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brother of rhomboid, a *rhomboid*-Related Gene Expressed during Early *Drosophila* Oogenesis, Promotes EGF-R/MAPK Signaling

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The *Drosophila rhomboid* (*rho*) gene participates in localized activation of EGF-receptor signaling in various developmental settings. The Rhomboid protein has been proposed to promote presentation and/or processing of the membrane-bound Spitz (mSpi) EGF-related ligand to generate an active diffusible form of the ligand. Here, we report on a new *rhomboid*-related gene identified by sequence similarity searching that we have named *brother of rhomboid* (*brho*). In contrast to *rho,* which is expressed in complex patterns during many stages of development, *brho* appears to be expressed only during oogenesis. *brho* transcripts are present in early oocytes and abut posterior follicle cells which exhibit high levels of MAPK activation. *brho,* like *rho,* collaborates with *Star* to promote signaling through the EGF-R/MAPK pathway, and genetic evidence indicates that Brho can activate both the mSpi and the Grk precursor EGF ligands in the wing. We propose that endogenous *brho* may activate the oocyte-specific Gurken ligand and thereby participate in defining posterior cell fates in the early follicular epithelium.

***Key Words: rhomboid; brother of rhomboid;* EGF-R; MAPK; *spitz; gurken; Star;* oogenesis.**

INTRODUCTION

Receptor tyrosine kinases (RTKs) constitute a large family of proteins that function through the highly conserved intracellular RAS/MAPK signaling cascade. These receptors are activated by extracellular signals such as growth factors and control various cell fate decisions during development. Members of the EGF-receptor subfamily of RTKs have been extensively studied for their critical roles in cell survival, proliferation, and differentiation (Moghal and Sternberg, 1999). The fruit fly provides a particularly good developmental and genetic system for understanding the complex mechanisms supporting EGF-receptor activation. Only one EGF-receptor gene (*Egfr*) has been identified so far in *Drosophila*. This single *Egfr* gene plays key roles in many different cell fate choices throughout the *Drosophila* life cycle, spanning oogenesis to imaginal disc development (Bier, 1998; Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997).

The participation of EGF-R signaling in a multitude of developmental processes is made possible by a combination of regulatory mechanisms controlling its localized activation and down-regulation (reviewed in Perrimon and Perkins, 1997). Several genes have been implicated in extracellular regulation of this ubiquitously expressed receptor. Among these genes, *spitz*, *gurken*, and *vein* encode EGF ligands that activate EGF-R in various developmental situations. The product of the *vein* gene (Vein) belongs to the neuregulin subfamily of EGF ligands and is directly secreted into the extracellular space (Schnepp *et al.*, 1996). In contrast, the product of the *spitz* gene is initially expressed as a membrane-bound inactive precursor (mSpi) (Rutledge *et al.*, 1992). Although the precise mechanism through which the mSpi precursor is activated remains unclear, it may involve proteolytic cleavage of mSpi to liberate a diffusible active peptide. In support of this possibility, an artificially secreted form of Spi lacking both cytoplasmic and transmembrane domains (sSpi) is much more active than the

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full-length mSpi form (Bang and Kintner, 2000; Golembo *et al.,* 1996; Schweitzer *et al.,* 1995). The Gurken (Grk) ligand is involved in several aspects of oogenesis (Van Buskirk and Schüpbach, 1999) and displays structural (Neuman-Silberberg and Schüpbach, 1993) and functional similarities to Spitz. For example, a truncated form of Grk lacking both transmembrane and cytoplasmic domains is highly active, in contrast to full-length Grk, which is inactive when misexpressed in the wing or in ovarian follicle cells (Queenan *et al.,* 1999). This finding suggests that Grk may require activation through a mechanism similar to that of Spi.

The *rhomboid* (*rho*) and *Star* (*S*) genes, which encode integral membrane proteins, also play important positive roles in regulating EGF-R signaling (Kolodkin *et al,* 1994; Bier *et al,* 1990). A variety of evidence indicates that *rho* and *Star* function in an interdependent fashion and act at least in part by promoting Spi activation. Consistent with this view, *rho, S,* and *spi* mutants have nearly identical embryonic phenotypes (Bier *et al.,* 1990; Mayer and Nüsslein-Volhard, 1988; Rutledge *et al.,* 1992). Analysis of gene expression and MAPK activation pattern indicates that Rho can activate EGF-R/MAPK signaling in neighboring cells (Golembo *et al.,* 1996; Guichard *et al.,* 1999; Sapir *et al.,* 1998). As the Rho protein is not observed to move from the cells in which it is expressed (Sturtevant *et al.,* 1996), the non-cell-autonomous activity of *rho* suggests that Rho participates in the production of a diffusible EGF signal. It has also been observed that sSpi can substitute for Rho in patterning the ventral epidermis (Schweitzer *et al.,* 1995), consistent with Rho acting to generate a sSpi-like activity. In addition, combined misexpression of *mspi* and *Star* in the developing wing leads to striking phenotypes, whereas neither of these genes has any effect when misexpressed alone (Pickup and Banerjee, 1999). Similarly, strong synergistic activation of EGF-R signaling is observed in the wing when *rho, Star,* and *mspi* are coexpressed (Guichard *et al.,* 1999). This striking synergy between misexpressed *rho, Star,* and *mspi* supports the idea that Rho and Star collaborate to convert mSpi into an active EGF ligand. More recently, Bang and Kintner used a frog heterologous assay system (Bang and Kintner, 2000) to demonstrate that Rho and Star are necessary for activating the mSpi ligand. Importantly, these investigators demonstrated that this activation is followed by, but does not require, proteolytic cleavage of the Spitz precursor.

In contrast to the *spi, Star,* and *Egfr* genes, which are expressed ubiquitously in most developmentally active tissues, *rho* is expressed in a spatially restricted and dynamic pattern (Bier *et al.,* 1990; Sturtevant *et al.,* 1993). Localized expression of *rho* correlates with cells requiring an elevated activity of EGF-R, as revealed by high levels of MAPK activation detected *in situ* (Gabay *et al.,* 1997a,b; Guichard *et al.,* 1999). In addition, as mentioned above, cells a short distance from *rho*-expressing cells often display elevated levels of MAPK activation. These observations suggest that the Rho protein provides the spatial and temporal clues necessary for restricting EGF-R activation to appropriate cells during development.

Although models in which Rho and Star collaborate to promote spatially restricted cleavage of mSpi, and hence EGF-R activation, account for many facts, several observations suggest that there may be additional missing components. For example, *rho* and *Star* are required for wing vein formation, while *spi* has no detectable function in the wing (Guichard *et al.,* 1999; Nagaraj *et al.,* 1999). One explanation for this observation is that Rho may act on a yet unknown EGF ligand during wing vein development. There also are examples in which *rho* does not appear to be required to activate EGF-R signaling. Thus, *rho* is dispensable in the female germ line, yet activation of EGF-R signaling by the Gurken ligand in the oocyte plays an essential role in the germarium and in establishing both the anterior–posterior (A/P) and the dorsal–ventral (D/V) axes of the oocyte (Nilson and Schüpbach, 1999). Similarly, during eye development, Spitz and Star are involved in recruiting undifferentiated cells to the forming ommatidium (Freeman, 1996), but *rho* has no detectable role in this process (Freeman *et al.,* 1992, see note added in proof). These latter observations raise the question of whether Spitz or Gurken might be activated by yet unidentified Rhomboid-like molecules.

