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Tre1, a G Protein-Coupled Receptor, Directs Transepithelial Migration of *Drosophila* Germ Cells

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In most organisms, germ cells are formed distant from the somatic part of the gonad and thus have to migrate along and through a variety of tissues to reach the gonad. Transepithelial migration through the posterior midgut (PMG) is the first active step during *Drosophila* germ cell migration. Here we report the identification of a novel G protein-coupled receptor (GPCR), Tre1, that is essential for this migration step. Maternal *tre1* RNA is localized to germ cells, and *tre1* is required cell autonomously in germ cells. In *tre1* mutant embryos, most germ cells do not exit the PMG. The few germ cells that do leave the midgut early migrate normally to the gonad, suggesting that this gene is specifically required for transepithelial migration and that mutant germ cells are still able to recognize other guidance cues. Additionally, inhibiting small Rho GTPases in germ cells affects transepithelial migration, suggesting that Tre1 signals through Rho1. We propose that Tre1 acts in a manner similar to chemokine receptors required during transepithelial migration of leukocytes, implying an evolutionarily conserved mechanism of transepithelial migration. Recently, the chemokine receptor CXCR4 was shown to direct migration in vertebrate germ cells. Thus, germ cells may more generally use GPCR signaling to navigate the embryo toward their target.

Introduction

Transepithelial migration is an important step during the immune response in order to accumulate leukocytes at inflamed sites. During this process, leukocytes tightly adhere to the blood vessel endothelium and emigrate from the blood vessel; subsequently, leukocytes invade the inflamed tissue by migrating across polarized epithelia (Springer 1994; Colgan et al. 1995; Parkos 1997; Huber et al. 1998, 2000; Johnson-Leger et al. 2000; Worthylake and Burridge 2001; Johnston and Butcher 2002; Zen and Parkos 2003). The processes that control leukocyte egress from the bloodstream have been well studied and have been shown to be highly regulated at the molecular level and to require a multistep process mediated by adhesion molecules and chemoattractants. Owing to a paucity of good in vitro and in vivo model systems, less is known about the subsequent migration of leukocytes through polarized epithelia (Li et al. 2002). Here we report on the identification of mutants that specifically affect transepithelial migration of *Drosophila* germ cells. Genetic analysis of this process in *Drosophila* may provide new insight into the molecular mechanisms that control transepithelial migration.

Several studies in *Drosophila* have identified genes that specifically affect separate steps in the germ cell migration and gonad formation processes (Warrior 1994; Williamson and Lehmann 1996; Moore et al. 1998a; Starz-Gaiano and Lehmann 2001). Primordial germ cells are formed at the posterior pole underlying somatic cells that give rise to the posterior midgut (PMG) anlage. During gastrulation, germ cells adhering to the PMG anlage are carried inside the embryo (for a summary of early migration events, see Figure 1A). From the blind end of the PMG primordium, germ cells start an active journey by transmigrating through midgut epithelium, moving from its apical to its basal side (Callaini et al. 1995; Jaglarz and Howard 1995). Once germ cells pass through the PMG, they migrate along the midgut toward the nearby mesoderm. From there, they transit from the midgut to the mesoderm, where they associate with three lateral clusters of gonadal mesoderm cells (somatic gonadal precursors [SGPs]). Germ cells adhere tightly to these clusters as they merge and coalesce into a gonad (Brookman et al. 1992; Moore et al. 1998b; Van Doren et al. 2003). Attractant and repellent germ cell guidance factors have been identified in genetic screens. During their migration on the midgut, germ cells move away from Wunen-expressing cells (Zhang et al. 1996, 1997; Starz-Gaiano et al. 2001). The two *wunen* genes encode homologs of lipid phosphate phosphatase 3 (LPP3) and are believed to catalyze phosopholipid dephosphoryla-

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Abbreviations: CNS, central nervous system; *cta*, *concertina*; GPCR, G protein-coupled receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMG-CoAR, HMG-CoA reductase; LPP3, lipid phosphate phosphatase 3; NCBI, National Center for Biotechnology Information; PMG, posterior midgut; *sctt*, *scattershot*; SGP, somatic gonadal precursor; *tre1*, *trapped in endoderm-1*; UAS, upstream activation sequence

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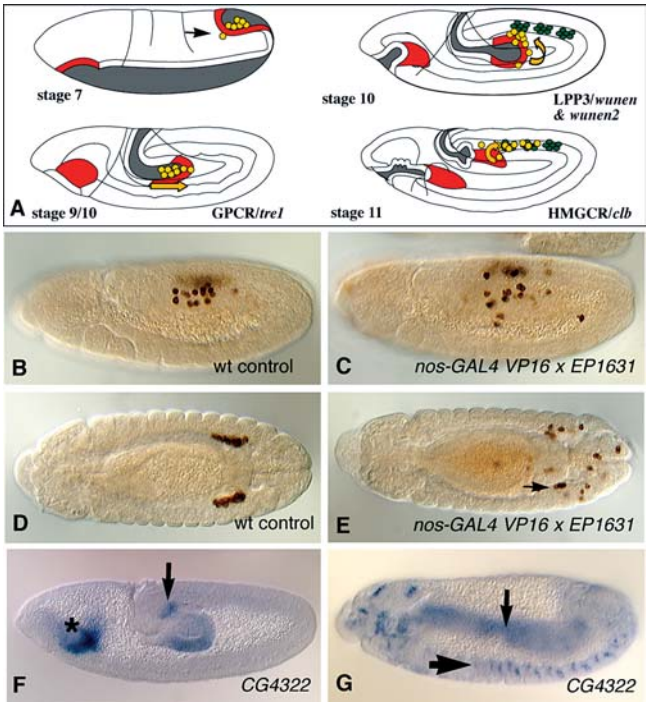

Figure 1. A Summary of Early Germ Cell Migration Steps and Genes

(A) For each stage, the position of germ cells (yellow) and midgut (red) is indicated. Yellow arrows point in the direction of migration. Note that in wild-type embryos, few germ cells can be observed on the basal side of the midgut anlage (black arrow, stage 7). Genes known for their specific role in germ cell guidance are indicated next to the step where the activity of the respective gene product is needed. In addition to the genes listed, a role for *hedgehog* has been suggested in germ cell migration (Desphpande et al. 2001); however, the exact step affected is unclear.

(B–G) Overexpression of *EP1631* in germ cells and expression pattern of *CG4322*. Anterior is to the left in all panels. (B), (C), (F), and (G) are lateral views; (D) and (E) are top views. Wild-type embryos (B and D) and embryos overexpressing *EP1631* (*CG4322* GPCR) in germ cells (C and E) were stained with anti-Vasa to mark germ cells. At stage 11 (B and C), germ cells in the wild-type (B) associate with the mesoderm, while germ cells expressing *EP1631* (*CG4322* GPCR) using the germ cell-specific *nos-GAL4* driver are disorganized (C). When the gonads are normally coalescing at stage 13 in wild-type control embryos (D), many germ cells expressing the *CG4322* GPCR remain lost and are found in ectopic locations (E; arrow points to few germ cells in gonad). (F and G) Expression pattern of *CG4322* RNA. *CG4322* RNA is detected in hemocytes (asterisk), in the caudal visceral mesoderm (arrow), and in the PMG at stage 9 (F) and in midgut (arrow) and glial cells (arrowhead) at stage 13 (G).

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tion (Zhang et al. 1997; Starz-Gaiano et al. 2001; Burnett and Howard 2003). It has been suggested that Wunen proteins act to produce a repellent signal or to destroy a phospholipid acting as an attractant. In the mesoderm, germ cells are attracted by gonadal mesodermal cells that express 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) and Hedgehog (Van Doren et al. 1998a; Deshpande et al. 2001).

The first step of active *Drosophila* germ cell migration, the migration through the PMG epithelium, has so far mostly been analyzed at the morphological level (Jaglarz and Howard 1994, 1995; Callaini et al. 1995), and mutations that specifically affect this step have not yet been described. Ultrastructural analysis of germ cell passage through the PMG epithelium revealed that the actin cytoskeleton rearranges in both the germ cells and the PMG. Coincident with germ cell passage, intracellular gaps form between the epithelial cells of the midgut. These rearrangements are an inherent property of the midgut cells, as they occur even in the absence of germ cells (i.e., in embryos from *oskar* and *tudor* mothers that lack germ cells). Furthermore, in *serpent* or *huckebein* mutant embryos, transmigration of germ cells is affected. These embryos lack a midgut; instead, the posterior invagination develops the epithelial character of the hindgut, which may cause a physical block to migrating germ cells (Reuter 1994; Warrior 1994; Jaglarz and Howard 1995; Moore et al. 1998a, 1998b). These observations are compatible with a passive model for germ cell transepithelial migration, in which germ cell exit is simply regulated by a change in midgut structure, or by more active models in which either mutual interactions between soma and germ line allow transgut migration or in which the expression of an attractive signal on the PMG directs germ cells through the epithelium. Heterochronic germ cell transplantation experiments seem consistent with all of these models, as they have shown that the timing of germ cell passage through the midgut is soma dependent and not cell autonomously programmed in the germ cells (Jaglarz and Howard 1994). However, transplanted somatic blastoderm cells are unable to migrate out of the PMG, indicating that the passage of cells through the PMG is germ cell specific and is not simply due to a mechanical displacement of the contents inside the midgut pocket toward the mesoderm. Until now, mutations that specifically affect this step without impairing the morphology or differentiation of the PMG have not been identified.

