaxis is studied in culture, cells are subject to artificial concentration gradients of attractants or repellants and the distance of action of a chemoattractant or repellant can be difficult to extrapolate to the *in vivo* situation.

The migration of germ cells that occurs during embryogenesis in *Drosophila* and other organisms provide an attractive experimental setting in which to study cell motility and chemotaxis *in vivo* (reviewed in Richardson and Lehmann, 2010). *Drosophila* germ cells are guided by the restricted expression of two lipid-modifying enzymes called Wunen (Wun) and Wunen2 (Wun2), hereafter collectively referred to as Wunens. The Wunens belong to a family of lipid phosphate phosphatases (LPPs), which are membrane-bound enzymes with their catalytic surfaces exposed to the extracellular or luminal surface of cells, depending on their subcellular localization. *In vitro* LPPs dephosphorylate a broad range of lipid phosphate substrates including S1P, LPA, PA and ceramide 1-phosphate (Morris et al., 2013).

*Drosophila* germ cells are found as a cluster of cells inside the blind end of posterior midgut pocket at the end of stage 9 of embryonic development. At stage 10 (6 hours 50 minutes after egg laying, AEL) they individualize and migrate across the midgut epithelium, which requires epithelial remodeling (Chanet and Schweisguth, 2012; Seifert and Lehmann, 2012). At stage 11 (9 hours AEL), the germ cells enter the mesoderm and while doing so partition bilaterally to associate with the somatic gonadal precursor cells (SGPs). These cells are specified at stage 11 as three bilateral clusters in the mesoderm in parasegments 10–12 (Brookman et al., 1992; Boyle and DiNardo, 1995). Males possess an additional SGP cluster originating in parasegment 13 (DeFalco et al., 2003). The two cell types align at stage 13 (11 hours 30 minutes AEL) then coalesce to form a compact spherical gonad at stage 14 (Boyle and DiNardo, 1995). The coalescence of the SGPs is, however, independent of the presence of germ cells (Brookman et al., 1992).

The genes encoding the Wunens show a dynamic expression pattern during *Drosophila* embryonic development. *wun* and *wun2* are expressed zygotically in somatic tissues, such as regions of the posterior midgut and the central nervous system (CNS), at stages 10 and 11, respectively, and the germ cells specifically avoid these tissues during their migration (Zhang et al., 1997; Starz-Gaiano et al., 2001). In the absence of somatic Wunens, germ cells scatter and are lost in the entire posterior of the embryo. These data suggest that expression of Wunens acts to repel germ cells.

Wunens could act at the molecular level by locally destroying an extracellular lipid phosphate attractant, creating a diffusible gradient that the germ cells detect (Renault et al., 2004). In this case, germ cells would move away from the somatic Wunen-expressing tissues

without the need for direct contact. An alternative model proposes that the extracellular part of Wunen acts as a membrane-bound ligand, therefore germ cells would have to make direct contact with the somatic Wunen-expressing cells in order to sense them (Zhang et al., 1997). Subsequent work has demonstrated that the catalytic activity of Wunen is essential for their effect on germ cells (Starz-Gaiano et al., 2001). Therefore a 'cell-contact dependent' model would be more likely to involve Wunen altering a lipid component of somatic cells, and thereby changing a surface property such that these cells become unattractive to migrating germ cells.

In order to distinguish between the cell-contact-dependent and contact-independent models and to understand how a specific pattern of expression of a LPP functions to affect cell migration in general, we combined ectopic Wunen expression with three-dimensional (3D) rendering, image segmentation (the automatic delineation of objects in the image) and live-imaging methods. We found that the Wunen-dependent signal is instructive to germ cells, and that germ cells do not need to make direct contact with Wunen-expressing somatic cells to detect their position, thereby substantiating the diffusible signal model. Furthermore, we have defined the distance up to which a Wunen-expressing somatic cell can influence a migrating germ cell and used this measurement to determine the extent to which Wunen contribute to the overall migratory path of the germ cells in wild-type embryos.

## Results

### Ectodermal expression of Wunen does not affect germ cell survival

Overexpression of Wunen in large regions of the embryo using a variety of different Gal4 drivers leads to germ cell death (Starz-Gaiano et al., 2001) making it difficult to determine the effect of Wunen specifically on migration. In order to circumvent this problem, we made use of drivers that express in the ectoderm in segmentally repeated domains. In particular we used Gal4 drivers that express under the control of the promoters of the pair-rule gene *hairy* (*h*) or the segment polarity gene *engrailed* (*en*) that express in 7 or 14 embryonic stripes respectively (Fig. 1A,B). In embryos zygotically null for *wun* and *wun2* (hereafter referred to as *wunen* somatic null embryos), germ cells are scattered throughout the posterior of the embryo and many enter the ectoderm (arrows, Fig. 1F; supplementary material Movie 5). We reasoned that by examining the response of germ cells to Wunen expressed in these domains in a *wunen* somatic null embryo we would be able to determine whether the signal is instructive, whether it is cell contact dependent or independent and measure the distance over which a Wunen-expressing somatic cell can influence a germ cell.

By examining the number of germ cells in late embryos (at stages 13–14, after germ cell migration is completed), we observed that expression of Wunen under the control of these drivers, either in a wild-type or *wunen* somatic null background (denoted *wun wun2* Z–), causes only a minor decrease in germ cell number (mean germ cell numbers shown in Fig. 1E–H) enabling us to uncouple the effect of Wunen in germ cell migration from germ cell death.

The two scenarios, involving a cell-contact-dependent or -independent signal, would predict that the germ cells would be positioned differently when Wun is expressed with these drivers in a *wunen* somatic null embryo. The model shown in Fig. 1C,D represents a lateral section of the dorsal half of an embryo with

two *en*-expressing Wun stripes. The arrow represents the direction of movement of the germ cells from the endoderm into the overlying germ layers. In case of a contact-dependent signal, we would expect germ cells to be distributed evenly throughout the mesoderm, with some germ cells ending up in the ectoderm between the stripes (interstripe domain) (Fig. 1C). However, in case of a contact-independent signal, we would expect germ cells to align in the interstripe domain (Fig. 1D). If the signal acts over a sufficient distance it could also exclude germ cells from the entire ectoderm by keeping the germ cells in the more distant mesoderm.

In a *wunen* somatic null background germ cells frequently migrate into the ectoderm (arrows, Fig. 1F). These lost ectodermal germ cells are completely absent when Wunen are expressed using either of the two ectodermal drivers in this background (Fig. 1G–I, for fluorescently stained 3D reconstructions, see supplementary material Movies 4–7). This indicates that segmental expression is sufficient to exclude germ cells from the entire ectoderm. At late stage 11 and early stage 12, the distance between the *h* stripes that overlie the germ cells (between parasegments 9 and 11, Fig. 1A) is 30  $\mu\text{m}$  (s.d. of 3.7  $\mu\text{m}$ , six measurements in three embryos), whereas for the *en* driver the distance between stripes that overlie the germ cells (between parasegments 10 and 11, and 11 and 12, Fig. 1A) is 31  $\mu\text{m}$  (s.d. of 3.5  $\mu\text{m}$ , eight measurements in three embryos) (supplementary material Fig. S1). Therefore, Wun-expressing ectodermal stripes can exert influence over the germ cells over a distance of at least 11  $\mu\text{m}$  (the distance between the *h* stripes minus the diameter of a germ cell divided by two, supplementary material Fig. S1) and indicates that repulsion is likely to be cell contact independent.