Here, we describe the identification of a novel *rhomboid*-related gene, *brother of rhomboid* (*brho*), from the *Drosophila* genome project (Adams *et al.,* 2000), that functions like *rho* to promote MAPK activation through the EGF-R pathway. Also like *rho, brho* interacts synergistically with *Star* to promote vein development. However, *brho* expression appears to be restricted to the female germ line, in contrast to *rho,* which is expressed in a complex pattern during many stages of development. *brho* expression in the oocyte abuts posterior follicle cells that exhibit MAPK activation during stages 5–8 of oogenesis when EGF-R signaling plays an essential role in determining fates of posterior follicle cells, which subsequently define the A/P axis of the embryo. The restricted pattern of *brho* expression suggests that it may participate in establishing the A/P axis of the egg chamber, possibly through activation of the EGF ligand Gurken in the oocyte. Consistent with this hypothesis, coexpression of *brho* and *mgrk* in the wing generates an ectopic vein phenotype much stronger than that caused by *brho* alone. As expression of mGrk alone cannot induce any visible effect, we propose that *brho* normally functions by activating mGrk during early oogenesis.

MATERIALS AND METHODS

Isolation and Characterization of brho Transcripts

RNA was extracted from flies at different developmental stages using TRIzol reagent (Life Technologies) according to manufacturer suggestions. To amplify an internal portion of the *brho* mRNA, primers surrounding the second intron were synthesized. The sense primer, designated RT *brhoS* (5′GCTGGGTTTCTTTGTTTA3′),

and the antisense primer, designated RT *brhoAS* (5′AGCATAGCGAGCATAGATG3′), were used on the RNA templates extracted from various tissues and stages of development using the Superscript One Step RT-PCR system (Life Technologies). PCR was carried out in a Perkin–Elmer Thermal Cycler 480, with the following parameters: 20 min at 50°C for synthesis of cDNA followed by 45 cycles (30 s at 94°C, 45 s at 55°C, 1 min 25 s at 72°C). To confirm that the observed PCR products corresponded to *brho,* a second antisense primer was designed, located internally to the first PCR product (RT*brhoAS2,* 5′GGCAAAGCCAAAGTCGAAGG 3′), and the first RT-PCR was used as template for the second PCR, with the same parameters. We determined that the full predicted open reading frame is contained in *brho* transcripts by using a sense primer containing the predicted ATG start codon (5′AATTCCAGATGAGCGGCAAG3′) and an antisense primer located downstream of the predicted STOP codon (5′CGACTGTTCTAGATCACCTGGGATTTCACACCTGGAA3′). A full cDNA product was amplified from RNA extracted from adult flies with these primers in a RT-PCR (reaction parameters were the same as those described above) and sequenced.

In Situ Hybridization to Ovaries

A *brho*-specific digoxigenin-labeled probe was synthesized from a *Hin*dIII-linearized *brho* genomic fragment cloned into the pCR-BluntII-TOPO vector (Invitrogen), using a T7 priming site. Ovaries were dissected and processed for *in situ* hybridization with the *brho* probe as described in Suter and Steward (1991).

Activated MAPK Staining*

Ovaries were fixed for 30 min in 8% formaldehyde, 0.05 M EGTA, 1× PBS, rinsed for an hour in PBS 0.1% Tween 20 (PBST), and stored overnight in methanol. After progressive rehydration in PBST, ovaries were incubated with 1/200 anti-dP-ERK antibodies (Sigma). Remaining steps were performed according to standard procedures (e.g., Gabay *et al.,* 1997a).

Fly Stocks and Crosses

Stocks and crosses were raised using standard methods. To determine genetic interactions between *brho* and known partners of the *rho/Egfr* pathway, UAS-*brho* flies were crossed to various test stocks, including GAL4 MS1096;UAS-*DN-Egfr,* GAL4 MS1096;UAS-*mspi,* GAL4 MS1096;UAS-*mgrk,* GAL4 MS1096; UAS-*Star,* and GAL4 MS1096;S^{X155} stocks.

Construction of UAS-brho Transgenic Lines

The genomic sequence of the *brho* gene comprising the complete open reading frame was amplified by PCR, with a sense primer located 5′ of the predicted ATG translation start codon (Drho2S, 5′GAGCAGCCTCGAGAACCTCCATACTTAATTCCAG3′) and an antisense primer containing the predicted STOP codon (Drho2AS, 5′CGACTGTTCTAGATCACCTGGGATTTCACACCTGGAA3′) from the DS 02734 P1 clone DNA using Vent DNA polymerase (New England Biolabs). The resulting PCR product was then cloned into the pCR-BluntII-TOPO vector (Invitrogen) and checked for proper orientation and sequence. The *brho* genomic fragment was excised with *Xho*I and *Xba*I sites and ligated into the *Xho*I–*Xba*I-digested pUAST vector. This construct was then introduced into flies by standard methods of P-element-mediated germline transformation of w^{1118} embryos.

Phylogenetic Reconstruction of Rho Family Proteins

Phylogenetic relationships were inferred using neighbor joining (NJ) and maximum likelihood (ML) from an ungapped 118-amino-acid subset of the alignment derived primarily from the second through the sixth transmembrane domains. Two methods of calculating the distance matrix for use in neighbor-joining tree reconstruction were used: the simple Poisson corrected proportional difference and Lake's paralinear distance for its robustness of inference on data sets having significant unequal rate effects (Lake, 1994). Support for NJ topologies was evaluated using bootstrap analysis with 1000 replicates. Support for maximum likelihood tree inference utilized quartet puzzling reliability values from 10,000 puzzling steps (analogous to bootstrap replicates). The NJ inference of tree topology and the subsequent bootstrap analysis were implemented in DAMBE (Xia, 2000). The quartet puzzling maximum likelihood analysis was preformed with PUZZLE (Strimmer and von Haesler, 1996). The NJ tree derived from the maximum likelihood distance matrix was used to compute the parameters for the models of substitution and rate heterogeneity. The ML model of evolution allowed for among-site rate heterogeneity with 1 invariant and 8 γ-distributed rate categories.

RESULTS

Identification of brother of rhomboid, a rho-Related Gene

We searched the *Drosophila* genome database made available by the Berkeley *Drosophila* Genome Project (Adams *et al.,* 2000) with the amino acid sequence of Rhomboid using the WU-BLAST program and identified five *rhomboid*-like genes (Fig. 1A). One of these *rho*-related genes, which we named *brother of rhomboid,* maps to cytological position 62A3 on the third chromosome. The complete *brho* transcription unit is contained within the P1 clone DS02734 and is located approximately 8 kb from *rho* in inverted orientation relative to *rho.* The Brho sequence clusters tightly with that of Rho in a cladogram of Rho-related proteins found in organisms from diverse kingdoms and phyla (Fig. 1B).

In order to confirm the computer-predicted coding sequence of *brho* experimentally, we amplified a *brho* cDNA using RT-PCR of RNA extracts from wild-type adult flies and sequenced the product. This analysis revealed that both the predicted translation start codon and the termination codons are included in the transcribed *brho* sequence and that the open reading frame of the cDNA exactly matches that of the predicted transcript. Sequence analysis of the cDNAs and alignment to *brho* genomic sequence revealed the presence of two small introns in *brho,* of 63 and 60 bp (Fig. 1C).

