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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 phospholipid dephosphorylation.

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Abbreviations: CNS, central nervous system; cta, concertino; 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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A Summary of Early Germ Cell Migration Steps and Genes

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 hgsheh has been suggested in germ cell migration (Deshpande et al. 2001); however, the exact step affected is unclear.

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 cells. Our results indicate 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.
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. Over-expression 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, trel (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 Anoph- eles 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 (ENSBARG07201) and Fugu (FuguGenscan3192I) 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). CG3171 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 trel (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.

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

Expression analysis of trel RNA showed a dynamic expression pattern (Figure 2B–2E). trel 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 trel expression in germ cells as they coalesced into the embryonic gonads. trel 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 trel function, we studied the mutant phenotype of AEP5, a trel mutation generated by imprecise excision of the EP line EP0496 and previously reported to lack trel RNA (Rorth 1996; Ishimoto et al. 2000; Dahanukar et al. 2001; Ueno et al. 2001) (see below). Embryos from AEP5 homozygous mothers that are also zygotically mutant (M;Z) show no specific trel RNA expression until stage 9 (Figure 2F), suggesting that the AEP5 deletion affects the regulation of maternal and early zygotic trel gene expression (Ueno et al. 2001). Embryos derived from AEP5 homozygous mothers (hereafter referred as trel mutant embryos) are defective in the first active step of germ cell migration, the transepithelial migration through 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 trel 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 S11–S1J) (Patel et al. 1987; Stein et al. 1994).
The lateral and gonadal mesoderm (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 Phylogenetic Typic 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 15 (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 tre1 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 AEP5 homozygous mothers (Figure 4C and 4E). In contrast, embryos from AEP5 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, AEP5, we analyzed two additional mutations that alter tre1 function (Figure 4F–4J). As described above, AEP5 is the strongest allele and its phenotype likely resembles the tre1 null phenotype. The P-element excision mutant AEP19 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 AEP19 homozygous mothers show a weaker germ cell migration phenotype than AEP5 (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 AEP19/AEP5 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 AEP5 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 AEP5 phenotype and more germ cells migrate correctly to the gonad (Figure 4F–4H). The phenotype of sctt mutants is enhanced in trans to AEP5, 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 AEP5 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 AEP5 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 89, 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{fis–LacZ} transgene, to distinguish the transplanted from the endogenous germ cells.
phenotypic rescue could be due to expression of transiently activates somatic expression in the PMG anlage at experiment lies in the fact that the zygotic rescue of the mutant phenotype as described above to D (Figure 6B). Embryos derived from crossing a complete rescue of the transepithelial migration phenotype nos-GAL4 D embryos obtained by crossing homozygous gene in the germ cells. In this experiment, 50% of the to carry a copy of on the X chromosome, only half of the embryos are expected to reach the gonad (arrows in [G]) compared to AEP5 mutants (Figure 3D). (G and H) At stage 13, most germ cells are passively carried into the blind pocket of the PMG during the blastoderm mutant 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). (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 trel embryos. Embryos from trel homozygous mothers that carried either the wild-type construct for both genes (T+) or the 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 trel 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 trel in the germ cells and tested whether the trel 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 trel promoter region and drive expression of trel RNA under GAL4 control. Since the trel 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 trel gene in the germ cells. In this experiment, 50% of the embryos obtained by crossing homozygous AEP5 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 AEP5 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 trel in the PMG during the blastoderm stage rather than due to germ cell expression. To rule out this possibility, we wanted to express trel 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 trel phenotype with this driver (Figure 6D; total number of embryos analyzed, n = 200). These experiments demonstrate that trel is required autonomously in germ cells for their migration through the PMG and that transcription of trel in early germ cells is sufficient to rescue the migration phenotype.

trel 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 trel phenotype more carefully. We observed that while most germ cells do not leave the midgut, in trel
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

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**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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**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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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 UASHos-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, Rho1N19, 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). Rho1N19-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 Rho1N19 transgene had successfully reached the gonad. Dominant-active Rho had a different effect. RhoV14-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 repellent 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 HMGC-CoAR (hmgcr) pathway to attract germ cells to the mesoderm, while the PMG produces a repellent signal produced by lipid phosphatase (wunen and wunen2) (for a
review of migration steps, see Figure 1A). At this point, neither the nature of the attractant or repellent produced by Cib/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 Cib/HMG-CoAR, Wunen, or both, this seems unlikely since germ cells can migrate successfully out of the PMG in these mutants and 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 \textit{Anopheles} proteins, which clearly aligned with the respective \textit{Drosophila} receptors, and there are also vertebrate members of this new family from human, mouse, zebrafish, and \textit{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, GP884, 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 \textit{Drosophila}, and chemokines like SDF1 have yet to be identified in the \textit{Drosophila} genome.