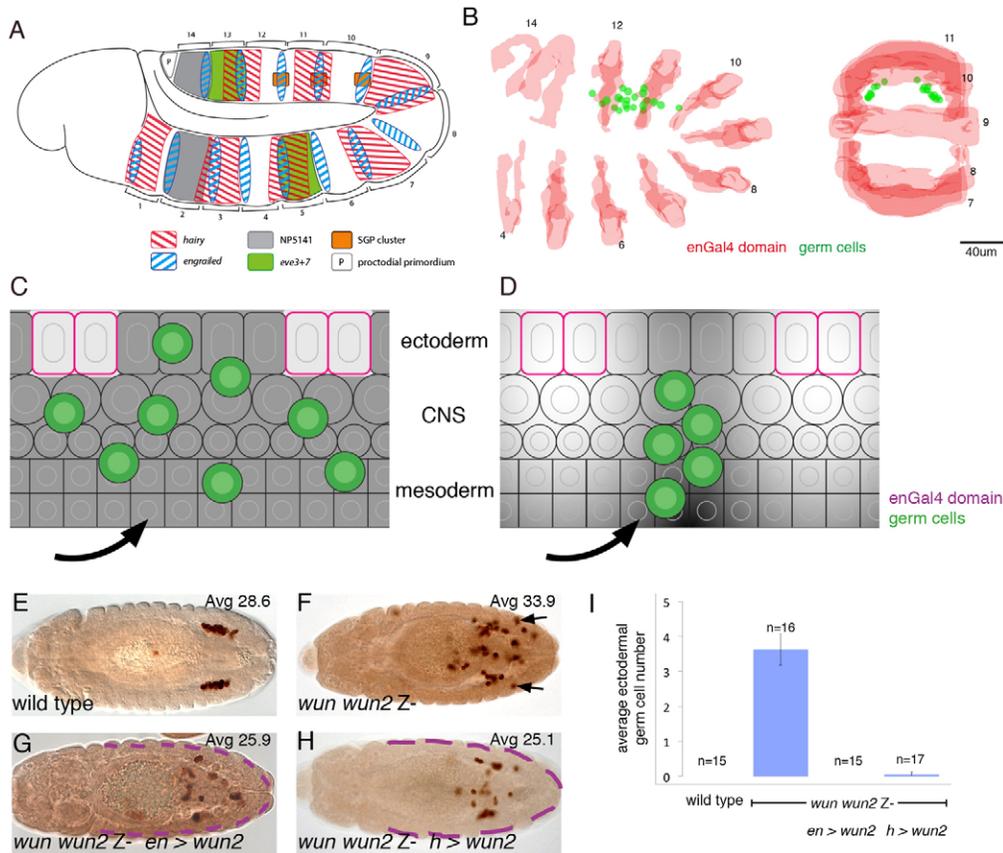
### Ectodermal expression of Wunen is instructive for germ cell migration

To examine how germ cells behaved with respect to the stripes during their migration we examined embryos at stages 10–12 and used fluorescent labeling and a benzyl-benzoate-containing mounting medium that allowed us to image through the entire embryo.

In wild-type stage 10 embryos, the germ cells are evenly spaced from each other on the basal side of the posterior midgut (endoderm) closest to the overlying mesoderm (Fig. 2A). In *wunen* somatic null embryos the germ cells are not confined and scatter over the entire basal surface of the posterior midgut with some germ cells present at the *en* stripe (arrowheads, Fig. 2B). In *wunen* somatic null embryos with *en*-driven Wun, the germ cells have already begun to align in a segmental fashion (Fig. 2C).

At stage 11 in wild-type embryos, the germ cells enter and align with the mesoderm, which runs perpendicular to segmental stripes (Fig. 2D). In *wunen* somatic null embryos there is no clear alignment with any tissue and some germ cells remain on the midgut (arrow, Fig. 2E) while others enter the ectoderm (arrowhead, Fig. 2E). In *wunen* somatic null embryos with *en*-driven Wun, germ cells align in the underlying mesoderm parallel to but between the Wun stripes (Fig. 2F, arrow shows some germ cells remaining on the basal side of the midgut).

In wild-type embryos at stage 12 the germ cells remain in the mesoderm and are loosely dispersed (Fig. 2G,J). In *wunen* somatic null embryos the germ cells are scattered between the ectoderm, mesoderm and endoderm (Fig. 2H,K, arrowheads show germ cells in the ectoderm). In *wunen* somatic null



**Fig. 1. Expression of Wun in ectoderm does not affect germ cell survival.** (A) Schematic map of a lateral stage 11 embryo showing the prospective parasegmental boundaries, the position of the three SGP clusters in the mesoderm, and the ectodermal expression domains that result from the Gal4 drivers of the pair rule genes *hairy* and *even skipped* (*eve3+7*), the segment polarity gene *engrailed* (*en*) and an insertion in the *ken and barbie* (*ken*) locus, *p{GawB}NP5141*. These drivers also express in the proctodeal primordium (marked P) and in domains in the head (not shown). Based on data from Carroll et al., 1988; Hooper et al., 1989; Kühnlein et al., 1998; Riechmann et al., 1998. (B) Posterior region of a stage 11 embryo with an *en*-driven catalytic dead version of Wun2 (Wun2H326K-Myc) stained with an anti-Vasa antibody to mark the germ cells and an anti-Myc antibody to mark the *en* stripes. The embryo was rendered in 3D and the germ cells (green) and *en* stripes (red) are shown following image segmentation. Left is the lateral view showing *en* stripes 4–14, right shows the posterior view, with *en* stripes 7–11. The germ cells in bisymmetrical clusters in the mesoderm do not contact the overlying ectodermal *en* stripes. (C,D) Cartoon illustrating a lateral section of a stage 11 embryo to show the predicted position of germ cells (green) relative to Wun-expressing ectodermal stripes (cells outlined in magenta) if the Wunen-dependent signal required cell–cell contact (C) or was cell-contact independent (D). Germ cells are migrating into the region from the endoderm (not shown) in the direction of the arrow. The light gray region in C indicates the region from which germ cells would be excluded and dark gray indicates the permitted area. In D the strength of gray shading represents the gradient of lipid phosphate (light gray, lowest levels of lipid phosphate; dark gray, higher levels). (E–H) Dorsal view of stage 13 embryos stained with an anti-Vasa antibody to mark the germ cells, with the following genotypes *wun<sup>CE</sup> UASwun2myc/+* (E), *wun<sup>CE</sup> UASwun2myc/Df(2R)wun<sup>GL</sup>* (F), *wun<sup>CE</sup> UASwun2myc/Df(2R)wun<sup>GL</sup> en > Gal4* (G), *wun<sup>CE</sup> UASwun2myc/Df(2R)wun<sup>GL</sup>; h > Gal4/+* (H). The mean total number of germ cells for each genotype is shown. Dashed lines indicate position of ectodermal driver expression. (I) Graph showing the mean  $\pm$  s.e.m. number of germ cells per embryo found in the ectoderm at stages 14–16 from fluorescently stained 3D reconstructed embryos of the genotypes indicated above. *n* indicates number of embryos scored.

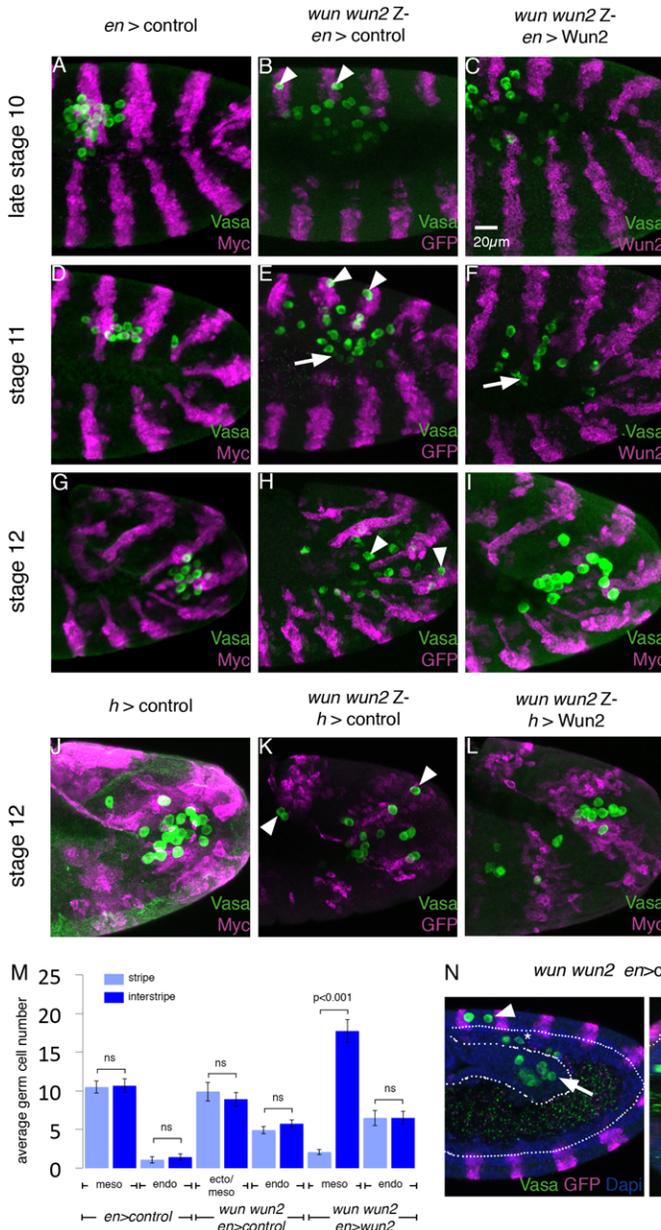
embryos with *en*- or *h*-driven Wun, germ cells avoid the ectoderm and the majority of them remain in the underlying tissue, and are well aligned parallel to, but between, the stripes (Fig. 2I,L).