Like *rho, brho* is predicted to encode a protein with seven transmembrane (TM) domains, with generally very short stretches of hydrophilic amino acids between the TM domains and a longer loop between TM1 and TM2. Comparison of the two proteins reveals that the strongest identities fall within the transmembrane domains (see Fig.

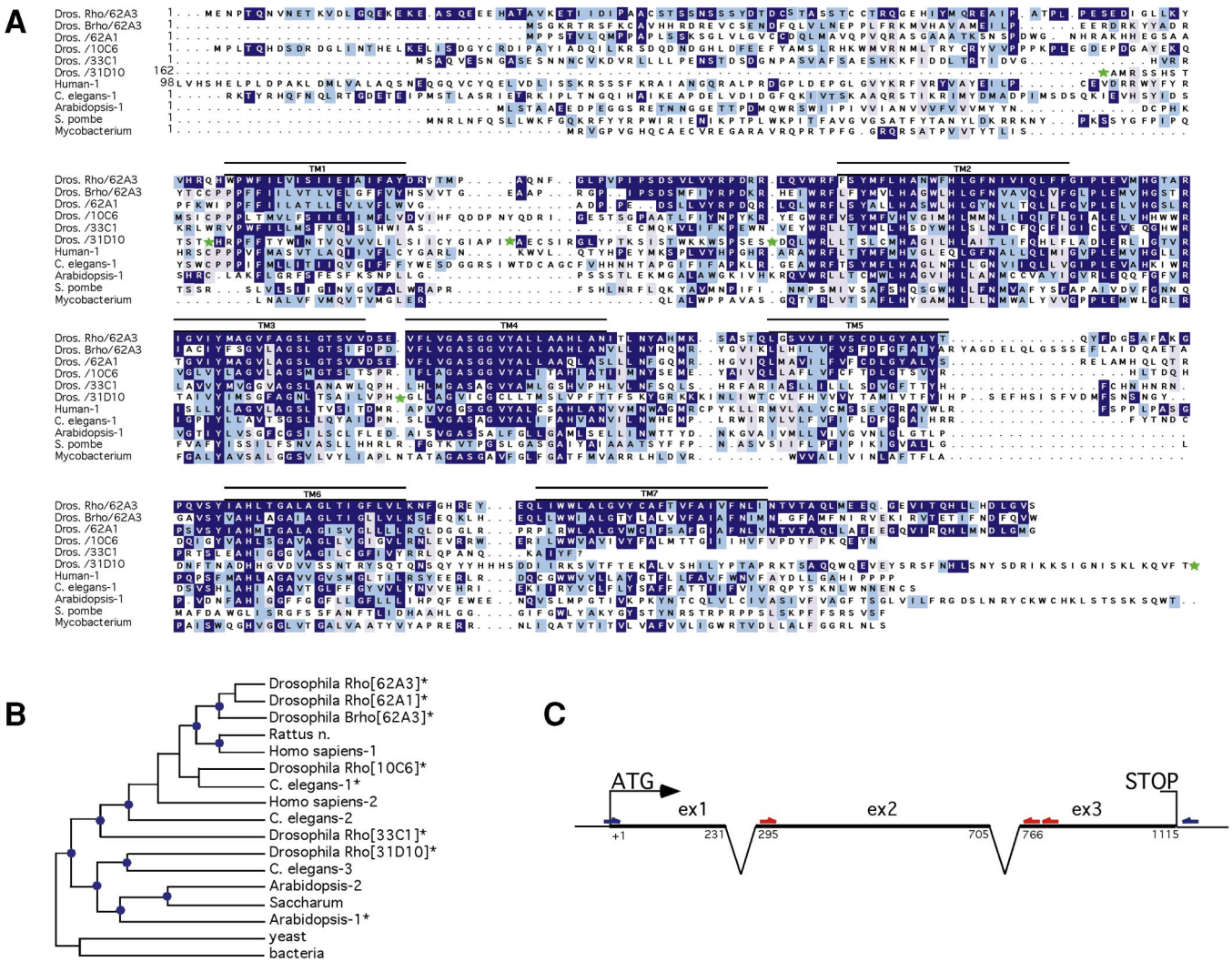

FIG. 1. Brho is a novel Rhomboid-related protein. (A) Alignment of Rho, Brho, and a sample of other Rho-related proteins. Identities to Rho are boxed in dark blue, similarities with Rho are boxed in medium blue, and similarities among Rho-related proteins other than Rho are boxed in mauve. Transmembrane domains, indicated with heavy lines as TM1 to TM7, are those predicted by the TMHMM program, but are in close agreement with predictions made by several other structure prediction programs, Top-Pred, Tmpred, and SOSUI, all accessed through the ExPasY Web site. For alignments of additional Rhomboid-related family members see aligned sequences online at http://dot.imgen.bcm.tmc.edu. The green stars indicate stretches of amino acids unique to *Drosophila* Rho-related [31D10] of 162, 39, 85, 119, 58, and 42 amino acids (in order). (B) Cladogram of Rho-related proteins in eukaryotes and prokaryotes. The inferences of phylogenetic relationships were constructed as described under Materials and Methods. All methods recovered the same topology, although the most basal bifurcations in the animal clade were not well supported by the maximum likelihood method. Asterisks indicate proteins that are also in the alignment in A. Blue dots indicate nodes significantly supported by all methods of tree reconstruction. Coding sequences of *Arabidopsis-1* (O82756), *Arabidopsis-2* (O81073), and *sugarcane* (*Saccharum*, Q43323) *rho*-related genes are reported in Roundsley *et al.* (1998), Peters *et al.* (1998), and Bugos *et al.* (1993), respectively. Coding sequences of *C. elegans-1* (Q19821), *C. elegans-2* (YLH2), and *C. elegans-3* (gi:5832784) *rho*-related genes, are reported in Wilson *et al.* (1994). Predicted protein sequences of Human-1 (NCBI gi:4506525) and a rat Rho(O88779) are reported in Pascall and Brown (1998). *Rho-2*, a second human *rhomboid*-related gene, was localized in genomic sequence mapping to chromosome 1p32.3–p34.1 (AL139260). A full-length EST for this gene has been sequenced by the NEDO human cDNA sequencing project (BAA91168). The *rho*-related gene of *Mycobacterium* (O53632) has been reported in Cole *et al.* (1998) and that of *Saccharomyces pombe* (YB4J) in Wood *et al.* (1997). The sequences of *Drosophila rho* (P20350) (Bier *et al.*, 1990) and *brho* were determined empirically. *Drosophila* Rho-related [62A1] (CT2274), *Drosophila* Rho-related [10C6] (CT4804), *Drosophila* Rho-related [31D10] (CG5364), and a complete protein sequence of *Drosophila* Rho-related [33C1] (CT34787) are available in GadFly, the annotated *Drosophila* genome. For *Drosophila* Rho-related [62A1] and *Drosophila* Rho-related [10C6], a second round of HMM-based gene prediction was preformed using FGENESH, available at http://genomic.sanger.ac.uk/, and for both of the genes an additional exon which encoded the putative start Met and the first transmembrane domain was found. All accession numbers provided refer to the SwissProt Database except the Human-1 and *C. elegans-3* Rho-related proteins, which are referred to by their NCBI numbers. The tree is rooted with the bacterial sequences. (C) The intron/exon structure of the *brho* locus, as determined by comparing full-length cDNA and genomic sequences. Red arrows indicate the positions of nested primers used for RT-PCR experiments (see Fig. 3). Blue arrows indicate the positions of primers used to amplify the full *brho* cDNA.