Here we report the identification of a gene encoding a novel G protein-coupled receptor (GPCR), *tre1* (*trapped in endoderm-1*), which is required for transepithelial migration of germ cells through the PMG epithelium in *Drosophila*. *tre1* RNA is localized to germ cells, and *tre1* acts cell autonomously in germ cells. We further identify the small GTPase Rho1 as a likely downstream target of Tre1 GPCR signaling. In *tre1* mutant embryos, most germ cells do not exit from the PMG. However, those that escape early from the midgut migrate normally to the gonad, suggesting that this gene is not required for normal motility and directionality of germ cells, but rather specifically functions during transepithelial migration. Recently, the chemokine receptor CXCR4 and its ligand SDF1 were shown to direct germ cell migration in zebrafish and mouse embryos (Doitsidou et al. 2002; Ara et al. 2003; Knaut et al. 2003; Kunwar and Lehmann 2003; Molyneaux et al. 2003). Tre1 belongs to a family of GPCRs related to CXCR4, thereby identifying GPCR signaling as a conserved mechanism for germ cell guidance.

Results

A Misexpression Screen Identifies a GPCR That Affects Germ Cell Migration

We conducted a gain-of-function screen using the GAL4/UAS (upstream activation sequence) system to upregulate genes specifically in the germ cells and then assaying for defects in germ cell migration, as previously described (Starz-Gaiano et al. 2001). To drive expression, the *nanos-GAL4-VP16* (*nos-GAL4*) transgene was used to maternally localize the *GAL4-VP16* transcriptional activator specifically to germ

plasm and primordial germ cells (Rorth 1996; Van Doren et al. 1998b). Of 2,300 lines screened, one, *EP1631*, gave the most striking phenotype, causing large numbers of germ cells to scatter throughout the embryo. At stage 11, when germ cells in the wild-type have largely associated with the mesoderm, germ cells expressing *EP1631* were very disorganized, and although many cells were near the SGPs, some cells migrated far past their mesodermal targets and into the ectoderm (Figure 1B and 1C). At later stages, many germ cells were found at ectopic locations, often resulting in gonads with as few as one germ cell, instead of the 12–15 found per gonad normally (Figure 1D and 1E). This phenotype was only observed when *EP1631* was expressed in germ cells. Overexpression of *EP1631* in a number of other migratory tissues, such as gut, mesoderm, central nervous system (CNS), trachea, or crystal cells, did not affect germ cell migration, nor were significant somatic defects observed in these embryos (data not shown).

EP1631 inserted upstream of the gene *CG4322*, which encodes a putative seven transmembrane GPCR. In situ hybridization analysis revealed that *CG4322* GPCR mRNA is expressed in a variety of migratory cells in the embryo, such as the hemocytes, PMG, caudal visceral mesoderm, and glia (Figure 1F and 1G). We did not, however, detect any *CG4322* mRNA in germ cells. To determine whether *CG4322* plays a role in normal germ cell migration, we generated deletion lines by imprecise P-element excision (see Materials and Methods). We found that tissues that endogenously express *CG4322* transcripts, such as midgut, visceral mesoderm, hemocytes, and glia, showed no gross abnormalities in these mutants (data not shown). Most importantly, we found no significant effect on germ cell migration (data not shown). In order to rule out a maternal contribution of *CG4322* to germ cell migration, we generated embryos that lacked both maternal and zygotic contribution of the *CG4322* GPCR by using the OvoD/Flp technique (see Materials and Methods). These embryos also showed normal germ cell migration. We therefore conclude that *CG4322*, while having a dramatic effect when misexpressed in germ cells, does not play a role normally in germ cells.

Identification of Tre1, a GPCR Expressed in the Germ Cells

The specific effect of *CG4322* misexpression on germ cell migratory behavior suggested to us that GPCR signaling may be important for normal germ cell migration in *Drosophila*, as was recently shown for zebrafish and mouse (Doitsidou et al. 2002; Ara et al. 2003; Knaut et al. 2003; Kunwar and Lehmann 2003; Molyneaux et al. 2003). However, as *CG4322* mutations did not affect germ cell migration, we reasoned that other, perhaps related GPCRs may play a role in germ cells either in concert with *CG4322* or on their own. To identify such a putative GPCR, we searched the *Drosophila* genome database for genes closely related to homologs of *CG4322*. *CG4322* belongs to the Rhodopsin receptor class of GPCRs. The closest homologs of *CG4322* in the fly genome are *CG4313*, the neighboring gene 2.5 kb upstream of *CG4322*, which has not yet been further characterized, and *CG3171*, which was previously thought to encode the receptor for Trehalose, *tre1* (see below) (Ishimoto et al. 2000; Dahanukar et al. 2001; Ueno et al. 2001). The National Center for Biotechnology Information (NCBI) database analysis identified three *Anopheles* proteins of unknown function, each most closely related to the respective fly GPCRs (Figure 2A). Several uncharacterized proteins from vertebrates, such as the human EX33 protein, which was found in a neutrophil cDNA library, the mouse GPR84 receptor, and a zebrafish (ENSDARG07201) and *Fugu* (FuguGenscan31921) putative GPCR, are the closest homologs to *CG3171*, *CG4322*, and *CG4313*. Our phylogenetic analysis suggests that this group may represent a new subclass of GPCRs. Among known ligand–receptor pairs, this group is most closely related to the vertebrate melatonin and histamine receptors and, more distantly, to vertebrate chemokine receptors (Figure 2A; data not shown).

We next determined the expression pattern of the two GPCR genes most closely related to *CG4322*. The neighboring GPCR gene, *CG4313*, is expressed weakly maternally and strongly later, at stage 13 of embryogenesis in the embryonic visceral mesoderm (data not shown). *CG4313* transcript was not detected in germ cells and a deletion, which affects both *CG4322* and *CG4313* transcripts, does not affect germ cell migration (see Materials and Methods; data not shown). The third GPCR gene, *CG3171*, however, showed clear expression in germ cells and a striking germ cell migration phenotype in mutant embryos (see below). This receptor had previously been misidentified as a receptor for Trehalose and named *tre1* (Ishimoto et al. 2000; Dahanukar et al. 2001; Ueno et al. 2001). We decided to change the meaning of the abbreviation to reflect the function of the gene and will refer to this gene as *trapped in endoderm-1*.

tre1 RNA Is Localized to Germ Cells and Required for Their Transepithelial Migration

Expression analysis of *tre1* RNA showed a dynamic expression pattern (Figure 2B–2E). *tre1* RNA is provided maternally, localized to the germ plasm, and can be detected clearly in germ cells until stage 9, when they initiate their migration through the PMG epithelium. We did not detect *tre1* expression in germ cells as they coalesced into the embryonic gonads. *tre1* RNA is also expressed in a variety of other tissues, including the amnioserosa, the developing CNS, the cardiac mesoderm primordium, midline glia, and (very prominently) the cuprophilic cells. To determine *tre1* function, we studied the mutant phenotype of *ΔEP5*, a *tre1* mutation generated by imprecise excision of the EP line *EP0496* and previously reported to lack *tre1* RNA (Rorth 1996; Ishimoto et al. 2000; Dahanukar et al. 2001; Ueno et al. 2001) (see below). Embryos from *ΔEP5* homozygous mothers that are also zygotically mutant (M^-Z^-) show no specific *tre1* RNA expression until stage 9 (Figure 2F), suggesting that the *ΔEP5* deletion affects the regulation of maternal and early zygotic *tre1* gene expression (Ueno et al. 2001). Embryos derived from *ΔEP5* homozygous mothers (hereafter referred as *tre1* mutant embryos) are defective in the first active step of germ cell migration, the transepithelial migration though the PMG (Figure 3A–3J). During stage 10 of embryogenesis, wild-type germ cells migrate from the apical side of the PMG epithelium to its basal side. In contrast, most germ cells in *tre1* mutant embryos do not transmigrate the PMG, but remain clumped together within the midgut pocket (Figure 3A and 3B). To follow germ cell and gut development in mutant and wild-type embryos, we double-labeled embryos with the germ cell marker Vasa and midgut-specific markers, such as *race*, and Fasciclin III, a visceral mesodermal marker (Figure S1A–S1D and S1I–S1J) (Patel et al. 1987; Stein et al.

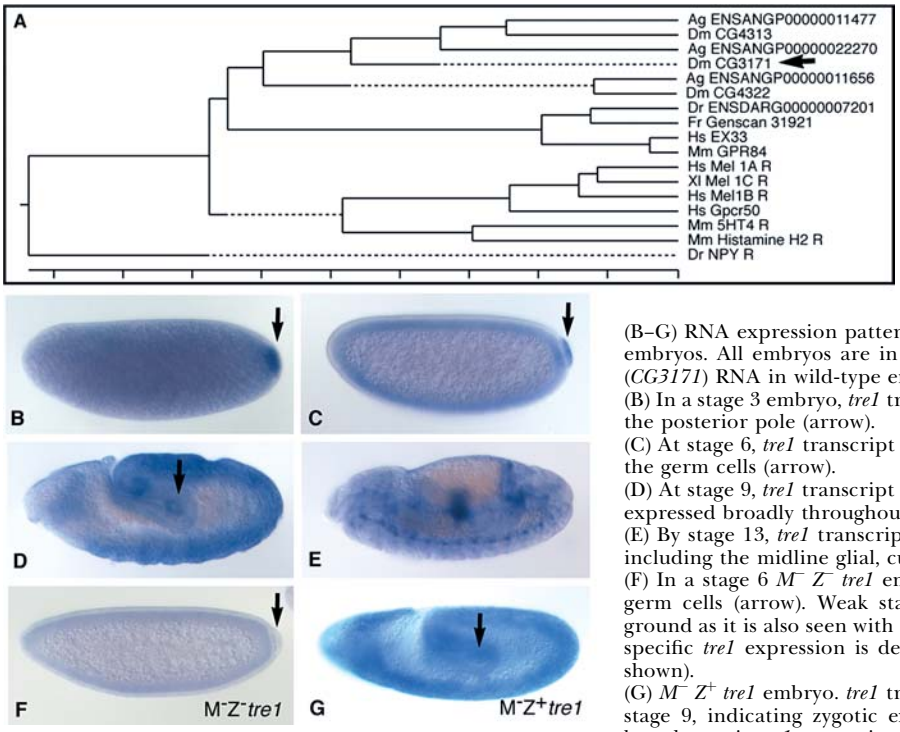

Figure 2. Phylogenetic Tree of Tre1 and Expression Patterns of *CG3171* (*tre1*)

(A) Phylogenetic tree of Tre1 protein with other closely related GPCRs (drawn using ClustalW of MegAlign program from DNASTAR). Tre1 (indicated by arrow) is closely related to a group of fly, *Anopheles*, and vertebrate GPCRs. Among known ligand–receptor pairs, this novel receptor group is most closely related to melatonin, histamine, and serotonin receptors. Abbreviations: Ag, *Anopheles gambiae*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Fr, *Fugu rubripes*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*.