We quantified the position of the germ cells in the endoderm, mesoderm and ectoderm relative to the overlying ectodermal stripes in wild-type, *wunen* somatic null and *wunen* somatic null embryos with *en*-driven Wun (Fig. 2M, a representative embryo illustrating the germ cell distribution across the germ layers is given in Fig. 2N and in 3D in supplementary material Movie 8). In wild-type embryos, the majority of germ cells are in the mesoderm and there is no difference in the number below and between the control stripes (interstripe). In *wunen* somatic null

embryos, germ cells are in all three tissues, but again there is no difference in the number in or below the control stripes and in the interstripe region. In *wunen* somatic null embryos with *en*-driven Wun, although the cells in the endoderm are equally distributed between the Wunen stripe and interstripe regions, the majority of germ cells in the mesoderm are in the interstripe region. This indicates that the signal from the ectoderm is able to affect the position of the germ cells in the mesoderm but not the endoderm.

#### Germ cells respond to a Wunen-dependent diffusible signal

We reasoned that if germ cells are responding to a diffusible signal and if that signal provides positional information in the form of a gradient, then the germ cells should be able to sense



**Fig. 2. Striped ectodermal expression of Wun2 causes germ cells to align in parallel and between the stripes.** (A–L) Maximum intensity projections of lateral views of embryos with genotypes *en>Gal4/UASwun2H326Kmyc* (A,D,G), *Df(2R)wun<sup>GL</sup>en>Gal4/Df(2R)wun<sup>GL</sup>UASLazaroGFP* (B,E,H), *wun<sup>CE</sup>UASwun2myc/Df(2R)wun<sup>GL</sup>en>Gal4* (C,F,I), *h>Gal4/UASwun2H326Kmyc* (J), *Df(2R)wun<sup>GL</sup>/Df(2R)wun<sup>GL</sup>UASLazaroGFP; h>Gal4/+* (K), *wun<sup>CE</sup>UASwun2myc/Df(2R)wun<sup>GL</sup>; h>Gal4/+* (L). Embryos are late stage 10 (A–C), stage 11 (D–F) and stage 12 (G–L) with germ cells stained with anti-Vasa antibody (green) and anti-GFP (B,E,H,K), anti-Myc (A,D,G,I,J,L) or anti-Wun2 (C,F) antibody (magenta) to visualize the *en* and *h* stripes, expressing Myc-tagged Wun2, or the non functional proteins Lazaro–GFP or Wun2H326K–Myc as controls. Arrowheads show germ cells in the ectoderm and arrows show germ cells that remained on the basal side of the midgut (endoderm). (M) Graph showing the mean  $\pm$  s.e.m. number of germ cells at different locations within stage 11–12 wild-type embryos with *en*-driven control stripes, *wunen* somatic null embryos and *wunen* somatic null embryos with *en*-driven Wun stripes (12 embryos were scored for each genotype). Germ cells were divided into those that had remained on the endoderm (endo) (which is deeper in the embryo and further away from the ectoderm) and those that had entered the mesoderm (meso) or ectoderm (ecto), and scored according to whether they were below an *en* stripe (stripe) or between an *en* stripe (interstripe). (N) Reconstructed sagittal (left) and transverse (right) views of a *wunen* somatic null embryo with control stripes (magenta) showing DAPI-stained nuclei (blue) and germ cells (green) that are in the different germ layers: endoderm (arrow), mesoderm (asterisk) and ectoderm (arrowhead). Boundaries between germ layers are indicated with dotted lines. The full embryo z-series is given in supplementary material Movie 8.

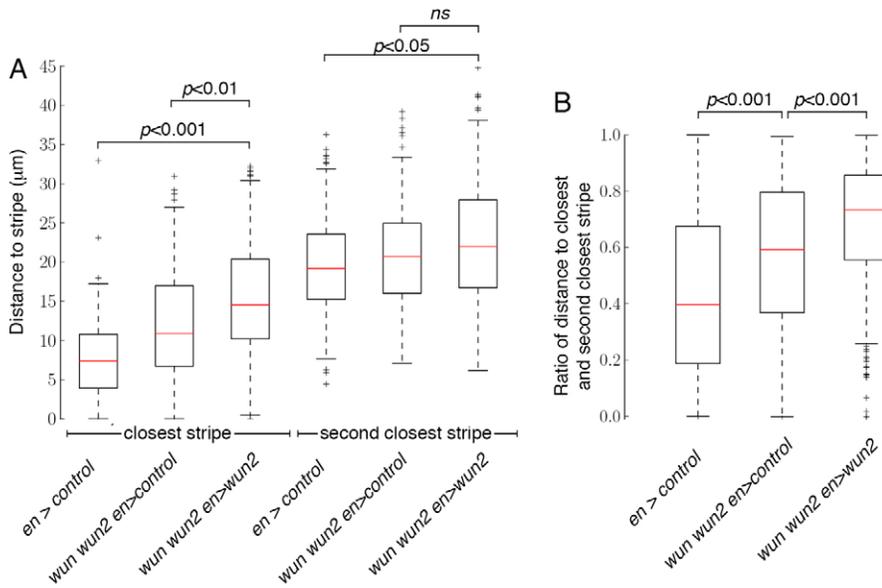
their position relative to their neighboring stripes and occupy the midpoint between them, i.e. the germ cells would be more likely to lie equidistant from their surrounding stripes.

To test this hypothesis, we determined the distances of the germ cells to the stripes that lie on either side of them (the closest and second-closest stripes). We focused on the *en*-driven stripes as their boundaries were better defined than those driven by *h*. We performed image segmentation on wild-type, *wunen* somatic null embryos with control stripes, and *wunen* somatic null embryos with *en*-driven Wunen stripes using a thresholding method to define the stripe domains. Each stripe was assigned a unique label and the distance of each germ cell to the nearest point of its closest stripe and the nearest point of its second closest stripe was calculated.

We found that the distribution of the distances of the germ cells to their closest stripe was narrow in wild-type embryos, with a median of 7  $\mu$ m (Fig. 3A). For *wunen* somatic null

embryos the distribution was broader, as would be expected owing to the scattered nature of the germ cells, with a median of 11  $\mu$ m. For *wunen* somatic null embryos with *en*-driven Wunen stripes the distribution was again broader but significantly shifted towards increased distances, with a median of 15  $\mu$ m ( $P<0.01$  by Mann–Whitney *U* test). For the second-closest stripe the distributions overlapped with each other and the medians were relatively similar, with germ cells lying between 19 and 22  $\mu$ m (Fig. 3A).

As a measure of the equidistance between neighboring stripes we used the ratio of the distance to the closest and second-closest stripes (Fig. 3B). A value of 1 indicates that a germ cell is perfectly equidistant. When we compared the distribution of ratios for the *wunen* somatic null embryos with Wunen stripes compared to either of the controls we found that there were significantly more germ cells with a ratio closer to 1 (Fig. 3B,  $P<0.001$ , see Materials and Methods). We conclude that germ



**Fig. 3. Germ cells are repelled from Wun-expressing *en* stripes and move to positions nearer to equidistant from their two nearest stripes.** Box-and-whisker plots showing (A) the distribution of the germ cell distance to closest and second-closest *en* stripe and (B) the ratio of the distance to closest and second-closest stripe in wild-type embryos with control stripes (*en*>control), *wun wun2* Z<sup>-</sup> embryos with control stripes (*wun wun2 en*>control) and *wun wun2* Z<sup>-</sup> embryos with stripes expressing Wun2 (*wun wun2 en*>*wun2*). Red lines indicate the median. Outliers, defined as being more than one interquartile distance from the box, are indicated as crosses.

cells can sense the stripes on either side of them, which is consistent with the presence of a diffusible signal.

#### Live imaging reveals that germ cells find the region between Wunen-expressing *en* stripes without making cell–cell contact

Although the fixed tissue analysis argues for a diffusible signal, it remains possible that the germ cells find an equidistant position by migrating back and forth and making repeated contacts with the stripes on either side of them. To exclude this hypothesis, we performed live imaging to monitor both the path of the germ cells as they find their position between the stripes and also how they maintain this position during later stages.

In wild-type stage 11 embryos, the germ cells remain motile but make only short movements within the mesoderm, whereas the embryo as a whole undergoes germ-band retraction, as visualized by the clockwise movement of the control stripes (Fig. 4A; supplementary material Movie 1). Interestingly, germ cells that appeared to have migrated far from the gonadal cluster (arrows in Fig. 4A), do finally move into the gonad (see supplementary material Movie 1), indicating that individual germ cells are capable of detecting the gonad, and the gonad is capable of accepting newly arriving germ cells at late stage 13.