1A). Rho and Brho are 41% identical and 64% similar overall and 62% identical and 83% similar within the transmembrane domains. This higher degree of conservation in predicted TM domains has also been observed for *rhomboid*-related genes found in the nematode, rat, human, plants, yeast, and bacteria (see Fig. 1A for examples). In addition to the TM domains, a stretch of 25 amino acids located at the end of the first loop between TM1 and TM2 is also very highly conserved. In contrast, the hydrophilic termini of the Rho and Brho proteins do not show any significant sequence similarity. Moreover, the N-terminal portion of *brho* is much shorter than that of *rho*. Some of the *rho*-like genes found in more distant organisms like plants, yeast, and bacteria have fewer or more predicted transmembrane domains. The most highly conserved regions in the Rhomboid family of proteins are the first loop between TM1 and TM2 and the TM2–TM6 transmembrane domains.

A phylogenic tree assembled from known Rho-related proteins (Fig. 1B) closely follows the ancestral relationships between organisms. This cladistic analysis reveals that gene duplications giving rise to new Rho proteins have occurred at different taxonomic levels. In the case of *Drosophila rhomboid*-related genes, there is clear evidence for several distinct rounds of gene duplication events that gave rise to the *Drosophila* Rho-related genes. The *rho-related [10C6], rho-related [31D10],* and *rho-related [33C1]* genes clearly arose before the divergence of arthropods and vertebrate lineages, whereas duplications that gave rise to the cluster consisting of *rho, brho,* and *rho-related [62A1]* evidently took place sometime after the divergence of the ecdysozoa and the lophotrochozoa.

brho Promotes EGF-R/MAPK Signaling in the Wing

As a first step in assessing the function of *brho,* we used the GAL4-UAS system (Brand and Perrimon, 1993) to misexpress a UAS-*brho* construct containing the full genomic sequence of *brho* in the wing. When this UAS-*brho* construct was expressed using the strong ubiquitous wing-specific GAL4 driver MS1096, we observed ectopic vein phenotypes similar to, although weaker than, those generated by misexpression of *rho* (Figs. 2C and 2B, respectively). This observation suggests that Brho functions like Rho by promoting EGF-R signaling. To test whether the induction of ectopic veins by *brho* misexpression requires EGF-R activity, we coexpressed UAS-*brho* with a dominant-negative EGF-R construct, UAS-*DN-Egfr* (Buff *et al.*, 1998). As previously observed for *rho* (Fig. 2E), *brho*-induced ectopic veins were entirely suppressed by DN-*Egfr* (Fig. 2F), resulting in narrow wings with missing veins typical of DN-*Egfr* misexpression (Fig. 2D). We also observed strong synergism between misexpressed *brho* and *Star*, as has been shown to be the case for *rho* (Guichard *et al.*, 1999), which is discussed further below. These results are consistent with *brho* functioning to promote EGF-R signaling.

Since EGF-R signaling results in MAPK activation, we assessed the activation state of MAPK following misexpression of *brho* in the wing disc. MAPK is an essential downstream component required to transduce signals from all RTKs to the nucleus. Activated MAPK (MAPK*) can be detected *in situ*, using an antibody directed against phosphorylated MAPK (anti dP-ERK antibodies) (Gabay *et al.*, 1997a,b). In wild-type wing discs, MAPK activation is restricted to vein primordia, as a consequence of endogenous localized *rho* expression (Fig. 2G and Gabay *et al.*, 1997a; Guichard *et al.*, 1999). In wing discs ubiquitously misexpressing *brho* (Fig. 2I), we observed a strong general activation of MAPK comparable to that found in discs ectopically expressing *rho* (Fig. 2H) or an activated form of EGF-R (data not shown). This observation provides independent support for *brho* activating the EGF-R/MAPK signaling pathway.

brho Expression Is Detected Only in the Early Oocyte

We surveyed *brho* expression during development by *in situ* hybridization in embryos, third-instar imaginal discs, and the female germ line and only observed expression in early stage oocytes (Fig. 3B). We also analyzed *brho* expression by performing PCR on various cDNA libraries and RT-PCR on RNA extracted from various developmental stages. Consistent with the very restricted expression of *brho* in the female germ line determined by *in situ* hybridization, we could amplify a *brho* RNA product from adult females by RT-PCR (Fig. 3A), but not from RNA extracted from embryos, larvae, pupae, or male adults. We also did not detect *brho* transcripts in cDNA libraries made from embryos or imaginal discs.

Expression of *brho* in the female germ line is restricted to the early developing oocyte between stage 5 and stage 8 (Fig. 3B). No *brho* staining was observed in the surrounding follicle cells, however. EGF-R signaling has been reported to play at least three separate roles during early and late oogenesis: first to package a single oocyte and its attached nurse cells in the germarium prior to formation of the egg chamber (Goode *et al.*, 1996), second to establish posterior follicle cell fates (stage 1–8 egg chambers) (González-Reyes *et al.*, 1995; Roth *et al.*, 1995), and third to establish the D/V axis in dorsal anterior follicle cells (stage 9–12 egg chambers), which is required to pattern the egg chamber and embryo (Neuman-Silberberg and Schüpbach, 1993, 1994). The restriction of *brho* expression to the narrow window of oocyte development between stages 5 and 8 suggests that Brho might be involved in activating EGF-R signaling in adjacent follicle cells required to specify posterior cell fates. As a direct measurement of EGF-R activity during oogenesis, we probed wild-type ovaries with anti-dP-ERK antibodies. During early stages (5–8), MAPK activation is detected only in posterior follicle cells abutting the oocyte in