\textbf{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 \textit{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 \textit{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 \textit{concertina} (\textit{cta}), the \textit{Drosophila} homolog of G protein \textit{q12/13} and a Rho guanine

\textbf{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 \textit{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\textsuperscript{WT}) migrate normally. (C and D) Germ cells expressing constitutively active Rho1 (Rho1\textsuperscript{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\textsuperscript{N19}) are still inside the PMG at stage 11 (E), and most germ cells remain “clumped” inside the gut (F).

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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 Trel (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 Trel subfamily share the NPxxY domain, suggesting that Rho1 might mediate Trel 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, β₁-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, Trel1, 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 UASHnos-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 trel1-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 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; Ushima 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. AEP5, AEP19, E09496, Tα Gα, Tα Gβ, and Tα Gδ were kindly provided by J. Carlson (Dahanukar et al. 2001; Ueno et al. 2001). sctt was kindly provided by M. Mlodzik (Lee et al. 2000). UAS-Rho1Δ'2, UAS-Rho1Δ'7 transgenic flies were kindly provided by M. Mlodzik (Lee et al. 2000). UAS-Rac-α, UAS-Rac-β, UAS-Rac-γ, UAS-Rac-δ, and UAS-Rho-α were kindly provided by P. Montell (Montell and Montell 1996; Barrett et al. 1997). UAS-CG4322 and UAS-CG4313 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).

Crossed to nos-GAL4 flies was kindly provided by J. Carlson (Dahanukar et al. 2001; Ueno et al. 2001). Embryos from males. Embryos from females were crossed to males. Embryos from sctt mutant background. Embryos from AEP5 homozygous females that also carried the genomic rescue transgene were crossed to AEP5 homozygous males and were tested for the transepithelial migration phenotype by anti-Vasa staining. For tissue-specific expression rescue experiments, AEP5 homozygous females, which carried one or two copies of the nos-GAL4 transgene, were crossed to EP16496 males. All embryos obtained from this cross showed the strong tre1 phenotype, and 50% (all female embryos) showed the weak zygotic rescued phenotype, which is clearly different from wild-type.

For analysis with Rhod. 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 (PB106 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 (Elidon 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 DNAs 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 (fat–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 supraperitoneal 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 trel 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 (H) and are tre1 mutant stage 10 (B), stage 13 (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 in situ hybridization to mark the anterior gut cells (arrow). The specification of midgut is not affected in trel 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 SGP5 (arrows). The lateral mesoderm and SGP5s are not affected in trel 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 trel mutant embryos, but note position of germ cells laterally in the gonad in wild-type (large arrow in [I] and in the center in trel mutants (large arrow in [J]).
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 ENSEANG00000011656 (GenBank XP_321621.1), A. gambiae ENSAANG000000162702 (GenBank XP_331683.1), Drosophila melanogaster CG3171 (GenBank NP_524792.1), D. melanogaster CG3413 (GenBank NP_569971.2), D. melanogaster CG3422 (GenBank NP_569970.2), Danio rerio ENSAANG00000007201 (Ensembl ENSAANG00000007201), D. rerio NPYR (GenBank NP_571312.1), Fugu rubripes (NCBI Blast: FuguGenomes3191), Homo sapiens EX37 (GenBank NP_065103.1), H. sapiens GPCR50 (GenBank NP_004125.1), H. sapiens Mel1AR (GenBank NP_005949.1), H. sapiens Mel1BR (GenBank NP_005950.1), M. musculus 5HT4 (GenBank NP_010645.1), M. musculus histamine H2R (GenBank NP_032512.1), and Xenopus laevis Mel1CR (GenBank BAB48391).

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GenBank accession numbers can be found at http://www.ncbi.nlm.nih.gov/, the Sanger Institute zebrafish Ensembl number at http://wwwensembl.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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