At stage 11 in *wunen* somatic null embryos with *en*-driven Wunen stripes, the germ cells also remain motile as shown by dynamicity of the actin enrichments in the germ cells as well as the movement of the two germ cells marked with an arrow and an arrowhead away from each other (Fig. 4B; supplementary material Movie 2). Overall, we do not see germ cells touching a stripe on one side and then on the other while aligning between them.

We also noticed that in the *wunen* somatic null embryos with *en*-driven Wunen stripes some germ cells form a gonad-like cluster. When we checked the path of the germ cells that reached the gonad, we noticed that several germ cells appear to take a shorter route without ever leaving the basal side of the midgut but finally joining the gonad cluster at stage 13 when the germ band is retracting (Fig. 4B, open arrow shows germ

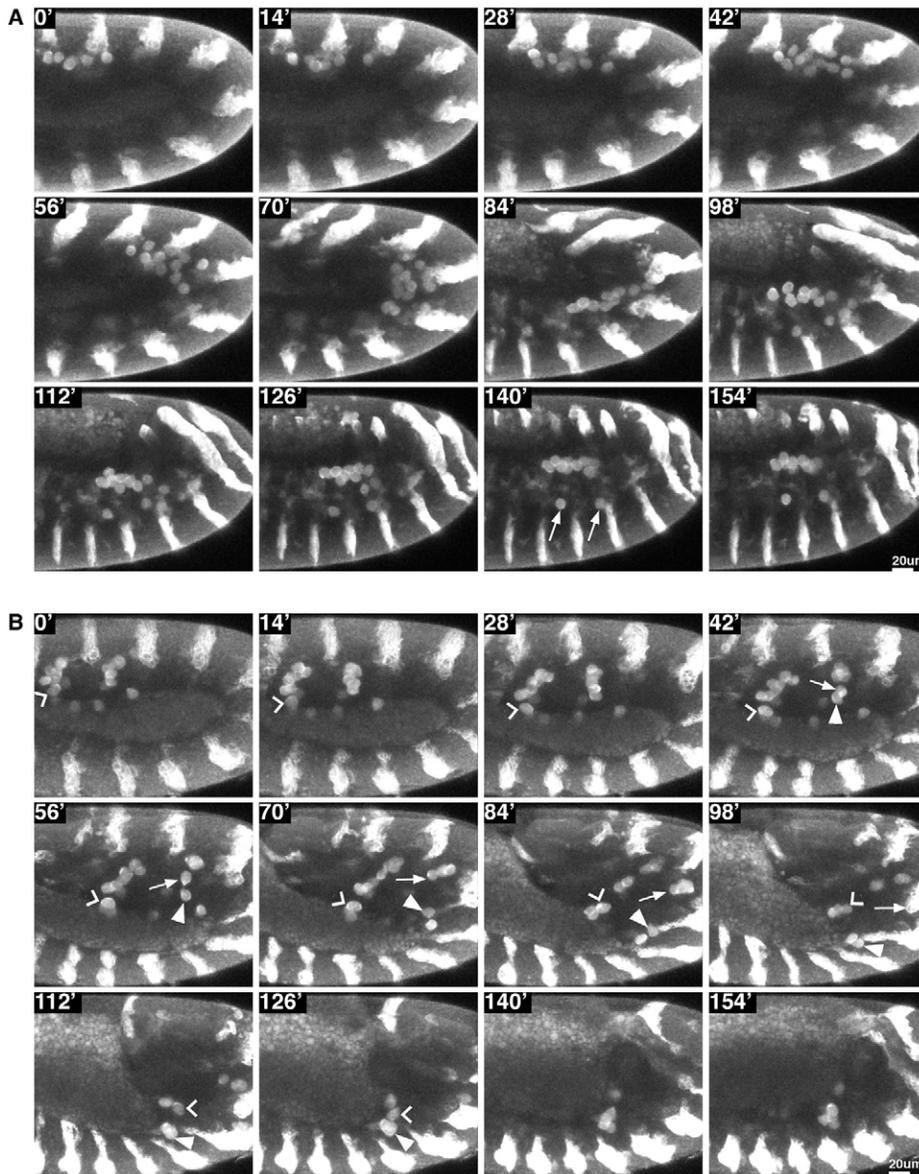
cells that sit on the midgut and ultimately join the gonad cluster).

If we look in stage 10 embryos at germ cells migrating to the region below and between the stripes (Fig. 5A; supplementary material Movie 3), we see that germ cells move directly to this position without touching the stripes anterior or posterior to their final location. Indeed one germ cell (tracked in green) passes underneath the stripe at a distance of 22  $\mu\text{m}$ . As germ band retraction occurred, we observed three germ cells migrating below and between the stripes, but without ever touching them (Fig. 5A). We also observed actin-rich accumulations at the leading edge of germ cells as they approached a stripe before making a turn and moving in the opposite direction (arrows in Fig. 5B and supplementary material Movie 3). This occurred when the germ cells were on average 15  $\mu\text{m}$  (s.d. of 4.6, five measurements made from two movies) from the overlying stripe and implies that the germ cells respond to the Wunen signal at least at a distance of 15  $\mu\text{m}$ . Such abrupt turns are rarely seen in post-stage-10 *wunen* somatic null embryos (Sano et al., 2005 and our unpublished results).

#### The maximum distance of the Wunen repulsion zone

The above data are based on the behavior of germ cells when confronted with stripes both anterior and posterior to their position. Thus, the germ cells might not be able to move as far away as they would normally and this would lead us to underestimate the distance at which a germ cell could sense a Wunen-expressing somatic cell. Therefore, we wanted to test how germ cells would respond to a single stripe.

We made use of a Gal4-containing P-element,  $p\{GawB\}NP5141$ , which drives expression in parasegments 2 and 14 (Fig. 1A; Kühnlein et al., 1998). The former domain is far enough anterior that it is unlikely to affect the germ cells, whereas the latter, at stages 10–11, lies above, but anterior to, where the germ cells would normally migrate. In a *wunen* somatic null background, a mean of 4.7 germ cells mis-migrate and enter parasegment 14 (Fig. 6A,E, ten embryos examined at stages 11 and 12). In contrast, when Wun is expressed in this domain, no germ cells were located



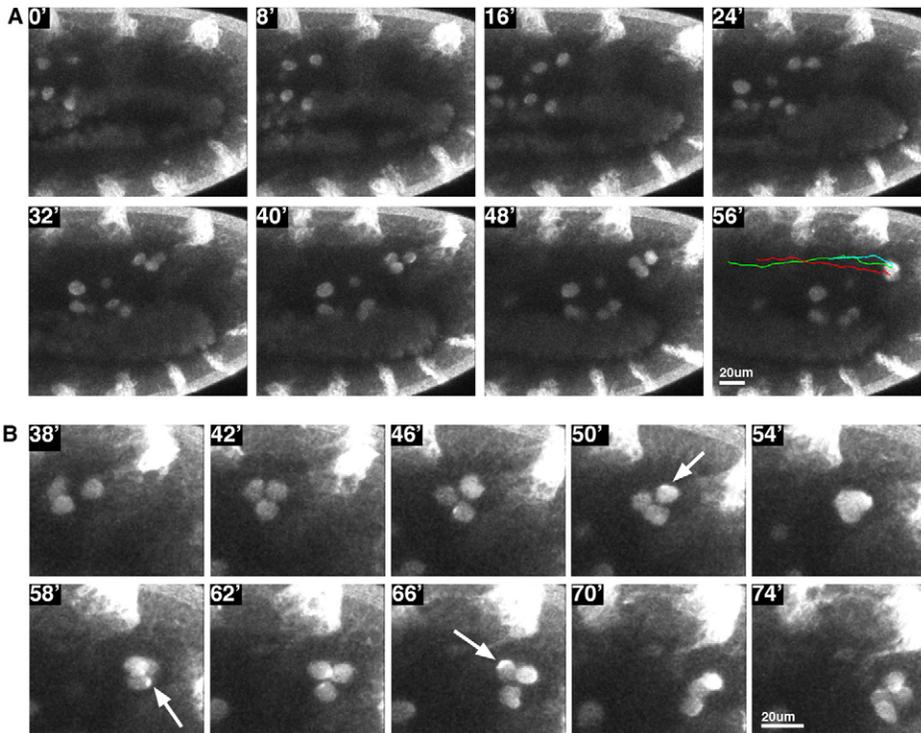
**Fig. 4. Germ cells align without touching the Wun stripes.** (A,B) Maximum intensity projections (10–12 slices, 2 µm apart) of lateral views of live stage 11 embryos with germ cells labeled with eGFP fused to an actin-binding domain of Moesin (*nos>moeGFP*) imaged every 2 minutes, with every seventh timepoint shown. The most-lateral confocal slices of the embryos, which would show the stripes wrapping around the lateral sides, are not included in the projections so as not to obscure the germ cells. The time in minutes after start point is given in the top left corner. (A) Control embryo with *moeGFP* stripes (*en>Gal4/UASmoeGFP*). Arrows show two germ cells, initially separated from the gonad, eventually migrating and joining the gonad at late stage 13 (see supplementary material Movie 1). (B) *wun wun2 Z-* embryo with Wun-GFP stripes (*Df(2R)wun<sup>GL</sup> en >Gal4/Df(2R)wun<sup>GL</sup> UASwunGFP*). Arrow indicates a germ cell that migrates to join a cluster of germ cells that ends up behind an *en* stripe, but this cluster does not move relative to the stripe and does not join the presumed gonad cluster. Arrowhead indicates a neighboring cell that stays in a more central location and later joins the presumed gonad cluster. Open arrow indicates an extreme lateral cell that joins to form the presumed gonad cluster.

in this region (Fig. 6B,F, ten embryos examined at stages 11 and 12), probably because the germ cells that would have entered this region have been repelled more posteriorly. The alternative explanation that these germ cells were eliminated by cell death is not supported because the total germ cell number in such embryos is not significantly less than in control embryos (Fig. 6I).