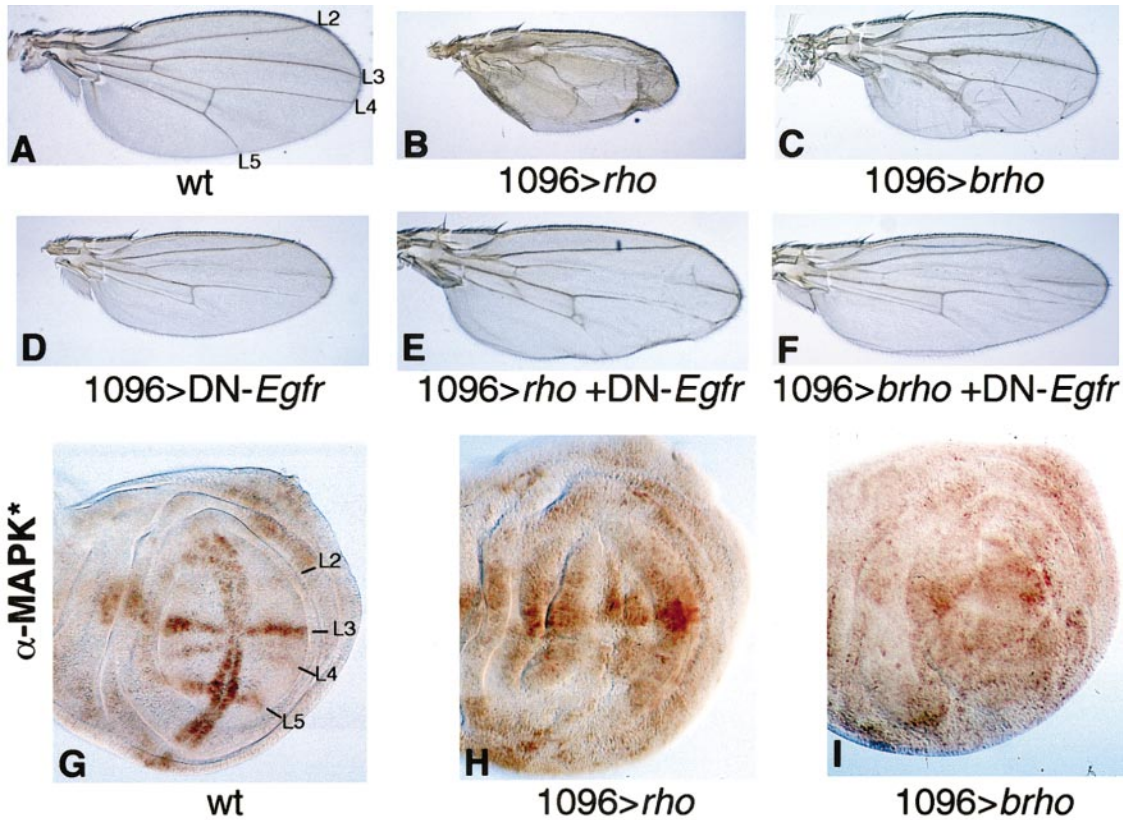

FIG. 2. Ectopic Brho activates EGF-R/MAPK signaling. (A) A wild-type wing. (B–F) Wings from flies expressing UAS transgenes under the control of the strong GAL4 wing driver MS1096. MS1096 GAL4 is ubiquitously expressed in the wing. It is expressed more strongly on the dorsal surface of the wing primordium during larval stages and becomes restricted to the dorsal surface during pupal stages (Capdevila and Guerrero, 1994; Lunde *et al.,* 1998). (B) Ectopic expression of *rho* induces a strong ectopic vein phenotype. (C) Expression of UAS-*brho* in the wing causes a similar, but weaker phenotype than that observed with *rho* (compare with B). (D) Expression of a dominant-negative EGF-receptor construct (UAS-*DN-Egfr*) causes vein-loss (particularly of dorsal veins L3, L4, and L5) and reduction in wing size. (E) Coexpression of UAS-*rho* and UAS-*DN-Egfr* results in a phenotype nearly identical to that of *DN-Egfr* alone. (F) Coexpression of UAS-*brho* and UAS-*DN-Egfr* results in a phenotype indistinguishable from that of *DN-Egfr* alone (compare with D). (G) A wild-type third-instar imaginal disc stained with the anti dP-ERK antibody. The longitudinal stripes are centered over vein primordia. (H) A MS1096 GAL4;UAS-*rho* imaginal disc, stained with the anti dP-ERK antibody. Ectopic *rho* expression causes ubiquitous activation of MAPK. (I) A MS1096 GAL4;UAS-*brho* imaginal disc, stained with the anti dP-ERK antibody. Ectopic expression of *brho* also causes ubiquitous activation of MAPK.

which *brho* and *gurken* (Neuman-Silberberg and Schüpbach, 1993) are expressed (Fig. 3C). This pattern of MAPK activation is temporally correlated with *brho* expression and is consistent with the hypothesis that *brho* participates in promoting EGF-R signaling in posterior follicle cells. It is noteworthy that *rho,* which activates EGF-R signaling in many other developmental settings, is not expressed in the oocyte or surrounding follicle cells during this period. During later stages of oogenesis (9–10), we observed that MAPK activation is restricted to follicle cells overlying the dorsal anterior end of the oocyte (Fig. 3D), consistent with previous reports (Peri *et al.*, 1999). This restricted activation of MAPK is believed to be the result of the asymmetrical localization of *gurken* transcripts to the dorsal–anterior portion of the oocyte, which then resolves into a double peak as a consequence of *rho, argos,* and *spitz* activity in the dorsalmost anterior follicle cells at stage 11 (Fig. 3D and Wasserman and Freeman, 1998). Interestingly, a trace of posterior activation of MAPK is also observed at stage 10, suggesting that sustained posterior EGF-R activity may maintain posterior fates of the egg chamber (data not shown).

We tested whether *brho* could activate the EGF-R pathway in the ovary by expressing the UAS-*brho* construct under the control of the CY2-GAL4 driver, which is expressed only in the follicular epithelium covering the oocyte (Queenan *et al.*, 1997). This ubiquitous follicle cell expression of *brho* caused dorsalization of the eggshell,