(B–G) RNA expression pattern of *CG3171* (*tre1*). Anterior is to the left in all embryos. All embryos are in lateral views. (B–E) Expression pattern of *tre1* (*CG3171*) RNA in wild-type embryos.

(B) In a stage 3 embryo, *tre1* transcript is provided maternally and enriched at the posterior pole (arrow).

(C) At stage 6, *tre1* transcript is degraded in somatic tissues, but protected in the germ cells (arrow).

(D) At stage 9, *tre1* transcript is still detected in germ cells (arrow), but is also expressed broadly throughout the soma.

(E) By stage 13, *tre1* transcript is highly expressed in several somatic tissues, including the midline glial, cuprophilic cells, glial cells, and CNS.

(F) In a stage 6 $M^- Z^-$ *tre1* embryo, no specific *tre1* transcript is detected in germ cells (arrow). Weak staining in somatic tissues represents the background as it is also seen with sense control RNA probe. At stage 13, weak but specific *tre1* expression is detected in cuprophilic cells and CNS (data not shown).

(G) $M^- Z^+$ *tre1* embryo. *tre1* transcript is detected weakly in the germ cells at stage 9, indicating zygotic expression in the germ cells (arrow). Note the broad zygotic *tre1* expression similar to (D).

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2002). While in wild-type embryos, germ cells migrate away from the gut during stage 11, associate with gonadal mesoderm, and eventually form two bilateral gonads, germ cells remained within the gut throughout development in *tre1* mutants (see Figure 3C–3F; Figure S1A–S1D and S1I–S1J). At the end of embryogenesis, *tre1* mutant embryos have on average one to two germ cells in either gonad, compared to about 12–15 germ cells per gonad in wild-type (see Figure 3E and 3F; see also Figure 7E and 7F). The overall number of germ cells seemed unaffected in the mutant. This phenotype is fully penetrant, and 100% of embryos derived from *tre1* mutant mothers show a strong germ cell migration defect.

To determine whether the germ cell migration defect observed in *tre1* mutants was due to a developmental defect in any of the somatic tissues known to be required for normal germ cell migration, we analyzed the expression of midgut and mesodermal markers in the mutants. Confocal analysis using anti-Vasa antibody to follow germ cells and anti-Neurotactin to mark the cell membranes of midgut epithelial cells during stages 9 and 10 revealed germ cells in a clump inside the midgut in the mutant at late stage 10 (Figure 3G–3J). Germ cells seemed to "avoid" the midgut, and very few cells were observed in close contact with midgut cells, suggesting that germ cells may be unable to penetrate the midgut epithelium. The morphology of the midgut cells, however, seemed unaffected. Analysis of expression patterns and overall morphogenesis in other somatic tissues, such as the midgut (*race*), the visceral mesoderm (Fasciclin III), and the lateral and gonadal mesoderm (*412*), revealed no difference between wild-type and mutants (see Figure S1A–S1J) (Patel et al. 1987; Brookman et al. 1992; Stein et al. 2002). We conclude that the transepithelial migration defect in *tre1* embryos is most probably not due to a secondary effect resulting from defects in the specification or morphogenesis of tissues lining the migratory pathway. Together with the expression pattern of *tre1* RNA in germ cells, these results suggest that *tre1* acts directly in the migrating germ cells.

tre1 Mutations Reveal Maternal Inheritance and a Phenotypic Series of Germ Cell Migration Defects

The *tre1* gene is located in polytene band 5A10 on the X chromosome and, as mentioned above, was initially identified as a GPCR thought to act as a taste receptor for Trehalose. Subsequently, however, a second GPCR, *Gr5a*, which maps adjacent to *tre1*, was shown to be the actual receptor of Trehalose, leaving the function of *tre1* and the nature of its ligand unknown (Ishimoto et al. 2000; Dahanukar et al. 2001; Ueno et al. 2001). The predicted transcription start sites of *tre1* and *Gr5a* are about 900 basepairs apart (Figure 4A). The deletion mutant *ΔEP5* extends from the first exon of *tre1* to the start of the *Gr5a* transcription unit. *ΔEP5* homozygous mutants are adult viable and were reported to lack both *tre1* and *Gr5a* transcripts (Ueno et al. 2001). To confirm that indeed loss of *tre1* and not loss of *Gr5a* gene function was responsible for the observed germ cell migration defect, we introduced into the deletion mutant genomic rescue constructs that contained a 10-kb genomic region, which covers both *tre1* and *Gr5a* (Dahanukar et al. 2001) (Figure 4B). In addition to the transgene that is wild-type for both genes ($T^+ G^+$), we tested two other transgenes, $T^- G^+$ and $T^+ G^-$ that carry a stop codon mutation near the N-terminus of *tre1* or *Gr5a*, respectively, and therefore supply a functional gene product for only one of the two genes (see Materials and Methods). The wild-type construct for both genes ($T^+ G^+$) and the construct carrying the wild-type copy for *tre1* ($T^+ G^-$)

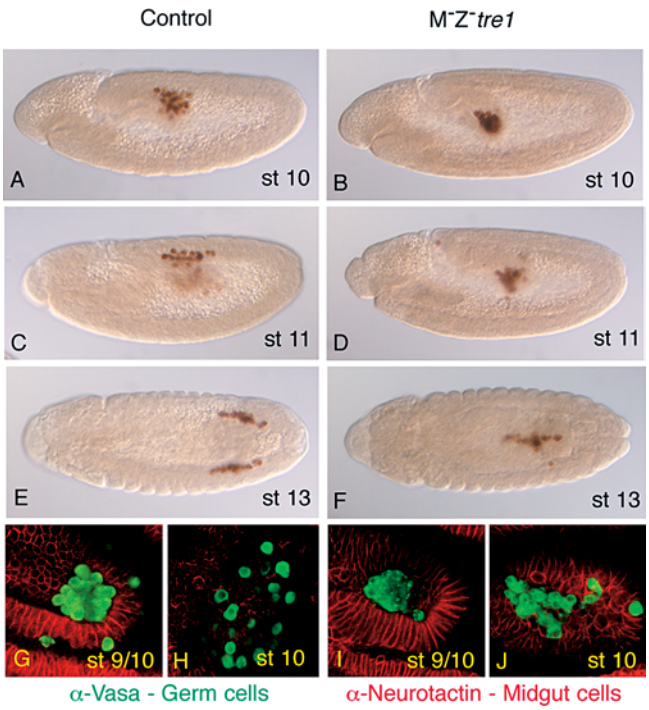

Figure 3. The Phenotype of M^{-} Z^{-} *tre1* Mutant Embryos

Anterior is left in all figures.

(A–F) Embryos are stained with anti-Vasa (brown) to mark germ cells. (A–D) Lateral views. (E–F) Top views. (A), (C), and (E) are wild-type embryos. (B), (D), and (F) are *tre1* mutant embryos. Wild-type germ cells migrate out of the PMG at stage 10 (A) and migrate toward mesoderm at stage 11 (C) and finally to the gonad at stage 13 (E), but in *tre1* mutant embryos, germ cells fail to leave the PMG ([B] shows stage 10 and [D] shows stage 11) and are mostly found "clumped" together in the middle of the gut at stage 13 (F).

(G–J) High magnification view of wild-type (G and H) and *tre1* mutant (I and J) embryos stained with anti-Neurotactin (red) to mark cell membranes of midgut epithelium and germ cell-specific anti-Vasa (green). Wild-type germ cells are migrating out of the PMG at early stage 10 (G) and are outside of the PMG and thus at a different optical plane than PMG at late stage 10 (H). *tre1* germ cells, in contrast, do not migrate out of the PMG at stage 9/10 (I) and are still left inside the PMG and thus at the same optical level as the PMG cells at late stage 10 (J). Punctate appearance of anti-Vasa staining in *tre* mutant germ cells is likely due to heat fixation protocol used as it can also be observed in wild-type germ cells (data not shown).

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rescued completely the migration phenotype of embryos from *ΔEP5* homozygous mothers (Figure 4C and 4E). In contrast, embryos from *ΔEP5* mothers carrying a nonfunctional copy of the *tre1* gene (T^{-} G^{+}) produced a strong migration phenotype, demonstrating that indeed *tre1*, and not *Gr5a*, is required for the migration of germ cells through the PMG (Figure 4D).

In addition to the *tre1* null allele, *ΔEP5*, we analyzed two additional mutations that alter *tre1* function (Figure 4F–4J). As described above, *ΔEP5* is the strongest allele and its phenotype likely resembles the *tre1* null phenotype. The P-element excision mutant *ΔEP19* partially deletes the putative promoter region of *tre1* and the promoter and the first exon of *Gr5a* (Figure 4A) (Dahanukar et al. 2001; Ueno et al. 2001), but still transcribes some *tre1* RNA (data not shown). Embryos from *ΔEP19* homozygous mothers show a weaker germ cell migration phenotype than *ΔEP5* (Figure 4J). While some germ cells remain in the midgut, the majority exits the gut and many germ cells migrate successfully to the gonad. Transheterozygous *ΔEP19/ΔEP5* embryos show an intermediate phenotype (data not shown). Finally, we found that the *scattershot* (*sctt*) mutation, which was isolated recently in a mutagenesis screen for X-chromosomal mutants with germ cell migration defects and was mapped to the same chromosomal region as *tre1* (Coffman et al. 2002), fails to complement the *ΔEP5* germ cell migration phenotype. In *sctt* mutants, the majority of germ cells remain in the gut; however, they seem less "clumped" compared to the strong *ΔEP5* phenotype and more germ cells migrate correctly to the gonad (Figure 4F–4H). The phenotype of *sctt* mutants is enhanced *in trans* to *ΔEP5*, suggesting that it is a partial loss-of-function mutation (Figure 4I; see Materials and Methods).