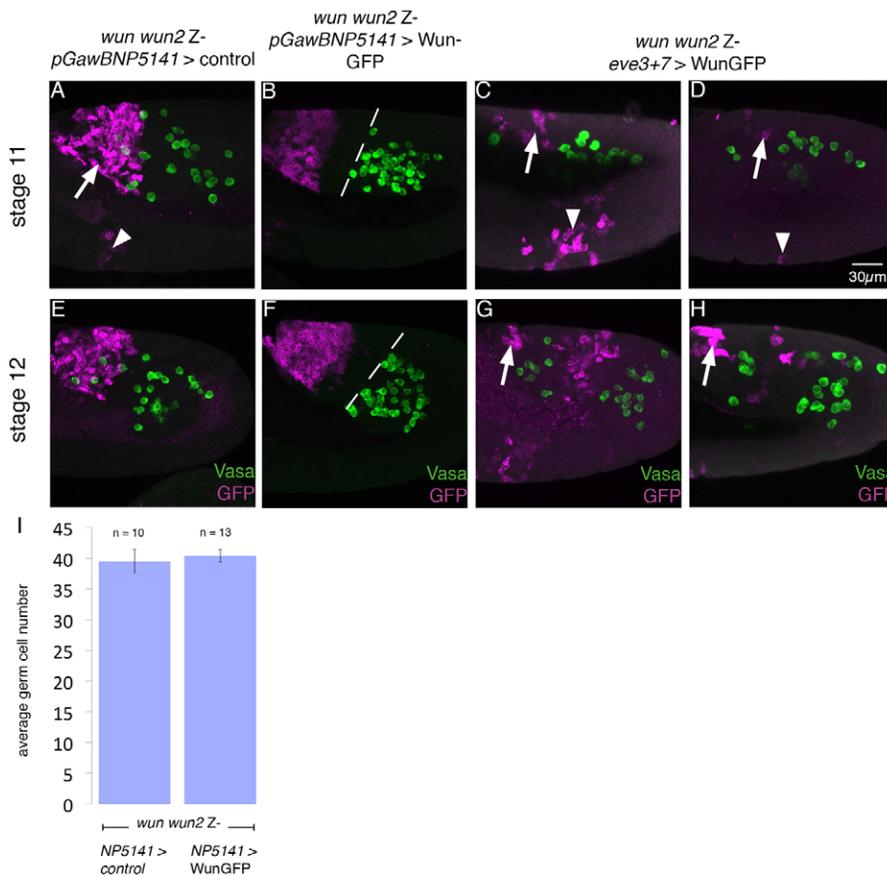
Given that 4.7 germ cells mis-migrate and enter parasegment 14 in *wunen* somatic null embryos, we measured the distance between Wun-expressing cells in parasegment 14 to the five nearest germ cells in *wunen* somatic null embryos with Wun expression driven by *p{GawB}NP5141*. We found that these germ cells were located on average 33 µm (s.d.=8.8, ten embryos with a total of 50 germ cells measured at stages 11 and 12) from the nearest point of Wun expression (Fig. 6). Thus, when faced with an expression domain of somatic *wunen* on one side with no restriction on the other, germ cells are repelled beyond 33 µm. This distance represents our estimate of the maximum range of influence of Wun-expressing somatic cells on germ cells.

#### Small expression domains of Wunen are sufficient to repel germ cells

The drivers used in the previous sections all result in expression in large stripes containing many hundreds of cells. We were interested to know whether small patches of cells would be sufficient to also cause repulsion. We therefore used a driver under the control of the promoter for the third and seventh stripe of the *even skipped* (*eve*) pair rule gene (*eve3+7*), which expresses in parasegments 5 and 13, respectively (Fig. 1A; Small et al., 1996). At stage 11 and 12, this driver gave small patches of expressing cells rather than a continuous stripe. Cells expressing within parasegment 5 are too far ventral to affect the germ cells and, although parasegment 13 is slightly anterior to where the germ cells would normally migrate, several germ cells enter this region in a *wunen* somatic null background (Fig. 6A,E). When Wunen is expressed using this driver in a *wunen* somatic null background we observed that no germ cells enter this region and that the expression of Wunen in the patches of cells in



**Fig. 5. Germ cells move directly into the region below and between *Wunen*-expressing *en* stripes.** (A,B) Maximum intensity projections (5–8 slices, 2  $\mu$ m apart) of lateral views of a living stage 10 embryo with the same genotype as in Fig. 4B imaged every 2 minutes. The time in minutes after the start point is given in the top left corner. (A) Projections are every fourth time point and are from slices from the deepest part of the embryo. Three germ cells show direct movement to the region between the stripes with no contact to either bounding stripe (the final time point includes the tracking of these germ cells). (B) Close up of the three germ cells tracked in A, shown every second time point. The germ cells move in the region below and between the stripes and the germ cells have actin-rich accumulations (arrows) as they approach and turn away from the stripes.



**Fig. 6. The Wun repulsion zone can extend up to 33  $\mu$ m and small patches of Wun expression are sufficient to repel germ cells.** (A–H) Lateral views of *wun<sup>GL</sup> UASLazaroGFP/wun<sup>GL</sup> p{GawB}NP5141* (A,E), *wun<sup>GL</sup> UASwunGFP/wun<sup>GL</sup> p{GawB}NP5141* (B,F) and *wun<sup>GL</sup> UASwunGFP/wun<sup>GL</sup> eve3+7>Gal4* (C,D,G,H). Embryos are stage 11 (A–D) and 12 (E–H) stained with anti-Vasa antibody (green) to mark germ cells and anti-GFP antibody (magenta) to visualize the *NP5141* or *eve3+7* domains, and are expressing Wun–GFP or the non-functional protein Lazaro–GFP. The arrowhead and arrow in A indicate expression in parasegments 2 and 14 respectively. The arrowhead and arrow in C indicate expression in *eve* stripes 3 and 7 respectively. The arrow in G and H indicates expression in the proctodaeum. Expression from the *eve3+7 Gal4* driver is heterogeneous; in some embryos defined stripes are observed (C,G), whereas in other embryos only small patches of cells express (D,H). However, in both cases germ cells avoid sites of expression. (I) Graph showing the mean  $\pm$  s.e.m. number of germ cells per embryo at stages 11 and 12 in fluorescently stained 3D reconstructed embryos of genotype *wun<sup>GL</sup> UASLazaroGFP/wun<sup>GL</sup> p{GawB}NP5141* and *wun<sup>GL</sup> UASwunGFP/wun<sup>GL</sup> p{GawB}NP5141*. *n* indicates number of embryos scored.