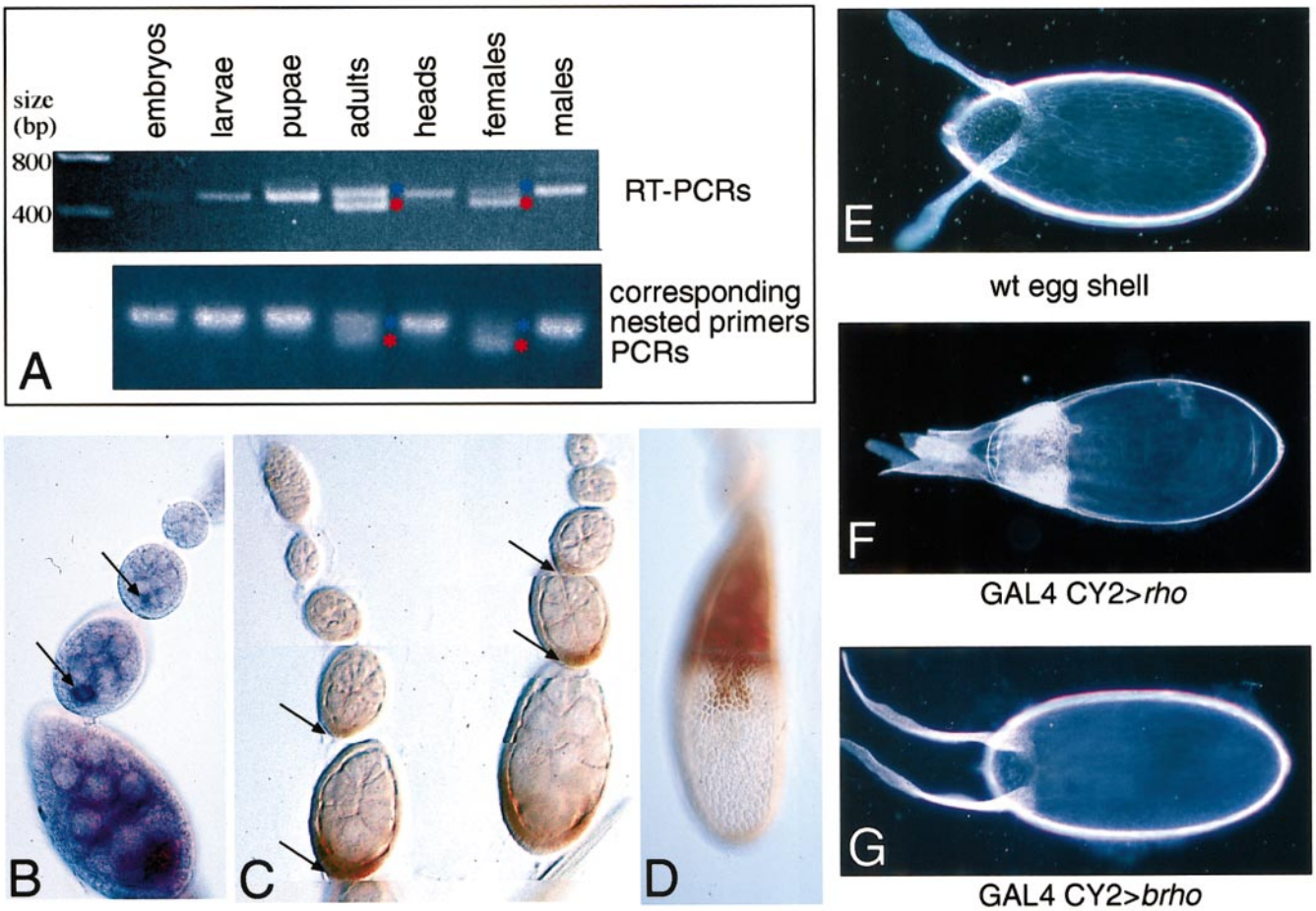

FIG. 3. *brho* is expressed in the female germ line. (A) Amplification of *brho* transcripts by RT-PCR. Top: RT-PCR on various RNA extracts from wild-type individuals with primers bracketing the second intron. Blue stars indicate the band amplified from contaminating genomic DNA and red stars indicate the smaller band amplified from spliced RNA. Bottom: Secondary PCR products amplified from the initial RT-PCR using an internal antisense primer. A specific *brho* signal is detected only in adult females. (B) *In situ* hybridization to wild-type ovaries with an antisense *brho* digoxigenin-labeled probe. Arrows indicate developing oocytes at stages 5 and 6. Transcripts are not detected after stage 8. The light purple stain seen in nurse cells is not specific as it is also observed using a *brho* sense control probe and other probes (data not shown). (C) Activation of MAPK in early wild-type egg chambers revealed by an anti-dP-erk antiserum specific for activated MAPK. MAPK activation is detected in posterior follicle cells abutting the oocyte (arrows). (D) Anti-dP-erk stain of a later wild-type egg chamber (stage 11), showing the double peak of nuclear MAPK activation in dorsal follicle cells. (E) A wild-type eggshell, with its dorsal appendages. (F) A CY2-GAL4 UAS-*rho* eggshell. Expression of *rho* in all the cells of the follicular epithelium causes dorsalization of the eggshell, leading to a single fused dorsal appendage covering the anterior end of the egg. (G) A CY2- GAL4 UAS-*brho* eggshell. Ectopic expression of *brho* in the follicle cells causes a weaker dorsalized eggshell phenotype. The dorsal appendages appear thicker and spread farther apart than in wild-type eggshells.

resulting in thickened dorsal appendages which were more spread apart in eggs from CY2;UAS-*brho* females (Fig. 3G) than in wild-type controls (Fig. 3E). In some more extreme cases, white appendage-like material filled in between the two appendages, as is typical of dorsalized eggshells (Nilson and Schüpbach, 1999). The average *brho* misexpression phenotype is similar to, but weaker than, that induced by ectopic *rho* in follicle cells using the same GAL4 driver (Fig. 3F).

brho Interacts Synergistically with Star

Star is expressed in the oocyte during a developmental window (stage 4 to 7) largely overlapping with *brho* expression (Pickup and Banerjee, 1999). As *Star* and *rho* act in concert during many stages of development (Mayer and Nüsslein-Volhard, 1988) and function in a strict interdependent fashion during wing vein development (Guichard *et al.*, 1999), we tested whether *brho* might also interact