The *tre1* phenotype is observed in the progeny of homozygous mutant mothers. To test for a zygotic requirement of *tre1*, we crossed embryos laid by *ΔEP5* homozygous mothers with wild-type males (M^{-} Z^{+} embryos). Although germ cell migration was clearly affected in M^{-} Z^{+} embryos, more germ cells crossed the midgut and migrated to the gonad compared to M^{-} Z^{-} embryos (Figure 4K). This observation is consistent with *tre1* RNA expression: while embryos from *ΔEP5* homozygous mothers crossed to wild-type males (M^{-} Z^{+}) lack maternal germ plasm, early germ cell, and cellular blastoderm expression, *tre1* RNA is expressed zygotically at low levels throughout the embryos, starting at stage 8/9, and is consistently seen in germ cells (see Figure 2G). Embryos only lacking zygotic *tre1* function (M^{+} Z^{-}) have no germ cell migration defect (data not shown). Thus, *tre1* has a maternal and zygotic component required for germ cell migration. The maternal component of *tre1* is critical for normal germ cell migration, while the zygotic component of *tre1* function is dispensable. The partial zygotic rescue of the maternal phenotype further suggests that zygotic *tre1* RNA transcription in germ cells may contribute to the ability of germ cells to migrate through the midgut epithelium.

tre1 Is Required Cell Autonomously in Germ Cells

tre1 mutants affect germ cell migration maternally, and *tre1* RNA is present in early germ cells; we therefore hypothesized that *tre1* may act specifically in germ cells to mediate their migration through the PMG. We used two experimental approaches to test whether *tre1* is required in the germ cells in a cell-autonomous manner. In the first approach, germ cells from wild-type or *tre1* mutant females were transplanted into *tudor* embryos (embryos produced by homozygous *tudor* mothers that lack germ cells) (Boswell and Mahowald 1985; Lehmann and Nüsslein-Volhard 1986, 1987). In the control experiments, wild-type germ cells migrated to the gonad in 41.6% (total number of embryos, $n = 36$) (Figure 5A–5B and 5G–5H). In total, 34% of all transplanted germ cells migrated successfully to the gonad (total number of germ cells transplanted, $n = 115$). In contrast, *tre1* germ cells transplanted into *tudor* embryos rarely migrated to the gonad. Only 11% of embryos had transplanted *tre1* germ cells in the gonad ($n = 38$) (Figure 5C–5D and 5G–5H), and only 9.1% of all transplanted germ cells successfully migrated to the gonad ($n = 87$). To test for a somatic role of *tre1*, we transplanted wild-type germ cells into *tre1* mutant embryos. We marked the transplanted germ cells with a *P[faf–LacZ]* transgene, to distinguish the transplanted from the endogenous germ cells

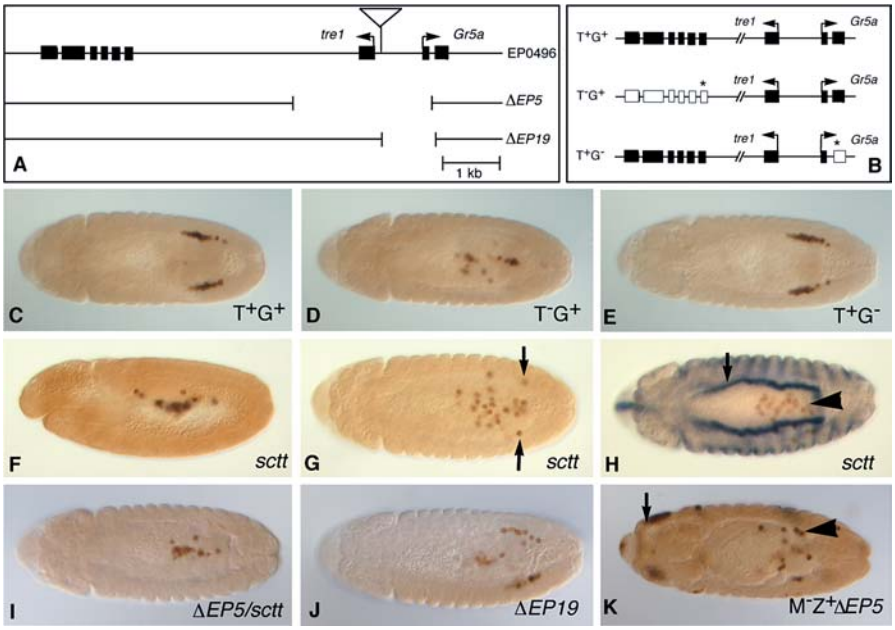

Figure 4. *tre1* Gene Structure, Genomic Rescue, and *tre1* Phenotypic Series

(A) Molecular structure of the *tre1* region (adapted from Dahanukar et al. 2001). The exons of *tre1* and *Gr5a* are shown as black boxes. Only two of seven exons are shown for *Gr5a*. The inverted triangle marks the insertion *EP(X)0496*. Deleted regions in *ΔEP5* and *ΔEP19* are shown by interrupted lines below.

(B) Genomic rescue constructs. Black filled boxes denote translated exons; white open boxes denote exons likely not translated because of stop codon mutation. $T^+ G^+$ contains both wild-type constructs for *tre1* (*T*) and *Gr5a* (*G*); $T^- G^+$ and $T^+ G^-$ contain a stop codon mutation (asterisk) for *tre1* and *Gr5a*, respectively.

(C–K) Anterior is to the left in all embryos. All embryos are at stage 13, except (F), which is at stage 11. Embryos are labeled with anti-Vasa (brown) to mark germ cells. The embryo in (K) is also stained for anti-β-galactosidase activity.

(C–E) Genomic rescued *tre1* embryos. Embryos from *tre1* homozygous mothers that carried either the wild-type construct for both genes ($T^+ G^+$) or the construct with a wild-type copy for *tre1* ($T^+ G^-$) rescued the *tre1* migration phenotype completely (C and E). However, embryos from a *tre1* mother carrying a nonfunctional copy of the *tre1* gene ($T^- G^+$) did not rescue the *tre1* migration phenotype (D).

(F–J) *tre1* phenotypic series. (F–H) $M^- Z^-$*sctt* embryos. (F) Stage 11 *sctt* embryos with strong transgut migration defect (for wild-type control, refer to Figure 3C); note that more germ cells have exited the gut compared to strong *ΔEP5* mutants (Figure 3D). (G and H) At stage 13, most germ cells remain inside the gut in *sctt* mutants, as judged by Fasciclin III staining (arrow in [H]; arrowhead points to germ cells), and more germ cells reach the gonad (arrows in [G]) compared to *ΔEP5* mutants (for wild-type and *ΔEP5*, refer to Figure 3E and 3F and Figure S1I and S1J). The phenotype is enhanced in embryos from *sctt/ΔEP5* females (I). *ΔEP19* embryos have weak phenotype (J).

(K) *tre1* phenotype can be rescued weakly by paternal zygotic copy. An increased number of germ cells migrates to the gonad (shown by arrowhead). The zygotic rescued embryos were identified by *deformed–LacZ* staining (arrow).

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by the presence of β-galactosidase activity (Fischer-Vize et al. 1992). In 54.4% ($n = 48$) of embryos examined, germ cells migrated to the gonad (Figure 5E–5F and 5G–5H) and 40.2% of the transplanted germ cells successfully migrated to the gonad ($n = 184$). These experiments suggest that *tre1* function is required within the germ cells for their normal migration.

In the second approach, we used tissue-specific gene expression to determine where Tre1 function is required. Using the germ cell-specific *GAL4* driver *nos-GAL4* and the EP line *EP0496*, we expressed *tre1* in the germ cells and tested whether the *tre1* mutant phenotype can be rescued (Figure 6A) (Van Doren et al. 1998b). In *EP0496*, the UAS sites required for *GAL4*-mediated transcriptional activation are inserted in the *tre1* promoter region and drive expression of *tre1* RNA under *GAL4* control. Since the *tre1* gene is located on the X chromosome, only half of the embryos are expected to carry a copy of *EP0496* and should thus express the *tre1* gene in the germ cells. In this experiment, 50% of the embryos obtained by crossing homozygous *ΔEP5* mothers carrying the *nos-GAL4* transgene to *EP0496* males showed a complete rescue of the transepithelial migration phenotype (Figure 6B). Embryos derived from crossing a *UAS-LacZ* line to *ΔEP5* mothers carrying *nos-GAL4* showed only the minor zygotic rescue of the mutant phenotype as described above (data not shown). A difficulty in the interpretation of this experiment lies in the fact that the *nos-GAL4* driver also transiently activates somatic expression in the PMG anlage at the blastoderm stage (Van Doren et al. 1998b). Thus, the phenotypic rescue could be due to expression of *tre1* in the PMG during the blastoderm stage rather than due to germ cell expression. To rule out this possibility, we wanted to express *tre1* in the PMG anlage at the blastoderm stage. Since there are no early *GAL4* drivers available that specifically express a reporter in the PMG anlage, we used a somatic driver, *nullo-GAL4*, which efficiently drives expression in all somatic tissues, including the PMG, during the blastoderm stage, but does not activate transcription in the germ cells (Figure 6C) (W. Gehring and E. Wieschaus, personal communication). We did not observe any rescue of the *tre1* phenotype with this driver (Figure 6D; total number of embryos analyzed, $n = 200$). These experiments demonstrate that *tre1* is required autonomously in germ cells for their migration through the PMG and that transcription of *tre1* in early germ cells is sufficient to rescue the migration phenotype.