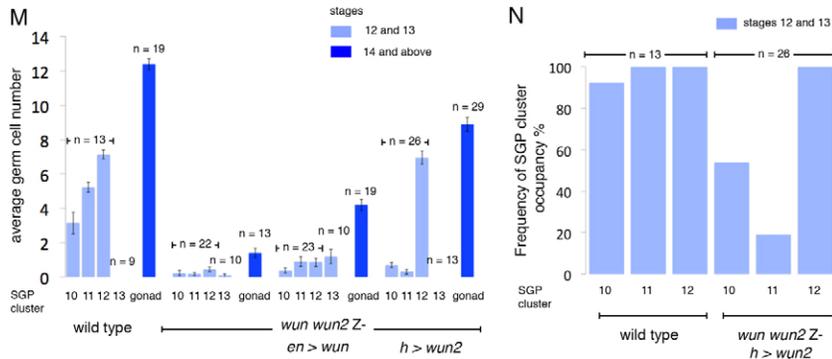
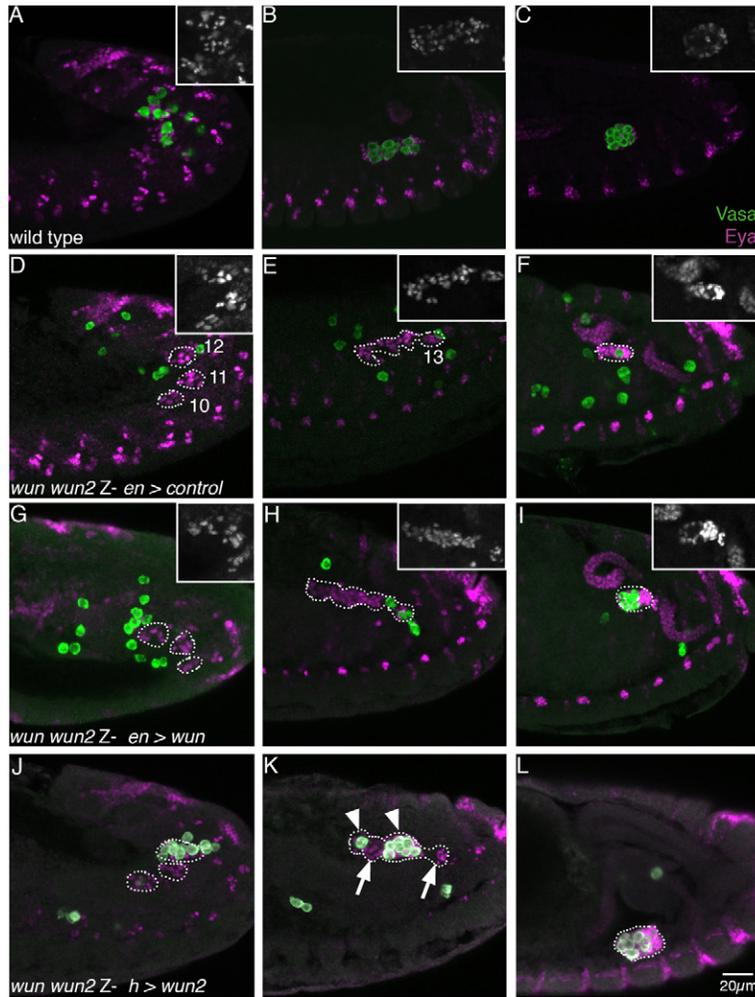
parasegment 13, splits the migrating germ cell population into two distinct clusters anterior and posterior to this region of expression at stages 11 and 12 (Fig. 6C,D,G,H, 18 embryos examined). This indicates that small expression domains of Wunen are sufficient to create a repulsion zone, which the germ cells avoid migrating into.

**Absence or overexpression of Wun does not affect SGPs, whereas ectopic Wun expression is sufficient to herd some germ cells to the gonad**

We noticed that in *wunen* somatic null embryos with *en*- or *h*-driven Wun, a number of germ cells clustered together in regions equating to the positions of the embryonic gonads (Fig. 1G,H).

We therefore examined how many germ cells were actually reaching the gonad in such backgrounds by staining embryos for the SGP marker, Eyes absent (*Eya*) (Fig. 7).

SGP behavior is unaffected by loss of somatic Wunens or segmental Wun misexpression (Fig. 7A–L). At stages 14–16, control embryos contained on a mean of 12 germ cells per gonad, whereas the gonads in *wunen* somatic null embryos contained on average just one germ cell (Fig. 7M). The number of germ cells reaching the gonad was indeed increased upon expression of *en*-driven Wun (a mean of four germ cells per gonad) and greatly increased upon expression of *h*-driven Wun (a mean of nine germ cells per gonad) (Fig. 7M).



**Fig. 7. Ectodermal Wunen expression does not affect SGP behavior and is sufficient to direct some germ cells to the gonad.** (A–L) Lateral views of stage 12 (A,D,G,J), 13 (B,E,H,K) and 15 (C,F,I,L) embryos of genotype *nos>moeGFP; en>moeGFP* (A–C), *Df(2R)wun<sup>GL</sup> en>Gal4/Df(2R)wun<sup>GL</sup> UASlazarogFP* (D–F), *Df(2R)wun<sup>GL</sup> en>Gal4/Df(2R)wun<sup>GL</sup> UASwunGFP* (G–I) and *wun<sup>CE</sup> UASwun2myc/Df(2R)wun<sup>GL</sup>; h>Gal4/+* (J–L) stained with antibodies against Vasa (green) to mark germ cells and against Eya (magenta), which marks the SGP nuclei but is also expressed in other tissues. Insets show SGP clusters in gray scale. Numbers indicate the parasegment identity for each of the SGP clusters. The arrows in K indicate unoccupied SGP clusters in parasegments 11 and 13; arrowheads indicate germ cells occupying SGP clusters in parasegments 10 and 12. (M) Graph showing mean ± s.e.m number of germ cells per SGP cluster in the indicated parasegments (at stages 12 and 13) and per gonad (at stages 14–16) in fluorescently stained 3D reconstructed embryos of the genotypes described above. *n* indicates number of gonads scored. (N) Graph showing the frequency of SGP cluster occupancy in *wun<sup>CE</sup> UASwun2myc/Df(2R)wun<sup>GL</sup>; h>Gal4/+* compared with sister control embryos. SGP cluster is scored as occupied based on presence of one or more germ cells. *n* indicates number of gonads scored.

We were intrigued as to why either *en*- or *h*-driven Wun should result in any rescue of germ cells to the gonad and why *h* should rescue better than *en* (Fig. 7M, dark blue bars). *en* ectodermal domains lie directly above all SGP clusters whereas *h* ectodermal domains sit above two of the SGP clusters (parasegment 11 and the male-specific cluster in parasegment 13) (Fig. 1A). We therefore examined the distribution of the germ cells associating with the individual clusters while they remained clearly distinguishable (during stages 12 and 13) to see if this was being affected by the distribution of Wun in the ectoderm.

In wild-type stage 13 embryos, germ cells have already associated with each of the clusters of SGPs in parasegments 10–12 (Fig. 7A,B,M). In *wunen* somatic null embryos, the few germ cells that do associate with the SGPs do not preferentially associate with any particular SGP cluster (Fig. 7D,E,M). This situation is similar in *wunen* somatic null embryos with *en*-driven Wun (Fig. 7G,H,M). At later stages, however, increased numbers of germ cells are found at the gonad (Fig. 7I,M), which correlates well with our live imaging in which we see some germ cells cluster laterally in the region of the gonad relatively late (Fig. 4B).

In comparison, with *h*-driven Wun in the same background we found that a large number of germ cells associated with the SGPs in parasegment 12 (Fig. 7J,K). Indeed these SGPs contained at least one germ cell in 100% of cases examined (Fig. 7M,N). The SGPs of parasegment 10 also had associated germ cells in 50% of cases, but the mean number of associated germ cells was far less than for parasegment 12 (Fig. 7M,N). In contrast, germ cells were very rarely associated with SGPs of parasegment 11 (Fig. 7M,N).

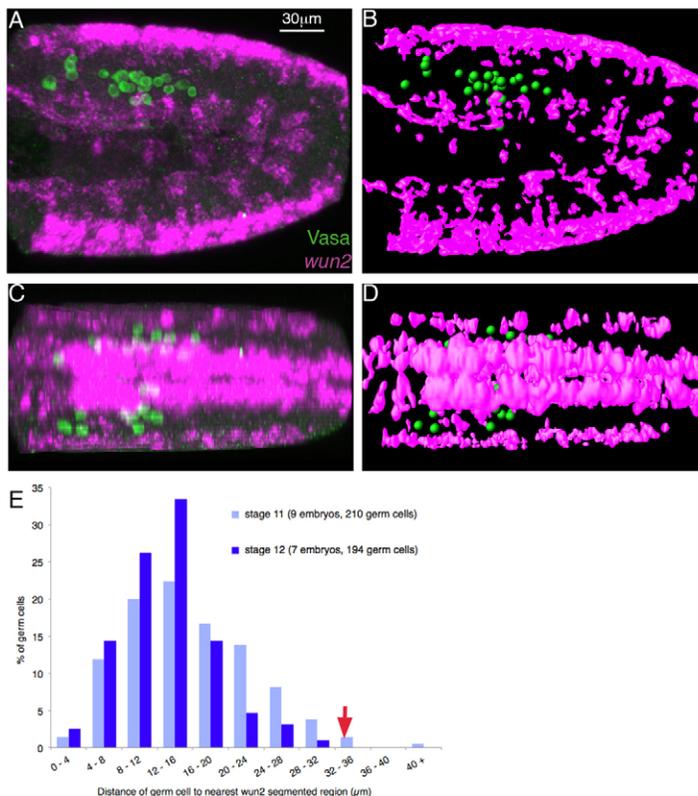
This bias in association of germ cells with different SGP clusters is consistent with the expression of Wun in the ectodermal *h* stripes that lie over the SGP clusters in

parasegments 11 and 13 repelling germ cells so that they associate with the SGPs in parasegment 12 and to a lesser extent parasegment 10. The distance of the ectodermal stripes to the underlying mesodermal SGP clusters is  $12.3 \mu\text{m}$  (s.d.=1.2, average over 15 SGP clusters), which is well within our estimate of the range of Wun action on germ cells.