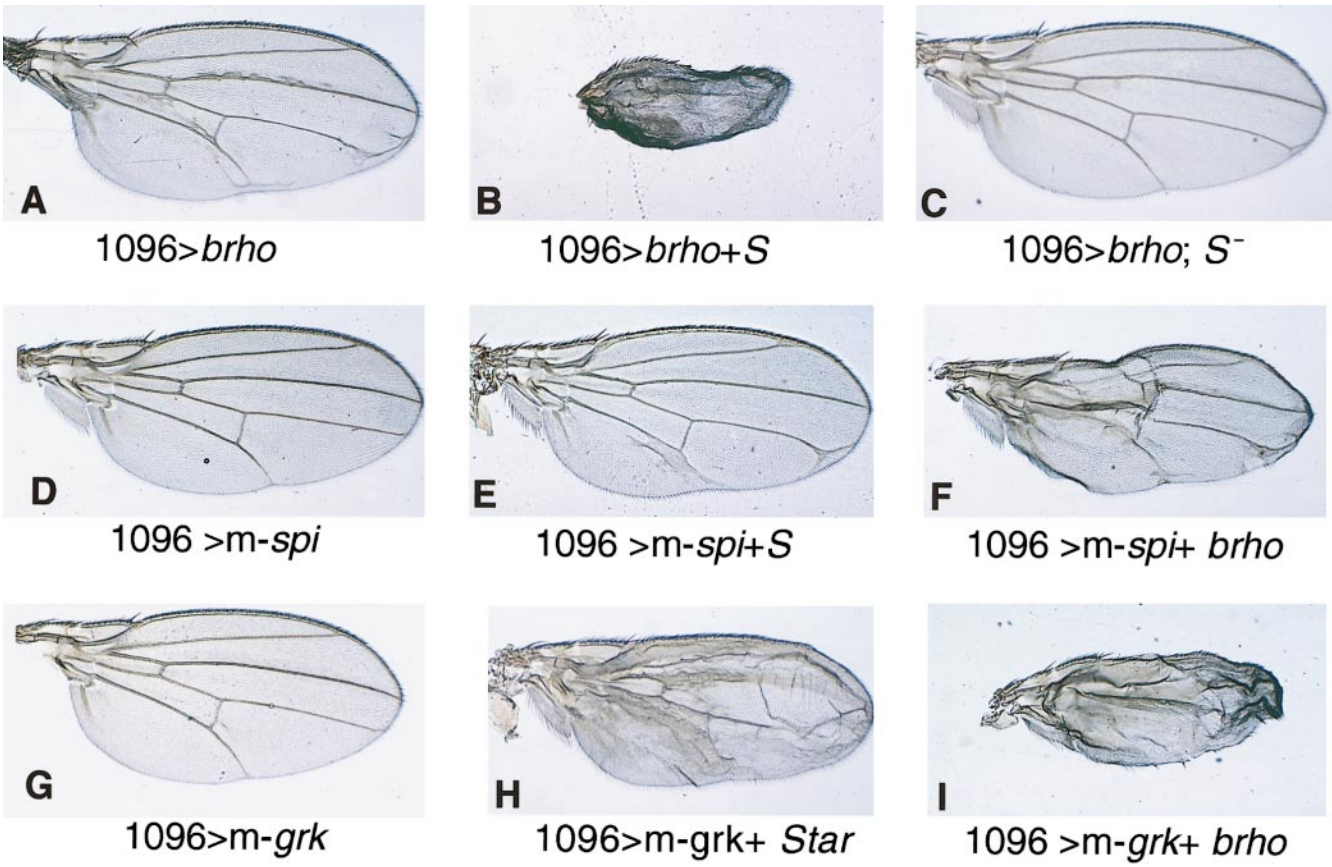

FIG. 4. Brho collaborates with Star and can activate both mGrk and mSpi. (A–I) Ubiquitous misexpression of various transgenes in the wing using the MS1096 GAL4. (A) Misexpression of UAS-*brho* during wing development results in a moderate ectopic vein phenotype. (B) Ubiquitous coexpression of UAS-*brho* and UAS-*Star* results in a strong synergistic ectopic vein phenotype. The UAS-*Star*/UAS-*brho* wing was excavated from a dead pupa. (C) Misexpression of UAS-*brho* in a fly lacking one functional copy of *Star* (e.g., $Star^{X155}$/+). The *brho* phenotype is almost completely suppressed in this *Star* heterozygous background. (D) Misexpression of UAS-*mspi* (e.g., the full-length Spi EGF ligand) has no detectable effect in the wing. (E) Ubiquitous coexpression of UAS-*Star* and UAS-*mspi* results in ectopic veins typical of EGF-R hyperactivation. (F) Ubiquitous coexpression of UAS-*brho* and UAS-*mspi* induces an ectopic vein phenotype much stronger than that induced by *brho* alone. (G) Misexpression of UAS-*grk* has no detectable effect in the wing. (H) Ubiquitous coexpression of UAS-*Star* and UAS-*grk* results in a strong ectopic vein phenotype. (I) Ubiquitous coexpression of UAS-*brho* and UAS-*grk* induces an ectopic vein phenotype much stronger than that induced by UAS-*brho* alone.

synergistically with *Star*. We coexpressed UAS-*brho* and UAS-*Star* constructs during wing development using the strong ubiquitous GAL4 driver MS1096 and observed highly penetrant pupal lethality, as has been previously observed for misexpression of *rho* and *Star* (Guichard *et al.*, 1999). Despite the pupal lethality, fully differentiated wings can be dissected from pupal cases, revealing a strong ectopic vein phenotype (Fig. 4B) which is much greater than that observed in response to ectopic *brho* alone (Fig. 4A). Since ectopic expression of *Star* alone has no detectable effect, this result reveals a potent synergism between Brho and Star in enhancing EGF-R activity during wing development. We also observed a strong effect on *brho* activity from reducing the dose of endogenous *Star* since *brho*-induced ectopic veins are almost completely suppressed in a *Star*−/+ heterozygous background (Fig. 4C). These results indicate that *Star* can collaborate with *brho*, as well as with *rho*, to activate EGF-R signaling.

Brho and Star Can Activate mSpi and mGrk in the Wing

The data presented thus far suggest that the Brho protein could function early during oogenesis by activating EGF-R signaling in follicle cells adjacent to the oocyte where *brho* is expressed. As a possible mechanism, we propose that Brho might promote processing or activation of the Grk protein in the oocyte to stimulate EGF-R expressed in adjacent follicle cells. Consistent with the idea that mGrk, like mSpi, requires activation, the mGrk protein does not

exhibit any activity when misexpressed in the wing (Fig. 4G and Queenan *et al.,* 1999). In contrast, an artificially truncated version of Grk, GrkΔTM, can activate EGF-R both in the wing and in follicle cells (Queenan *et al.,* 1999). In order to determine whether activation of mGrk involves Star, as has been observed for mSpi (Pickup and Banerjee, 1999), we coexpressed *Star* and *gurken* during wing development. Coexpression of *mgrk* and *Star* results in a strong ectopic vein phenotype (Fig. 4H), which is greater than that caused by coexpression of *mspi* and *Star* (Fig. 4E). This finding supports the view that the Grk EGF ligand can be activated through a mechanism similar to that of mSpi.

To determine whether Brho can also participate in activating Grk, we coexpressed UAS-*brho* and UAS-*mgrk* in the wing. The ectopic vein phenotype resulting from the coexpression of *brho* and *mgrk* is significantly stronger than that caused by *brho* alone (Figs. 4I and 4A, respectively), indicating that Brho can activate the mGrk precursor. We also observed a synergistic effect between *brho* and *mspi,* similar to that which has been previously observed between *rho* and *mspi* (Fig. 4F and Guichard *et al.,* 1999). The phenotypes resulting from coexpressing *brho* + *mgrk* (Fig. 4I) is significantly stronger than that from coexpressing *brho* + *mspi* (Fig. 4F); however, we do not believe that this necessarily reflects a preference of Brho for activating Grk versus Spi, since coexpression of UAS-Star with these ligands also results in a much stronger phenotype with Grk than Spi. These data suggest rather that the UAS-mGrk construct may be expressed more efficiently or at higher levels than the UAS-*mspi* construct. Also, it was not possible in these experiments to determine whether there was a significant increase in the severity of the phenotype resulting from coexpression of UAS-*rho* with either UAS-*mspi* or UAS-*mgrk* in the wing since UAS-*rho* generates a very strong ectopic vein phenotype when misexpressed alone.

DISCUSSION

We report on the identification and characterization of a novel *rhomboid*-related gene, *brother of rhomboid,* which is expressed specifically in the female germ line. We demonstrate that *brho* functions like *rho* by collaborating with *Star* in activating EGF-R/MAPK signaling. Brho can potentiate the activity of both mSpi and mGrk EGF ligands, consistent with the possibility that Brho may activate Grk to promote EGF-R/MAPK signaling and define posterior fates in the early follicular epithelium.

A Family of Rho-Related Proteins in Drosophila and Other Eukaryotes

Rho-related genes have been found in organisms from diverse kingdoms including the nematode *Caenorhabditis elegans,* rat, human, *Arabidopsis,* sugar cane, yeast, and bacteria. The existence of this family of *rhomboid*-related genes raises the question whether these genes all fulfill similar biochemical functions in these widely divergent species. In the case of Brho, which clusters tightly with Rho in evolutionary cladograms (Fig. 1B), our analysis strongly suggests that it functions like Rho to promote EGF-R signaling, possibly by activating the TGFα-like ligand Gurken. It is unclear, however, whether other *rho*-related genes found in *Drosophila* or different organisms will also activate the EGF-R pathway. No function has yet been assigned to any of the vertebrate Rho proteins. In the nematode, EGF-R plays a prominent role in vulval development (Sternberg and Han, 1998), but no mutation has been identified in either of the two nematode *rho*-related genes in comprehensive screens designed to identify genes involved in vulval development (Herman and Horvitz, 1997). It is possible that these two genes have redundant functions or that they are involved in regulating EGF-R activity during other developmental processes (Aroian and Sternberg, 1991). Since RTKs have not been found in plants (Satterlee and Sussman, 1998), yeast, or bacteria, the *rho*-related genes in these organisms presumably serve other functions. It will be interesting to determine whether the activities of these Rho-related proteins are similar to those of Rho and Brho, such as promoting the processing or activation of transmembrane polypeptides.

A striking feature of *rho*-related genes is that amino acid sequence conservation is most prominent in the predicted transmembrane regions. This is also true for other seven-pass transmembrane proteins such as G-protein-coupled receptors (GPCRs), although Rho proteins show no direct homology to any of the known GPCR subfamilies. Interestingly, a GPCR protein has recently been shown to be required for EGF-R signaling in a mammalian system and does so by promoting processing of an EGF ligand (Prenzel *et al.,* 2000). Similarly, presenilin-related molecules, which form another subfamily of seven-pass transmembrane proteins, have been shown to function by activating intramembrane proteolysis of Notch (Struhl and Greenwald, 1999; Ye *et al.,* 1999; De Strooper *et al.,* 1999). These observations raise the question of whether these seven-pass transmembrane proteins and Rho-related proteins may be generally involved in promoting proteolysis of other integral membrane proteins.