tre1 Mutant Germ Cells Are Motile and Can Migrate to the Gonad

Migration through the PMG is thought to be the first stage at which germ cells are actively migrating; thus, a failure to pass through the PMG might be due to a failure to respond to a specific guidance signal. Alternatively, since the germ cells are passively carried into the blind pocket of the PMG during gastrulation movements, the step of transepithelial migration would be the first step to be affected if germ cells were immotile. To distinguish between these two possibilities, we examined the *tre1* phenotype more carefully. We observed that while most germ cells do not leave the midgut, in *tre1*

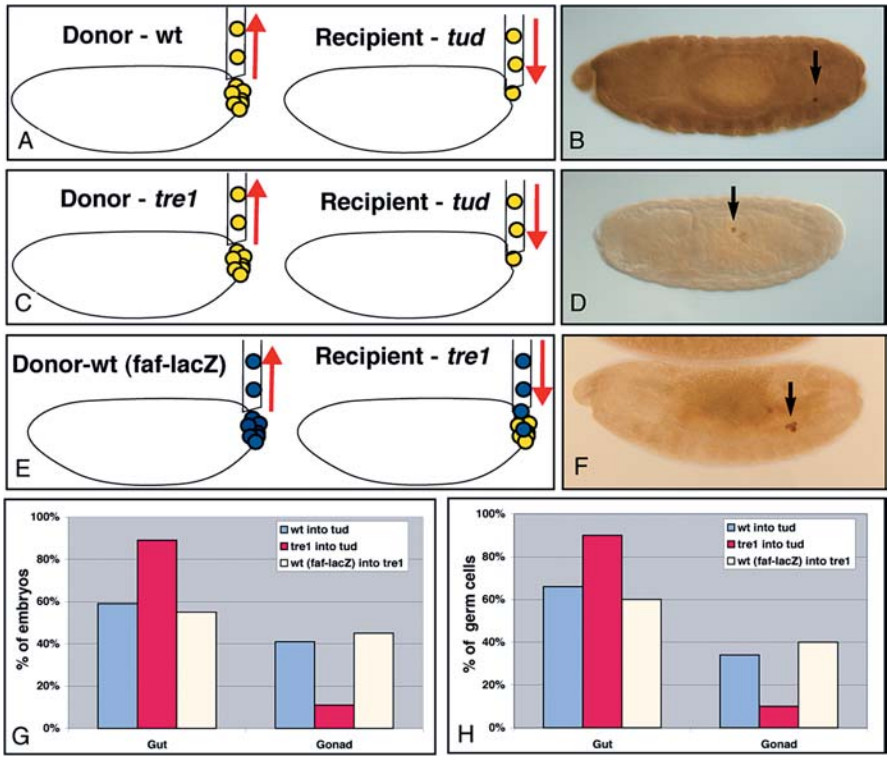

Figure 5. Germ Cell-Specific Requirement of *tre1* by Germ Cell Transplantation

(A), (C), and (E) depict the experimental scheme for germ cell transplantation. Germ cells (yellow) were transplanted from wild-type (A) or *tre1* (C) stage 6 embryos to same stage embryos from *tudor* mothers, which do not have germ cells. In (E), germ cells (blue) labeled with *LacZ* (*faf–LacZ*) transgene were transplanted to the same stage *tre1* embryos, to distinguish donor and host germ cells by β-galactosidase activity. (B), (D), and (F) are examples of transplanted, fixed, and stained embryos. Anterior is left in all embryos. Embryos in (B) and (F) are at stage 13; embryo in (D) is at stage 14. Embryos in (B) and (D) are stained with anti-Vasa (brown), and the embryo in (F) is stained with anti-β-galactosidase (brown). Arrow points to transplanted germ cells. (G–H) Summary of transplantation experiments. The bar graph in (G) summarizes the position of germ cells in embryos with successful transplantation ($n = 36$ for wild-type germ cells, $n = 38$ for *tre1* mutant germ cells, and $n = 48$ for *faf–LacZ*-labeled wild-type germ cells). The bar graph in (H) summarizes the number of germ cells from successful transplantations at particular locations ($n = 115$ for wild-type germ cells, $n = 87$ for *tre1* mutant germ cells, and $n = 184$ for *faf–LacZ*-labeled wild-type germ cells). Note that even in wild-type control transplantations, most germ cells, which do not reach the gonad, remain associated with the gut.

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mutant embryos, a few germ cells are consistently found in the gonad in most embryos (Figure 7A–7F), indicating that *tre1* germ cells were motile and were able to follow guidance signals to reach the embryonic gonad. Careful counting showed that the number of *tre1* germ cells that had passed through the PMG anlage at the blastoderm stage, prior to midgut pocket formation (1.27 germ cells per embryo, $n = 50$), correlated with the number of germ cells on the basal side of the PMG at stage 10 (1.47 germ cells per embryo, $n = 50$) and the number of germ cells in the gonad at stage 13 (1.2 germ cells per embryo, $n = 50$). This indicates that germ cells that migrated to the gonad in *tre1* embryos might have originally crossed the PMG anlage prior to midgut specification and may thus not require a Tre1-mediated signal. This phenotype cannot be explained by incomplete penetrance of the mutant for two reasons. First, we observe the same average number of germ cells on the basal side of the blastoderm in wild-type and mutant embryos, suggesting that even in wild-type some "pioneer" germ cells take an "earlier" route, one that does not require transepithelial migration through the midgut (see Figure 1A, stage 7 arrow). Second, the majority of germ cells that pass through the blastoderm prior to PMG specification seem to migrate correctly to the gonad, which would not be expected if passing through the blastoderm were the consequence of a partially penetrant migration phenotype. This suggests that *tre1* germ cells are defective in a migratory

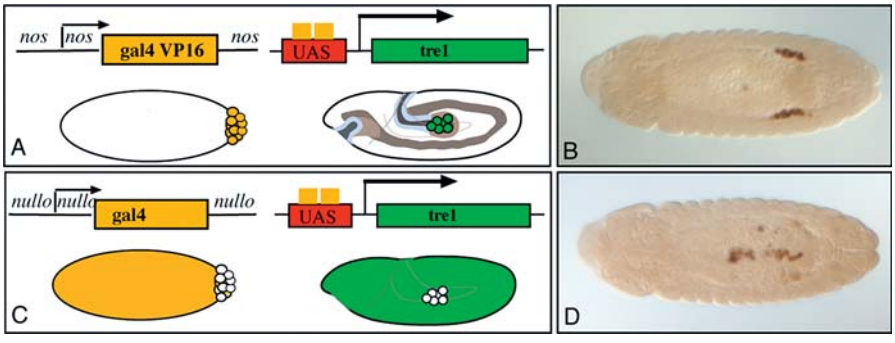

Figure 6. Germ Cell-Specific Rescue of *tre1* Phenotype

(A) and (C) depict the experimental rationale for the tissue-specific gene expression experiment. *EP(X)0496*, which drives expression of *tre1* RNA, was expressed either in the germline by the germline-specific driver *nos-GAL4* (A) or in the soma by somatic blastoderm cell-specific driver *nullo-GAL4* (C). *nos-GAL4* (yellow) is maternally localized to the posterior pole (yellow) and drives expression in germ cells (green), starting at stage 7 and persisting through embryogenesis and transiently in posterior somatic tissues at blastoderm stage (data not shown). *nullo-GAL4* (C) (yellow) drives expression in all somatic cells at blastoderm stage except for germ cells (green). (B and D) Embryos at stage 13 (top view) stained with anti-Vasa. Anterior is left. Expression of *tre1* only in the germ cells rescued the *tre1* phenotype (B). Expression of *tre1* in somatic tissues did not rescue the *tre1* mutant phenotype (D).

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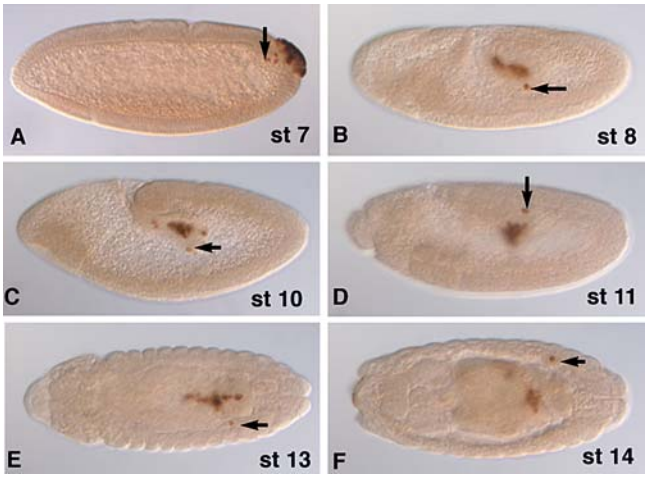

Figure 7. *tre1* Germ Cells Can Migrate to the Gonad

(A–F) *tre1* embryos labeled with anti-Vasa to mark germ cells. Anterior is left. Embryos in (A)–(D) are lateral views, and embryos in (E) and (F) are top views. Arrows point to the few germ cells that migrate correctly. Arrows in (A) and (B) point to a few germ cells that are clearly on the basal side of PMG anlage (on average, 1.27 germ cells per embryo). Arrow in (C) points to germ cells on the basal side of the PMG (on average, 1.47 germ cells per embryo). The arrow in (D) points to a germ cell that migrated successfully into the mesoderm. Arrows in (E) and (F) mark single germ cells that reached the embryonic gonad (on average, 1.2 germ cells per embryo).