We conclude that the replacement of the intricate endogenous expression pattern of the *wunens* by ectopic domains is sufficient to herd a surprisingly large proportion of the germ cells to the gonad but with clear effects on the relative occupancy of SGP clusters. The local attraction of germ cells probably occurs through an additional signal emanating from the SGPs via an HMGCoA reductase and ABC transporter pathway (Van Doren et al., 1998; Ricardo and Lehmann, 2009).

### Correlating the position of germ cells to Wunen dependent repulsion in wild-type embryos

Our estimate of the maximum range of influence of Wun-expressing somatic cells is based on protein misexpression and overexpression. To determine whether overexpression has a disproportionate influence on the degree of repulsion, we overexpressed Wun and Wun2 under the control of the Gal4-containing *wun2* driver line, *p{GawB}wun2<sup>9-175</sup>*, which recapitulates much of the endogenous expression pattern of *wun2* (supplementary material Fig. S2). We found that germ cell survival and migration were identical to that in control embryos (supplementary material Fig. S2), indicating that Wun overexpression can be tolerated in cells that endogenously express Wun2 and that spatial expression outweighs protein levels in determining the path of germ cell migration and level of survival. We conclude that our distance estimates based on Wun overexpression are likely to be relevant to the wild-type Wun levels.



**Fig. 8. All germ cells in wild-type embryos lie within the Wun repulsion zone.** (A) Lateral and (C) dorsal view of a 3D reconstruction of a stage 11 wild-type embryo stained for Vasa, to mark the germ cells (green), and for *wun2* mRNA expression (magenta). Image segmentation using Imaris was used to determine the position of the germ cells and *wun2* expression domains (B,D) and the distance of each germ cell to its nearest *wun2* domain was determined (E). The red arrow marks the  $33\text{-}\mu\text{m}$  distance of Wun action, calculated from Fig. 6, showing that more than 99% of germ cells lie within this range.

To determine the extent to which the path of migrating germ cells can be explained by Wunen-dependent repulsion we examined the position of germ cells in embryos fluorescently stained to highlight endogenous *wun2* mRNA expression (Fig. 8A,C). We performed image segmentation on the *wun2* expression domains and measured the distance of germ cells to their nearest Wun2-expressing domain in stage 11 and 12 embryos (Fig. 8B,D). We find that these germ cell distances are normally distributed with median values of 15 and 12  $\mu\text{m}$  at stages, 11 and 12 respectively. This decrease is largely due to the proximity of small patches of *wun2*-positive cells in the lateral mesoderm and ectoderm at stage 12. Because small patches of *wunen*-positive cells can repel germ cells (Fig. 6D,H) we consider these areas functionally relevant. Our experiment using the single-stripe NP5141 driver demonstrated that germ cells can be affected up to a distance of 33  $\mu\text{m}$ , therefore we considered this distance to be the effective range of Wunen action. From our results on the distribution of the distance of germ cells from the Wun2-expressing segmented regions in wild-type embryos, we find that more than 99% of germ cells are located closer than 33  $\mu\text{m}$  to their nearest Wun2-expressing domain (Fig. 8E). We conclude that all germ cells are influenced by Wunen expression for the entire duration of their migration.

## Discussion

In this paper, we have explored the nature of the signal that regulates germ cell migration and survival in *Drosophila* embryos. Germ cells are excluded from Wunen-expressing somatic domains in wild-type embryos suggesting that Wunen destroys an attractive signal. We have used ectopic misexpression of Wunen in embryos otherwise somatically null for Wunens to show that Wunens are instructive for dictating the germ cell migration path. In particular, Wunen expression in *en* or *h* ectodermal stripes causes germ cells to align parallel to and between the stripes. In such embryos, germ cells have an equidistance ratio closer to 1 as compared to control embryos, indicating that they are probably integrating a signal from both sides. By live imaging, we observed that germ cells reach and maintain these parallel positions without making any direct contacts with the stripes. Taken together, our data strongly argue that the signal modulated by Wunen-positive somatic cells is cell contact independent.

The exclusion of germ cells from the entire ectoderm when Wun is expressed using the *en* or *h* drivers might result from germ cells being repelled by the ectodermal stripes or from death of germ cells that have entered the ectoderm. We favor the former because in live imaging studies (Fig. 4B; Fig. 5) we do not see germ cells enter the ectoderm and we do not see ectodermal Vasa-positive cell remnants, which are indicative of germ cell death (Sano et al., 2005). In both scenarios however, our conclusion that the mechanism is cell-contact independent remains valid.

When presented with a single Wun stripe, using the NP5141 driver, the germ cells were repelled at distances up to 33  $\mu\text{m}$ . This represents our estimate of the maximal effective range of the Wun signal. This distance is comparable to assessments of the effective range of Wingless (Wg) and Hedgehog (Hh), which can form gradients over at least 50  $\mu\text{m}$  in wing imaginal discs (Strigini and Cohen, 2000; Zhu and Scott, 2004). When germ cells are faced with multiple *wunen* domains, such as *wun*-expressing *en* stripes, then germ cells can tolerate being much

closer, but make avoidance turns when 15  $\mu\text{m}$  away (Fig. 5). In wild-type embryos, 99% of germ cells are located up to 33  $\mu\text{m}$  from a *wun2*-expressing domain (Fig. 8E). Therefore, although germ cells are probably influenced by Wunen expression for the entire duration of their migration, 59% of them are closer than 15  $\mu\text{m}$ . We postulate that the small patches of wild-type *wun2*-positive cells in the lateral mesoderm and ectoderm might not repel as far as the much larger ectopic *wunen*-expressing *en* stripes.

Membrane-bound ligands can also act as long-range signals through their presence on long actin-rich cytoplasmic extensions. For example, lateral inhibition of *Drosophila* sensory organ precursor (SOP) fate is mediated by the transmembrane Notch ligand Delta, which can signal three to five cell diameters away through cytoplasmic extensions of up to 20  $\mu\text{m}$  (de Jossineau et al., 2003). Therefore, we considered whether germ cells could use cytoplasmic projections to make direct contact with Wun-expressing stripes. We and others see germ cell filopodia at their leading edge and often a longer lagging tail (or uropod) in both live and fixed tissue analysis (Sano et al., 2005; Brown et al., 2006). The filopodia are generally no longer than 2  $\mu\text{m}$ , whereas the tail can be up to 8  $\mu\text{m}$  (Sano et al., 2005; Brown et al., 2006). Therefore, these projections are not sufficiently long to make contact with the stripes. Furthermore, we used a constitutively active form of Moesin, MoeT559D, which disrupts the actin cytoskeleton in *Drosophila* photoreceptors (Karagiannis and Ready, 2004) and suppresses filopodia from the leading edge during dorsal closure (data not shown). Expression of this construct in germ cells caused only minor defects in germ cell survival indicating that germ cells do not rely on Moesin-dependent filopodia for migration in wild-type embryos (data not shown).

Another possibility is that expression of Wunens stimulates the somatic cells to make extensions that contact the germ cells. We looked for such extensions from *en* stripes expressing GFP-tagged catalytically active and dead forms of Wun but we did not observe any such extensions (data not shown). Taken together these data suggest that cytoplasmic extensions cannot account for the ability of germ cells to avoid Wun-expressing cells and supports the model that the migration is not dependent on cell contact.

We have used *wun2* overexpression in its endogenous pattern to evaluate the relative importance of somatic Wun expression levels versus its spatial distribution in permitting germ cell survival. Our data show that the level of Wunen protein in endogenously Wunen-expressing cells is not crucial to regulate germ cell survival. Ectopic expression can be tolerated if it is spatially separated from the germ cells. Therefore, it is the location of the Wunen expression, and not its overall level, which is crucial for germ cells. This is similar for other signaling systems, for example ubiquitous misexpression of Hh leads to patterning defects but overexpression in its normal locations (using the *en* driver) has little effect on segmentation (Porter et al., 1996). Insensitivity to overexpression in endogenous domains might well be a common feature of signaling molecules.