In contrast to GPCR subfamilies, no significant homologies have been found in the N-terminal or C-terminal tails among the Rho-related proteins so far identified. Consistent with the absence of sequence conservation in the N-terminal domains of Rho-related proteins, an N-terminal truncated version of Rho is fully active in the *Drosophila* wing (Guichard *et al.,* manuscript in preparation). This observation, together with the conservation of amino acid residues in the transmembrane domains, has important functional implications for the family of Rhomboid-related proteins. It may reflect the presence of a conserved active site within the plasma membrane, where Rho proteins interact with potential partner(s). The recent finding that the transmembrane domain of Spi is important for its

functional interaction with Rho and Star (Bang and Kintner, 2000) supports this view.

It is noteworthy that the *Drosophila rho-related [31D10]* and *C. elegans rho3* genes contain an additional insert of amino acids between TM1 and TM2. This insert, which is highly conserved between these two proteins (data not shown), contains a cluster of cysteine residues typical of growth factor-like domains in extracellular proteins. This observation suggests that the loop between TM1 and TM2 lies outside of the cell and consequently that the N-terminus of Rho proteins should be cytoplasmic and the C-terminus extracellular. It will be interesting to determine whether the additional conserved domains of amino acids in the Rho-related [31D10] and *C. elegans* Rho3 proteins interact with other extracellular components involved in the function of these proteins.

The Gurken Ligand Can Be Activated by Star and Brho

The Spitz and Gurken proteins both have single transmembrane domains and belong to the TGFα subfamily of EGF ligands (Neuman-Silberberg and Schüpbach, 1993; Rutledge *et al.*, 1992). As observed for Spitz (Schweitzer *et al.*, 1995), misexpression of the full-length Grk protein in the wing or in follicle cells has no effect. In contrast, truncated versions of Spi and Grk missing the transmembrane and cytoplasmic domains (sSpi and GrkΔTC, respectively) are potent activators of EGF-R in these same assays (Queenan *et al.*, 1999). Curiously, GrkΔTC protein is not localized at the apical pole of the follicle cells and unlike wild-type Gurken does not get transported into adjacent follicle cells when expressed in the oocyte (Neuman-Silberberg and Schüpbach, 1996). One explanation for these results is that GrkΔTC activates EGF-R intracellularly (Queenan *et al.*, 1999). This mechanism differs from that proposed for the Spitz ligand since a similarly truncated version of Spitz (sSpi) can be detected as a secreted product in the medium of cells expressing the *sspi* construct (Schweitzer *et al.*, 1995). Also, sSpi is able to activate EGF-R a few cell diameters away from its site of expression (Golembo *et al.*, 1996), in further support of the idea that sSpi is secreted into the extracellular space where it can diffuse or be transported to adjacent cells.

In this study, we demonstrate that membrane-bound Grk ligand can be activated by Star and Brho. This finding strongly supports the idea that endogenous mGrk is activated by a mechanism similar to that for mSpi, involving Star and a Rhomboid-related protein. This proposal is further supported by the fact that the transmembrane domain, which in Spi mediates the interaction with Star and Rho (Bang and Kintner, 2000), is highly conserved between Grk homologues of *Drosophila melanogaster* and of *D. virilis* (Peri *et al.*, 1999). Moreover, there is a significant homology between the transmembrane domains of Grk and Spi (25% identity and 65% similarity) (Neuman-Silberberg and Schüpbach, 1993). On the other hand, no significant homology between Grk and Spi is observed in the cytoplasmic domain. This latter fact is not surprising, considering that the cytoplasmic domain of Spi can be replaced by an unrelated sequence and still have an inhibitory effect on Spi activity, suggesting that its presence but not its primary sequence is important (Bang and Kintner, 2000). The apparent uncoupling of Spi or Gurken activation from the production of a secreted cleaved product (suggested by the analysis of truncated Grk function in flies and Spi function in the heterologous *Xenopus* assay system) raises the possibility that the primary function of Rho and Star may not be to promote Spi or Grk cleavage, but rather to modify its presentation, which in some cases may also lead to ligand processing.

A Model for brho Function during Oogenesis

Three temporally distinct functions of EGF-R signaling can be distinguished during oogenesis (Nilson and Schüpbach, 1999; Van Buskirk and Schüpbach, 1999). First, very early (in the germarium), EGF-R activity is required for the correct packaging of a single oocyte and its attached nurse cells into a single egg chamber by follicle cells (Goode *et al.*, 1996). Second (in stage 1–8 egg chambers), the Grk ligand is expressed in the oocyte and signals to adjacent posterior follicle cells, through activation of the EGF-R, to establish anterior–posterior polarity of the egg chamber (González-Reyes *et al.*, 1995; Roth *et al.*, 1995). Finally, EGF-R functions in follicle cells (in stage 9–12 egg chambers) to establish the dorsoventral polarity of the egg chamber and embryo. During stages 9–10, *grk* mRNA is restricted to the dorsal anterior corner of the oocyte, where it produces a localized source of Grk protein at the apical surface of the oocyte that activates the EGF receptor in adjacent follicle cells. Ultimately, dorsal EGF-R activation is refined into a pair of peaks by which it promotes differentiation of the dorsal appendages (Wasserman and Freeman, 1998). A variety of evidence indicates that *rho* plays a critical role in this later process (Ruohola-Baker *et al.*, 1993; Wasserman and Freeman, 1999). In contrast, there is no evidence that *rho* participates in the earlier EGF-R-dependent events. Furthermore, *rho* expression cannot be detected in stage 1–8 egg chambers. *brho*, on the other hand, is expressed during stages 5–8 and may function to activate Grk-mediated EGF-R signaling during this intermediate period.

This current work indicates that Brho can activate EGF-R signaling and suggests that it may have a role in defining posterior fates of the egg chamber since MAPK is activated exclusively in the posterior follicle cells abutting the oocyte at stages 5–8. Although it has been proposed that posterior EGF-R activation occurs during stages 1–6 (González-Reyes *et al.*, 1995), or at stage 7 (Roth *et al.*, 1995), the data presented in this study are consistent with this event occurring any time prior to stage 8. Our direct observation of MAPK activation is most consistent with the later time window. It is possible, however, that EGF-R signaling is initiated earlier, but that the levels of MAPK activation are

not easily detectable during these stages. Since the Grk ligand requires activation in order to signal to neighboring cells, we propose that endogenous Brho participates in activating Grk during midoogenesis, which then activates EGF-R signaling to define posterior cell fates in the follicular epithelium. Confirmation of this hypothesis awaits the isolation of *brho* loss-of-function mutants or antisense analysis.

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Note added in proof. After this manuscript was accepted for publication another study has been published which reports that the *rho-related* gene *roughoid* (designated *rho-related 62A6* in our study) functions in collaboration with *rho* to promote EGF-R signaling during eye development (Wasserman *et al.*, 2000). It is noteworthy that *rho*, *brho*, and *roughoid* cluster within the same evolution clade (see Fig. 1B).

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