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step that allows them to pass through the PMG epithelium, but that they are otherwise motile and able to respond to other guidance signals to reach the gonad.

Intracellular Cascades of Tre1 May Involve Rho Signaling

GPCR signal transduction is often mediated by members of the Rho family of small GTPases. These GTPases play major roles in the reorganization of the actin cytoskeleton to promote adhesion and movement (Mitchell et al. 1998; Fukuhara et al. 1999; Ridley 2001; Neves et al. 2002; Pierce et al. 2002). To test the involvement of these proteins in transepithelial migration, we used the *UAS/nos-GAL4* system to express wild-type, constitutively active, or dominant-negative forms of small GTPases in the germ cells. Rac, RhoL, and Cdc42 expression had no effects on transepithelial migration of germ cells, while later aspects of germ cell migration were affected by expression of constitutively active and dominant-negative forms of Rac in germ cells (data not shown; Starz-Gaiano 2002). Interference with normal Rho1 function, on the other hand, caused a consistent and penetrant transepithelial migration phenotype (Figure 8A–8F). Overexpression of a dominant-negative form of Rho1, $Rho1^{N19}$, in germ cells caused many of them to remain inside the PMG of stage 10 embryos, closely resembling the phenotype observed in *tre1* mutant embryos (Figure 8E) (Barrett et al. 1997). $Rho1^{N19}$-expressing germ cells were clumped in the middle of embryos at stage 13 (Figure 8F). At stages 13–14, when wild-type germ cells assemble into gonads, very few germ cells expressing the $Rho1^{N19}$ transgene had successfully reached the gonad. Dominant-active Rho had a different effect. Rho^{V14}-expressing germ cells successfully transmigrated the PMG during stages 9 and 10 of embryogenesis, but subsequently some germ cells failed to move from the PMG into the mesoderm (Figure 8C) (Lee et al. 2000). As a consequence, these germ cells also remained associated with the PMG (Figure 8D). Expression of wild-type Rho1 had no effect on germ cell migration (Figure 8A–8B) (Prokopenko et al. 1999). The fact that a dominant-negative form of Rho1 caused a similar migration defect as that observed in *tre1* mutant embryos and that expression of other GTPases either showed no or a different migration defect strongly suggest that Tre1-dependent transepithelial migration is mediated by Rho GTPase in germ cells.

Discussion

We have identified a novel *Drosophila* GPCR, Tre1, that is required for transepithelial migration of germ cells through the PMG epithelium. *tre1* RNA is expressed in germ cells, and *tre1* acts cell autonomously in germ cells. Transmigration of germ cells through the PMG epithelium is the first active stage of germ cell migration, and specific mutations had previously not been identified for this step. Tre1 GPCR function specifically affects this stage, as "pioneer" *tre1* germ cells that bypass the requirement for transepithelial migration through the PMG are motile and can follow other, later-acting migratory cues. These results suggest that GPCRs play an important role in transepithelial migration of germ cells and lead us to speculate that Tre1 might function in a manner equivalent to the chemokine receptors required for transepithelial migration of leukocytes.

Tre1 and Directed Transepithelial Cell Migration

Previous models for transgut migration of germ cells relied on the study of wild-type germ cell migration and analysis of mutants that affect PMG specification (Jaglarz and Howard 1994, 1995; Callaini et al. 1995). Most of these observations—including the fact that the midgut epithelium reorganizes independently of germ cells, that genes that disrupt PMG specification block germ cell transgut migration, and that either retarded or precocious germ cells would transmigrate the gut in accord with gut morphology—were compatible with a passive model. In this model, germ cells would pass through the gut merely as a consequence of the reorganization of the gut epithelium. Furthermore, this model would predict that, except for their ability to be motile, germ cells would not require any specific functions to pass the midgut epithelium. In contrast, our analysis of *tre1* gene function demonstrates that the Tre1 GPCR acts in germ cells to specifically promote transepithelial migration. Thus, alternative models have to be considered in which gut rearrangements, while being a prerequisite for transgut migration, would not be sufficient to trigger the migration event per se. One possibility is that Tre1 mediates the initial interactions between germ cells and PMG cells, which may facilitate the passage of germ cells. Alternatively, Tre1 may mediate the directed migration of germ cells through the PMG. According to this latter model, migration may be directed by the expression of a ligand on the basal side of the midgut.

Both attractant and repellant guidance signals for germ cells have already been identified in *Drosophila* (Zhang et al. 1996, 1997; Van Doren et al. 1998a; Starz-Gaiano et al. 2001). The gonadal mesoderm produces an attractant mediated by the HMG-CoAR (*hmgcr/clb*) pathway to attract germ cells to the mesoderm, while the PMG produces a repellent signal produced by lipid phosphatase (*wunen* and *wunen2*) (for a

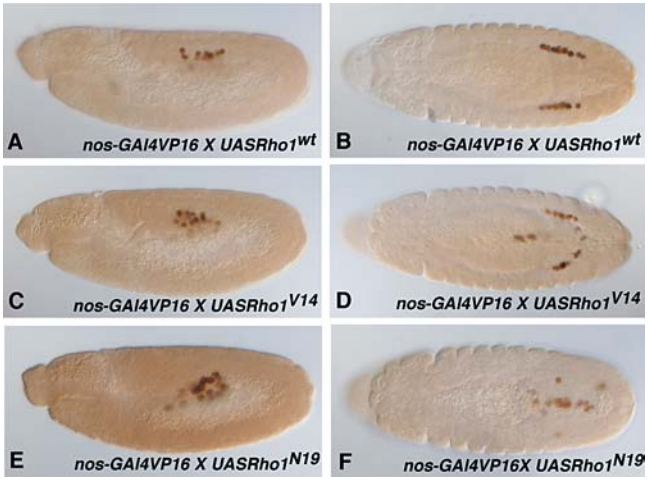

Figure 8. Rho GTPase Is Required for Transepithelial Migration of Germ Cells

(A–F) Wild-type, constitutively active, and dominant-negative Rho1 constructs under UAS promoter control were expressed in germ cells using the *nos-GAL4* driver. Embryos are stained with anti-Vasa (brown) to mark germ cells. Anterior is left. Embryos in (A), (C), and (E) are lateral views at stage 11. Embryos in (B), (D), and (F) are top views, stage 13.

(A and B) Germ cells expressing wild-type Rho1 ($Rho1^{wt}$) migrate normally.

(C and D) Germ cells expressing constitutively active Rho1 ($Rho1^{V14}$) successfully transmigrated the PMG (C), but some germ cells fail to move from the PMG into the mesoderm and remain associated with the PMG (D).

(E and F) Germ cells expressing dominant-negative Rho1 ($Rho1^{N19}$) are still inside the PMG at stage 11 (E), and most germ cells remain "clumped" inside the gut (F).

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review of migration steps, see Figure 1A). At this point, neither the nature of the attractant or repellent produced by Clb/HMG-CoAR and Wunen nor the receptors in germ cells that mediate these signals have been identified. While it may be tempting to speculate that Tre1 could respond to signals produced by Clb/HMG-CoAR, Wunen, or both, this seems unlikely since germ cells can migrate successfully out of the PMG in these mutants and *tre1* "pioneer" germ cells can migrate successfully to the gonad. Thus, a Tre1 ligand remains elusive.

Tre1 Is the Founding Member of a New Class of GPCRs

The Tre1 GPCR belongs to a new subclass of Rhodopsin family GPCRs. Within this subclass we have identified three fly homologs. Indeed the striking phenotype of one of these homologs, *CG4322*, when misexpressed in germ cells, led us to the discovery of Tre1's role in transepithelial migration. The fact that all three homologs are expressed in migratory cell populations, such as germ cells, hemocytes, glia, and midgut cells, raises the possibility that they may have conserved functions in directional cell migration. While only *tre1* mutants cause the transgut migration defect, *CG4322* but not *tre1* overexpression in germ cells produces a germ cell migration phenotype (data not shown). These receptors may thus activate different downstream signaling cascades. Alternatively, differences in the extent of their expression levels or their ability to activate the same downstream pathway independent of ligand may cause the differences in migratory response we observed. Given the expression patterns of the three homologs, it is possible that they function in a partially redundant manner and that they respond to the same ligand. NCBI database analysis identified three uncharacterized *Anopheles* proteins, which clearly aligned with the respective *Drosophila* receptors, and there are also vertebrate members of this new family from human, mouse, zebrafish, and *Fugu*. These are largely uncharacterized GPCRs, and we do not know their exact expression pattern, function, or mode of activation. It is interesting to note, however, that the human family member, EX33, and the mouse homolog, GPR84, are expressed in migratory tissues, including leukocytes, which undergo transepithelial migration (Wittenberger et al. 2001; Yousefi et al. 2001). Based on these observations, it is tempting to speculate that this new group of GPCRs might be required for a variety of migratory functions, including transepithelial migration. It will be interesting to see whether these GPCRs also play an important role in germ cell development in other organisms.

Related to this family are GPCRs responding to nonpeptide ligands such as melatonin, histamine, and serotonin. In mammals, two receptors for melatonin, Mel1a and Mel1b, have been identified, and nonmammalian vertebrate species have, in addition, a third melatonin receptor, Mel1c (Figure 2A) (Dubocovich 1995; Reppert et al. 1996; von Gall et al. 2002; Jin et al. 2003). In the fly genome, melatonin receptors have not yet been identified, even though melatonin and the enzymes required to produce melatonin are present (Finocchiaro et al. 1988; Hintermann et al. 1996; Amherd et al. 2000). Melatonin may not be a good candidate for the ligand, however, as Tre1 seems to be more closely related to a separate group of vertebrate GPCRs and lacks key motifs conserved among the melatonin receptors (Reppert et al. 1996). More distantly related to the Tre1 family of GPCRs are the chemokine receptors, including CXCR4 (data not shown). This receptor has been shown to control the migratory behavior of many different cell types. Most importantly, in zebrafish, one of the two *CXCR4* genes is expressed in germ cells, and expression of the ligand SDF1 along the migratory path directs germ cells toward their target (Doitsidou et al. 2002; Knaut et al. 2003; Kunwar and Lehmann 2003). Mouse knockout mutations of *CXCR4* and *SDF1* were also shown to affect germ cell migration and survival (Ara et al. 2003; Molyneaux et al. 2003), suggesting a conserved mechanism guiding vertebrate germ cells. While related, Tre1 is not the closest homolog to CXCR4 in *Drosophila*, and chemokines like SDF1 have yet to be identified in the *Drosophila* genome.