Our data are consistent with a model in which the somatic Wunen expression sets up relatively short-range repulsion zones within the embryo. We envisage these domains result from a local depletion of a lipid phosphate substrate. This could take the form of a discrete change in levels ('all or nothing') or a gradient. The ability of cells to find equidistant positions between Wunen

stripes and to be repelled over many cell diameters when faced with a single Wunen expressing stripe strongly favors a gradient (depicted schematically in Fig. 1D).

The canonical model of morphogen gradient formation is that the morphogen is secreted locally and diffuses to create a gradient. However, morphogens can also be restricted to a narrower area by localized depletion accomplished by receptor-mediated endocytosis or sequestration (Incardona et al., 2000; Boldajipour et al., 2008; Yu et al., 2009). Although we do not know the source of the ligand in the case of germ cells, our data are consistent with gradient formation based on dephosphorylation of an extracellular lipid phosphate by an LPP.

What would be the nature of the lipid gradient? S1P is present in human plasma and serum bound to low- and high-density lipoprotein and albumin (Murata et al., 2000; Yatomi et al., 2000). LPA is also found in human serum bound to albumin (Tigyi and Mileli, 1992). It is possible that a similar protein-binding partner is present in *Drosophila* embryos. The extracellular movement of morphogens, such as Hh and Wg, has been proposed to occur on membranous vesicles (also called argosomes or exosomes) or lipoprotein particles (Greco et al., 2001; Panáková et al., 2005). It is possible that similar particles are important for the formation of a lipid gradient that affects germ cell migration.

*Dictyostelium* cells can respond at a distance of  $\sim 70 \mu\text{m}$  away from a micropipette containing LPA (Jalink et al., 1993). S1P is important for the movement of heart progenitor cells from bilateral positions to the midline in zebrafish (Kupperman et al., 2000), which involves a distance of  $\sim 100 \mu\text{m}$ . S1P also regulates the circulation of T-lymphocytes in mouse, in particular allowing T-cells to exit from lymph nodes, which are several millimeters in length (Matloubian et al., 2004). In spite of these essential roles, it is not always clear whether absolute levels or gradients of S1P are required. Drug treatments that increase the S1P levels of the lymphoid organs, but not the levels in the circulatory system (effectively reversing the normal difference in S1P levels between these locations), are sufficient to block T-cell exit (Schwab et al., 2005), which is suggestive that a gradient is required in this circumstance, however its contour is not known. Our work has shown that lipid gradients in a *Drosophila* embryo can exist over distances comparable to their protein counterparts. Whether such distances are scaled up in the larger embryos and tissues of other species remains an open question.

## Materials and Methods

### Fly stocks

The following *Drosophila* lines are as described previously: *wun<sup>CE</sup>*, a chromosomal aberration resulting in loss of *wun* and *wun2* expression (Zhang et al., 1996); *Df(2R)wun<sup>GL</sup>*, a deficiency removing *wun* and *wun2* (Zhang et al., 1996); *UAS wun2-myc*, *UAS wun2-H326K-myc* and *UAS wun-GFP* (Burnett and Howard, 2003); *UAS lazaro-GFP* (Garcia-Murillas et al., 2006, does not affect germ cells, our unpublished results); *eve3+7>Gal4* (Small et al., 1996); and *UAS moeT559Dmyc* (Karagiosis and Ready, 2004). *en>Gal4 (P{en2.4-GAL4}e16E)* and *h>Gal4 (P{GawB}h1J3)* were from the Bloomington Stock Center.

*p{GawB}wun2<sup>9-175</sup>* was identified as an insertion of the Gal4-containing *pGawB* vector into the 5'UTR of *wun2* by Daria Siekhaus (Institute of Science and Technology, Austria) from a collection of enhancer trap lines provided by Ulrike Heberlein (University of California, San Francisco). *p{GawB}NP5141* is an insertion 5' of the gene *ken* and was provided by the *Drosophila* Genetic Resource Center, Kyoto.

### Immunohistochemistry and fluorescent *in situ* hybridization

Embryos were laid at room temperature, dechorionated in 50% bleach for 3 minutes, fixed for 20 minutes in 4% formaldehyde in PBS/heptane, devitellinized using heptane/methanol, and stained using standard protocols. For

fluorescent *in situ* hybridization, embryos were fixed in 37% formaldehyde and *in situ* was carried out as described previously (Lécuyer et al., 2008) using a DIG-labelled RNA probe transcribed from the full-length *wun2* cDNA. Primary antibodies were as follows: rabbit anti-Vasa (1:10,000) courtesy of Ruth Lehmann, rat anti-Vasa (1:40) and mouse anti-Eya (1:12) from the Developmental Studies Hybridoma Bank (DSHB), rabbit anti-Myc (ab9106, 1:1000) from Abcam, rabbit anti-Wun2 (1:100), chicken anti-GFP (ab13970, 1:1000) from Abcam, sheep anti-Dig:POD (1:250) from Roche. Secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen), Cy3, Cy5 and biotin (Jackson ImmunoResearch) were used at 1:500. Fluorescent *in situ* signals were detected using the tyramide signal amplification kit (Invitrogen).

Fluorescently stained embryos were either mounted in aquamount (Polysciences) or dehydrated in methanol and mounted in benzylbenzoate:benzyl alcohol (2:1) and visualized using an Olympus FV1000 confocal microscope with a UPlanSApo 60 $\times$  water (NA 1.2) objective or a Leica SP2 confocal microscope with an HCX PL APO CS 40 $\times$  oil (NA 1.25) objective. Biotinylated secondary antibodies were visualized using a Vectastain ABC Kit (Vector Labs) and 3,3'-diaminobenzidine, dehydrated and mounted in Epon resin and viewed on a Zeiss AxioImager.

Live imaging of GFP-expressing embryos was performed using the UPlanSApo 60 $\times$  water objective on an Olympus FV1000 confocal microscope. Maximum intensity projections were made in ImageJ 1.45 (National Institutes of Health, USA). Live imaging movies were subject to a despeckle filter. 3D reconstructions, measurements and segmentation of the fluorescent *wun2* *in situ* hybridization pattern were made using Imaris 7.4.0 (Bitplane).

To distinguish between ectoderm, mesoderm and endoderm for the quantification of germ cell positions in Fig. 2M, we used the nuclear stain DAPI to visualize the internal structures of the embryo, in particular to distinguish the endoderm, which takes a characteristic shape below the mesoderm (Fig. 2N; supplementary material Movie 8). We used the *h-* or *en-*driven stripes to assess the depth of the ectoderm, with the intervening space defined as the mesoderm.

### Segmentation of ectodermal stripes

We manually defined the positions of the germ cells using the Point Picker plugin (Philippe Thévenaz, Biomedical Imaging Group, Swiss Federal Institute of Technology Lausanne) in ImageJ. To identify the stripes expressing the fluorescent marker, the fluorescence intensity was first standardized along the *z*-axis by determining the 90th percentile of expression in each *z*-slice, fitting a line (slope + intercept) to these values and using the line fit to normalize fluorescence intensities. The normalized image stack was then thresholded at the 80th, 85th, 90th and 95th percentile. The resulting binary stack was then smoothed by five iterations of binary erosion in the *x-y* directions, followed by three iterations of binary dilation. Subsequently, all objects with a pixel volume of  $>1000$  were labeled. The labeled image stacks were manually inspected to identify the appropriate threshold (85 in most cases). Oversegmentation was corrected by merging disjointed parts of the same stripe. Given the curated segmentation, the distance of each germ cell from all stripes was determined using the `distance_transform_edt()` function from `scipy.ndimage` (<http://www.scipy.org/>). Analysis scripts, written in Python, are available on request.

To investigate the hypothesis that germ cells migrate to locations as far as possible from Wun2-expressing stripes, we compared their distances to the closest and next-closest stripes in wild-type embryos with *en*-driven control stripes, *wun wun2* somatic null embryos with *en*-driven control stripes, and *wun wun2* somatic null embryos with *en*-driven *wun2* stripes. Because different germ cells in the same embryo are not statistically independent observations, we assessed statistical significance by Mann-Whitney *U*-test on the mean results from different embryos. In addition, we produced 1000 boot-strap data sets by sampling embryos with replacement and measured the boot-strap distribution of the Kolmogorov-Smirnov statistic (maximal vertical difference of the cumulative histograms). If this statistic is consistently either positive or negative, we considered our observations robust across all embryos examined. Our observations are statistical significant using both criteria. Analysis scripts, written in Python, are available on request.

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**Author contributions**

A.M. and A.D.R. designed the experiments; A.M. made the *Drosophila* stocks and performed the fixed tissue experiments, A.D.R. performed the live imaging; R.A.N. performed the image segmentation and ratio analysis, A.M. and A.D.R. wrote the paper.

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