Intracellular Cascade of Tre1 for Transepithelial Migration

Our studies also identified a likely downstream target of Tre1 GPCR activity. We find that the ability of germ cells to transmigrate the PMG is affected by mutations in *tre1* and by inhibiting Rho1 function. Rho GTPase family members have been shown to mediate GPCR responses through both G protein-dependent and G protein-independent mechanisms (Mitchell et al. 1998; Fukuhara et al. 1999; Ridley 2001; Neves et al. 2002; Pierce et al. 2002). Generally, Rho GTPase mediates signals from G proteins to regulate the actin cytoskeleton to promote adhesion and movement. In *Drosophila*, Rho1 has been intensively studied for its effect on cell shape changes during gastrulation (Barrett et al. 1997; Leptin 1999). Here Rho1 acts downstream of *concertina* (*cta*), the *Drosophila* homolog of G protein α12/13 and a Rho guanine

exchange factor, RhoGEF2 (Parks and Wieschaus 1991; Barrett et al. 1997; Prokopenko et al. 1999, 2000). Rho1, Cta, and another RhoGEF (Pebble) are present in early germ cells and are thus likely targets to mediate transepithelial migration affected by Tre1 (Parks and Wieschaus 1991; Prokopenko et al. 2000; Magie et al. 2002). However, because of the maternal-effect gastrulation defect observed in *cta* mutants and the role of Pebble in blastoderm cytokinesis, we have not yet been able to investigate their roles in germ cell migration. Interestingly, a few mammalian GPCRs in the Rhodopsin class mediate a response by directly associating with monomeric GTPases, such as Rho1 and ARF, which are involved in the regulation of endocytosis and phagocytosis (Mitchell et al. 1998). This interaction is dependent upon an NPxxY motif in the seventh transmembrane domain of the receptor. All GPCRs of the Tre1 subfamily share the NPxxY domain, suggesting that Rho1 might mediate Tre1 signals through this motif.

Transepithelial Migration of Leukocytes and Germ Cells

Leukocyte infiltration of lumen or mucosal surfaces is a common aspect of inflammation. The inflammatory response consists of multiple steps: transendothelial migration through the endothelium, subsequent migration of leukocytes across the extracellular matrix, and finally transepithelial migration into the affected tissue (Springer 1994; Colgan et al. 1995; Parkos 1997; Huber et al. 1998, 2000; Johnson-Leger et al. 2000; Worthylake and Burridge 2001; Johnston and Butcher 2002; Zen and Parkos 2003). Although much is known about the initial recognition process and the interactions of leukocytes with endothelial cells, less is known about the molecular mechanism that regulates transepithelial migration of leukocytes. It has been proposed that chemokine-activated, β_2-integrin-dependent adhesion between leukocytes and epithelia is largely responsible for initial adhesive interaction (Colgan et al. 1995). Ultimately, leukocytes cross the epithelia by migrating along the normally sealed paracellular pathway to the luminal side, which involves a rapid and highly coordinated opening and closing of epithelial intracellular junctions (Huber et al. 1998; Zen and Parkos 2003).

Similarities between transepithelial migration of leukocytes and germ cells are evident. Like leukocytes, germ cells form large pseudopodia, which interact transiently with the protrusions formed by midgut cells (Callaini et al. 1995; Jaglarz and Howard 1995). Similar to the opening within epithelia to permit leukocyte passage, rearrangement of adherens junctions in the midgut epithelium takes place and intracellular gaps form between these cells, which permits passage of germ cells (Zen and Parkos 2003). Despite this apparent similarity in the migratory mode of germ cells and leukocytes, significant differences exist. For example, it seems clear that, unlike transepithelial migration of leukocytes, integrin signaling is not involved in transepithelial migration of *Drosophila* germ cells. Integrins are heterodimers that consist of an α and a β subunit. Removal of both β subunits in *Drosophila* does not affect germ cell migration (D. Devenport and N. H. Brown, personal communication). This finding is particularly surprising because integrins are required for mouse germ cell migration (Anderson et al. 1999). In transepithelial migration of leukocytes, integrins are required for stable adhesion of migrating leukocytes to epithelial cells. In *Drosophila*, germ cells are already in proximity to the midgut cells; thus, integrin function may be dispensable. An alternative possibility is that germ cells and midgut cells use different sets of molecules for their initial attachment. Another interesting difference is that, unlike the transepithelial migration of leukocytes, germ cells are not required for the breakdown of cellular junctions in the midgut cells (Callaini et al. 1995; Jaglarz and Howard 1995). Defining more clearly the signaling pathways during germ cell and leukocyte migration may provide further evidence regarding the conservation between these two systems.

GPCR Signaling and Germ Cell Migration

In this study, we identified a GPCR, Tre1, required for transepithelial migration. We found that receptor activity is provided maternally to the germ cells, but that the phenotype can also be partially rescued by zygotic expression of the receptor or completely restored by zygotic overexpression of the receptor using the *UAS/nos-GAL4* transcription system. While it has been firmly established that the onset of zygotic expression in germ cells is delayed with respect to zygotic expression in the soma, our results suggest that zygotic gene expression is activated in germ cells prior to the onset of germ cell migration (Seydoux and Dunn 1997; Van Doren et al. 1998b; Seydoux and Strome 1999). This result, as well as the phenotypes observed after overexpression of Rho1 or the *tre1*-related gene *CG4322*, further demonstrates the usefulness of the *nos-GAL4* system for the analysis of even very early aspects of germ cell migration and development. The analysis of early germ cells has been hampered by the pleiotropic effects that many of the known signaling molecules exert on oogenesis and early embryogenesis, making it often difficult to assess germ cell migration in an embryo with defective somatic patterning. In the course of our studies using the *nos-GAL4* system, we have expressed many constitutively activated and dominant-negative forms of GTPases. While other GTPases, such as activated Rac and Rho1, affected the actin cytoskeleton of germ cells and led to migration defects, only dominant-negative Rho1 GTPase gave us a specific transepithelial migration defect (this study; Starz-Gaiano 2002). We also tested receptors and transducers for most signaling pathways that control many aspects of development, such as FGF, EGF, Notch, Wingless, Hedgehog, Pten, and PI3 kinase in germ cells. Except for the GPCR Tre1 and *CG4322*, none of them resulted in any type of germ cell migration defect (M. Starz-Gaiano, P. S. Kunwar, A. Santos, J. Stein, and R. Lehmann, unpublished data; Starz-Gaiano 2002). Our data suggest that GPCR signaling is a major determinant in the guidance of *Drosophila* germ cells. Given the role recently shown for the GPCR CXCR4 in zebrafish and mouse germ cell migration (Doitsidou et al. 2002; Ara et al. 2003; Knaut et al. 2003; Kunwar and Lehmann 2003; Molyneaux et al. 2003), GPCR signaling may indeed be an evolutionarily conserved aspect of germ cell development.

We show here that in addition to providing directional cues for germ cell guidance along somatic tissue, GPCRs play an important role in the transepithelial migration of germ cells. *Drosophila* germ cells are not unique with regard to transepithelial migration. Primordial germ cells in chick embryos migrate into the vasculature, where they are passively transported by the bloodstream until they trans-

migrate the endothelium and invade the gonad (Fujimoto et al. 1976; Ukeshima et al. 1991). Mouse germ cells also undergo transepithelial migration as they move out of the hindgut toward the mesentery (Wylie 1999; Molyneaux et al. 2001). Very little is known about the molecules required for these early migratory events in vertebrates. Our study of transepithelial migration in *Drosophila* may provide the first molecular insight into this process.

Materials and Methods

Fly stocks. *ΔEP5*, *ΔEP19*, *EP0496*, T^+ G^+, T^- G^+, and T^+ G^- were kindly provided by J. Carlson (Dahanukar et al. 2001; Ueno et al. 2001). *sctt* was kindly provided by C. R. Coffman (Coffman et al. 2002). *UAS-Rho1*^{wt}, *UAS-Rho1*^{V14} transgenic flies were kindly provided by M. Mlodzik (Lee et al. 2000). *UAS-Rho1*^{N19}, *UAS-Rac*^{wt}, *UAS-Rac*^{V12}, *UAS-Rac*^{N17}, *UAS-RhoL*^{N25}, and *UAS-RhoL*^{V12} were kindly provided by D. Montell (Murphy and Montell 1996; Barrett et al. 1997). *UAS-Cdc42*^{N17} and *UAS-Cdc*^{V12} were provided by L. Luo (Luo et al. 1994). *nullo-GAL4* flies were a kind gift from W. Gehring and E. Wieschaus, and *nos-GAL4* was as described elsewhere (Van Doren et al. 1998b). *Dfd-LacZ.4xE2* was a kind gift from W. McGinnis (Zeng et al. 1994). All EP lines used for the misexpression screen were provided by the Berkeley *Drosophila* Genome Project. Selected lines were later obtained from Exelixis (South San Francisco, California, United States).

Misexpression screen, cloning, and genetic analysis of *CG4322*. The misexpression screen for germ cell migration has been described (Starz-Gaiano et al. 2001). *nos-GAL4* females were crossed to a collection of 2,300 independent lines containing the UAS randomly inserted in the genome (Rorth 1996). Progeny of such crosses each have a random gene highly and specifically expressed in germ cells, prior to their active migration. *EP1631* is inserted on the X chromosome. Using plasmid rescue, we cloned the genomic region flanking the insert, which then was used to identify cDNAs from embryonic libraries. To confirm that the phenotype observed with the *EP1631* line was indeed caused by overexpression of *CG4322*, we expressed *CG4322* cDNA under the control of UAS regulatory sequences and observed a germ cell mismigration phenotype very similar to that of *EP1631*. For functional analysis, we used two deficiencies generated by imprecise excision of the *EP1529* P-element, located in the 5′ UTR of the *CG4322*. *ΔC17* deletes the coding region of *CG4322*, while *ΔD18* deletes *CG4322* as well as the neighboring gene, *CG4313*. Both lines are homozygous and male larval semilethal, but are rescued to full viability by *Tp(1;3)w*^{vco} or by a *CG4322* genomic rescue construct. By in situ hybridization, we showed that *CG4322* RNA is not detected in *ΔD18* and is detected in only a small fraction of *ΔC17* mutant embryos (about 2%). For germline clones, we introduced FRT recombination sites into the *ΔD18* and *ΔC17* strains and generated embryos that lacked both maternal and zygotic contribution of *CG4322* and *CG4313* using the OvoD/Flp technique.

***tre1* genetics and expression analysis.** In accordance with *Drosophila* nomenclature rules, the original abbreviation *tre1* has been maintained, but the gene name has been changed to *trapped in endoderm-1* to reflect the gene's mutant phenotype. If not otherwise stated, all studies describing the *tre1* mutant phenotype were carried out with embryos from *ΔEP5* homozygous females crossed to *ΔEP5* mutant males. *tre1* mutations were generated by imprecise excision of the EP line *EP0496*, which is inserted in the promoter region of *tre1/tre1* (Ueno et al. 2001). Embryos from *ΔEP5* homozygous mothers that are also zygotically mutant (M^-,Z^-) show no specific *tre1* RNA expression, but still have strong staining in cuprophilic cells and weak staining in the CNS. Embryos from *ΔEP5* homozygous mothers crossed to wild-type males (M^- Z^+) express *tre1* RNA in germ cells, CNS, cardiac mesoderm, and other tissues, suggesting that this aspect of expression is under zygotic control.

For complementation analysis with *sctt*, homozygous *sctt* flies were crossed to *ΔEP5* males. Embryos from *sctt/ΔEP5* females were crossed with *sctt* males and analyzed for germ cell migration phenotype. Based on map position (Coffman et al. 2002), phenotype, and complementation analysis (this study), *sctt* is likely a hypomorphic allele of *tre1*. However, by sequence analysis we have not been able to identify a mutation in the Tre1 coding region of *sctt* mutant males, nor have we detected significant changes in *tre1* RNA expression in *sctt* mutant embryos (P. S. Kunwar and R. Lehmann, unpublished data); thus, allelism is not yet unequivocally established.

For the zygotic rescue experiment, homozygous *ΔEP5* mothers were crossed to males carrying the X-linked *P[Dfd-LacZ.4xE2]* marker. Thus, female embryos, which received a *tre1*$^+$ copy from their father, were identified by anti-β-galactosidase staining. For the genomic rescue, the respective genomic constructs were crossed into the *tre1/ΔEP5* mutant background. Embryos from *ΔEP5* homozygous females that also carried the genomic rescue transgene were crossed to *ΔEP5* and were tested for the transepithelial migration phenotype by anti-Vasa staining. For tissue-specific expression rescue experiments, *ΔEP5* homozygous females, which carried one or two copies of the *nos-GAL4* transgene, were crossed to *EP(X)0496* males. Of the embryos obtained from this cross, 50% (females) showed complete rescue of the *tre1* phenotype. *ΔEP5* mothers carrying one or two copies of *nullo-GAL4* were crossed to *EP(X)0496* males. All embryos obtained from this cross showed the *tre1* phenotype; 50% (all male embryos) showed the strong *tre1* phenotype, and 50% (all female embryos) showed the weaker zygotic rescued phenotype, which is clearly different from wild-type.

For analysis with Rho1, Rac, Cdc42, and RhoL, *nos-GAL4* mothers were crossed to males carrying the respective transgene under UAS control. The embryos obtained from these crosses were analyzed for germ cell migration phenotype.

In situ hybridization and immunohistochemistry. The following antibodies were used for immunostaining of embryos: rabbit anti-Vasa (1/2,500; a gift from A. Williamson and H. Zinszner), rabbit anti-β-galactosidase (Cappel, 1/20,000), mouse anti-Neurotactin (BP106 Hybridoma Bank, 1/200), and mouse anti-Fasciclin III (7G10 Hybridoma Bank, 1/300). Immunohistochemistry was as described earlier (Stein et al. 2002). For staining with anti-Neurotactin, embryos were heat fixed as described elsewhere (Eldon and Pirrotta 1991; Stein et al. 2002). For double-labeling of embryos with an antibody and RNA in situ hybridization, embryos were first carried through the antibody procedure and then hybridized with in situ probe as described elsewhere (Manoukian and Krause 1992). In situ hybridization was performed as described in Lehmann and Tautz (1994). The following cDNAs were used to transcribe probes: *race*, *412*, *CG4322*, *CG4313*, and *tre1* (*CG3171*).

Germ cell transplantation. For germ cell transplantation, embryos from *tudor* females, which lack germ cells, or *tre1* mutant embryos were used as hosts. Germ cells from *OregonR* embryos, *tre1* mutant embryos, and embryos derived from otherwise wild-type females carrying a *fat facets–LacZ* (*faf–LacZ*) transgene (Fischer-Vize et al. 1992) were used as donors. The germ cell transplantation technique has been described elsewhere (Lehmann and Nüsslein-Volhard 1986, 1987; Ephrussi and Lehmann 1992). In brief, germ cells were taken from donor embryos at early- to mid-blastoderm stage and about one to five germ cells were injected into the posterior pole of recipient embryos at late-blastoderm stage, which were dried briefly to reduce turgor. Embryos were covered with halocarbon oil and host embryos were left to develop until they reached stages 13–14. Host embryos were removed from coverslip, fixed, and devitellinized by hand. To improve antibody staining reaction with a small number of experimental embryos, *tudor* embryos were used as "carriers" mixed with the recipient embryos. The transplanted germ cells were identified by immunostaining with anti-Vasa or anti-β-galactosidase, depending on the experiment.

Supporting Information

Figure S1. Specification and Morphogenesis of Somatic Tissues Required for Germ Cell Migration Are Normal in *tre1* Mutant Embryos

Anterior is left in all pictures. (A), (B), (E), and (F) are lateral views; (C), (D), and (G)–(J) are dorsal views. Embryos shown are wild-type stage 10 (A), stage 13 (C), stage11 (E), stage 14 (G), and stage 13 (I) and are *tre1* mutant stage 10 (B), stage13 (D), stage 12 (F), stage 14 (H), and stage 13 (J). All the embryos are labeled with anti-Vasa (brown) to mark germ cells (arrowhead). (A–D) Embryos are labeled with *race* RNA (blue) to mark the midgut cells (arrow). The specification of midgut is not affected in *tre1* mutant embryos, but germ cells are found inside the midgut, as shown in (B) and (D). (E–H) Embryos are labeled with *412* retrotransposon RNA (blue) to mark lateral mesoderm and SGPs (arrows). The lateral mesoderm and SGPs are not affected in *tre1* mutant embryos. (I and J) Embryos are stained with anti-Fasciclin III (blue) to mark visceral mesoderm. The visceral mesoderm (small arrow) is not affected in *tre1* mutant embryos, but note position of germ cells laterally in the gonad in wild-type (large arrow in [I]) and in the center in *tre1* mutants (large arrow in [J]).

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Accession Numbers

The accession numbers of the other closely related GPCRs in the phylogenetic tree of Tre1 protein, as shown in Figure 2A, are *Anopheles gambiae ENSANGP00000011477* (GenBank XP__321623.1), *A. gambiae ENSANGP00000011656* (GenBank XP__321622.1), *A. gambiae ENSANGP00000022270* (GenBank XP__315017.1), *Drosophila melanogaster CG3171* (GenBank NP__524792.1), *D. melanogaster CG4313* (GenBank NP__569971.2), *D. melanogaster CG4322* (GenBank NP__569970.2), *Danio rerio ENSANGP00000007201* (Ensembl ENSANGP0000007201), *D. rerio* NPYR (GenBank NP__571512.1), *Fugu rubripes* Genscan 31921 (NCBI Blast FuguGenscan31921), *Homo sapiens EX33* (GenBank NP__065103.1), *H. sapiens Gpcr50* (GenBank NP__004215.1), *H. sapiens* Mel1AR (GenBank NP__005949.1), *H. sapiens* Mel1BR (GenBank NP__005950.1), *Mus musculus 5HT4* (GenBank NP__032339.1), *M. musculus GPR84* GenBank (NP__109645.1), *M. musculus* histamine H2R (GenBank NP__032312.1), and *Xenopus laevis* Mel1CR (GenBank AAB48391).

GenBank accession numbers can be found at http://www.ncbi.nlm.nih.gov/Genbank/, the Sanger Institute zebrafish Ensembl number at http://www.ensembl.org/Danio_rerio/, and the NCBI Blast *Fugu* number at www.ncbi.nlm.nih.gov/BLAST/Genome/fugu.html.

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Conflicts of interest. The authors have declared that no conflicts of interest exist.

Author contributions. PSK, MS-G, and RL conceived and designed the experiments. PSK, MS-G, and RL performed the experiments. PSK, MS-G, and RL analyzed the data. PSK, MS-G, RJB, and UH contributed reagents/materials/analysis tools. PSK, MS-G, and RL wrote the paper. ∎

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